JHR 95: 1–12 (2023)
doi: 10.3897/jhr.95.96631
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Oviposition experience promotes active reproductive behaviour in a synovigenic parasitoid

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Academic editor: Zachary Lahey | Received 25 October 2022 | Accepted 6 December 2022 | Published 17 February 2023

https://zoobank.org/F134A655-AB53-4393-82A5-79AD9B54DD98

Citation: Wang Z-Y, Wang Y-F, Yin S-Y, Liu P-C, Hu H-Y (2023) Oviposition experience promotes active reproductive behaviour in a synovigenic parasitoid. Journal of Hymenoptera Research 95: 1–12. https://doi.org/10.3897/jhr.95.96631

Abstract

Parasitoids are important insects that are commonly released into the environment to reduce the population sizes of pest species. The lifetime reproductive success of parasitoids mainly depends on host availability and the availability of mature eggs. Consequently, it is predicted that female wasps must balance the risk of egg or host (time) limitation with maximized lifetime fecundity. Typically, synovigenic females, which continue to mature eggs throughout their lifetime, have been shown to adjust their egg production rate in response to environmental variations in host availability to reduce the risk of egg limitation. In this study, we found that in a synovigenic egg parasitoid, *Anastatus japonicus* (Hymenoptera: Eupelmidae), the oviposition experience of *Ana. japonicus* females significantly enhanced the egg load and increased the rate of mature egg production. However, in contrast to other studies, the experience of contact with a host did not significantly affect the egg load in females. This result suggests that the overall oviposition experience might induce an adjustment and accelerate egg maturation in *Ana. japonicus* and is likely more important in egg maturation than transitory host contract. In addition to affecting the egg load, oviposition experience influenced *Ana. japonicus* female reproductive behaviour, which shifted virgin female behavioural preferences from mating to oviposition and laying more eggs per clutch. Our study provides an optimal strategy for the post-oviposition release of *Ana. japonicus*, an egg parasitoid of several lepidopteran forest pests, to improve biocontrol effectiveness.

Keywords

Anastatus japonicus, biological control, egg limitation, egg load

Introduction

Reproduction is crucial for all animals. Insect parasitoids are insects that parasitise other organisms, and all invertebrate life stages, including the egg, larval/nymphal, pupal, and adult stages are susceptible to parasitisation. Because of their parasitic nature, an increasing number of species have been extensively released to reduce the population sizes of pest species (Hassan 1993; Zhishan et al. 2003; Asgari and Rivers 2011; Wang et al. 2019). Many studies on parasitoid wasps have focused on applied research, with the intent to improve the attack and control of pest populations by such species (Powell 1986; Wajnberg et al. 2008; Yang et al. 2014). Generally, the lifetime reproductive success of parasitoids mainly depends on host availability and the availability of mature eggs (Jervis et al. 2001; Tylianakis et al. 2004; Hougardy et al. 2005). In the field, parasitoids generally experience one of the two following situations. Either the number of mature eggs available for laying exceeds the number of oviposition opportunities or the number of oviposition opportunities exceeds the number of mature eggs available for laying; these are defined as host/time limitation and egg limitation, respectively (Godfray 1994; Richard and Casas 2012). However, a perfect match between the number of hosts and the availability of mature eggs is rare. Consequently, to maximize reproductive success, insect parasitoids are hypothesized to balance the risk of egg limitation and time limitation (Minkenberg et al. 1992; Rosenheim 1996; Heimpel et al. 1998; Rosenheim et al. 2008).

According to the type of egg production, parasitoids can be classified as pro-ovigenic or synovigenic. Pro-ovigenic species mature all or most of their lifetime complement of eggs prior to emergence from the host, whereas synovigenic species emerge with very few or no mature eggs, and egg maturation begins at eclosion and continues throughout adult life (Flanders 1950; Jervis et al. 2001). A number of internal and environmental factors have been shown to influence egg production and maturation in both pro-ovigenic and synovigenic species. Host resources greatly influence synovigenic females that adjust their egg production rate in response to host availability (Papaj 2000). For example, in the parasitoid *Eupelmus vuilleti* (Crawford) (Hymenoptera: Eupelmidae), contact with a host by the female antennae can accelerate egg maturation (Casas et al. 2009), and in the aphid parasitoid *Aphelinus albipodus* (Hayat & Fatima) (Hymenoptera: Aphelinidae), females mature eggs faster in the presence of preferred hosts (Wu and Heimpel 2007). In those studies, females were usually manipulated by a single contact with the host (i.e., antennal) to perceive the host's presence, and then egg loads were counted. However, contact or perceived contact with a host is only one component of complete oviposition behaviour. Therefore, this study mainly focused on the effect of complete oviposition behaviour on egg loads in female wasps.

Lymantria dispar (L.) (Lepidoptera: Erebidae) is a leaf-feeding insect that causes the large-scale defoliation of forest and urban trees worldwide during intermittent population outbreaks. *Anastatus japonicus* Ashmead (formerly *Anastatus disparis* Ruschka) (Hymenoptera: Eupelmidae) is a recorded egg parasitoid of *L. dispar* (Crossman 1925; Kurir 1944; Avci 2009; Alalouni et al. 2013) and is widely distributed in Europe

and Asia. In addition, due to its potential as a biological control agent of *L. dispar*, *Ana. japonicus* was imported to North America in the early 1900s (Crossman 1925; Yan et al. 1989) and achieved effective control efficiency. Field investigations revealed that, together with *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae), which is also a primary egg parasitoid of *L. dispar*, parasitism rates might reach 20–40% (Hoy 1976; Reardon 1981; Brown and Cameron 1982; Brown 1984). In addition to *L. dispar*, several other noxious lepidopteran species that are primarily forest pests, including *Malacosoma neustria testacea* (Motschulsky), *Odonestis pruni* (Linnaeus), *Antheraea pernyi* (Guerin-Meneville), *Dendrolimus punctatus tabulaeformis* (Walker), and *Actias selene ningpoana* (Felder), can be parasitised (Yan et al. 1989; Li and Lou 1992; Li et al. 2001). Great efforts have been made to mass rear *Ana. japonicus* for the control of *Caligula japonica* (Moore) in China, as the eggs of *Ant. pernyi* are suitable factitious hosts (Yan et al. 1989). The lifetime fecundity of a female *Ana. japonicus* is several hundred offspring (Liu et al. 2015), and the offspring sex ratio is mainly influenced by host quality (i.e., size) (Liu et al. 2017). The egg maturation mode of *Ana. japonicus* is typically synovigenic, and previous studies found that females usually contained tens of mature eggs by dissecting the abdomen (Liu and Hao 2019). Hosts of *Ana. japonicus*, i.e., *L. dispar*, typically lay clutches containing hundreds or thousands of eggs; thus, the lifetime reproductive success of a female parasitoid is limited by the supply of mature eggs, i.e., egg limitation. Several synovigenic females have been shown to adjust their egg production rate in response to environmental variations in host availability (Papaj 2000; Wu and Heimpel 2007; Casas et al. 2009). During oviposition in a host, parasitoids may learn to recognize particular visual and olfactory host stimuli and use these cues to modify subsequent behaviours (Vet et al. 1995; Vinson 1998). Thus, in this study, the effect of the complete oviposition experience on egg load was studied in *Ana. japonicus*. In addition, the effect of contact with a host on egg load was tested, as well. Finally, the effects of oviposition experience on subsequent behaviour and capacity, e.g., behavioural choice and host exploration capacity, were also studied in *Ana. japonicus*.

Materials and methods

Host and parasitoid species

Ana. japonicus colonies were first established from a population reared on *L. dispar* egg masses collected in Tongliao city, China (43°62'N, 122°25'E) in December 2019, and the colony was subsequently maintained on *Ant. pernyi* eggs. Eggs of *Ant. pernyi* were obtained by laparotomizing adult female abdomens, and they were maintained at 0 °C (Wang et al. 2014). We isolated the parasitised host eggs of *Ant. pernyi* individually in polyethylene tubes (height: 7.5 cm; diameter: 1 cm), with the openings covered by a cotton ball to prevent any mating behaviour before the experiment began. A previous study showed that mating behaviour significantly increased the egg load (Liu and Hao 2019).

Egg loads in females with host contact experience and oviposition experience

A single, one-day-old, newly emerged *Ana. japonicus* virgin female was introduced into a Petri dish (height: 1.5 cm, diameter: 5 cm) with four eggs of *Ant. pernyi*; the Petri dishes were maintained at 26 ± 0.5 °C with 70 ± 5% relative humidity (RH). When the female made antennal contact with one of the hosts for more than 30 s, the behaviour was interrupted and the female was placed into a cylindrical plastic box (diameter: 5.0 cm, height 5.0 cm) for rearing. As a control treatment, no host eggs were provided to females. Female wasps aged 2 to 10 days were dissected for the determination of egg loads. Egg loads were measured in terms of the number of mature eggs in the ovaries. Honey water (honey:water = 4:6) was supplied on cotton balls as nutrition for adult females until dissection (Yan et al. 1989). The selected adults were euthanized by freezing at -80 °C, and then the abdomens were placed into a Petri dish with a saline solution. We counted the number of mature eggs by dissecting the abdomens using forceps under a microscope (Leica M205A, Germany). In total, 13–17 females were dissected for each treatment.

To acquire oviposition experience, a one-day-old virgin female was introduced into a Petri dish (height: 1.5 cm, diameter: 5 cm) with four host eggs. After completing an oviposition event in a host, the female was removed and reared in a cylindrical plastic box (diameter: 5.0 cm, height 5.0 cm); the treatment was considered "oviposition experience". Based on preliminary experiments, an oviposition event was considered finished when the female completed oviposition (i.e., the female initiated parasitisation of a new host or moved away from the previously parasitised host for at least 1 min), and the duration of the oviposition event lasted for more than 10 min. As a control treatment, no host eggs were provided to females. Females aged 2 to 10 days and exposed to the above two treatments were dissected for the determination of egg loads, and they were fed honey water (honey: water = 4: 6) daily. In total, 13–17 females were used for each treatment.

Effect of oviposition experience on mate and oviposition choice behaviours

Previous studies revealed that newly eclosed females rarely lay offspring on the first day. Thus, in this study, two-day-old virgin females (with oviposition experience or without any experience) were introduced into a Petri dish (height: 1.5 cm, diameter: 5 cm) containing one newly eclosed virgin male and four fresh *Ant. pernyi* eggs for 60 min. The entire process was video recorded. In each dish, the first mating and oviposition events in the female were recorded.

Effect of oviposition experience on subsequent host exploration capacity

In this experiment, an artificial host clutch containing nine *Ant. pernyi* eggs was offered to two virgin females (with oviposition experience and without any experience); the females and egg clutch were placed into a Petri dish (height: 1.5 cm, diameter: 5 cm) to allow oviposition for three hours. In each dish, each host egg in the clutch was marked with a number (e.g., 1, 2….9). For easy differentiation, one randomly selected female

in each dish was marked with white (the other female was marked with green) acrylic paint on the back of the thorax. In total, the sample size was 16, and the entire process was video recorded. Similarly, successful oviposition in a host was considered when the oviposition process was complete and the duration of the oviposition event lasted for more than 10 min. In each dish, successful oviposition and the number of host eggs in which the marked wasps oviposited were determined by reviewing the recorded videos.

Statistical analysis

All analyses were performed with *R* software (version 2.14.1). In our study, the effects of female age and experience on egg load were analysed with generalized linear mixed models (GLMMs, lme4 package, Bates et al. 2018) with a Poisson distribution and log link function (Crawley 1993). When the ratio of residual deviance and residual *df* were >1, the data exhibited overdispersion. Under this scenario, significance testing was performed using quasi-Poisson regression, and significance was assessed based on the F statistic (Crawley 1993, 2007). In each model, egg load was considered the response variable, and the factors of female age and oviposition experience were considered fixed effects. The criterion for significance was a p value < 0.01 when testing interactions (Crawley 2007). For the behavioural choice experiment, the preferences for mating and oviposition were analysed using sign tests, and a chi-square test was employed to determine the effect of the oviposition experience of females on preference. Finally, a paired-samples *t*-test was used to analyse the difference in the number of host eggs parasitised by females with oviposition experience and without oviposition experience.

Results

Egg loads in females with host contact experience and oviposition experience

On the second day, a few mature eggs (virgin females without any experience: 5.93 ± 1.22; virgin females with host contact experience: 6.08 ± 0.83) were observed in the ovaries. The GLMM analysis showed that the number of mature eggs in the females was significantly influenced by individual age ($F = 29.034$, $df_1 = 8$, $df_2 = 209$, $p < 0.001$) but not by contact with the host ($F = 0.034$, $df_1 = 1$, $df_2 = 209$, $p = 0.854$). In addition, there were no interaction effects of female experience status and age on the number of mature eggs ($F = 0.187$, $df_1 = 8$, $df_2 = 209$, $p = 0.992$). As shown in Fig. 1, daily egg loads increased from day 2 until day 5 and then plateaued from days 6–10.

The results showed that both age ($F = 10.653$, $df_1 = 8$, $df_2 = 214$, $p < 0.001$) and oviposition experience ($F = 39.891$, $df_1 = 1$, $df_2 = 214$, $p < 0.001$) had significant effects on the number of mature eggs in females. There were significant interaction effects between female experience status and age on the number of mature eggs ($F = 5.52$, $df_1 = 8$, $df_2 = 214$, $p < 0.001$). As shown in Fig. 1, daily egg loads in females with oviposition experience peaked at day 3, with 16.7 ± 1.12 eggs per female, while in females with and without host contact experience, the egg load peaked at approximately day 5.

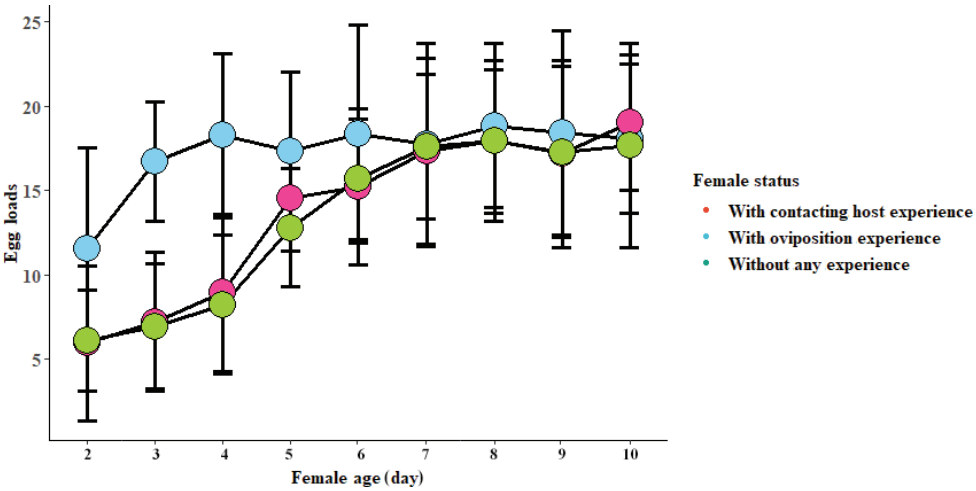

Figure 1. Mean egg loads (+SEs) over time of *Anastatus japonicus* females. Egg loads were measured in terms of the number of mature eggs in the ovaries. The age of measured females ranged from 2 to 10 days old.

Effect of oviposition experience on mate and oviposition choice behaviours

When mating and oviposition choices were presented to females without any experience, most females (29/30) successfully made a choice that preferred for mating (n = 21) (Z=-2.228, p = 0.026) (Fig. 2). In addition, all females with oviposition experience successfully made a distinct choice, while those without experience did not show any preference for mating (n = 13) or oviposition (n = 17) (Z=-0.548, p = 0.584) (Fig. 2). Thus, oviposition experience had a significant effect on the above choice results (c^2 = 5.107, df = 1, p = 0.024).

Effect of oviposition experience on subsequent host exploration capacity

When an artificial host clutch containing nine *Ant. pernyi* eggs was offered for oviposition, females with oviposition experience produced a mean of 1.78 ± 0.15 offspring (Fig. 3), which was significantly more than that produced by females without any oviposition experience (0.92 ± 0.12) (paired-samples t test, t = 3.515, df = 8, p = 0.001). In addition, among all the oviposition behaviours, conspecific superparasitism occurred only once (1/43) when two females in a single dish laid eggs in the same host.

Discussion

Anastatus japonicus is a typically synovigenic parasitoid that continuously matures eggs throughout its lifetime. Synovigenic females have been shown to adjust their egg production rate in response to host availability (Papaj 2000). Our study provides

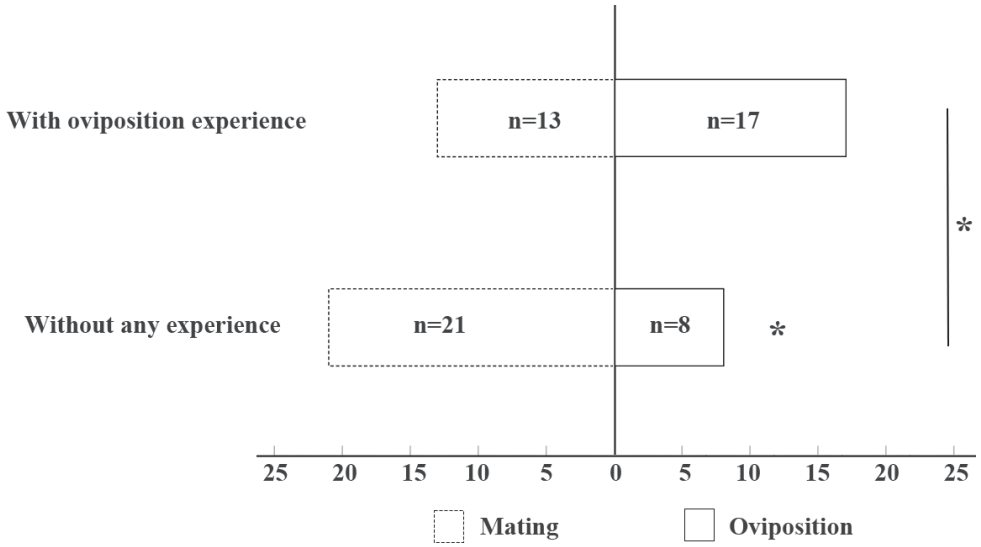

Figure 2. Effect of oviposition experience on mate and oviposition choice behaviours. * $p < 0.05$.

further evidence that the oviposition experience of *Ana. japonicus* females significantly enhances female egg load and rapidly increases the number of mature eggs (Fig. 1). Hosts are important resources for reproduction, and it is predicted that female wasps must balance the risks of egg and time limitation with maximum lifetime fecundity (Minkenberg et al. 1992; Rosenheim 1996; Heimpel et al. 1998; Rosenheim et al. 2008). Thus, *Ana. japonicus* females with oviposition experience are likely to reduce the risk of egg limitation by rapidly increasing their egg loads. Similar to the study in the parasitoid *E. vuilleti* (Crawford) (Casas et al. 2009), oviposition experience might initiate a hormonal cascade leading to egg maturation in *Ana. japonicus* and should be studied further. In contrast to a study in the parasitoid *E. vuillet*, host contact experience in *Ana. japonicus* did not affect the egg loads in females. This result suggests that oviposition experience is likely more important in egg load maturation than transitory host contact in *Ana. japonicus*. A limitation of this study was that it did not consider the effects of host density in *Ana. japonicus*, as a fixed number of host eggs was provided. The density of hosts in the environment has been suggested to be directly linked to egg maturation rates in many studies (Rosenheim 1996; Rosenheim et al. 2000; Segoli and Rosenheim 2013). Usually, a high host density elicits production of a larger egg load (Bodin et al. 2009; Casas et al. 2009). However, a negative effect of host density on egg load was observed in the soybean aphid parasitoid *Binodoxys communis* (Gahan) (Dieckhoff et al. 2014). Thus, the effect of host density on egg load should be studied and considered in further research.

Hymenopterans are haplodiploid; thus, virgin females can produce male offspring before mating (Cook 1993; Heimpel and de Boer 2008). Hypothetically, virgin females need to evaluate the following trade-offs: (1) search for hosts and produce sons

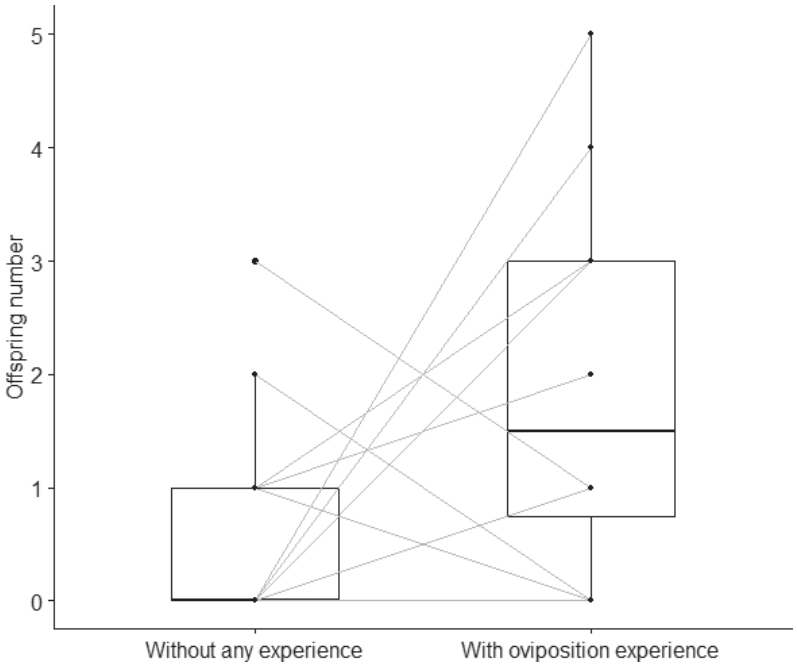

Figure 3. Box plot for paired data of offspring number laying by female with oviposition experience and female without any oviposition experience in a dish. Boxes: 25th and 75th percentiles; heavy line: median; whiskers: 1.5 times the interquartile range of the data.

immediately or (2) search for mates and perhaps produce both sons and daughters later in life (Godfray 1990; Godfray and Hardy 1993; Godfray 1994; Fauvergue et al. 2008). In *Ana. japonicus*, the results showed that virgin females preferred to complete mating first. A specific local mating system likely contributed to the preference for mating. It may be a result of long-term evolution that female dispersal occurs before mating. In addition, virgin *Ana. japonicus* may have lower fitness than mated females, that only produces male offspring. However, our study showed that the oviposition experience of females changed the behavioural preference from mating to oviposition. A potential explanation for oviposition experience influencing subsequent oviposition behaviour in *Ana. japonicus* females is that females with oviposition experience may fine-tune behaviours closely associated with reproduction (Pyle et al. 1991). Alternatively, compared to females without experience, those with oviposition experience had larger egg loads, indicating that these individuals had a higher risk of time limitation. Time-limited species often have large egg loads or the ability to quickly replenish their egg supply, so their reproductive success is proportional to the number of hosts they are able to attack during their lifetime (Stephens and Krebs 1986; Charnov and Stephens 1988).

Thus, oviposition experience of females changed the behavioural preference from mating to oviposition in our species, may be more expected to achieve the reproductive success in response to the risk of time limitation.

Generally, in addition to egg load, oviposition experience influenced female *Ana. japonicus* reproductive behaviour, shifting virgin female behavioural preference from mating to oviposition, allowing more eggs to be laid per host clutch. The change in behavioural preference from mating to oviposition may be a direct effect of oviposition experience in females. In our study, when an artificial host clutch containing nine *Anth. pernyi* eggs was offered for oviposition, females with oviposition experience laid a mean of 1.78 ± 0.15 eggs, which was significantly more than that laid by females without oviposition experience. In addition, during oviposition in a host, parasitoids learn to recognize particular visual and olfactory stimuli of the host and use these cues to modify subsequent behaviours (Vet et al. 1995; Vinson 1998). Thus, oviposition experience might also be associated with an increased hatch rate and increased host acceptability, resulting in more eggs laid per clutch. In addition, among all the oviposition behaviours observed in the current study, conspecific superparasitism occurred only once (1/43), as two females in a dish oviposited in the same host. Females exhibited a host discrimination ability and could identify parasitised hosts to avoid wasting eggs (Liu et al. 2018). Our experimental design including a large host number for females may also have contributed to the general absence of conspecific superparasitism.

Anastatus japonicus is an egg parasitoid of *L. dispar* (Crossman 1925; Kurir 1944; Avci 2009; Alalouni et al. 2013) and a potential biological control agent of this species in North America (Crossman, 1925; Yan et al. 1989). Great efforts have been made to mass rear *Ana. japonicus* for the control of *C. japonica* in addition to *L. dispar* in China, and the eggs of *Ant. pernyi* are suitable factitious hosts (Yan et al. 1989). Our study suggested that females with oviposition experience had a higher reproductive value, with larger egg loads and a preference for oviposition. Therefore, in the rearing of *Ana. japonicus* for biological control, hosts can be provided before the release of wasps so that the female wasps can gain oviposition experience. This will likely improve the reproductive value of the released female wasps so that the wasps can parasitize more hosts, improving the efficacy of biological control.

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JHR 95: 13–30 (2023)
doi: 10.3897/jhr.95.89678
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Ovipositor characteristics differ between two parasitoids (Hymenoptera, Figitidae) of *Drosophila suzukii* (Diptera, Drosophilidae) in an adventive landscape

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Academic editor: Elijah Talamas | Received 8 September 2022 | Accepted 31 January 2023 | Published 17 February 2023

https://zoobank.org/D4BF1C35-568A-4116-8890-B83B85D2B923

Citation: Earley NG, Abram PK, Lalonde RG, Moffat CE (2023) Ovipositor characteristics differ between two parasitoids (Hymenoptera, Figitidae) of *Drosophila suzukii* (Diptera, Drosophilidae) in an adventive landscape. Journal of Hymenoptera Research 95: 13–30. https://doi.org/10.3897/jhr.95.89678

Abstract

Different ovipositor characteristics among parasitoid species that share similar niches are associated with different wasp life histories and selective pressures. The length of wasp ovipositors, for example, can determine the accessibility of hosts that feed at different depths within food substrates. Two parasitoids, *Ganaspis brasiliensis* and *Leptopilina japonica* (Hymenoptera, Figitidae), which attack *Drosophila suzukii* (Diptera, Drosophilidae) in their native range, have been investigated for their suitability for the global biological control of the small fruit pest. Despite their sympatry in microhabitat, the parasitoids have differing host ranges, and *D. suzukii* parasitism rates by each parasitoid species appear to depend on the fruit species occupied by the host species. Adventive populations of both parasitoids have been detected in the Pacific Northwest of Canada and the United States where they can be found parasitizing *D. suzukii* larvae in crop and non-crop fruits. We dissected and measured the ovipositors of parasitoids reared from three species of fresh fruits at three sites in southwestern British Columbia, Canada, and investigated the influence of parasitoid species, fruit type, and collection site on ovipositor characteristics. We found that ovipositor length differed markedly between the two parasitoid species and between sites while ovipositor width, and stoutness, differed only between the two parasitoid species, but did not vary among sites or fruit hosts. We discuss how ovipositor morphology traits could be associated with differences in life history and host ranges in the two parasitoid species.

Keywords
biological control, competition, *Ganaspis brasiliensis*, *Leptopilina japonica*, morphology, niche partitioning, spotted-wing drosophila

Introduction

Parasitoid reproductive success depends on access to and successful exploitation of available hosts. For parasitoid Hymenoptera, the morphology of ovipositors used to lay their eggs into or onto hosts, is critical for the effective exploitation of hosts within structural niches (Le Ralec et al. 1996; Quicke et al. 1999; Vilhelmsen and Turrisi 2011). One way that insect herbivores can reduce attack by parasitoids is by feeding deep within the confines of plant structures like galls, fruits, or tree trunks. Select parasitoid species are able to circumvent this type of host defence by accessing their hosts earlier in host development in smaller plant structures (a phenological adaptation, e.g. Weis and Abrahamson 1985), while others overcome this defence using long ovipositors that can access hosts deep inside larger plant structures (a morphological adaptation, e.g. Weis and Abrahamson 1985). Hosts can further escape pressures imposed by previously associated parasitoids by occupying larger plant structures like cultivated fruits (e.g. Feder 1995) or feeding on earlier phenological stages of their host plants (e.g. ripening as opposed to rotting fruit). Some of these shifts in host feeding behaviour could engender costs for the host, which would be balanced against the benefit of decreased parasitism risk (Gratton and Welter 1999). In addition, these differences in feeding niche could hypothetically spur the evolution of modified ovipositors in parasitoids that allow them to effectively access hosts in these novel environments (Vermeij 1999; Sivinski et al. 2001; Sivinski and Aluja 2003).

Constraints imposed by parasitoid ovipositor length can affect the success of biological control of non-native pests such as the olive fruit fly *Bactrocera oleae* Rossi (Diptera: Tephritidae) in the California olive agricultural system (Sime et al. 2007; Wang et al. 2008, 2009). Here, larger fruits are preferred by growers and this bias in cultivar choice has been shown to prevent certain parasitoids (Hymenoptera: Braconidae) with shorter ovipositors from accessing their hosts (Sime et al. 2007; Wang et al. 2008, 2009). Parasitoid species with shorter ovipositors are less effective at controlling the pest larvae in larger fruits, while those species with longer ovipositors can access host larvae more effectively in larger fruits (Sime et al. 2007; Wang et al. 2008, 2009). Parasitoids in this system with longer ovipositors also have broader host ranges; a consideration that complicates the biological control of *B. oleae* in an agricultural system that selects for larger fruits (Sime et al. 2007; Wang et al. 2008, 2009). Thus, the success of a biological control programme could be reduced by physical barriers to parasitism.

The behaviour of host larvae can also influence the success of parasitism, by evading detection by parasitoids or by allowing larvae to escape parasitism when first encountered by a parasitoid (Gross 1993; Kacsoh et al. 2013; Robertson et al. 2013; Singh et al. 2015). Larvae living in semi-concealed environments may escape

parasitism by diving deeper into their developmental substrate (van Lenteren et al. 1998). In order to parasitize mobile hosts that attempt to escape parasitism, Figitidae (Hymenoptera) that parasitize semi-concealed dipterous larvae have an ovipositor clip (Buffington 2007), a morphological feature that restrains host larvae prior to their envenomation (van Lenteren et al. 1998). *Drosophila* Fallén (Diptera: Drosophilidae) larvae have differing foraging behaviours within their developmental substrate where some (e.g. *D. melanogaster* Meigen) feed relatively close to the surface while others (e.g. *D. suzukii* Matsumura), that typically develop in deeper substrates such as ripening fruits, dive deeper into the substrate more frequently and for longer (Kim et al. 2017). Differential diving behaviours of *Drosophila* larvae could have implications for parasitoid access to hosts.

Two parasitoid wasp species, *Leptopilina japonica* Novković & Kimura and *Ganaspis brasiliensis* Ihering (Hymenoptera: Figitidae), have been proposed as candidate classical biological control agents for the small fruit pest *D. suzukii* (Lee et al. 2019). These parasitoids have recently been detected in North America, specifically in southwestern British Columbia (BC), Canada (Abram et al. 2020) and northwestern Washington State, USA (Beers et al. 2022). *Leptopilina japonica* has the broader host range of the two parasitoids and successfully parasitizes several drosophilid species in four genera in laboratory trials (Kimura and Novković 2015; Girod et al. 2018b; Daane et al. 2021). The lineage of *G. brasiliensis* recorded in BC and Washington State ("G1"; Beers et al. 2022) has a narrower host range, primarily attacking flies in the subgenus *Sophophora* Sturtevant in laboratory trials (Girod et al. 2018b; Giorgini et al. 2019; Daane et al. 2021). These parasitoids are adventive in the fruit growing regions of southwestern BC but have evidently not dispersed into the province's interior (Abram et al. 2022a, b).

The sympatry of parasitoids exploiting the same host in the same habitat can lead to competition between parasitoids, which typically manifests as either competition between adult parasitoids for mating or oviposition resources (extrinsic competition) and/or competition between immature parasitoids competing for host resources (intrinsic competition) (Harvey et al. 2013; Ode et al. 2022). The inability of a developing parasitoid to compete in intrinsic competition within the host can be mitigated by extrinsic competition by the adult, and vice versa (Hood et al. 2021). Extrinsic and intrinsic competition may influence niche partitioning by parasitoids that exploit the same host in the same habitat by influencing their exploitation of hosts in unrecognized microhabitats (e.g. Heatwole et al. 1964; Heatwole and Davis 1965). The competitive interactions between *L. japonica* and *G. brasiliensis* are beginning to be characterized. While *G. brasiliensis* avoids hyperparasitizing hosts (Wang et al. 2019), *L. japonica* does not avoid such intrinsic competition (Böttinger et al. 2019) and is a superior intrinsic competitor in hyperparasitized larvae than is *G. brasiliensis* if hyperparasitism occurs within 24 hours of the initial parasitism event (Wang et al. 2019). Therefore, extrinsic competition by *G. brasiliensis* through the exploitation of host larvae in media that is not accessible to ovipositing *L. japonica* could facilitate host partitioning in this system.

In a field survey of fresh fruit collections in southwestern BC, *L. japonica* and *G. brasiliensis* made up 67.2% and 32.0% of the larval parasitoid community, respectively (Abram et al. 2022a). In the adventive range, *L. japonica* appears to cause higher percent parasitism of *D. suzukii* in larger fruits (e.g. strawberries, *Fragaria* spp.; blackberries, *Rubus* spp.) whereas *G. brasiliensis* appears to impose higher percent parasitism than *L. japonica* in smaller fruits (e.g. elderberries, *Sambucus* spp.; Abram et al. 2022a). In fresh fruit collections in China, *G. brasiliensis* was better able to parasitize *D. suzukii* in smaller fruits than in larger fruits with a parasitism rate that was higher in *Sambucus adnata* Wallich than in *Rubus foliosis* Weihe or *Rubus niveus* Thunberg, respectively (Giorgini et al. 2019). Abram et al. (2022a) observed differences in larval *D. suzukii* attack by adventive parasitoids that were similar to those observed in California olives (Sime et al. 2007; Wang et al. 2008, 2009), where higher parasitism rates were observed in parasitoid species with broader host ranges, and these tended to be associated with larger fruits. Additionally, Fellin et al. (2023) found that the G1 lineage of *G. brasiliensis* that was released to control *D. suzukii* in Italy have significantly shorter ovipositors than the adventive Italian *L. japonica*. It is possible that different ovipositor characteristics may affect host-choice and facilitate coexistence through niche partitioning (Price 1972) in the *D. suzukii* biological control system.

The objective of this study was to quantify differences in ovipositor characteristics of *L. japonica* and *G. brasiliensis* in BC, Canada. First, based on previously described associations between host range breadth and ovipositor length in other parasitoid guilds, we predicted that *L. japonica* would have longer ovipositors than *G. brasiliensis*, as longer ovipositors may be more efficient for indiscriminately parasitizing larvae regardless of their species, or level of prior parasitism. Additionally, we predicted that the apparently more specialized *G. brasiliensis* would have significantly stouter ovipositors (ovipositor width/ovipositor length) than do *L. japonica*. Stouter ovipositors may be better able to puncture fruits at an earlier stage of ripeness with *D. suzukii* developing inside; thus, further reducing the probability of mortality through lethal intra- and interspecific competition not only spatially (within a fruit) but also temporally (over the ripening period of a fruit).

Methods

Field sampling

Our samples were taken from fresh fruit collections by Abram et al. (2022a) at field sites throughout the lower mainland of southwestern BC, Canada, from late May to late October, 2020 (Fig. 1). Fruit collections at a site began when the first ripe fruit of the target fruit species or variety were available to pick and ended when fruit was scarce and remaining fruit was rotten or desiccated. We collected samples of three fruit species (cultivated blackberry, *R. fruticosus* agg. L.; Himalayan blackberry, *R. armeniacus* Focke; and red elderberry, *S. racemosa* L.). The number of berries collected was not constant for each host plant because fruit size varied from plant to plant and the aim

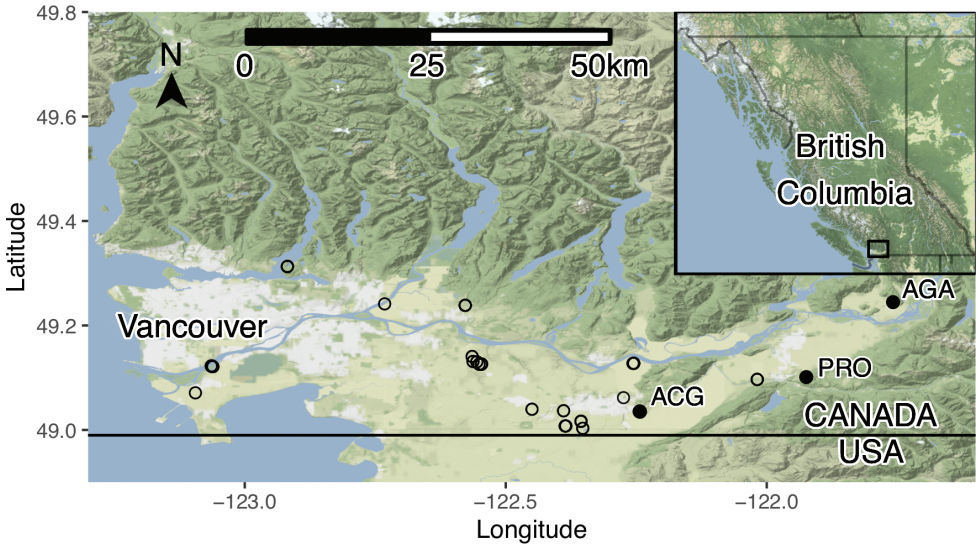

Figure 1. Map of sites in the lower mainland, British Columbia, Canada, where fresh fruits were sampled and insect inhabitants were reared and identified throughout the growing season of 2020. Solid circles with site IDs represent collection sites where the ovipositors of a subsample of female figitid emergents were removed and measured. Open circles represent all collection sites in Abram et al. (2022b). The box in the inset shows where the study area is located in British Columbia, Canada. WGS84 projected map made in ggmap v.3.0.0 (Kahle and Wickham 2013) with map tiles by Stamen Design, under CC BY 3.0.

of each collection was to collect a similar net fruit weight. See Abram et al. (2022a) for more detailed collection procedures, but briefly, we made approximately weekly fresh fruit collections at sites over the fruiting period of a mixture of cultivated and non-cultivated plants from a range of habitat types (e.g. community garden, experimental farm, mid-elevation forest). We did not record individual fruit characteristics (size, weight, depth, etc.).

Fruit rearing

Fruit samples were held in ventilated 12 × 12 × 8 cm plastic containers (Ziploc, SC Johnson, Racine, WI) as described in Abram et al. (2022b) and monitored every 2–3 days for adult *D. suzukii*, *L. japonica*, and *G. brasiliensis* emergence. Due to SARS-CoV-2 pandemic restrictions, samples were reared in uncontrolled rearing rooms where temperatures ranged from 15 °C to 25 °C (ACG rearing room: 19 °C, 21 °C, 20 °C; PRO and AGA rearing room: 15 °C, 25 °C, 22 °C; minimum, maximum, and mean temperatures, respectively). Held at constant temperature, the developmental extremes of *D. suzukii* are close to 10 and 30 °C (Tochen et al. 2014) and *L. japonica* and *G. brasiliensis* have been shown to complete development between 17.2 and 27.5 °C (Hougardy et al. 2019). Abram et al. (2022a) reported no evidence for parasitoids having entered diapause and the PRO and AGA rearing room only dropped below 17 °C for 2 days. See Abram et al. (2022b) for more detailed rearing procedures.

Dissections

We selected wasps from fruit collections with high female emergence of both parasitoid species at locations with collections of *Rubus armeniacus* Focke, *R. fruticosus* agg. L., or *Sambucus racemosa* L. These collections were chosen to limit potential variation in collection date as many collections had few females of both species. All females from each selected collection were dissected but only those specimens that yielded measurable ovipositors were included in our data. We selected wasps from three sites in southwestern BC: *i*) Abbotsford Community Garden, Abbotsford (ACG), *ii*) Agassiz Research and Development Centre, Agassiz (AGA) and *iii*) Promontory Park, Chilliwack (PRO) (Table 1). We identified wasps emerging from fresh fruit collections based on scutellar plate morphology as the shapes of the scutellar plates of *L. japonica* and *G. brasiliensis* are distinctive enough to determine species identity (see Abram et al. 2020, 2022a, b). Representative vouchers of both species were identified by M. Buffington (USDA-ARS) and are deposited in the National Insect Collection, National Museum of Natural History, Smithsonian Institution, Washington D.C (Abram et al. 2022a, b).

We point-mounted, labelled, and photographed the identified wasps, removed the metasoma, and dissected out ovipositors using insect pins and fine-tipped forceps. We photographed ovipositors through a dissecting microscope at 50× magnification and glued the ovipositor to collection paper affixed to the specimen pin. We measured ovipositor characteristics from the photographs using the straight-line tool, the freehand line tool, and the measure function in ImageJ v1.53a (Schneider et al. 2012) to transform pixels to mm (Lue 2017). All measurements were calibrated to the ocular micrometer. We measured ovipositor length along the inside curve of each ovipositor from the base of the egg sac to the tip of the ovipositor (Fig. 2). We measured the width of each ovipositor where the picture was the clearest nearest the middle. We calculated ovipositor stoutness as the quotient of the ovipositor's width divided by its length.

Tibial length measurements

To test whether some intraspecific variation in ovipositor length could be due to a positive association with body size (Niklas 1994), we measured hind tibia length as a proxy of body size (Van Alpen and Thunnissen 1983; West et al. 1996; Nicol and Mackuer 1999) of a random subset of wasps from both species, and from all sites, *post hoc*. We removed the left hind leg from point mounted specimens and photographed the inside of the tibia bent at approximately 90° at the femorotibial joint through a dissecting microscope at 80X magnification. We measured the tibia from the photographs using the straight-line tool and the measure function in ImageJ v1.53a (Schneider et al. 2012). We measured the straight line distance from the base of the tibial spur to the far edge of the femorotibial joint (Fig. 3).

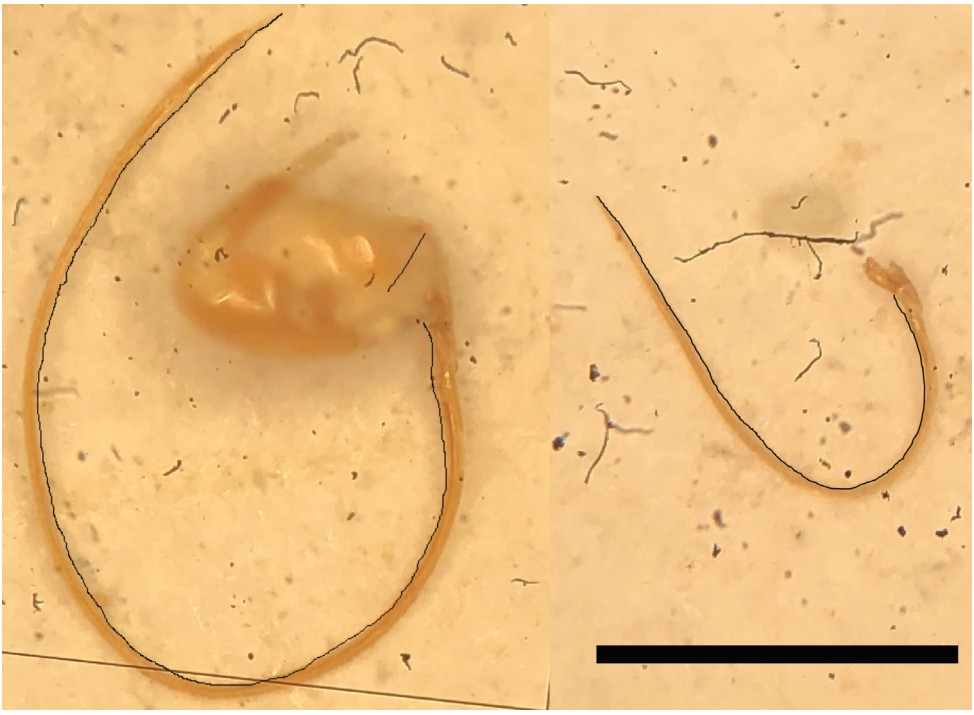

Figure 2. Comparison of ovipositors dissected out of female *L. japonica* (left) and *G. brasiliensis* (right) collected in southwestern British Columbia, Canada. Images were taken through a dissecting microscope at ×50 magnification. Scale bar represents 0.5mm.

Table 1. The number of measured ovipositors dissected from parasitoids reared from collections of fresh fruits at different sites in British Columbia, Canada.

Collection site	Plant species	Collection date	Wasp species	Number dissected
Abbotsford Community Garden, Abbotsford	*S. racemosa*	2020-06-22	*L. japonica*	2
		2020-07-10	*L. japonica*	6
			G. brasiliensis	7
	R. armeniacus	2020-07-24	*L. japonica*	4
			G. brasiliensis	5
		2020-08-07	*L. japonica*	3
			G. brasiliensis	2
Promontory Park, Chilliwack	*S. racemosa*	2020-07-24	*L. japonica*	3
			G. brasiliensis	8
	R. armeniacus	2020-08-31	*L. japonica*	7
			G. brasiliensis	7
		2020-09-07	*L. japonica*	2
			G. brasiliensis	2
Agassiz RDC, Agassiz	*R. fruticosus*	2020-07-25	*L. japonica*	5
			G. brasiliensis	4

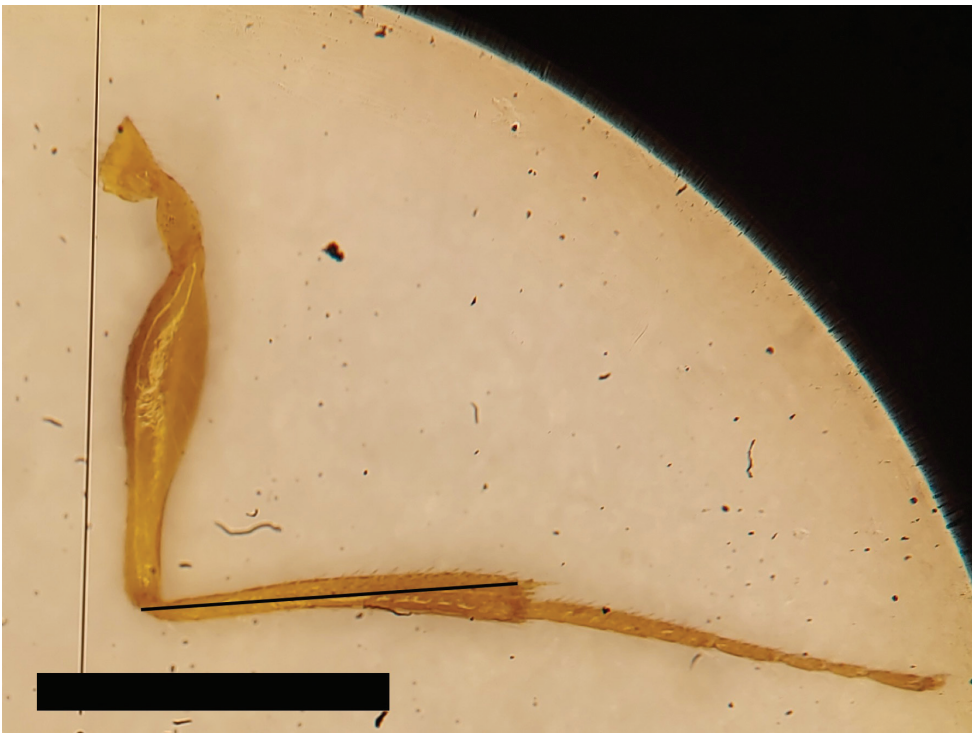

Figure 3. Left hind leg of a female *L. japonica* collected in southwestern British Columbia, Canada. We measured tibia length as the straight line distance of the inside of the tibia from the base of the tibial spur to the far edge of the femorotibial joint. Image taken through a dissecting microscope at ×80 magnification. Scale bar represents 0.5 mm.

Statistical analysis

We investigated the influence of wasp species (*G. brasiliensis*, *L. japonica*), collection site (ACG, PRO), and fruit collected (red elderberry, Himalayan blackberry) as candidate categorical predictors for the response variables ovipositor length, ovipositor width, and ovipositor stoutness using Gamma family generalized linear models (GLMs) estimated using F-tests. We used Gamma distributed GLMs because the linear model variance across the predictors were unequal. In all cases, we inspected residual plots to assess adequacy of model fit. We did not consider interactions between covariates in our statistical analyses. Since the AGA site had different fruit species from the other two sites, we removed specimens collected from the AGA site from our GLM analyses. For specimens collected from the AGA site we fit two sample t-tests with ovipositor length, ovipositor width, and ovipositor stoutness as response variables and wasp species (*G. brasiliensis*, *L. japonica*) as categorical predictors for each response variable with Bonferroni-adjusted alpha. We also investigated associations between tibia length on ovipositor length within each of the two parasitoid species using generalized linear models with Gaussian error distributions. We conducted all statistical analyses in R software, version 4.0.3 (R Core Team 2020). See Suppl. material 1 for the data used to perform these analysis.

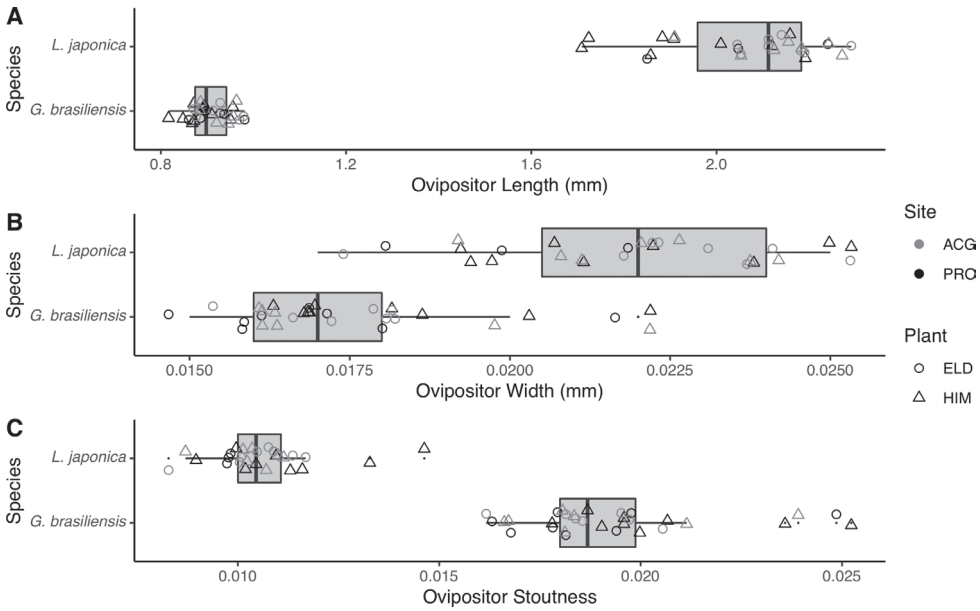

Figure 4. A Ovipositor length, **B** ovipositor width, and **C** ovipositor stoutness measured from representative samples of *L. japonica* (N=27) and *G. brasiliensis* (N=31) reared from *D. suzukii* infested fruit collected in southwestern British Columbia in 2020. Black markers represent specimens reared from fruits collected at Promontory Park, Chilliwack (PRO), while gray markers represent specimens reared from fruits collected at the Abbotsford Community Garden, Abbotsford (ACG). Circular markers represent specimens reared from collections of fresh red elderberry (*Sambucus racemosa* L.; ELD), while triangular markers represent specimens reared from collections of fresh Himalayan blackberry (*Rubus armeniacus* Focke; HIM). Vertical lines in boxes represent the first, second, and third quartiles while whiskers represent the 1.5 inter-quartile range.

Results

Ovipositor length

For specimens reared from fruits collected at the ACG and PRO sites, *L. japonica* had 77.8% longer ovipositors than *G. brasiliensis* (Fig. 4A, $F_{1,55} = 2882.36$, $P < 0.0001$) and wasps, regardless of species, collected from the ACG site had 14.3% longer ovipositors than those collected from the PRO site ($F_{1,55} = 14.24$, $P < 0.0001$). Fruit species was not associated with any differences in ovipositor length ($F_{1,54} = 1.27$, $P = 0.26$). For specimens reared from fruits collected at the AGA site, *L. japonica* had 79.6% longer ovipositors than *G. brasiliensis* ($t_{6.67} = -34.9$, $P < 0.0001$).

Ovipositor width

For specimens reared from fruits collected at the ACG and PRO sites, *L. japonica* had 21.9% wider ovipositors than *G. brasiliensis* (Fig. 4B, $F_{1,56} = 61.65$, $P < 0.0001$). Fruit species and collection site were not associated with any differences in ovipositor width

($F_{1,54}$ = 0.65, P = 0.42 & $F_{1,54}$ = 0.34, P = 0.56, respectively). For specimens reared from fruits collected at the AGA site, *L. japonica* had 16.9% wider ovipositors than *G. brasiliensis* ($t_{5.81}$ = -2.55, P = 0.0447).

Ovipositor stoutness

For specimens reared from fruits collected at the ACG and PRO sites, *G. brasiliensis* had 58.2% stouter ovipositors than *L. japonica* (Fig. 4C, $F_{1,56}$ = 341.7, P < 0.0001). Fruit species and collection site were not associated with any differences in ovipositor stoutness ($F_{1,54}$ = 2.35, P = 0.13 & $F_{1,54}$ = 1.61, P = 0.21, respectively). For specimens reared from fruits collected at the AGA site, *G. brasiliensis* had 65.3% stouter ovipositors than *L. japonica* ($t_{4.42}$ = 7.76, P < 0.0001).

Tibia length

Ovipositor length was positively correlated with tibia length (Fig. 5) for both *L. japonica* ($F_{1,13}$ = 145.5, P < 0.0001) and *G. brasiliensis* ($F_{1,12}$ = 10.9, P < 0.007).

Discussion

We demonstrated that ovipositor length, width, and stoutness differed between *L. japonica* and *G. brasiliensis* in southwestern BC. Our findings are consistent with our predictions that *G. brasiliensis* have significantly shorter and stouter ovipositors than do *L. japonica*. Additionally, our findings align with those of Fellin et al. (2023) as *G. brasiliensis* in both studies had shorter ovipositors than *L. japonica*.

The absolute difference in ovipositor lengths between the *G. brasiliensis* and *L. japonica* is most relevant for determining how far their ovipositors could penetrate into substrates harboring their shared hosts, regardless of the intraspecific relationship between hind tibia length and ovipositor length. Our findings align with those in the California olive biocontrol system (Sime et al. 2007; Wang et al. 2008, 2009); in both systems multiple parasitoids attack hosts in the same developmental substrate (Giorgini et al. 2019; Wang et al. 2021) and those parasitoids with broader host ranges have longer ovipositors than do those parasitoids with more restricted host ranges that have shorter ovipositors.

The intraspecific variation in *G. brasiliensis* and *L. japonica* ovipositor characteristics is associated with variation in intraspecific body size. This may be driven by environmental factors such as fruit characteristics or rearing temperature. Although fruit type did not appear to influence ovipositor characteristics in our study, fruit characteristics (size, weight, depth, ripeness, etc.) may influence host size (Poças et al. 2022) and host size can affect parasitoid size (Nicol and Mackauer 1999; Harvey et al. 2004). Additionally, temperature variation can cause size variation in insects (Atkinson 1994) and thus could be a source of variation in our results.

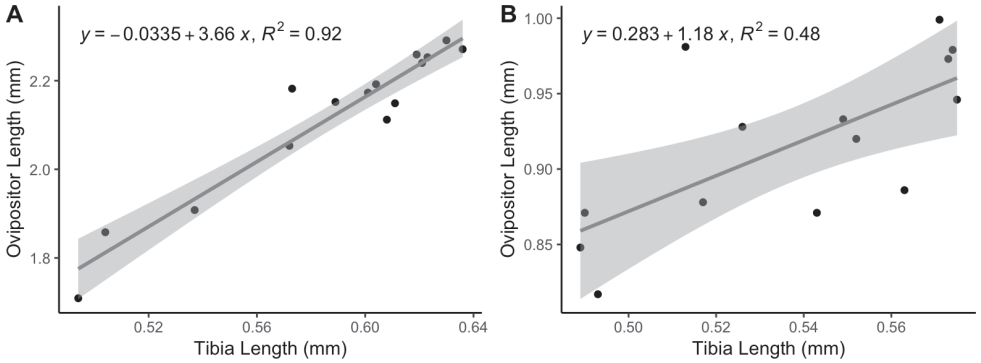

Figure 5. Relationship between ovipositor length and tibia length for a random subset of **A** *L. japonica* (N = 15) and **B** *G. brasiliensis* (N = 14). Trendlines represent the linear regression and shading represents the 95% confidence interval.

The interspecific variation in ovipositor length that we quantified here may be associated with different host searching behaviours (e.g. Compton et al. 2009). The oviposition behaviour of a subset of *Leptopilina* and *Ganaspis* species were described by Vet and Bakker (1985); they showed that the *Leptopilina* species in their study search for hosts using a "walk and probe" method where they walk over likely host substrate while constantly probing it with their ovipositors and rarely stopping. Conversely, *Ganaspis* species were shown to search for hosts using a "walk then probe" method where they take prolonged walks then stop and stand still for extended time periods before probing (Vet and Bakker 1985). The apparently more methodical host searching technique used by the *Ganaspis* species, when compared to the *Leptopilina* species described by Vet and Bakker (1985) may be related to their ability to sense hosts, likely through host movement, within the substrate. Vet and van Alphen (1985) assessed the importance of vibrotaxis (host vibrational cues), ovipositor searching, and antennal searching in a suite of *Leptopilina* and *Ganaspis* species and found that the *Ganaspis* investigated relied entirely on vibrotaxis. Conversely, the *Leptopilina* species investigated by Vet and van Alphen relied primarily on ovipositor searching, while vibrotaxis and antennal searching were used to limited and varying degrees depending on the *Leptopilina* species. Assuming that, like its congeners (Vet and van Alphen 1985), *G. brasiliensis* relies on vibrotaxis to locate high quality (i.e. unparasitized, early instar) hosts in their oviposition substrate, we speculate that *G. brasiliensis* is less likely to find hosts deeper in their oviposition substrate, so a longer ovipositor may have less utility. Future work should assess the importance of vibrotaxis, ovipositor searching, and antennal searching for *L. japonica* and *G. brasiliensis* while searching for *D. suzukii* larvae.

Leptopilina japonica larvae generally outcompete *G. brasiliensis* in *D. suzukii* hosts that have been parasitized by both parasitoids within 24 hours (Wang et al. 2019). Because of this, *G. brasiliensis* may find competitor free space by attacking *D. suzukii* larvae that are present in less ripe fruits. A stouter ovipositor may, for example, facilitate oviposition by *G. brasiliensis* through tougher fruit skins of undamaged fruits as a

stouter ovipositor could support puncturing fruit with a higher required penetration force without the ovipositor buckling (Vincent and King 1995; Quicke et al. 1999; Cerkvenik et al. 2017) although Figitids searching for hosts in damaged fruits are known to wander inside of fruits through holes made by other insects (e.g. Guimarães and Zucchi 2004). Abram et al. (2022a) found that parasitism by the parasitoids in southwestern BC typically lagged behind *D. suzukii* presence in fruits by approximately two weeks, and that *L. japonica* appeared earlier in the ripening season in collections of *R. armeniacus*, while *G. brasiliensis* appeared earlier in the ripening season in collections of *S. racemosa*. However, Abram et al. (2022a) did not account for variation in fruit ripeness within samples so their dataset is likely inadequate to describe the effect of fruit ripeness on parasitism by either species. In nature, *D. suzukii* oviposit into and develop from wild fruits of differing size, depth, and ripeness, which can be present simultaneously (e.g. Ulmer et al. 2022).

The relationship between fruit characteristics and the relative abundance of larval parasitoids of *D. suzukii* has not yet been quantified. The apparent affinity of *L. japonica* for larger fruits and *G. brasiliensis* for smaller fruits observed in fruit collections from both China (Giorgini et al. 2019) and BC (Abram et al. 2022a) invites further research. In the laboratory, *D. suzukii* larvae spend more time diving in fruits than do *D. melanogaster* Meigen larvae (Kim et al. 2017), which could be a behavioural response by *D. suzukii* to pressures imposed by the specialist *G. brasiliensis* and could present an open niche for *L. japonica* to exploit with its longer ovipositor. Future research should investigate how *L. japonica* and *G. brasiliensis* search for host larvae in fruits, the depths at which they parasitize host larvae, and the relationship between parasitoid ovipositor characteristics and fruit characteristics like fruit diameter, depth, and skin thickness. Future work should also focus on the parasitoid communities within *D. suzukii* infested fruits throughout the fruit's phenology (from ripening through decomposition) as *D. suzukii* infestation opens novel niches for other frugivorous insect species (e.g. Chamberlain et al. 2020) and by extension their associated parasitoids.

The narrow host range of *G. brasiliensis* has led to its recent approval for field release in the United States of America (Beers et al. 2022). *Leptopilina japonica*, however, has not yet been approved for release in the USA due to its broader host range. This presents an interesting opportunity to identify the realized niche of *G. brasiliensis* in the presence of the superior intrinsic competitor *L. japonica* where both species co-occur compared to the realized niche of *G. brasiliensis* where it is released for biological control. In its introduced range, the poorer intrinsic competitor (s. str. Harvey et al. 2013; Ode et al. 2022) will not be subject to the competitive pressures of *L. japonica*. This competitive release could impact the parasitoid's realized niche wherein *G. brasiliensis* may be better able to make use of its fundamental niche in North America and may result in differing ovipositor characteristics between populations that are and are not in direct competition with *L. japonica*.

The clear differences in ovipositor length, width, and stoutness between *G. brasiliensis* and *L. japonica* demonstrated in our study, and the apparent use of

semiochemical cues for competitive avoidance by *G. brasiliensis* but not *L. japonica* (Böttinger et al. 2019; Wang et al. 2019) offers an interesting opportunity to investigate the evolutionary drivers of these biological and ecological differences. Such insights are likely important for our understanding of the usefulness of *G. brasiliensis* and *L. japonica* for the biological control of *D. suzukii* globally.

Acknowledgements

We thank T. Hueppelsheuser, M. Franklin, J. Sherwood, E. Grove, and P. Eraso for their efforts in fruit collection and insect rearing. We thank D. Iritani and Y. Watanabe for their help with microscopy and C.-H. Lue and M. Buffington for their wisdom regarding ovipositor measurements. Finally, we thank C. Cock, T. Nelson, and J. Sherwood for their technical support, D. Ensing for his support with model selection, and W. Wong for his participation in brainstorming ovipositor questions and future research. This research (funding to P.K.A., C.E.M., N.G.E.) is part of Organic Science Cluster 3, led by the Organic Federation of Canada in collaboration with the Organic Agriculture Centre of Canada at Dalhousie University, supported by Agriculture and Agri-Food Canada's Canadian Agricultural Partnership-AgriScience Program. P.K.A. and C.E.M. were also supported by funding from Agriculture and Agri-Food Canada, A-BASE #2955. N.G.E. was additionally supported by Graduate Student Fellowships from the University of British Columbia-Okanagan.

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Supplementary material 1

Raw data from figitid collections and ovipositor and tibia measurements

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Data type: table (excel file)

Link: https://doi.org/10.3897/jhr.95.89678.suppl1

JHR 95: 31–44 (2023)
doi: 10.3897/jhr.95.97654
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

The genome of the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera, Scelionidae), a model organism and biocontrol agent of stink bugs

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Academic editor: Elijah Talamas | Received 18 November 2022 | Accepted 12 January 2023 | Published 17 February 2023

https://zoobank.org/D4BCA9D4-A91B-4965-A7A0-8034808639C4

Citation: Lahey Z, Chen H, Dowton M, Austin AD, Johnson NF (2023) The genome of the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera, Scelionidae), a model organism and biocontrol agent of stink bugs. Journal of Hymenoptera Research 95: 31–44. https://doi.org/10.3897/jhr.95.97654

Abstract

Trissolcus basalis (Wollaston) is a minute parasitic wasp that develops in the eggs of stink bugs. Over the past 30 years, *Tr. basalis* has become a model organism for studying host finding, patch defense behavior, and chemical ecology. As an entry point to better understand the molecular basis of these factors, in addition to filling a critical gap in the genomic resources available for parasitic Hymenoptera, we sequenced and assembled the genome of *Tr. basalis* using short (454, Illumina) and long read (Oxford Nanopore) sequencing technologies. The three sequencing methods produced 32 million reads (4.10 Gb; 27.9×), which were assembled into 7,586 scaffolds. The 147 Mb (N50: 42.8 kb) assembly contains complete sequences for 93.1% of the insect BUSCO dataset, and an extensive annotation protocol resulted in 14,158 protein-coding gene models, 12,197 (86%) of which had a blast hit in GenBank. Repetitive elements comprised 13.8% of the genome, and a phylogenomic analysis recovered *Tr. basalis* as sister to Chalcidoidea, a result in line with other studies. We identified 174 rapidly evolving gene families in *Tr. basalis*,

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including olfactory receptors and pheromone/general odorant binding proteins. These genetic elements are an obligatory portion of the parasitoid-host relationship, and the draft genome of *Tr. basalis* has and will continue to be useful in elucidating these relationships at finer resolution.

Keywords
assembly, biological control, insect genomics, nanopore, Telenominae

Introduction

Trissolcus basalis (Wollaston) (Hymenoptera: Scelionidae) is a minute, solitary parasitoid of stink bug eggs (Hemiptera: Pentatomoidea), principally the cosmopolitan pest *Nezara viridula* (L.) (Pentatomidae). This parasitoid is found primarily in tropical and subtropical regions, where it has been used effectively in the biological control of its host (Davis 1964; Clarke 1990; Corrêa-Ferreira and Moscardi 1996). Given the economic importance of its host, considerable effort has been expended to elucidate how female *Tr. basalis* locate *N. viridula* eggs in the narrow window of time during which they are susceptible to attack (Bin et al. 1993; Colazza et al. 1999, 2004; Salerno et al. 2006; Laumann et al. 2009). Host location and acceptance by female wasps are known to be mediated by chemical cues, some of which have been isolated and identified (Mattiacci 1993; Colazza et al. 2004, 2007). This effort to sequence the genome of *Tr. basalis* was undertaken as a step in characterizing its repertoire of chemoreceptor proteins (Chen et al. 2021a) and to better understand the mechanisms of host finding in platygastroid wasps and their evolutionary consequences.

Methods

Whole-genome sequencing

454 Life sciences

Sequencing followed the protocol of Mao et al. (2012). Briefly, DNA was extracted from 25 adult male *Tr. basalis* from a colony maintained at the Università di Perugia (Perugia, Italy). Sequencing was conducted at the University of Pennsylvania Perelman School of Medicine on a Roche/454 GS FLX sequencer using Titanium chemistry, which generated 5,080,113 reads (1,535,920,544 bp).

Illumina

To correct homopolymer errors in the 454 reads, an Illumina sequencing library was prepared from five female *Tr. basalis* in the same culture. The DNA extract was prepared for Illumina sequencing using a Nextera DNA Sample Preparation Kit (Epicen-

tre Biotechnologies, Madison, Wisconsin, USA). Sequencing was conducted on an Illumina Genome Analyzer IIx (Illumina, San Diego, California, USA) at the Nucleic Acid Shared Resource (College of Medicine, The Ohio State University, Columbus, Ohio, USA). In total, 29,780,645 51-bp reads (1,518,812,895 bp) were generated.

Oxford nanopore

High molecular weight DNA was extracted from approximately 100 unsexed *Tr. basalis* using a Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA quality was estimated using an Agilent Bioanalyzer. The DNA library was prepared using a Ligation Sequencing Kit 1D. Sequencing was performed on a R9.5 flow cell using an Oxford Nanopore MinION (Oxford Nanopore, Oxford, United Kingdom). The 48-hour MinION sequencing run generated 341,751 reads (1,047,061,835 bp). All steps, excluding DNA extraction, were conducted at The Molecular and Cellular Imaging Center (MCIC; The Ohio State University, Wooster, Ohio, USA).

Processing of sequencing reads

Pyrosequencing reads are particularly susceptible to the accumulation of homopolymer errors (Huse et al. 2007). These were corrected using HECTOR (version 1.0.0; Wirawan et al. 2014). Adapter sequences were removed from nanopore reads with Porechop (version 0.2.3; https://github.com/rrwick/Porechop) and reads with internal adapter sequences were split into two reads.

Genome assembly

The *Tr. basalis* genome was assembled following a hybrid approach that utilized short (454, Illumina) and long read (Oxford Nanopore) sequencing technologies. 454 and nanopore reads were assembled with SPAdes (version 3.11.1; Bankevich et al. 2012), with the initial assembly (assembled in 2010 by NFJ) treated as 'trusted contigs' and the '-careful' flag turned on to minimize misassemblies. The assembly was polished with single-end 51 bp Illumina reads for 4 iterations using Pilon (version 1.22; Walker et al. 2014). The polished assembly was then scaffolded with RNA-seq reads from pooled tissues of male and female *Tr. basalis* using rascaf (version 20161129; Song et al. 2016; Chen et al. 2021a) to produce the final assembly.

Assembly statistics and quality

Genome statistics were calculated with QUAST (version 4.5; Gurevich et al. 2013). Genome assembly completeness was assessed with BUSCO (version 4.0.6; Simão et al. 2015) using the Metazoa, Arthropoda, Insecta, Endopterygota, and Hymenoptera datasets.

Genome annotation

The *Tr. basalis* genome was annotated following the protocol of Daren Card (Department of Organismic & Evolutionary Biology, Harvard University), with modifications (https://gist.github.com/zjlahey/3c400c3039eef674e335d3d850ad595f).

Repetitive elements

Repetitive elements were identified and annotated with RepeatModeler (version open-2.0.1; Flynn et al. 2020) and RepeatMasker (version 4.1.0; Smit et al. 2014). First, a custom repeat library was generated for *Tr. basalis* using RepeatModeler. This repeat library was then combined with a curated arthropod repeat library from RepBase (Bao et al. 2015), which was used to mask complex repetitive elements in the *Tr. basalis* genome using RepeatMasker.

Protein-coding genes

Protein-coding genes were annotated in an iterative fashion with MAKER (version 3.01.03; Campbell et al. 2014). MAKER utilizes external evidence in the form of protein and transcript sequences from other organisms to train *ab-initio* gene prediction software to annotate genes within a genome. In the first iteration, external evidence was supplied to MAKER as (1) TransDecoder-derived coding sequences (CDS) from each *Tr. basalis* transcriptome assembly; (2) CDS from *Telenomus remus* Nixon (Huayan Chen, unpublished data); (3) TransDecoder-derived protein sequences from each *Tr. basalis* transcriptome assembly; (4) all 170 arthropod proteomes in OrthoDBv10.1 (http://www.orthodb.org/); and (5) the UniProtKB/Swiss-Prot protein database (Bateman et al. 2020). Subsequent rounds utilized SNAP (version 2006-07-28; Korf 2004) and Augustus (version 3.3.3; Stanke and Waack 2003) to improve the gene models from the first iteration and identify new genes in the assembly. MAKER was run for three iterations, until the number of gene models and average length of each gene declined. Conserved domains within proteins of the final gene set were identified using InterProScan (version 5.46-81.0; Blum et al. 2020), and conserved functions were determined by performing a BLASTp (version 2.6.0) of the gene set against all metazoan proteins in the Swiss-Prot Uniprot database (Bateman et al. 2020). Finally, COGNATE (version 1.0; Wilbrandt et al. 2017) was employed to generate summary statistics of the annotated protein set (no. of exons/introns, avg. gene length, etc.).

Non-coding RNAs

We followed the protocol on Rfam (https://docs.rfam.org/en/latest/genome-annotation.html) to identify and annotate non-coding RNAs with Infernal (version 1.1.3; Nawrocki and Eddy 2013) and Rfam (version 13.0; Kalvari et al. 2018). Nuclear transfer RNAs were annotated with tRNAscan-SE (version 2.0.6; Lowe and Eddy 1997).

Gene family analysis

Taxon sampling and protein datasets

To estimate gene gains, losses, and rapidly evolving gene families within *Tr. basalis*, we conducted a gene family analysis using the *Tr. basalis* proteome and the protein sequences of six additional hymenopterans. Taxa were chosen based on the availability of hymenopteran proteomes and included three members of Proctotrupomorpha [*Belonocnema kinseyi* Weld (Cynipidae), *Nasonia vitripennis* (Walker) (Pteromalidae), and *Trichogramma pretiosum* Riley (Trichogrammatidae)]; one member of Ichneumonoidea [*Microplitis demolitor* Wilkinson (Braconidae)]; one member of Orussoidea [*Orussus abietinus* (Scopoli) (Orussidae)]; and the turnip sawfly, *Athalia rosae* (L.) (Tenthredinidae). Protein sequences of *A. rosae*, *O. abietinus*, *M. demolitor*, *N. vitripennis*, and *T. pretiosum* were downloaded from OrthoDB v10 (Kriventseva et al. 2019). The *B. kinseyi* proteome (then under the name *B. treatae* (Mayr) (Zhang et al. 2021)) was downloaded from NCBI. Redundant isoforms of multicopy genes in the *B. kinseyi* proteome were removed prior to analysis. Proteomes downloaded from OrthoDB did not require this step.

Gene family identification and clustering

Orthogroup inference was conducted with OrthoFinder (version 2.5.2; Emms and Kelly 2019) at default parameters (DIAMOND, MAFFT, FastTree). Due to computational limitations associated with using IQ-TREE at the tree inference step of OrthoFinder, we performed a separate phylogenetic analysis on the same 4,510 orthologues (SpeciesTreeAlignment.fa) identified during the initial run using IQ-TREE (version 2.1.2; Minh et al. 2020). The final step of OrthoFinder was then rerun with the species tree produced by IQ-TREE as input (orthofinder.py -ft RESULTS_DIR -s IQ-TREE_SPECIES_TREE). We then converted the species tree to a time-calibrated ultrametric tree using the OrthoFinder accessory script make_ultrametric.py, with the root node calibrated at 265 mya based on the estimated divergence time between *Athalia* Leach and *Orussus* Latreille in the TimeTree database (Kumar et al. 2017).

Gene family evolution

Rates of gene gain and loss (λ) were estimated with CAFE (version 4.2.1; Han et al. 2013) using the orthogroup count data and ultrametric time-tree produced by OrthoFinder as input. Prior to running CAFE, we modified the orthogroup count data file by removing gene family clusters where only a single species was present (Prost et al. 2019). This step reduced the number of gene families from 11,205 to 10,190. Finally, we accounted for possible deviation in the number of observed vs true gene family counts by estimating an error model (ε) to optimize the value of λ.

Data availability

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAAMPD000000000. Raw DNA sequencing reads (454, Illumina, Nanopore) are available at the Sequence Read Archive by searching for BioProject Accession PRJNA49235.

Results and discussion

Genome sequencing and assembly statistics

We assembled the genome of *Tr. basalis de novo* using sequence data from second- and third-generation sequencing technologies. The combined read output from all sequencing platforms totaled 4.10 Gb (27.9× coverage). These reads were assembled into 7,568 scaffolds, totaling 147 Mb in length (34.7% GC content). The scaffold N50 was 42.8 kb, and the longest scaffold measured 349,262 kb. Given low read coverage, we were unable to estimate genome size *in silico*. However, the *Tr. basalis* draft assembly size falls within the range of genome size estimates of other platygastroids, which are typically between 200 and 400 Mb (data not shown), in addition to the average genome size range of other hymenopterans. We assessed genome assembly completeness with BUSCO, using the Insecta odbv10 database (N = 1,367) in genome mode and with the 'long' flag enabled to perform a more thorough search. We recovered 93.1% complete, 1.8% duplicated, 1.4% fragmented, and 3.7% missing Insecta BUSCOs in the *Tr. basalis* genome. These values compare favorably with other parasitoid Hymenoptera with more contiguous genome assemblies (Fig. 1).

Genome annotation

Repetitive elements

RepeatMasker annotated 13.8% of the *Tr. basalis* genome as composed of repeats, approximately half of which were unclassified repeats (7.1%). The most abundant classified repetitive elements were various LINE and LTR retroelements (3.0%); DNA transposons (1.3%); and simple repeats (1.5%). The repeat landscape of *Tr. basalis* shows a relatively uniform distribution of repeat classes, with a gradual decline in the proportion of LTR retroelements and an increase in the proportion of DNA transposons (Fig. 1). Subtle increases in the proportion of LINEs and rolling circle transposons are evident between Kimura distances 0.04 and 0.12. SINEs contribute little to the overall repeat content in *Tr. basalis*, and other Hymenoptera, in general (Petersen et al. 2019). A complete list of the repeats found in the *Tr. basalis* genome is in the Suppl. material 1.

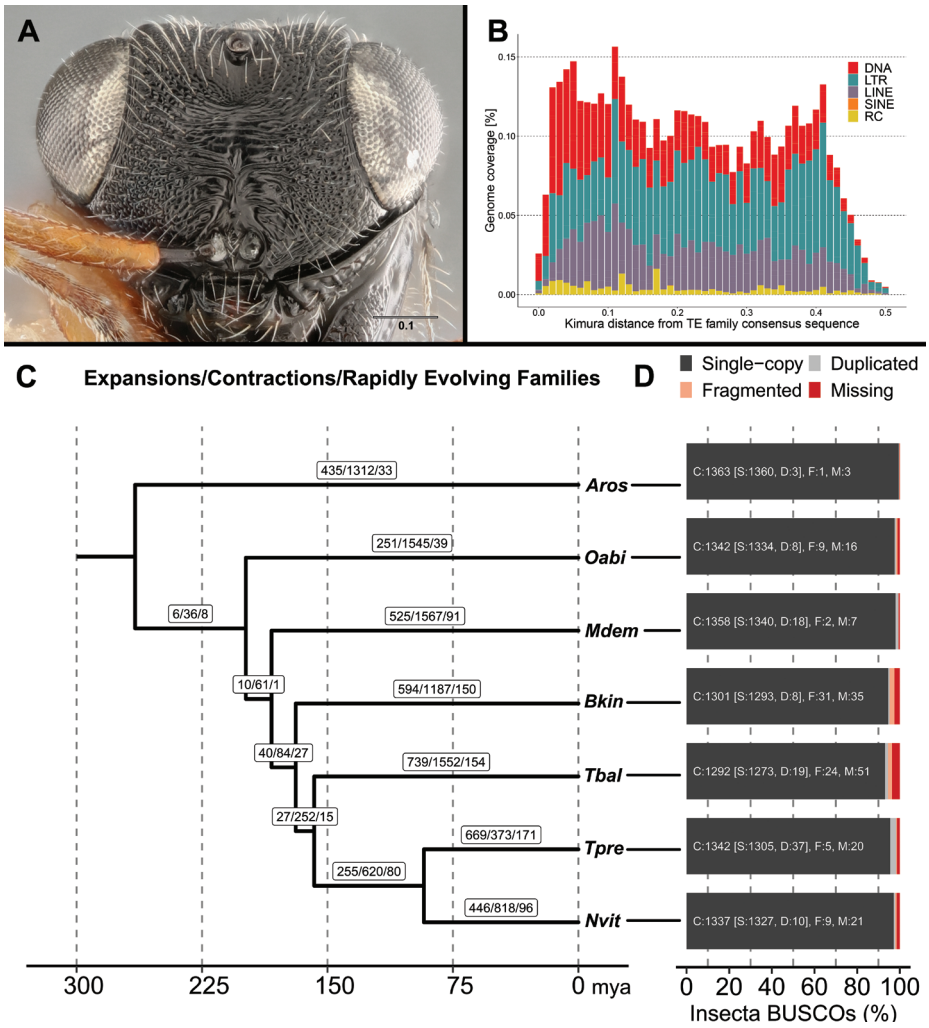

Figure 1. Morphological and genomic traits of *Trissolcus basalis* **A** head of female *Tr. basalis* reared from BMSB eggs in Tuscaloosa, Alabama, USA (FSCA 00090269 **B** repeat landscape plot of different TE classes within the *Tr. basalis* genome. Nucleotide sequence divergence in each TE copy was calculated as the Kimura distance between the annotated TE copies in the genome and the consensus sequence of each TE family **C** ultrametric timetree depicting the position of *Tr. basalis* relative to six other hymenopterans inferred from a phylogenetic analysis of 4,510 single-copy protein-coding genes identified by OrthoFinder. Numbers above branches (left to right, separated by forward slashes) indicate gene family expansions, gene family contractions, and the number of rapidly evolving gene families in each lineage. Each branch received 100% SH-aLRT and UFBoot2 support values **D** genome assembly completeness comparison based on the proportion of BUSCOs recovered in each genome using the Insecta odbv10 dataset (N = 1367). Abbreviations: BMSB, brown marmorated stink bug; C, complete; D, duplicated; DNA, DNA transposon; F, fragmented; LINE, long interspersed nuclear element; LTR, long terminal repeat; M, missing; mya, million years ago; RC, rolling circle transposon; S, single-copy; SINE, short interspersed nuclear element; TE, transposable element; Aros, *Athalia rosae*; Bkin, *Belonocnema kinseyi*; Mdem, *Microplitis demolitor*; Nvit, *Nasonia vitripennis*; Oabi, *Orussus abietinus*; Tbal, *Trissolcus basalis*; Tpre, *Trichogramma pretiosum*.

Protein-coding genes

The MAKER genome annotation pipeline resulted in 14,158 protein-coding gene models. Approximately 95% (13,507) of the 14,158 gene models have an annotation edit distance (AED) score of less than 0.5, and 70% (9,915) contain at least one recognizable InterPro domain. AED is a quality control metric that explains how well the gene annotations produced by MAKER match external evidence (i.e., proteomes from other species). The AED values and proportion of gene annotations with a recognizable InterPro domain for the *Tr. basalis* genome are indicative of a well-annotated assembly (Holt & Yandell, 2011). In addition, nearly half of the protein set (6,929 or 48.9%) was assigned at least one gene ontology (GO) term. To determine how well our annotated protein set compares with external protein databases, we queried our protein annotations against those of the metazoan portion of the Swiss-Prot/UniProt database and all Hymenoptera protein sequences deposited in GenBank (last accessed March 17, 2021). A total of 9,303 (65%) and 12,197 (86%) of the annotated proteins in *Tr. basalis* were supported by a best BLASTp hit in the Swiss-Prot/UniProt database and GenBank, respectively. A table of the most frequently recovered InterPro domains, GO terms, and Pfam entries associated with the *Tr. basalis* protein set is available in the Suppl. material 1.

Non-coding RNAs

Seventy-five different RNA families were annotated in the *Tr. basalis* genome. The top 5 most common families belong to the tRNA (RF00005), Histone3 (RF00032), 5S_rRNA (RF00001), SSU_rRNA_eukarya (RF01960), and LSU_rRNA_eukarya (RF02543) RNA sequence families. We also identified both conserved regions of the *Sphinx* long non-coding RNA gene, which plays a role in the regulation of male mating behavior in the fruit fly *Drosophila melanogaster* Meigen (Wang et al. 2002; Dai et al. 2008). Within Hymenoptera, *Sphinx* has been reported from 15 taxa including three species of parasitoid in the pteromalid genus *Nasonia* Ashmead (Werren et al. 2010). Its role in regulating mating behavior in Hymenoptera is not known. Additional statistics of the ribosomal DNA within the *Tr. basalis* genome are in the Suppl. material 1.

Gene family analyses

We compared the annotated proteome of *Tr. basalis* with those of six other hymenopterans with well-annotated genomes. Orthogroup clustering performed with OrthoFinder assigned 81,474 (93.4%) of the 87,222 protein sequences into 11,205 orthogroups. The number of orthogroups with all species present was 6,295 and 4,510 of these were identified as single-copy orthologues. Regarding *Tr. basalis*, 81.8% (11,582) of its genes were assigned to an orthogroup, and 76.7% (8,599) of orthogroups contained *Tr. basalis*. The number of orthogroups specific to *Tr. basalis* was 173, and the number of genes within these 173 species-specific orthogroups was 1,026 (7.2% of the 11,582

genes assigned to an orthogroup). The number of unassigned genes in *Tr. basalis* was much higher than the taxa with which it was compared. Potential explanations for this discrepancy are (1) the fragmentary nature of the *Tr. basalis* draft assembly leading to truncated protein models and (2) inaccurate gene annotations. Increasing genome contiguity using additional long-read sequencing technologies and chromosome confirmation capture would decrease the incidence of truncated protein models, and manual curation of the gene models would aid in the identification of false positives.

The orthogroup count data and ultrametric timetree produced by OrthoFinder were used to estimate the rate of gene family evolution with CAFE. We estimated the rate of gene family evolution (gains and losses) in this group of Hymenoptera at 0.0008, after accounting for possible genome assembly/annotation error. This result is in line with a recent multi-order gene family analysis that reported the rate of gene family gain and loss in 24 hymenopteran taxa at 0.0009 (Thomas et al. 2020), a gene turnover rate slower than Coleoptera (0.001), Diptera (0.001), and Lepidoptera (0.0014). In total, 638 gene families were identified as rapidly evolving among the 7 hymenopterans included in this study.

We identified 174 (99 expansions and 55 contractions) rapidly evolving gene families in *Tr. basalis*, with most (91) rapidly expanding families containing at least one member with an InterPro, PANTHER, or Pfam annotation (Suppl. material 1), and slightly fewer than half with at least one corresponding GO term (48). Notable examples of gene families undergoing rapid evolution in *Tr. basalis* are three groups of olfactory receptors (contracting in OG0000089; expanding in OG0000163 and OG0000567), one group of 7-transmembrane chemoreceptors (expanding, OG0000365), and one group of pheromone/general odorant binding proteins (expanding, OG0009810). The chemoreceptor repertoire of *Tr. basalis* was recently treated by Chen et al. (2021a) who employed sex- and tissue-specific transcriptome assemblies, in addition to the *Tr. basalis* genome, to annotate its gustatory, olfactory, and ionotropic receptor genes. One family of proteins not treated by Chen et al. (2021a), yet integral in the recognition and delivery of odorant molecules to their respective odorant receptors, are the odorant binding proteins (OBPs) (Pelosi and Maida 1995). OBPs are small, water-miscible polypeptides that solubilize and deliver volatile, hydrophobic compounds to the membrane of chemosensory receptor neurons for further processing (Pelosi et al. 2018). Therefore, OBPs are the first component in a multistep process that begins with semiochemical binding and culminates in a behavioral response. We are only beginning to investigate the OBP repertoire in *Tr. basalis*; however, given the quality of the *Tr. basalis* draft genome, we have identified, annotated, and characterized 18 putative OBPs, and determined those that exhibit antennal-biased expression patterns (King et al. 2021).

Author's note

While this manuscript was in preparation, Xu et al. (2021) published a highly contiguous, chromosome-scale genome assembly of *Te. remus* (Platygastroidea: Scelionidae), a telenomine egg parasitoid of the fall armyworm *Spodoptera frugiperda* (J. E. Smith)

(Lepidoptera: Noctuidae). *Telenomus* Haliday occupies an important phylogenetic position within the family Scelionidae as the sister taxon to *Trissolcus* Ashmead (Taekul et al. 2014; Chen et al. 2021b), and thus serves as an ideal candidate with which to compare the *Tr. basalis* genome assembly reported here. A preliminary investigation into some commonly reported genome metrics corroborates several features that may be characteristic of telenomine genomes: (1) small genome size (< 150 Mb); (2) low repetitive element content; (3) approximately 15,000 protein-coding genes; and (4) similar rates of gene family evolution (Xu et al. 2021). These differences were discernible between the two genomes despite the stark contrast in assembly contiguity (i.e., 11.9 Mb scaffold N50 for *Te. remus*; 42.8 kb scaffold N50 for *Tr. basalis*). This suggests that even highly fragmented genome assemblies can be of sufficient quality to infer genome-scale parameters accurately. We anticipate comparative genomic analyses between *Te. remus* and *Tr. basalis* will result in major discoveries related to genome evolution within Hymenoptera and the genomic factors implicated in host location, host acceptance, and the biological control potential of both species.

Acknowledgements

We thank Dr. Malte Petersen (Max Planck Institute of Immunobiology and Epigenetics) for sharing the R script used to generate the repeat landscape plot. Thanks to Dr. Jason Mottern (USDA-APHIS) and an anonymous reviewer for their careful and thoughtful review of the manuscript. This material is based upon work supported in part by the National Science Foundation under grant No. DEB-0614764 to N.F. Johnson and A.D. Austin and by funding from The Ohio State University, and the National Natural Science Foundation of China (31900346) to Huayan Chen.

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Supplementary material 1

Genome of the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera, Scelionidae), a model organism and biocontrol agent of stink bugs

Author: Zachary Lahey

Data type: genomic (excel document)

Explanation note: Bioinformatic data associated with the annotated *Trissolcus basalis* genome assembly.

Link: https://doi.org/10.3897/jhr.95.97654.suppl11

JHR 95: 45–58 (2023)
doi: 10.3897/jhr.95.97527
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

A new species and two new records of the genus *Alysia* Latreille (Hymenoptera, Braconidae, Alysiinae) from South Korea

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Academic editor: J. Fernandez-Triana | Received 14 November 2022 | Accepted 13 January 2023 | Published 17 February 2023

https://zoobank.org/CF9F06EC-165D-428F-9192-95F0C5FC2926

Citation: Sohn J-H, van Achterberg C, Kim H (2023) A new species and two new records of the genus *Alysia* Latreille (Hymenoptera, Braconidae, Alysiinae) from South Korea. Journal of Hymenoptera Research 95: 45–58. https://doi.org/10.3897/jhr.95.97527

Abstract

In the genus *Alysia* Latreille, 1804 (Braconidae: Alysiinae), a new species, *Alysia erecta* **sp. nov.**, and two new records, *Alysia hebeiensis* Zhu, van Achterberg & Chen, 2018 and *A. sirin* Belokobylskij, 1998, are described and illustrated. In addition, the DNA barcode region of the mitochondrial *subunit I* (*COI*) of these species have been sequenced. An identification key for all *Alysia* species officially recorded from Korea is provided.

Keywords

Alysiini, COI, Hymenoptera, new combination, new record, new species, taxonomy

Introduction

The subfamily Alysiinae is a large taxon of the family Braconidae, consisting of over 2,440 valid species worldwide (Yu et al. 2016). Among them, 180 species in 21 genera are recorded in Korea (NIBR 2021). Alysiinae is generally distinguished from other subfamilies morphologically by having the exodont (= non-overlapping in closed condition) mandibles. Alysiinae includes two tribes, Alysiini and Dacnusini, which

are distinguishable each other in most cases by having vein r-m of fore wing present (Alysiini) or absent (Dacnusini). Alysiinae belongs to the cyclostome clade, of which members are koinobiont endoparasitoids for dipterous larvae. They use the outward-curved teeth of the exodont mandibles to break out the host puparium (Docavo et al. 2002). Some species have been used for biological control (Ozawa et al. 2001; Chabert et al. 2012).

The genus *Alysia* Latreille, 1804, is a large group in the subfamily Alysiinae, including 125 species worldwide (Yu et al. 2016; Zhu et al. 2018). This genus can be diagnosed by having the first flagellomere longer than the second flagellomere (but not over 1.5 times), the comparatively short vein 3-SR of fore wing, the posterior position of vein r of fore wing, the propodeum more or less wrinkled or rugose and usually lacking an areola and vein m-cu of the hind wing distinct. According to Bartlett et al. (1978), *Alysia manducator* has been introduced to control the sheep blowfly, *Calliphora stygia* (Fabricius, 1781) in eastern Australia and New Zealand, but became established only in the latter country.

Comparatively, few papers are dealing with the eastern Palaearctic species: Two new species from Mongolia by Papp (1991); 14 new species from Far East Russia by Belokobylskij (1998); six new species from China by Zhu et al. (2018). Since seven species had been known in Korea, one species was recently transferred to the genus *Cratospila* (Sohn et al. 2022). Although Papp (1994) has been reported *A. brachycera*, *A. lucia*, *A. nigritarsis*, *A. sophia*, *A. tipulae* and *A. truncator* from Ryanggang, North Korea, the national checklist of South Korea (NIBR 2021) lists only two species (*A. sophia* and *A. tipulae*). Therefore, five species officially are recorded in South Korea from this study.

In this study, we present new morphological characters and the *COI* barcoding sequences of one new and two newly recorded species (*A. hebeiensis* and *A. sirin*). This study also provides descriptions, diagnosis, identification key and photographs of the diagnostic characters for the three species.

Materials and methods

Samples used in this study were collected with Malaise traps in South Korea at the Dodae-ri, buk-myeon, Gapyeong-gun, Gyeonggi-do. Sorting and preparation were done at the Animal Systematics Lab. (ASL), Department of Biological Science, Kunsan National University (KSNU). For morphological identification, Wharton et al. (1997) and Zhu et al. (2017) were used. Morphological characters were observed with a Leica M205C stereo microscope. The Taxapad database (Yu et al. 2016) was used for references. The terminology was followed of Wharton (2002) and van Achterberg (1993). The holotype of new species is deposited in the NIBR (National Institute of Biological Resources, Incheon) collection.

A Leica DMC2900 digital camera and a Leica M205 C microscope (Leica Geosystems AG, Mannheim, Germany) were used for photography; several pictures

were taken for each final photo using multi-focusing technology. LAS V4.11 (Leica Geosystems AG, St. Gallen, Switzerland) and HeliconFocus 7 (Helicon Soft, Kharkiv, Ukraine) software were used for stacking the photos. The final illustrations were created using Adobe Photoshop CS6.

Extraction of DNA was done in ASL, KSNU. Whole genomic DNA was extracted from the specimens by using a Labopass Tissue kit (COSMOgenetech, Daejeon, Korea) following the manufacturer's protocol. In order to conserve morphologically complete voucher specimens, DNA extraction method was used slightly modified from 'non-destructive method' by Favret (2005) and 'freezing method' by Yaakop et al. (2009). In the original protocol, the sample was crushed or wounded, and then soaked with 180 µl of buffer ATL + 20 µl of proteinase, following by three hours over incubation at 55 °C. In the slightly modified DNA extraction methods, samples were soaked with 180 µl of buffer ATL + 20 µl of proteinase K without destroying the sample, followed by 10 minutes incubation at 55 °C and then kept in a freezer at -22 °C overnight. After that the general protocol was used for the remaining steps. The primer set of LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') was used to amplify approximately 658 bp as the partial front region of the *COI*. The polymerase chain reaction (PCR) products were amplified by using AccuPowerH PCR PreMix (BIONEER, Corp., Daejeon, Korea) in 20 µl reaction mixtures containing 0.4 µM of each primer, 20 µM of the dNTPs, 20 µM of the $MgCl_2$, and 0.05 µg of the genomic DNA template. PCR amplification was performed using a GS1 thermo-cycler (Gene Technologies, Ltd., Somerset, U.K) according to the following procedure: initial denaturation at 95 °C for 5 min, followed by 34 cycles at 94 °C for 35 sec; an annealing temperature of 48 °C for 25 sec; an extension at 72 °C for 45 sec, and a final extension at 72 °C for 5 min. The PCR products were visualized by electrophoresis on a 1.5% agarose gel. A single band was observed and then sequenced using an automated sequencer (ABI Prism 3730 XL DNA Analyzer, California, USA) at Macrogen Inc. (Seoul, South Korea).

Sequence alignment was performed in MEGA version 7 (Kumar et al. 2016) with ClustalW method. To estimate the pairwise genetic distances, the *P*-distance model was conducted using MEGA version 7.

Results and discussion

A total of 621 bp of the *COI* barcode region were sequenced for *Alysia erecta* sp. nov. (GenBank accession no. OP391515), *A. hebeiensis* Zhu & van Achterberg, 2018 (GenBank accession no. OP391514), and *A. sirin* Belokobylskij, 1998 (GenBank accession no. OP391516) Pairwise genetic distances were calculated by using '*P*-distance' model with option for pairwise deletion; *A. erecta* differed by 6% from *A. hebeiensis* and by 9% from *A. sirin*; *A. hebeiensis* 10% from *A. sirin*.

Taxonomy

Alysia Latreille, 1804

Figs 1–3

Alysia Latreille, 1804: 173–174; Shenefelt 1974: 939; Wharton 1980: 458; Chen and Wu 1994: 28; Belokobylskij 1998: 170; Zhu et al. 2018: 2. Type species: *Ichneumon manducator* Panzer, 1799.

Cechenus Illiger, 1807: 54; Type species: *Ichneumon manducator* Panzer, 1799. Synonymized by Curtis 1826.

Bassus Nees, 1812: 201; Type species: *Ichneumon manducator* Panzer, 1799. Synonymized by Nees 1819.

Anarcha Foerster, 1863: 265; Ashmead 1900: 105; Baltazar 1962: 759. Type species: *Anaraha notabilis* Foerster, 1863. Synonymized by Fischer 1971.

Goniarcha Foerster, 1863: 265; Marshall 1872: 125; Ashmead 1900: 105. Type species: *Alysia lucicola* Haliday, 1838. Synonymized by Marshall 1894.

Strophaea Foerster, 1863: 265; Marshall 1872: 127; Ashmead 1900: 105. Type species: *Alysia rufidens* Nees, 1834. Synonymized by Marshall 1894.

Diagnosis. First flagellomere longer than second (Figs 1B, J, 2B, J, 3B, J), not over 1.6 times, face granulate (Figs 1E, 2E, 3E) or largely smooth, eye slightly oval, clypeus triangularly shaped, wide and protruding anteriorly (Figs 1E, 2E, 3E); mandible (Figs 1K, L, 2K, L, 3K, L) with 3 teeth, third mostly lobe-shaped. second tooth narrow and sharp; pronope absent, notauli present, precoxal sulcus distinct, medially deeply impressed (Figs 1G, 2G, 3G), scutellar sulcus distinct, propodeum more or less rugose and usually without areola, sometimes with enlarged spiracles; pterostigma robust, fore wing (Figs 1C, 2C, 3C) vein 2-SR slightly bent, first discal cell shorter than wide in median length. vein 3-SR usually shorter than vein 2-SR; veins 2-SR+M and r-m not sclerotized, hind wing vein 1r-m shorter than vein M+CU, vein m-cu distinct; first tergite with dorsope (Figs 1H, 2H, 3H).

Biology. Endoparasitoids of larval Calliphoridae, Sarcophagidae, Tephritidae, Anthomyiidae, Agromyzidae and Mycetophylidae (Yu et al. 2016).

Distribution. Cosmopolitan, but most *Alysia* species occur in the northern part of the Northern Hemisphere and many are Holarctic. About 70% of the species have most or all of their range within the boreal coniferous biome (Wharton 1986).

Key to species of *Alysia* Latreille from South Korea

1 Antenna with 5–10 white segments subapically (Fig. 3B); first metasomal tergite 2.4–3.0 times longer than its apical width (Fig. 3H) ..... ***A. sirin* Belokobylskij, 1998**

– Antenna without white segments (Fig. 2B); first tergite 1.0–1.7 times longer than its apical width (Fig. 2H) **2**

2 First flagellomere about 1.5 times longer than second; setose part of ovipositor sheath 1.6–1.7 times longer than hind tibia; eye in dorsal view 1.1–1.2 times as long as temple.....***A. tipulae* (Scopoli, 1763)**

– First flagellomere 1.2–1.3 times longer than second; setose part of ovipositor sheath 0.5–1.3 times as long as hind tibia; eye in dorsal view 1.2–1.4 times as long as temple.....**3**

3 Setose part of ovipositor sheath 1.2 times longer than hind tibia (Fig. 2A); hind femur 4.4–4.6 times longer than its maximum width .... ***A. hebeiensis* Zhu, van Achterberg & Chen, 2018**

– Setose part of ovipositor sheath 0.5–0.7 times as long as hind tibia; hind femur 3.9–4.0 times longer than its maximum width.....**4**

4 First antennal flagellomere about 2.5 times longer than wide (Fig. 2J); mandible 1.2 times longer than its maximum width (Fig. 1K); pterostigma dark brown; metasoma after first tergite dark brown (Fig. 1A) ....***A. erecta* sp. nov.**

– First flagellomere about 3.5 times longer than wide; mandible 1.6–1.7 times longer than its maximum width; pterostigma pale yellowish brown to brown; metasoma after first tergite usually yellow or orange..... .....***A. sophia* (Haliday, 1838)**

***Alysia erecta* Sohn & van Achterberg, sp. nov.**
https://zoobank.org/78EBA32D-8A2D-4D4E-8C95-904A7289EA87
Fig. 1A–L

Type material. ***Holotype***, ♀ (NIBR), **South Korea**, Dodae-ri, buk-myeon, Gapyeong-gun, Gyeonggi-do, 37°56'11.8"N, 127°28'50.2"E, 05.IV.2018, Sohn. GenBank accession no. OP391515.

Comparative diagnosis. The new species is recognizable by its comparatively short ovipositor sheath (setose part 0.6 times longer than mesosoma *versus* 1.0–1.7 times in other S Korean species), the short first flagellomere (2.5 times longer than wide versus 3.0–4.5 times) and robust mandible (1.2 times longer than wide *versus* 1.4–1.7 times). The new species runs in the key by Zhu et al. (2018) to *A. hebeiensis* Zhu & van Achterberg, 2018 and differs from this species by having the first flagellomere less slender (2.5 times *versus* 3.8–4.0 times in *A. hebeiensis*) and the first metasomal tergite shorter (1.1 times longer than its apical length *versus* 1.2–1.7 times).

The new species runs in the key by Belokobylskij (1998) to *A. masneri* Wharton, 1988 based on the colour of the clypeus, but to *A. vladik* Belokobylskij, 1998 if only morphological characters are used. The new species differs from *A. masneri* by the robust first tergite (1.1 times longer than its apical width *versus* 1.6 times in *A. masneri*), the shorter antenna (0.9 times as long as body *versus* distinctly longer than body) and the shorter ovipositor sheath (0.6 times as long as mesosoma *versus* 0.9 times). The new species differs from *A. vladik* by having the first flagellomere less slender (2.5 times versus 4.0 times in *A. vladik*), the hind femur less slender (3.9 times *versus* 4.3 times), the first

metasomal tergite shorter (1.1 times longer than its apical width *versus* 1.2–1.3 times), the clypeus black (similar to colour of face *versus* yellowish, contrasting with black face).

According to the key by Wharton (1986) the new species belongs to his *Alysia tipulae* group of the subgenus *Anarcha* Foerster. It runs in the key by Wharton (1988) to *A. umbrata* Stelfox, 1941 and differs by having the mandible less slender (1.2 times versus 1.6–1.7 times in *A. umbrata*).

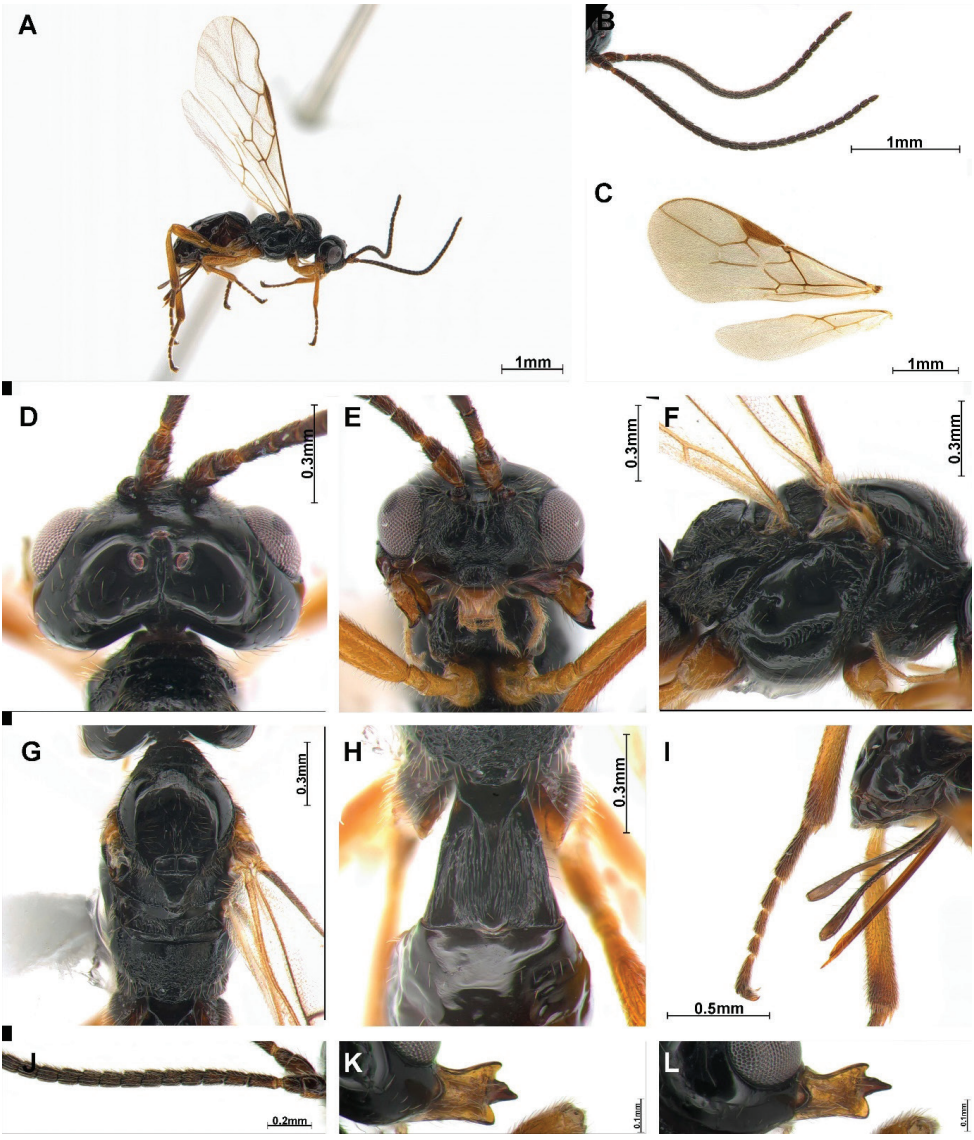

Figure 1. A–L *Alysia erecta* sp. nov., ♀ **A** habitus, lateral view **B** antennae **C** wings **D** head, dorsal view **E** head, front view **F** mesosoma, dorsal view **G** mesosoma, lateral view **H** anterior half of metasoma, dorsal view **I** ovipositor sheath, lateral view **J** basal part of antenna **K, L** mandible.

Description. Holotype, ♀, length of body 3.1 mm in lateral view, length of antenna 3.0 mm and of fore wing 3.6 mm.

***Colour*:** Body (Fig. 1A) black, first tergite and mesonotum entirely reddish brown; antenna dark brown; mandible pale yellow; leg yellowish brown basally, tarsus brown.

***Head* (Fig. 1D):** Width of head 1.9 times its median length in dorsal view. Antenna 0.9 times longer than body, 31 segmented. First flagellomere 1.3 times longer than second and 2.5 times longer than wide. Compounded eye slightly oval, in lateral view 1.6 times as long as wide. Minimum width of face (Fig. 1E) 1.6 times its height (measured from ventral rim of antennal sockets to upper margin of clypeus); face setose, wrinkled and rather mat. Eye in dorsal view 1.4 times as long as temple. Frons nearly entirely glabrous. Ocello-ocular line (OOL) 4.1 times longer than diameter of anterior ocellus; OOL: antero-posterior ocellar line (AOL): postero-ocellar line (POL) = 24 : 7 : 10. Stemmaticum with deep and long median groove. Vertex smooth and with sparse setae. Mandible 1.2 times longer than wide, with three teeth; first tooth curved, with distinct incision between first and second tooth; second tooth reddish brown, narrow and sculptured; second tooth 1.5 times longer than first tooth. Maxillary palp pale yellow and 0.4 times longer than mesosoma.

***Mesosoma*:** In dorsal view mesosoma 1.9 times longer than wide, 1.4 times longer than wide in lateral view. Mesoscutum (Fig. 1G) with oval medio-posterior depression and with setae; notauli impressed anteriorly, not reaching medio-posterior depression; anterior mesosoma crenulated; scutellar sulcus with two carinae; in lateral view, apical part of mesopleuron and metapleuron with long setae. metanotum sculptured. Propodeum (Fig. 1G) entirely wrinkled, 0.4 times longer than wide in maximum length; precoxal sulcus (Fig. 1F) deep and distinct, occupying entire length of mesopleuron; propodeum curved dorsally in lateral view. Fore wing (Fig. 1C) 2.3 times as long as wide in maximum length; pterostigma 3.9 times as long as wide; vein r of fore wing 2.6 times longer than wide and issued from distal third of pterostigma; vein 2-SR slightly bent; vein 3-SR 1.1 times longer than vein 2-SR ; vein 2-SR+M and r-m not sclerotized; 2-SR: r : 3-SR = 28: 5: 25; first discal cell of fore wing approx. 0.8 times longer than wide in median length; first subdiscal cell of fore wing approx. 3.4 times as long as wide in median length. Hind wing vein M+CU: vein 1r-m = 9: 7.

***Leg*:** Hind coxa reddish brown apically, compressed and 1.1 times longer than hind trochanter; hind femur brownish yellow, 3.9 times as long as wide and 0.7 times longer than hind tibia; hind tibia as long as hind tarsus.

***Metasoma*:** First tergite (Fig. 1H) striate, 1.1 times longer than its apical width and blackish. Setose part of ovipositor sheath (Fig. 1I) 0.6 times as long as mesosoma and 0.5 times as long as hind tibia. Ovipositor without subapical dorsal notch (Fig. 1I).

Male. Unknown.

Distribution. South Korea.

Etymology. Named after the erect setae on the flagellomeres: "*erecta*" is Latin for erect.

***Alysia hebeiensis* Zhu, van Achterberg & Chen, 2018**
Fig. 2A–L

Alysia hebeiensis Zhu, van Achterberg & Chen, 2018: 4

Material. 1♀ (NIBR), **South Korea**, Dodae-ri, buk-myeon, Gapyeong-gun, Gyeonggi-do, 37°56'11.8"N, 127°28'50.2"E, 04.IV.2018, Sohn. GenBank accession no. OP391514.

Redescription. ♀, length of body in lateral view 3.4 mm, length of antenna 4.0 mm and of fore wing 4.1 mm.

***Colour*:** Body (Fig. 2A) black, but metasoma entirely reddish brown; antenna dark brown basally, leg yellowish brown basally, tarsus brown.

***Head* (Fig. 2D):** Width of head 2.0 times its median length in dorsal view. Antenna 1.2 times longer than body, 38 segmented. First flagellomere 1.2 times longer than second and 3.8 times longer than wide. Compounded eye slightly oval, in lateral view 1.4 times as long as wide. Minimum width of face (Fig. 2E) 1.8 times its height (measured from ventral rim of antennal sockets to upper margin of clypeus); face wrinkled with long setae. Eye in dorsal view 1.3 times as long as temple. Ocello-ocular line (OOL) 6.1 times longer than diameter of anterior ocellus; OOL: antero-posterior ocellar line (AOL) : postero-ocellar line (POL) = 23 : 6 : 7. Vertex smooth and with sparse long setae. Mandible 1.4 times longer than wide, with three teeth; first tooth lobe–shaped; second tooth reddish brown, wide and sculptured; second tooth 1.3 times longer than first tooth. Maxillary palp pale yellow and 0.5 times longer than mesosoma.

***Mesosoma*:** In dorsal view mesosoma 2.0 times longer than wide, 1.6 times longer than wide in lateral view. Mesoscutum (Fig. 2G) with oval medio-posterior depression and long setae; notauli impressed anteriorly, not reaching medio-posterior depression; anteriorly mesosoma crenulated widely; scutellar sulcus with four carinae; in lateral view, mesopleuron and metapleuron with long setae. Metanotum sculptured. Propodeum (Fig. 2G) entirely wrinkled, 0.5 times longer than wide in maximum length; precoxal sulcus (Fig. 2F) distinct, without setae, occupying entire length of mesopleuron; propodeum curved dorsally in lateral view. Fore wing (Fig. 2C) 2.2 times as long as wide in maximum length; pterostigma 3.4 times as long as wide; vein r of fore wing 2.1 times longer than wide; vein 2-SR slightly bent; vein 2-SR+M and r-m not sclerotized; 2-SR: r : 3-SR = 6: 1: 5; first discal cell of fore wing approx. 0.8 times longer than wide in median length; first subdiscal cell of fore wing approx. 3.6 times as long as wide in median length. Hind wing vein M+CU: vein 1r-m = 4: 1.

***Leg*:** Hind coxa apically pale yellow and 1.2 times longer than hind trochanter; hind femur 4.4 times as long as wide and 0.7 times longer than hind tibia; hind tibia 1.2 times longer than hind tarsus.

***Metasoma*:** First tergite striate and narrow, 1.7 times longer than its apical width and dark brown. Setose part of ovipositor sheath (Fig. 2I) 1.2 times longer than mesosoma and 1.2 times longer than hind tibia.

Male. Unknown.

Distribution. China (Zhu et al. 2018), South Korea (new record).

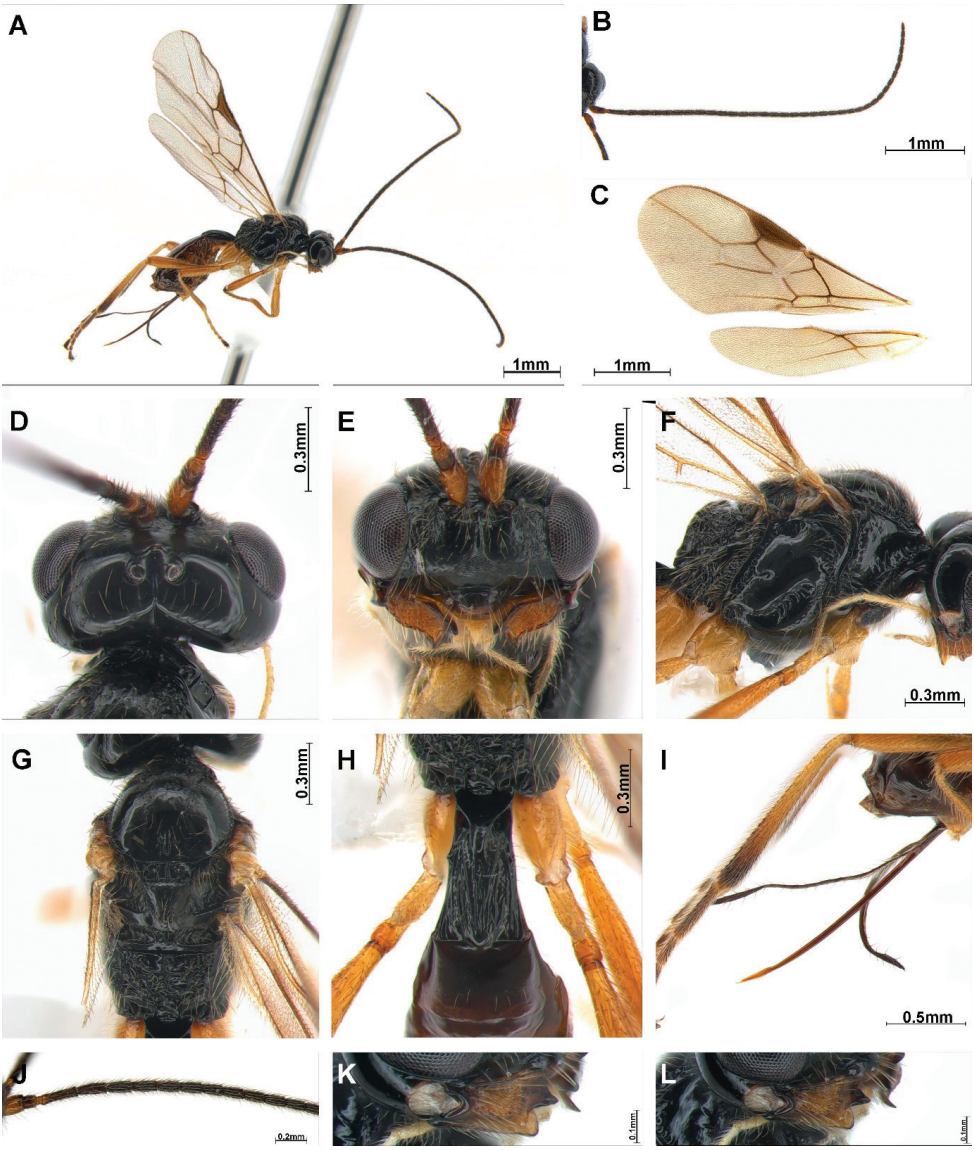

Figure 2.A–L *Alysia hebeiensis* Zhu, van Achterberg & Chen, 2018 ♀ **A** habitus, lateral view **B** antennae **C** wings **D** head, dorsal view **E** head, front view **F** mesosoma, dorsal view **G** mesosoma, lateral view **H** anterior half of metasoma, dorsal view **I** ovipositor sheath, lateral view **J** basal part of antenna **K, L** mandible.

Table 1. COI pairwise genetic distances between the three *Alysia* spp. from South Korea.

	A. erecta	*A. hebeiensis*	*A. sirin*
A. erecta	0.000		
A. hebeiensis	0.061	0.000	
A. sirin	0.094	0.098	0.000

***Alysia sirin* Belokobylskij, 1998**
Fig. 3A–L

Alysia sirin Belokobylskij, 1998: 178.

Material. 1♀ (NIBR), **South Korea**, Dodae-ri, buk-myeon, Gapyeong-gun, Gyeonggi-do, 37°56'11.8"N, 127°28'50.2"E, 05.IV.2018, Sohn. GenBank accession no. OP391516.

Description. ♀, length of body in lateral view 3.9 mm, length of antenna 4.0 mm and of fore wing 3.5 mm.

***Colour*:** Body (Fig. 3A) black, but metasoma entirely pale yellow; antenna dark brown basally, apical parts pale yellow (two apical segments missing); hind leg basally tri-coloured, coxa pale yellow, apical part of hind femur and hind tibia yellowish brown, posterior part of hind femur, hind tibia and hind tarsus reddish brown.

***Head* (Fig. 3D):** Width of head 2.1 times its median length in dorsal view. Antenna incomplete, remaining part as long as body and 29 segmented. First flagellomere 1.4 times longer than second and 4.4 times longer than wide. Compounded eye slightly oval, in lateral view 1.2 times as long as wide. Minimum width of face (Fig. 3E) 1.8 times its height (measured from ventral rim of antennal sockets to upper margin of clypeus); face granulate and with long setae; labrum wrinkled. Eye in dorsal view 2.4 times as long as temple. Ocello-ocular line (OOL) 5.4 times longer than diameter of anterior ocellus; OOL: antero-posterior ocellar line (AOL): postero-ocellar line (POL) = 22: 5: 7. Vertex with long setae. Mandible 1.6 times longer than wide, first tooth with setae; first tooth lobe–shaped; second tooth reddish brown, narrow and sharp; second tooth 1.5 times longer than first tooth; apical part of third tooth reddish brown, short and flat. Maxillary palp pale yellow and 0.8 times longer than mesosoma.

***Mesosoma*:** In dorsal view mesosoma 1.9 times longer than wide, 1.4 times longer than wide in lateral view. Mesoscutum (Fig. 3G) with slightly oval medio-posterior depression and long setae; notauli impressed anteriorly, not reaching medio-posterior depression; mesosoma crenulated anteriorly; scutellar sulcus with four carinae; in lateral view, apical part of mesopleuron and metapleuron with long setae. Metanotum rugose. Propodeum (Fig. 3G) entirely rugose, 0.4 times longer than wide in maximum length; precoxal sulcus (Fig. 3F) distinct, apical part with setae, occupying entire length of mesopleuron; propodeum curved dorsally in lateral view. Fore wing (Fig. 3C) 2.2 times as long as wide in maximum length; pterostigma 3.3 times as long as wide; vein r of fore wing 3.6 times longer than wide; vein 2-SR slightly bent; vein 2-SR+M and r-m not sclerotized; 2-SR: r : 3-SR = 10: 2: 7; first discal cell of fore wing approx. 0.9 times longer than wide in median length; first subdiscal cell of fore wing approx. 4.1 times longer than wide medially. Hind wing vein M+CU: vein 1r-m = 16: 5.

***Leg*:** Hind coxa apically pale yellow; hind coxa 1.2 times longer than hind trochanter; hind femur 4.6 times as long as wide and 0.7 times longer than hind tibia; hind tibia as long as hind tarsus.

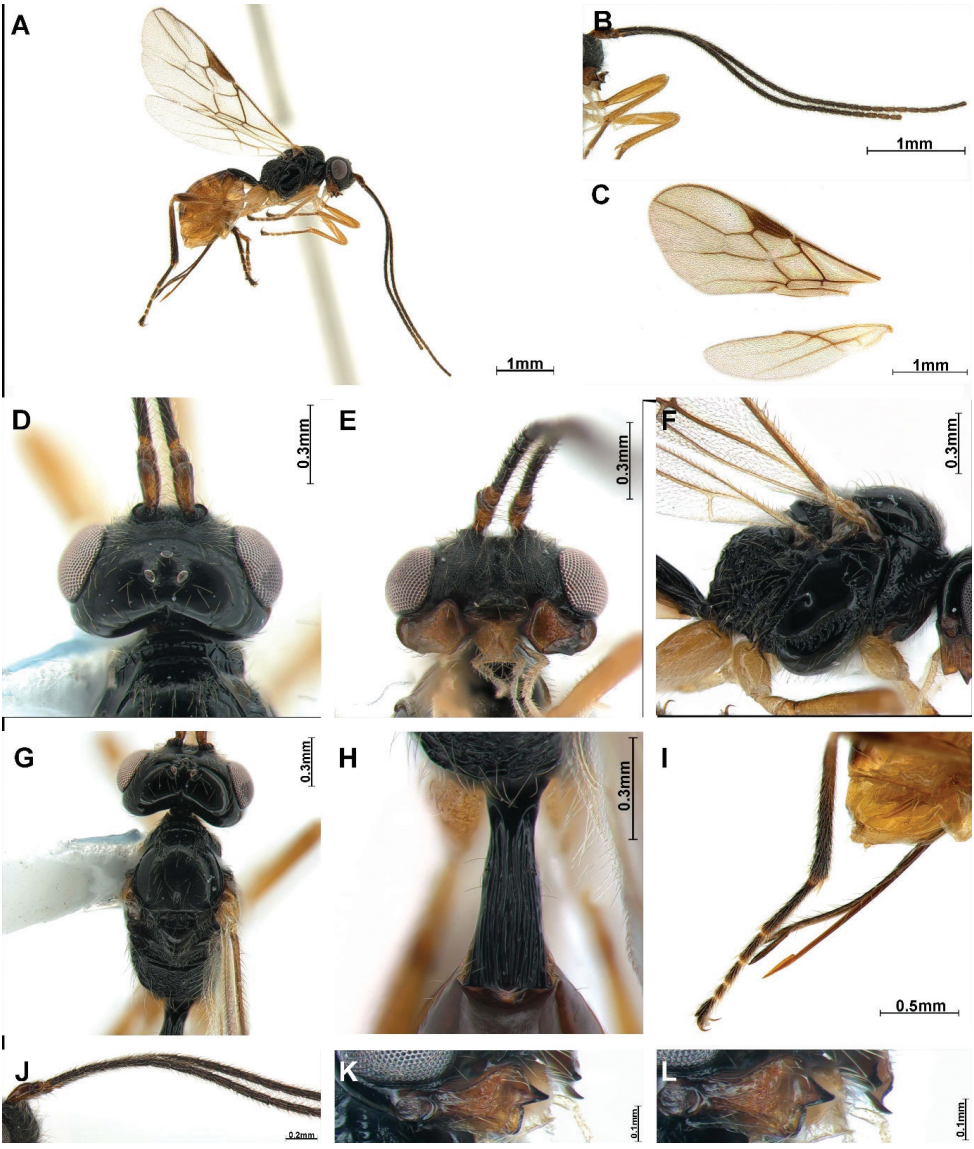

Figure 3. A–L *Alysia sirin* Belokobylskij, 1998♀ **A** habitus, lateral view **B** antennae **C** wings **D** head, dorsal view **E** head, front view **F** mesosoma, dorsal view **G** mesosoma, lateral view **H** anterior half of metasoma, dorsal view **I** ovipositor sheath, lateral view **J** basal part of antenna **K, L** mandible.

***Metasoma*:** First tergite striate and narrow, 2.5 times longer than its apical width and dark brown. Setose part of ovipositor sheath (Fig. 3I) 1.3 times longer than mesosoma and 1.3 times longer than hind tibia.

Male. Unknown.

Distribution. Eastern Palaearctic, Japan, Russia (Yu et al. 2016), South Korea (new record).

Acknowledgements

This work was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR202203201). It was also supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2022R1A2C1091308). It was also supported by a grant from the Korea Environment Industry & Technology Institute (KEITI) through Exotic Invasive Species Management Program (2018002270005) funded by Korea Ministry of Environment (MOE) of the Republic of Korea.

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JHR 95: 59–72 (2023)
doi: 10.3897/jhr.95.96784
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

First record of the parasitoid subfamily Doryctinae (Hymenoptera, Braconidae) in Rovno amber: description of a new genus and species with stigma-like enlargement on the hind wing of the male

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Academic editor: J. Fernandez-Triana | Received 27 October 2022 | Accepted 28 December 2022 | Published 17 February 2023

https://zoobank.org/DEAA4D16-C248-4EA8-B26C-83133240F854

Citation: Belokobylskij SA, Simutnik SA, Vasilenko DV, Perkovsky EE (2023) First record of the parasitoid subfamily Doryctinae (Hymenoptera, Braconidae) in Rovno amber: description of a new genus and species with stigma-like enlargement on the hind wing of the male. Journal of Hymenoptera Research 95: 59–72. https://doi.org/10.3897/jhr.95.96784

Abstract

A new genus and species of the braconid parasitoid subfamily Doryctinae, *Eocenhecabolus kotenkoi* **gen. et sp. nov.**, from the late Eocene Rovno amber are described and illustrated. *Eocenhecabolus* **gen. nov.** is the first unambiguously extinct Doryctinae genus. This genus is described from the male and characterised by the following features: in the fore wing by the postfurcal position of the recurrent vein (m-cu) relatively to the first radiomedial vein (2-SR), and a distally open brachial (second subdiscal) cell; in the hind wing by the presence of the elementary stigma-like enlargement on the distal half of the costal (1-SC+R) vein. The different types of stigma-like enlargements found in the hind wings of males in the subfamily Doryctinae are discussed.

Keywords

Coleoptera, description, Eocene, fossil, Hecabolini, *Hemidoryctes*, stigma-like enlargement

Introduction

The subfamily Doryctinae is morphologically one of the most diverse groups of idiobiont parasitoids of the family Braconidae (Zaldivar-Riverón et al. 2008; Quicke 2015). For development, they predominantly use the larval stages of the hosts from the order Coleoptera and rarely Lepidoptera, Hymenoptera and perhaps Isoptera; however, a few tropical (especially Neotropical) taxa are known as phytophages (gall-associated – inquiline or inducer) (Zaldivar-Riverón et al. 2008, 2014; Belokobylskij and Maetô 2009; Yu et al. 2016).

The unambiguous doryctine fossil taxa mainly have been described or recorded as inclusions in fossil resin (Taimyr retinite, Baltic, Mexican and Dominican ambers) (Brues 1933; Muesebeck 1960; Zherikhin 1978; Zuparko and Poinar 1997), and only a few are known from rock fossils (Statz 1936, 1938; Belokobylskij 2014). The most common fossil doryctine genus is *Doryctes* Haliday, 1836 (~ 14 species), although some species may belong to *Ontsira* Cameron, 1900 because sometimes it is very difficult to separate the fossil representatives of these genera (Brues 1933; Statz 1938; Belokobylskij 2014). One species of the genus *Rhaconotus* Ruthe, 1954 (described as *Ichneumon petrinus* Scudder, 1877 (Scudder 1890) and later (Brues 1910) transferred to *Hormiopterus* Giraud, 1869) was recorded from a rock fossil of the Florissant Lagerstätte (latest Eocene), but this determination is doubtful given the short and character's reduced description and incomplete illustrations. Additionally, three late Oligocene species of the genus *Spathius* Nees, 1818 have been described by Statz (1936, 1938) from rock fossils of Rott Lagerstätte, but its descriptions are ambiguous and they could be representatives of other doryctine genera or even non-doryctines.

Four reported extant genera with described fossil species belong to the doryctine tribe Hecabolini, but the taxonomic positions of all these records are questionable. The morphological characters of *Hecabolus gladiator* Statz, 1936 (rock fossil from Rott) indicate that it is likely a member of the brachistine genus *Eubazus* Nees, 1814. The extinct *Promonolexis klebsi* Brues, 1933 (Baltic amber) is probably a synonym of the brachistine genus *Blacus* Nees, 1818 (Belokobylskij 2014). The fossil *Polystenus obduratus* Brues, 1933 (Baltic amber) is actually not a representative of the genus *Polystenus* Foerster, 1862 and perhaps not Doryctinae, but its status is difficult to justify based on the description and requires re-examination of the type (which is perhaps lost) or additional specimens. According to the description, the extinct *Semirhytus caudatus* Brues, 1933 (Baltic amber) is probably a member of the subfamily Rogadinae rather than Doryctinae. Additionally, there is a very doubtful record of the specialised doryctine genus *Heterospilus* Haliday, 1836 from the Late Cretaceous (Santonian) Taimyr amber (Zherikhin 1978) and this hypothesis requires verification.

The real taxonomic position of the fossil species *Doryctomorpha tertiaria* Brues, 1933 (Baltic amber), described originally in the New Zealand endemic genus *Doryctomorpha* Ashmead, 1900 (currently considered to be within the subfamily Mesostoinae: Quicke et al. 2020; Jasso-Martinez et al. 2022) remains unclear. Unfortunately, the original description and illustration (Brues 1933) are insufficient for an accurately taxonomic placement of this species, so the type must be found and verified or new material made available for certainty.

Unlike the fossil doryctine braconids listed above, the systematic position of two other taxa belonging to the tribe Ecphylini is beyond doubt. The discovery of two specialised doryctine genera *Ecphylus* Foerster, 1862 with *E. oculatus* Muesebeck, 1960, and *Aivalykus* Nixon, 1938 with *A. dominicanus* Zuparko & Poinar, 1997 in Miocene Mexican and Dominican ambers is interesting and valuable (Muesebeck 1960; Zuparko and Poinar 1997). The extant members of both these genera are known as parasitoids of predominantly bark beetles (Curculionidae, Scolytinae) larvae.

Rovno amber is coeval with late Eocene Baltic amber, which has yielded more than 310 new arthropod species, and nearly all are unknown from Baltic amber (Makarkin et al. 2022). Recently, Varash District localities have yielded dozens of taxa unknown from the better studied Klesov deposit (Telnov et al. 2022; Dietrich et al. 2023), many of which were found in Velyki Telkovichi (e.g. Simutnik et al. 2020; Legalov et al. 2022a).

Only two recently published records exist for Rovno amber braconids: description of a new species of *Microtypus* Ratzeburg, 1848 (Belokobylskij et al. 2021), and report about the presence of the aphidiine genus *Toxares* Haliday, 1840 (Kalyuzhna and Perkovsky 2021).

This paper provides an illustrated description of the male of a new doryctine genus and species discovered in late Eocene Rovno amber which is characterized by the presence of a stigma-like enlargement on the hind wing and an open distally brachial (first subdiscal) cell of the fore wing.

Materials and methods

A well preserved, mainly complete parasitoid was found in the clear piece VT-729 (36 × 27 × 17 mm, weight 7 grams before primary treatment) of the collection from Velyki Telkovichi, Varash District, Rovno Oblast.

The specimen was examined using the equipment and techniques described in Simutnik et al. (2022a). Photographs were taken using a Leica Z16 APO stereomicroscope equipped with a Leica DFC 450 camera and processed with LAS Core. The final plates were prepared in Adobe Photoshop CS6.

The terminology employed for morphological features and sculpture, as well as body measurements follow Belokobylskij and Maetô (2009). Wing venation nomenclature also follows Belokobylskij and Maetô (2009), with the terminology of van Achterberg (1993) shown in parentheses.

The specimen used for this study is deposited in the collection of the I.I. Schmalhausen Institute of Zoology of the National Academy of Sciences of Ukraine, Kiev (**SIZK**).

Systematic part

Class Insecta Linnaeus, 1758
Order Hymenoptera Linnaeus, 1758
Family Braconidae Nees, 1811
Subfamily Doryctinae Foerster, 1863

Genus *Eocenhecabolus* Belokobylskij, gen. nov.
https://zoobank.org/3E9686FC-03E0-4910-B954-E7FEBAC094F0

Type species. *Eocenhecabolus kotenkoi* Belokobylskij, gen. et sp. nov., by present designation and monotypy.

Etymology. Named after "Eocene" from the geological epoch dated to the Rovno amber and the generic name of its extant type genus *Hecabolus* of the tribe Hecabolini from subfamily Doryctinae. Gender: masculine.

Description. ***Head*** (Fig. 1E, F, H) not depressed, weakly transverse. Ocelli medium-sized, weakly convex, arranged in triangle with base 1.3 times its sides. Frons almost not convex, without lateral protuberances. Eyes large, oval, glabrous. Face distinctly convex. Malar suture present, but weak. Clypeus relatively high, with distinct lower visor. Clypeal suture fine laterally, absent on wide distance dorsally. Anterior tentorial pits small. Occipital carina present and distinct at least laterally and dorsally. Mandibles robust. Maxillary palpus medial length. ***Antenna*** (Fig. 1C, E, H) mostly missing, only four segments present. Scape short and wide, approximately as long as maximum width. Pedicel relatively short and thick, about as long as scape. First flagellomere long, subcylindrical, weakly curved and without any modifications. ***Mesosoma*** (Fig. 1C, G) not depressed. Pronotum convex in posterior half, with distinct short longitudinal lateral carinae. Sides of pronotum mainly smooth with short rugae on oblique furrow. Mesoscutum distinctly (but not highly) roundly convex above pronotum, densely and rather distinctly granulate-punctate. Notauli present, deep and complete, reaching prescutellar furrow. Scutellum convex. Prepectal carina present, distinct. Mesopleuron mainly smooth. Precoxal sulcus present, but short (not more than half of mesopleuron length below), rather deep, almost straight, finely crenulate. Metascutum without dorsal tooth (lateral view). Propodeum evenly curved in lateral view, with areas delineated but distinct carinae, with wide, sub-round and smooth basolateral areas, with narrow and long areola, distinctly separated petiolate area and relatively short basomedial carina; without lateral tubercles; propodeal spiracle subcircular. ***Wings*** (Figs 1A, B, 2A). Fore wing relatively wide, evenly faintly infuscate; pterostigma rather long and wide. Radial (marginal) cell not shortened, closed distally, wide, about 3.5 times longer than its maximum width. Metacarp (1-R1) 1.2 times longer than pterostigma. Radial vein (r) arising weakly before middle of pterostigma. First medial abscissa (1-SR+M) present and weakly sinuate. Both radiomedial veins (2-SR and r-m) present. Second radiomedial (submarginal) cell relatively long, pentagonal. Discoidal (first discal) cell petiolate anteriorly; petiole (1-SR) short. Recurrent vein (m-cu) distinctly postfurcal, weakly convergent posteriorly with basal vein (1-M). First mediocubital vein (M+CU1) well sclerotised and straight. Nervulus (cu-a) distinctly postfurcal. Brachial (first subdiscal) cell open posteriorly; brachial vein (CU1b) absent. Transverse anal veins (2A and a) absent. Hind wing. Second abscissa of costal vein (1-SC+R) with elementary elliptic stigma-like enlargement. Radial vein (SR) unsclerotised and transparent. Nervellus (cu-a) present. Submedial (subbasal) cell large. First abscissa of mediocubital vein (M+CU) more than twice longer than second abscissa

(1-M). ***Legs*** (Figs 1C, D, 2B) rather robust and short. Fore tibia with distinct spines arranged almost in single line. Hind coxa elongate, without ventro-basal tubercle and corner, weakly shorter than propodeum. Hind femur short and wide, 0.7 times as long as hind tibia. Hind tibia weakly thickened distally, with at least two distinctly visible spines on its dorsal margin in distal quarter. Hind tibial spur glabrous, relatively short, about 0.3 times as long as hind basitarsus. Hind basitarsus short, about half as long as second to fifth segments combined. Tarsal claw medium size, simple and evenly curved. ***Metasoma*** (Figs 1C, D, G, 2B) elongate, oval in dissection, not pressed, segments behind third one distinctly exposed posteriorly. First metasomal tergite not wide, weakly widened distally, with deep dorsope, with distinct dorsomedial carinae situated closed to each other, with distinct lateral carinae, striate medially and smooth laterally, with spiracles situated on basal third of tergite, spiracular tubercles small, weakly shorter that second and third tergites combined. Suture between second and third tergites absent. Second tergite mainly smooth, with shallow and short sublateral depression. Laterotergites (epipleura) of segments behind first one perhaps not separated; spiracles placed on the lateral part of tergites. Genitalia distinctly visible from below.

Comparative diagnosis. This new genus belongs to the tribe Hecabolini based on the fore wing with a distally open brachial (subdiscal) cell and the hind wing of male with an elementary stigma-like enlargement. The latter character is similar to that found in the extant doryctine genera *Hemidoryctes* Belokobylskij, 1992, *Dendrosoter* Wesmael, 1838, *Bracocesa* Koçak & Kemal, 2008, and *Doryctophasmus* Enderlein, 1912.

Eocenhecabolus gen. nov. is most similar to the Pantropical *Hemidoryctes* Belokobylskij from the subtribe Stenocorsina (Doryctinae: Hecabolini) by the wing venation and analogous enlargement on the hind wing. However, the new genus differs from *Hemidoryctes* by the very short antennal scape, approximately as long as its maximum width (elongated, about 1.5 times longer than the maximum width of that in *Hemidoryctes*), the enlarged pedicel, about as long as the scape (not enlarged and only about 0.5 times as long as the scape in *Hemidoryctes*), the mostly smooth temple with additional sparse punctuation (densely granulate-striate in *Hemidoryctes*), the mostly smooth side of the mesosoma (basically densely granulate in *Hemidoryctes*), the propodeum with areas delineated by distinct carinae (without areas delineated by carinae in *Hemidoryctes*), the fore wing not maculate, but only faintly infuscate (distinctly maculate in *Hemidoryctes*), the distinctly postfurcal recurrent vein (m-cu) of the fore wing (usually distinctly antefurcal in *Hemidoryctes*), the relatively short discoidal (discal) cell of the fore wing (distinctly elongate in *Hemidoryctes*), the weakly postfurcal nervulus (cu-a) in the fore wing (strongly postfurcal in *Hemidoryctes*), the first abscissa of the mediocubital vein (M+CU) of the hind wing distinctly longer than the second abscissa (1-M) (distinctly shorter in *Hemidoryctes*), the smooth and less thick hind femur, 3.0 times longer than its maximum width (densely granulate-reticulate and thicker, 2.5 times longer in *Hemidoryctes*), the hind tibia with relatively long setae and at least two distinct spines on its dorsal margin (with very short setae and without spines on the dorsal margin in *Hemidoryctes*), the shortened hind tarsus with the segment not narrowed toward its distal margin (elongate and segments distinctly narrowed distally in

Hemidoryctes), and the smooth metasoma behind the first tergite (the second and part of third tergites heavily sculptured in *Hemidoryctes*).

Apart from several individual differences, the new genus differs from other three extant genera exhibiting stigma like enlargement on hind wing (*Dendrosoter* Wesmael, *Bracocesa* Koçak & Kemal and *Doryctophasmus* Enderlein) in having the open distally brachial (first subdiscal) cell and no brachial vein (CU1b) in the fore wing (this cell closed distally and the brachial vein present in all latter genera), and large submedial (subbasal) cell in the hind wing with the first abscissa of the mediocubital vein (M+CU) distinctly longer than the second abscissa (1-M) (this cell small and the first abscissa short in all three latter genera).

Among known fossil Doryctinae genera, *Eocenhecabolus* gen. nov. is superficially similar to the extinct *Doryctomorpha tertiaria* Brues, 1933 described based on a female from Baltic amber (Brues, 1933). However, the assignment of this species to the peculiar endemic New Zealand genus *Doryctomorpha* Ashmead, 1900 from the subfamily Mesostoinae is very doubtful and unsupported by known morphological characters. The female of *D. tertiaria* Brues perhaps may belong to the new genus described here, but absence of important information in this species description (especially regarding wing venation and legs) and uninformative figure together with the loss of the type specimen prevent us to form a reliable opinion about its placement. Anyway, *Eocenhecabolus kotenkoi* gen. et sp. nov. differs from *D. tertiaria* Brues by having the head transverse in dorsal view, with a transverse diameter of eye 1.5 times longer than the temple (head subquadrate and with a transverse diameter of eye 2.0 times longer than the temple in *D. tertiaria*), the vertex transversely and sinuately striate (smooth in *D. tertiaria*), the propodeum with areas distinctly delineated by carinae (without areolation in *D. tertiaria*), and the hind coxa suboval and without a prominent lower corner (subtriangular and with a prominent lower corner in *D. tertiaria*).

***Eocenhecabolus kotenkoi* Belokobylskij, sp. nov.**
https://zoobank.org/CAE0E88C-095C-47A4-ABB5-ECFF03CE9082
Figs 1, 2

Type material. *Holotype*: male, SIZK VT-607, Velyki Telkovichi, Varash District, Rovno amber, late Eocene.

Description. Male. Body length 1.5 mm; fore wing length 1.3 mm.

***Head*:** Head relatively high, its width about 1.3 times medial length. Occiput weakly concave. Transverse diameter of eye 1.5 times longer than temple (subdorsal view). POL 1.3 times Od, approximately 0.5 times OOL. Eye about 1.2 times as high as broad (lateral view). Malar space 0.3 times height of eye, almost equal to basal width of mandible. Face width 0.9 times height of eye, 1.3 times medial height of face and clypeus combined. Hypoclypeal depression subround, its transverse width 0.9 times distance from edge of depression to eye, 0.4 times width of face.

***Antenna*:** First flagellomere almost 7.0 times longer than its apical width, approximately twice longer than scape. Second segment present only basally, remaining part missing.

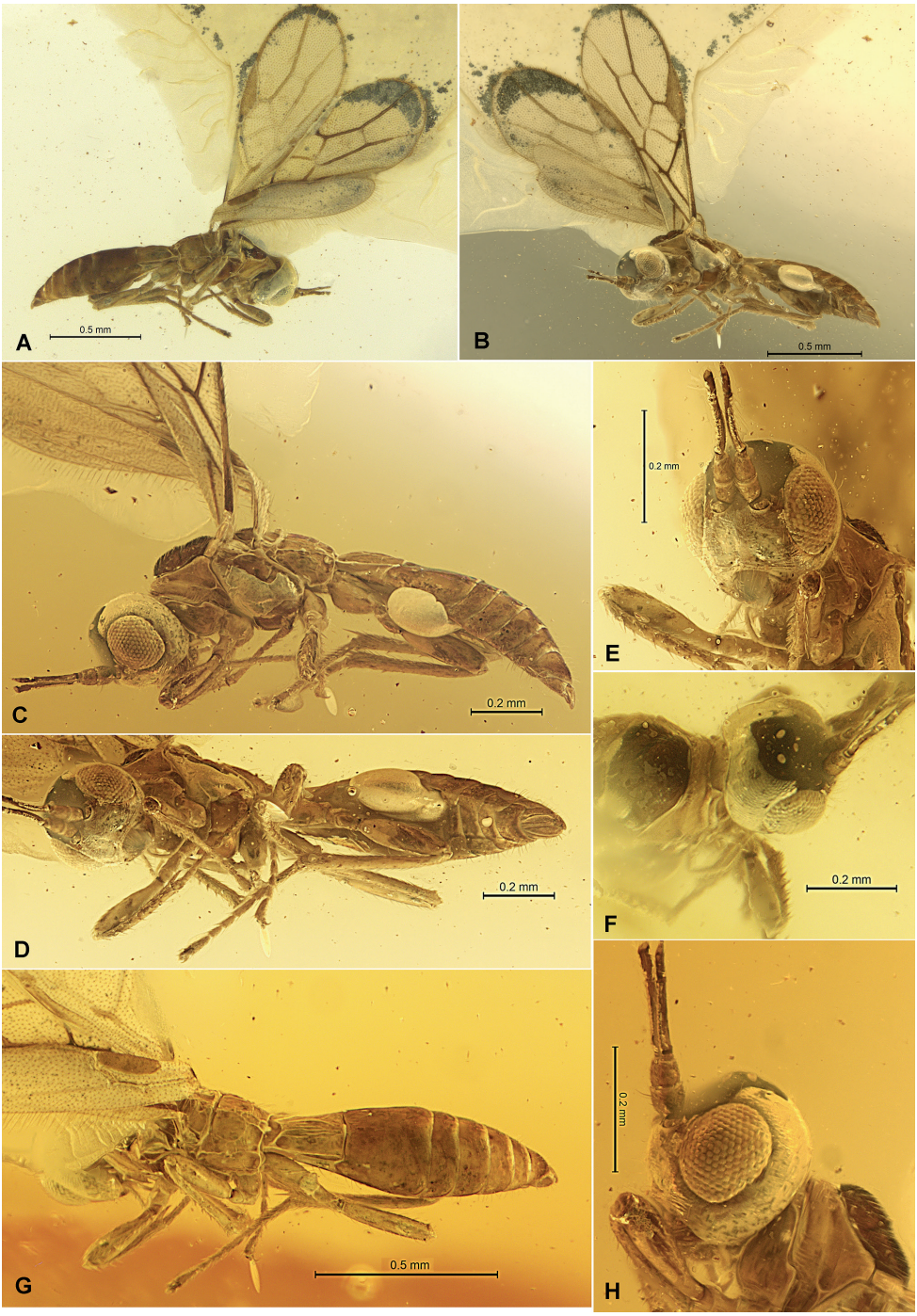

Figure 1. *Eocenhecabolus kotenkoi* gen. et sp. nov. (male, holotype, Rovno amber, # SIZK VT-607) **A** habitus, right dorso-lateral view **B** habitus, left ventro-lateral view **C** body, lateral view **D** body, ventro-lateral view **E** head and antenna, fronto-lateral view **F** head, dorsal view **G** propodeum and metasoma, dorsal view **H** head and antenna, lateral view.

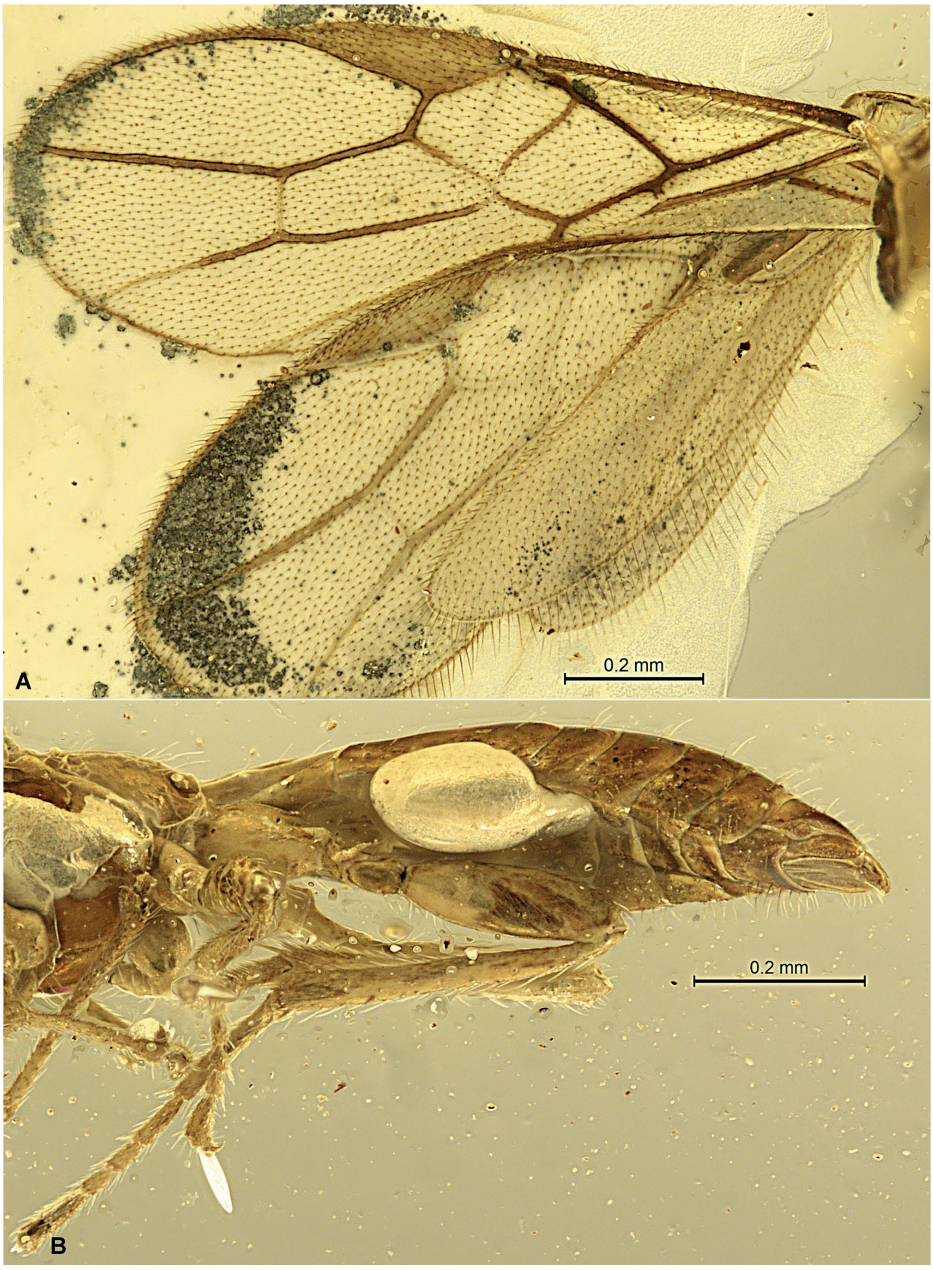

Figure 2. *Eocenhecabolus kotenkoi* gen. et sp. nov. (male, holotype, Rovno amber, # SIZK VT-607) **A** wings **B** metasoma and hind leg, lateral view.

***Mesosoma*:** Mesosoma long, its length 1.8 times height. Neck of prothorax relatively short. Pronotal carina absent, dorsal pronotal lobe distinctly convex. Median lobe of mesoscutum convex, distinctly protruding forward, without anterolateral

corners. Prescutellar depression relatively long. Subalar depression shallow and mainly smooth. Lateral carinae between propodeum and metapleuron strong and complete.

***Wings*:** Fore wing wide, 2.6 times longer than its maximum width. Pterostigma wedge-shaped, 3.7 times longer than its width. Radial vein (r) arising from basal 0.4 of pterostigma. First (r) and second (3RSa) radial abscissae forming obtuse angle; first abscissa (r) 0.7 times as long as maximum width of pterostigma. Second radial abscissa (3RSa) 3.0 times first abscissa (r), 0.5 times as long as the straight third abscissa (3RSb), 1.3 times longer than the straight first radiomedial vein (2RS). Second radiomedial (submarginal) cell relatively wide and long, 2.7 times longer than its maximum width, 1.8 times longer than the narrow brachial (first subdiscal) cell. Recurrent vein (1 m-cu) 0.75 times as long as first radiomedial vein (2RS), 0.6 times as long as basal vein (1M). Discoidal (first discal) cell rather short, 1.7 times longer than its maximum width. Nervulus (1cu-a) 0.6 times as long as distance between basal (1M) vein and nervulus (1cu-a). Parallel vein (2CUb) weakly curved basally. Brachial (second subdiscal) cell relatively short and narrow. Hind wing almost 4.5 times longer than its maximum width. Stigma-like enlargement 3.5 times longer than maximum width. First abscissa of mediocubital vein (M+CU) almost twice longer than second abscissa (1-M).

***Legs*:** Fore femur about 4.5 times longer than maximum width. Fore tarsus 1.2 times longer than fore tibia. Hind coxa almost 1.5 times longer than its maximum width, 0.8 times as long as propodeum. Hind femur 3.0 times longer than its width. Hind tarsus almost as long as hind tibia. Second segment of hind tarsus 0.4 times as long as basitarsus, weakly longer than fifth segment (without pretarsus).

***Metasoma*:** Length 1.2 times larger than length of head and mesosoma combined. First metasomal tergite 1.4 times longer than distal maximum width, 1.3 times longer than propodeum; apical width of first tergite about 1.6 times its basal width. Second and third tergites combined 1.3 times longer than basal width of second tergite, 0.9 times as long as their maximum width.

***Sculpture and pubescence*:** Temple densely transversely and sinuately striate with additional reticulation laterally. Face weakly transversely striate, smooth medially. Frons and most part of temple perhaps mainly smooth. Propodeum mostly smooth, only sometimes with short and sparse rugae along carinae; areola almost 2.5 times longer than its width; basomedial carina present in basal 0.3 of propodeum. Hind coxa and femur smooth. First metasomal tergite striate medially, weakly rugose sublaterally, almost smooth laterally. Second tergite mainly smooth, finely striate in small basolateral areas. Remaining part of metasoma smooth. Hind tibia with rather dense and short semi-erect setae, its length 0.4–0.6 times maximum width of tibia.

***Colour*:** Body almost entirely brown. Legs mainly reddish brown to pale reddish brown. Fore wing almost entirely faintly evenly infuscate. Pterostigma entirely brown.

Female. Unknown.

Etymology. This species is named in honour of the well-known Ukrainian braconidologist, Dr Anatoly Grigorievich Kotenko.

Discussion

The fossil braconid taxa from the subfamily Doryctinae are relatively common in the Paleogene and Neogene compared to the members of many other braconid subfamilies. Most of these taxa have been attributed to extant genera (*Doryctes* Haliday, *Ontsira* Cameron, *Rhaconotus* Ruthe, *Spathius* Nees, *Polystenus* Foerster, *Ecphylus* Foerster, *Aivalykus* Nixon, *Hecabolus* Wesmael, *Semirhytus* Szépligeti and *Heterospilus* Haliday). Only one genus known from a fossil, monotypic *Promonolexis* Brues, 1933 from Baltic amber (the type species *P. klebsi* Brues, 1933), was described in Doryctinae (Brues 1933), but actually it may belong to the genus *Blacus* Nees (Brachistinae) (Belokobylskij 2014). *Eocenhecabolus* gen. nov. is the first unambiguously extinct genus of Doryctinae.

Eocenhecabolus gen. nov. is the first recorded extinct doryctine representative with a stigma-like enlargement on the hind wing. Similar structures on the hind wing are known in numerous males of extant genera, predominantly from the tribes Hecabolini and Heterospilini, but a few taxa with such enlargement of an elementary type also have been recorded in the tribe Doryctini. The functional role of this structure in males is not fully understood, but it may have sensory or sexual attraction functions.

According to the morphological investigation of this structure in extant Doryctinae (Belokobylskij 1983) three types of hind wing stigma-like enlargement in males are known (Fig. 3):

1. elementary, "*Dendrosoter*" type (Fig. 3A) – relatively flat widened distal part of the second costal vein (1-SC+R) with its ventral (and dorsal) surface entirely evenly covered by short setae; the hind wing usually with a recurrent vein (m-cu), and a nervulus (cu-a) arising from the mediocubital vein (M+CU) and not connected to the enlargement (*Dendrosoter*, *Bracocesa*, *Doryctophasmus*, *Hemidoryctes*).
2. moderately modified, "*Hecabolus*" type (Fig. 3B, C) – dorsally convex stigma-like enlargement of the hind wing connected not only to the costal vein (1-SC+R), but also to the mediocubital (M+CU) and basal (1r-m) veins; the enlargement weakly bent downward only anteriorly and its margin without eyelash-like setae; with setae on the ventral surface rather evenly distributed; the nervulus (cu-a) arising from the posterior margin of enlargement, and the recurrent vein (m-cu) often absent (many members of the tribe Hecabolini).
3. complex, "*Heterospilus*" type (Fig. 3D, E) – dorsally convex stigma-like enlargement of the hind wing connected to three veins of the hind wing (costal (1-SC+R), mediocubital (M+CU) and basal (1r-m)); most of it margins are bent downward (except places where the veins originate), especially anteriorly; the margins of the curved parts covered by eyelash-like setae; additionally present small and setose ear-shaped process inside of the lower (inner) surface of the enlargement; wide large area of the ventral surface of the enlargement glabrous; the nervulus (cu-a) arising from posterior margin of the enlargement, and the recurrent vein (m-cu) always absent (most members of the tribe Heterospilini).

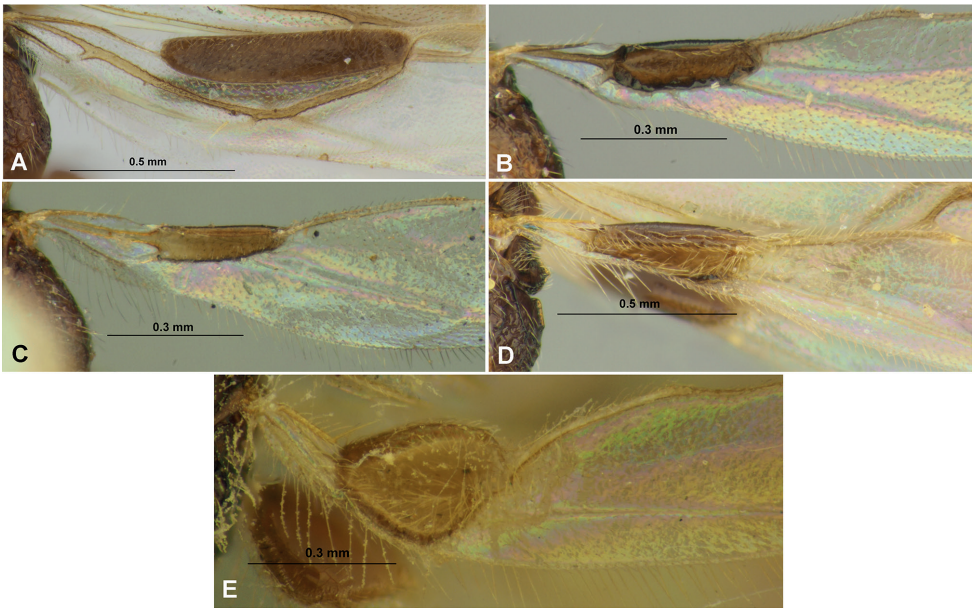

Figure 3. Stigma-like enlargement on the male hind wing **A** *Dendrosoter middendorffii* (Ratzeburg, 1848) **B** *Leluthia hungarica* (Szépligeti, 1900) **C** *Leluthia transcaucasica* (Tobias, 1976) **D** *Heterospilus tauricus* Telenga, 1941 **E** *Heterospilus* sp.

The host of *Eocenhecabolus kotenkoi* gen. et sp. nov. is unknown. However, it perhaps belongs to the tribe Hecabolini, the members of which are predominantly known as ectoparasitoids of coleopteran larvae. Coleopteran larvae in Rovno amber are abundant (Perkovsky 2016; Haug et al. 2022), but largely understudied. Many of the better studied Rovno amber flat wasps (Bethylidae) as well as extant ones also are often known as beetle parasitoids, and nearly all Rovno bethylids (14 of 15) are unknown in the Baltic amber fauna (Colombo et al. 2021 and references therein) as are 85% of Rovno amber beetle species (Legalov et al. 2022b). Thus, it is assumed that the braconid ectoparasitoids of coleopteran larvae in Rovno amber could be mostly different from those in Baltic amber.

Eocenhecabolus kotenkoi gen. et sp. nov. is the 24th non-ant hymenopteran genus (from 58, 41.4%) and 51st non-ant hymenopteran species (from 74, 68.9%) found in Rovno amber but unknown from Baltic amber (Simutnik et al. 2022a, b).

Acknowledgements

We are grateful to Nikolai R. Khomich (Rovno, Ukraine) for help in obtaining the specimens studied in this paper, to Anatoly P. Vlaskin (SIZK) for cutting and polishing the sample, and to Sarah C. Crews (California Academy of Sciences, USA) for editing

of the English text. The authors are also very thankful to Dr. A.P. Ranjith (Bangalore, India), Dr. Angelica M. Penteado-Dias (São Carlos, Brazil) and Dr. Jose Fernandez-Triana (Ottawa, Canada) for their useful suggestions and comments on the first version of the manuscript.

This study was performed as part of the State Research Project No 122031100272–3 for SAB; work of SAS was supported by grant NRFU No 2020/02/0369.

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JHR 95: 73–83 (2023)
doi: 10.3897/jhr.95.97490
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Laotris luzulae (Hymenoptera, Braconidae, Alysiinae, Dacnusini), a new species from the southwest of England

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Academic editor: J. Fernandez-Triana | Received 12 November 2022 | Accepted 11 January 2023 | Published 17 February 2023

https://zoobank.org/877F1129-B440-4EAF-9744-9E8A9999776F

Citation: Godfray HCJ (2023) *Laotris luzulae* (Hymenoptera, Braconidae, Alysiinae, Dacnusini), a new species from the southwest of England. Journal of Hymenoptera Research 95: 73–83. https://doi.org/10.3897/jhr.95.97490

Abstract

Laotris luzulae Godfray, **sp. nov.** is described in the small genus *Laotris* Nixon, 1943, (Braconidae, Alysiinae, Danusini) from five specimens reared from *Cerodontha silvatica* (Groschke, 1957) (Diptera, Agromyzidae) mining *Luzula sylvatica* (Huds.) Gaudin (Juncaceae) in Devon and Gloucestershire in the southwest of Great Britain. Six further specimens from Somerset caught as adults in the 1950s are also noted. It differs morphologically from the three described species of *Laotris* and shows a 4.2% and 6.6% genetic distance at the CO1 barcode locus from an undescribed North American species and from the European *L. striatula* (Haliday, 1839), respectively.

Keywords

Alysiinae, Braconidae, Dacnusini, Europe, *Laotris*, new species, United Kingdom

Introduction

Laotris Nixon, 1943, is a small genus of Alysiinae (Braconidae) in the tribe Dacnusini; it was established by Nixon (1943, 1954) to accommodate the rather anomalous species, *striatula*, which Haliday (1839) had described in *Alysia* Latreille, 1804, with Marshall (1891) moving it to *Dacnusa* Haliday, 1833. In general shape and body size, this

species resembles the many species of *Dacnusa*, *Chorebus* Haliday, 1833, and *Exotela* Förster, 1863, that largely parasitise Agromyzidae (Diptera), and it was with these genera that Nixon associated *Laotris*. However, Griffiths (1964) argued that it shared derived characters (in particular the striate second metasomal tergite and the mandible with a fourth small tooth dorsal to the large middle tooth) with members of the *Coelinius* genus-group and he considered *Laotris* a basal member of that assemblage. Wharton (1994) agreed with this placement, and considered *Laotris* to be most closely related to, and possibly congeneric with, *Synelix* Förster, 1863 (=*Ectilis* Nixon, 1943). Keys to the genera that have at some time been included in *Coelinius* Nees, 1819, are provided by van Achterberg (2014) and Zheng et al. (2017) which include *Laotris* because of its similar mandibles.

Griffiths (1968) described a second species, *L. rupestris*, from Poland and Tobias (1998) a further species, *L. minuscularia*, from far-east Asian Russia. *Laotris striatula* is a parasitoid of *Cerodontha* (*Dizygomyza*) *luctuosa* (Meigen, 1830) (Agromyzidae) which mines *Juncus* (Juncaceae) reeds while the single specimen of *L. rupestris* was reared from an unidentified species of *Cerodontha* (*Dizygomyza*) mining *Carex sempervirens* Vill. (Cyperaceae) (Griffiths 1968).

Laotris luzulae is described here based on five specimens reared from *Cerodontha* (*Dizygomyza*) *silvatica* (Groschke) (Agromyzidae) mining *Luzula sylvatica* (Huds.) Gaudin (Juncaceae) in Devon and Gloucestershire in the southwest of Great Britain. A further six swept specimens were found in unsorted material in the Natural History Museum, London, UK (NHMUK), also from southwest Britain (Somerset). It differs in morphology and biology from the previously described species and shows a genetic divergence of 6.5% from *L. striatula* at the CO1 mitochondrial barcode locus.

Methods

Material examined

Five specimens of an undescribed species of *Laotris* were reared from *Cerodontha silvatica* in south Gloucestershire and south Devon (full details in type designation below).

In addition, six specimens (three males and three females) were found amongst unsorted Dacnusini in the NHMUK's collections. They too come from the southwest of England and were collected in the Haddeo Valley, Brompton Regis, Somerset (Grid Reference, SS9529) by JF & DMS Perkins on 13.viii.1952. They are included in the NHMUK's digital catalogue with codes NHMUK010885170 to NHMUK010885175.

Material of *L. striatula* used for genetic analysis was from a collection of five males and a female reared from *Cerodontha luctuosa* on *Juncus effusus*, four from Hessle (Grid Reference, TA033272; collected 6.xii.2020) and two from Anlaby (Grid Reference, TA033278; collected 18.i.2021 & 7.ii.2021); both near Hull, Yorkshire in the north

of England (BP Warrington, codes A1292-5 [Hessle] and A1303 & A1308 [Anlaby]). Here and below, "codes" refer to entries in a database of those parasitoid wasps in the collection of the National Museum of Scotland (NMS, Edinburgh) that have been examined by the author. Three non-reared specimens in the NMS, and 32 specimens in the NHMUK, including material studied by Nixon and Griffith, were also examined.

Photographs were taken through a Leica M125C microscope with focus stacking using Leica LAS X software and final processing in Photoshop.

CO1 sequencing was carried out on legs removed from specimens by the Biodiversity Institute of Ontario at the University of Guelph. To obtain maximum sequence information, initial Sanger sequencing was supplemented by "next generation" short read sequencing. Sequence analysis was carried out on the BOLD platform and using the programme MEGA11 (Molecular Evolutionary Genetics Analysis version 11, Tamura et al. (2021)). The sequence data is publicly available at http://v4.boldsystems.org/.

Systematics

***Laotris luzulae* Godfray, sp. nov.**
https://zoobank.org/9803187F-B3AF-4EE8-BA3C-853C59EBE858

Type material. *Holotype*: female (code: NK64), Hencliffe Woods, south Gloucestershire, Grid Reference, ST6371, emerged 13.v.2003, in NMS.

***Paratypes*:** female (code: NK62, in NHMUK with digital catalogue number NHMUK010885191), emerged 27.iv.2002, and male (code: NK63, in NMS), emerged 7.v.2003, otherwise same data as holotype; two females (codes: NK195, NK196, deposited in NMS), Steps Bridge, South Devon, Grid Reference, SX801884, both collected 15.iv.2004 deposited in NMS.

NK62, NK63 & NK64 were reared by DJ Gibbs and NK195 & NK196 by M. Storey.

Description of female (Fig. 1). *Size*: body length (excluding antennae), 2.3–2.5 mm; wing length 2.7–3.0 mm.

***Colour*.** Head black except for labrum, mandibles and part of cheek immediately above insertion of mandibles which are brown. Antennal scape, pedicel and proximal part of first flagellar segment brown with rest of flagellum black; palps pale yellow. Mesosoma black except for small brown patches near base of wings. Legs including coxae light yellow-brown (in some specimens with base of hind coxa dark) with only fifth tarsal segment infuscated. Petiole black with rest of metasoma shading from dark brown anteriorly to black posteriorly.

***Head*.** Antennae with 30 or 31 segments (3 specimens each), approximately equal in length to wing; first flagellar segment 3.4 times as long as maximally wide, 0.75 times as long as scape plus pedicel; second flagellar segment 2.8 times as long as maximally wide; distal flagellar segments approximately two times as long as maximally wide. Head 1.7 times as wide as long in dorsal view, with sparse forward-directed setae

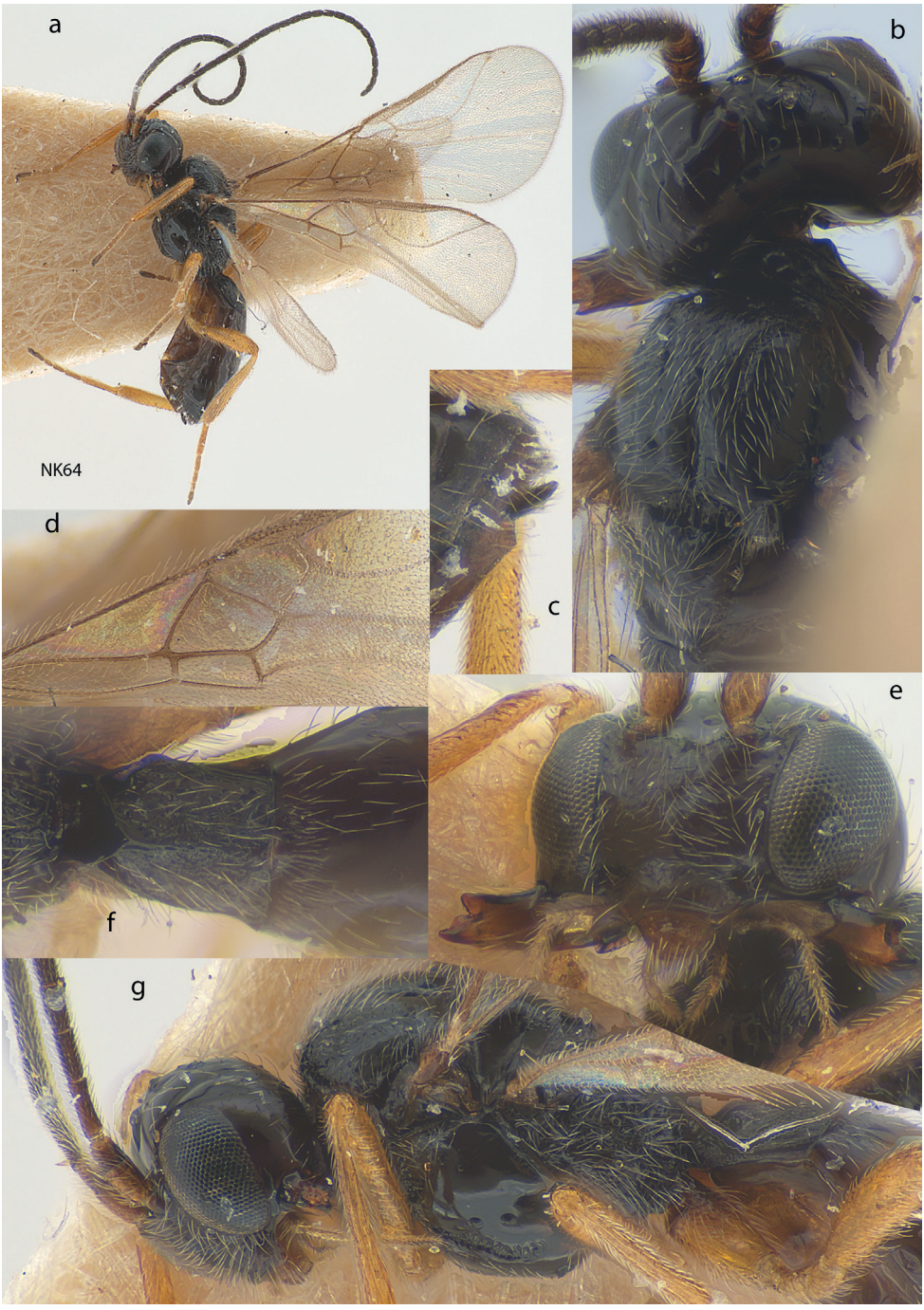

Figure 1. *Laotris luzulae* sp. nov. female **a** general habitus **b** dorsal view of head and mesosoma **c** ovipositor **d** basal part of forewing **e** face and mandibles **f** dorsal view of first and second metasomal tergites **g** lateral view of head and mesosoma.

on occiput and temples; ocelli arranged in an equilateral triangle with OOL almost exactly twice POL; OOL 2.3 times posterior ocellar diameter, a small medial depression on frons dorsal to the antennal insertions. In lateral view, width of eye approximately equal to width of cheek with frons slightly protruding beyond eye; clypeus distinctly protruding. Face at narrowest point half width of head and width and height (measured from ventral labrum rim to antennal insertion) equal, slightly roughened with extensive but not dense setae which are ventrally directed along eye margin, medially directed above labrum, dorso-medially directed on lateral panels of face, and dorsally directed near mid-line. Clypeus 0.4 times as high as maximum width, its ventral margin with a narrow carina which is shallowly concave medially and forms distinct angles at ventral-lateral corners. The clypeus is largely bare or with sparse ventrally directed setae. Labrum ventrally with dense setae which are approximately equal in length to height of labrum. Maxillary palps six-segmented and reaching to a little before middle of mesopleuron, last segment 6 times as long as wide and 1.3 times length of penultimate segment. Labial palps four-segmented. Mandible more or less parallel-sided, 1.9 times as long as medially wide, with a long central tooth and subequal first and last teeth; a small fourth tooth appears as an outgrowth on anterior edge of central tooth (not posterior edge as in many *Chorebus* spp.).

Mesosoma. Mesosoma in lateral view 1.4–1.5 times as long as high. Pronotum dorsally with a medial pit; laterally largely hairless, smooth and shining above oblique suture, with some rugosity around its margins. The oblique suture is rugose with some setae below. Mesoscutum dorsally 1.1–1.2 times as broad as long, anteriorly roughened, its surface covered with backwardly directed setae except for posterior lateral margins. Notaulices well-developed as a series of connected pits that converge on, but do not quite meet, the postero-medial fovea. Fovea elongate, 6–7 times as long as wide. Prescutellar furrow 3 times as broad as long, with a medial longitudinal costa and indistinct sub-medial costae. Scutellum smooth with backwardly directed setae, especially at margins and posterior apex. Postscutellum with backwardly pointed setae and a median carina that bluntly protrudes in lateral view. Propodeum strongly sculptured with backwardly directed setae, most dense posteriorly. Mesopleuron bare and shining centrally; epicnemial (antero-dorsal) area with setae and rugosity, a small patch of setae at postero-ventral corner; pleural suture and episternal scrobe smooth. Precoxal suture strong and ribbed, extending from anterior margin about four-fifth of way to posterior margin. Area below precoxal suture with setae. Metapleuron with rugose sculpture similar to propodeum, covered with quite dense postero-ventrally directed setae.

Legs. Hind coxa dorsally somewhat rugose. Hind femur 4.5 times as long as maximum width. Hind tarsus approximately same length as hind tibia. Ratio of hind tarsal segment lengths (from base): 1:0.58:0.39:0.31:0.38.

Wings. Pterostigma elongate, approximately 7 times longer than broad, more or less parallel-sided beyond origin of radius (*r*) (at 1/5) until 4/5 when it tapers to metacarp (*R1a*). First segment of radius (*r*) slightly longer than width of pterostigma; radius distally (*RS*) somewhat sinuate reaching wing margin before wingtip; metacarp half

Figure 2. *Laotris luzulae* sp. nov. male **a** dorsal view of head and mesosoma **b** general habitus **c** mandible **d** face **e** dorsal view first and second metasomal tergites **f** lateral view of head and mesosoma.

length of pterostigma. Vein *m-cu* received into first submarginal cell (antefurcal condition). First subdiscal cell closed at postero-ventral corner by *2cu-a*.

***Metasoma*.** Metasoma 1.2–1.3 times length of mesosoma. First tergite (petiole) 1.4 times as long as its posterior width, initially widening from base but approximately parallel-sided in posterior two thirds. At base, two carinae run from margins diagonally to join before centre and continue as a short, indistinct, medial ridge. Surface of first tergite rugose with some indistinct longitudinal ridges, covered in sparse backward-directed setae except for central area which is largely bare. Lateral and posterior edges of first tergite are margined by a narrow carina. Second tergite with an area of longitudinal striae that extends only over its basal 1/3–1/4. Most of tergite covered with sparse setae, except for posterior central area and posterior margin. There are rows of setae at bases of posterior metasomal tergites. Ovipositor does not project beyond apical tergite in retracted position.

Description of male (Fig. 2). *Size*: body length (excluding antennae), 2.3–2.7 mm; wing length 2.7–2.8 mm.

The single reared male specimen has 33 antennal segments while the three swept male specimens have 29, 30 & 31 segments. Otherwise, the male is very similar to the female with no obvious sexual dimorphism.

Molecular analysis. Sequence data from the mitochondrial CO1 gene (the standard barcode locus) were obtained from three specimens each of the new species and *L. striatula.* Full barcode sequences (658bp) were obtained from five specimens and 513bp from one specimen of *L. luzulae*. The BOLD database contains no further European specimens of *Laotris* but ten specimens of a species of wasp collected in the Yukon (Canada) and assigned the Barcode Index Number (BIN) ADV6100 which showed a 4.2% genetic distance from *L. luzulae*. The new species was placed in BIN AEO8807 and showed a 6.6% genetic distance from *L. striatula* (placed in BIN AEO8806) (Fig. 3). The magnitude of the genetic divergence supports the hypothesis based on morphology that *L. luzulae* is distinct from *L. striatula*, and also from the undescribed North American species.

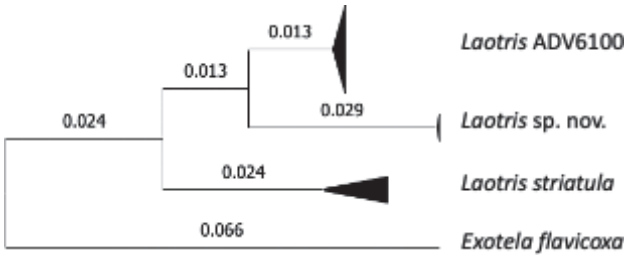

Figure 3. Neighbour-joining tree (CO1 gene, Kimura 2-parameter model) of *Laotris* species with an *Exotela flavicoxa* (placed by some authors in the genus *Antrusa*) sequence used as an outgroup. The height of the triangular wedges represents sample size and their horizontal width the genetic variation within the species.

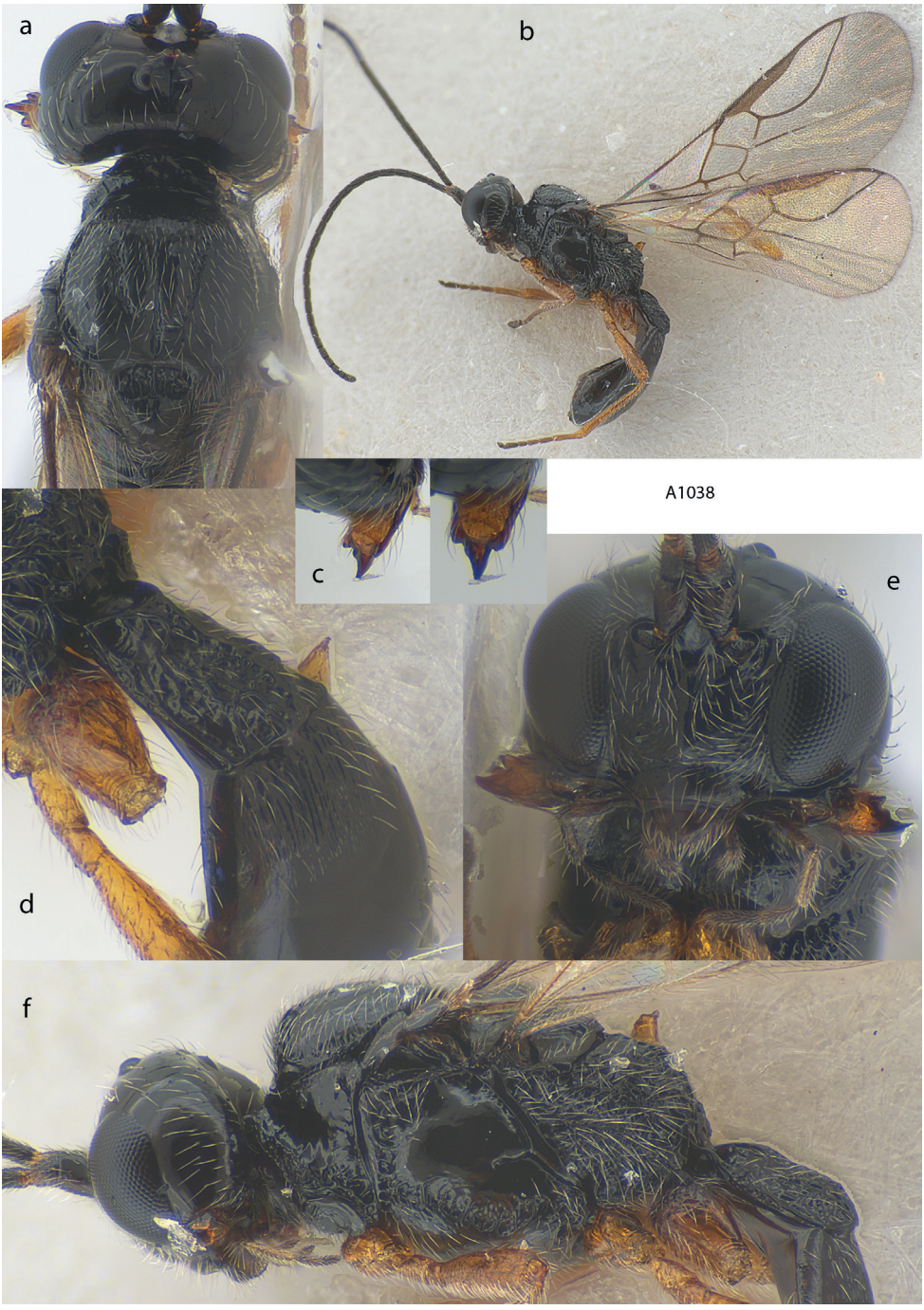

Figure 4. *Laotris striatula* Haliday female **a** dorsal view of head and mesosoma **b** general habitus **c** two views of mandible **d** dorso-lateral view of first and second metasomal tergites **e** face **f** lateral view of head and mesosoma.

Discussion

The new species differs from *Laotris striatula* (Fig. 4), the only common and widespread *Laotris* species in Europe, in morphology, biology and DNA sequence. The main morphological differences are:

- Striations on second metasomal tergite restricted to anterior half or less; in *L. striatula* the striations typically cover the whole surface though in some specimens the striations cover just the anterior three quarters.
- Normally over 30 antennal segments in both sexes while in *L. striatula* there are normally fewer than 28. Of the *L. luzulae* material examined one (out of 10 with intact antennae) had 29 antennal segments as did one (out of 35) *L. striatula*.
- Legs largely light yellow-brown with only the base of the hind coxae infuscated; the majority of *L. striatula* have greater infuscation, and in lighter specimens the legs are red-brown rather than yellow-brown.
- Pubescence of the mesoscutum (especially the lateral lobes), propodeum and metapleuron more extensive than in *L. striatula.*

I have not seen the single specimen and holotype of *L. rupestris*, which is in the Museum of the Polish Academy of Science, Warsaw, but Griffith's description clearly demonstrates it is morphologically distinct. In comparison with the new species, *L. rupestris* has very dark legs, fewer antennal segments (28), a less developed additional tooth on the mandible and a largely smooth metapleuron. It shares with *L. luzulae* more restricted striations on the second metasomal tergite compared with *L. striatula.*

I have also not seen the type of *Laotris minuscularia* Tobias (in the Zoological Institute, Academy of Sciences, St Petersburg) which was described from the Vladivostok region (Primorsky Krai) of the Russian Far East. However, its small number of antennal segments (20) strongly suggests it is distinct from the species described here.

The three *Laotris* whose life history are known all attack agromyzid flies in the genus *Cerodontha*, subgenus *Dizygomyza*, though different host species on different host plant genera. *Laotris* is regarded as a relatively plesiomorphic member of the *Coelinius* genus group (Griffiths 1964; Wharton 1994) and the only member of the group that attacks Agromyzidae. Griffith (1966) points out that the most plesiomorphic members of the *Dacnusa* genus group, which has radiated greatly on Agromyzidae, also attack *Cerodontha* (and *Agromyza*) species feeding on monocots and suggested this may reflect the life history of the first Dacnusini. Unfortunately, there was insufficient phylogenetic signal in the barcode DNA sequence to explore this hypothesis further.

It is striking that all three collections of *L. luzulae* come from south-west England, though of course this may reflect sampling bias. The host is not common in the UK and in addition to records from the south-west it has been recorded from Wales, Northern Ireland and Berkshire (https://agromyzidae.myspecies.info/node/1499). A second species of *Cerodontha*, *C. luzulae* (Groschke), also feeds on *Luzula*, but has a

more northerly distribution and is recorded from North Wales and Scotland. I have seen 45 parasitoids reared from this host in Scotland by KP Bland which were 33 *Chorebus merellus* (Nixon, 1937) (Dacnusini), 9 *Apodesmia* nr. *similis* (Szépligeti, 1898) (Braconidae, Opiinae), 2 *Phaedrotoma reptantis* (Fischer, 1957; *sensu* C van Achterberg, in litt.) (Opiinae) & 1 *Pediobius metallicus* (Nees, 1834) (Chalcidoidea, Eulophidae). Griffiths (1968) also notes 11 German and Polish records of *C. merellus* from *C. luzulae*. The absence of *Laotris luzulae* from these rearings suggest it may be specific to *Cerodontha silvatica* rather than to *Cerodontha* feeding on *Luzula.* The only other parasitoids of *C. silvatica* I am aware of are a single specimen of *C. merellus* reared by JP Day in Devon, UK (this parasitoid is recorded from a number of *Cerodontha* spp.) and a single specimen of the relatively polyphagous (within the Agromyzidae) *Grammospila rufiventris* (Nees, 1812) (Braconidae, Alysiinae, Alysini) reared by DJ Gibbs in Gloucestershire, UK. Apart from the Griffiths records, all the other wasps mentioned here are in the NMS collection.

Wharton (1994) stated he had examined five specimens of *Laotris* from North America (three from Colorado and singletons from the Yukon and British Columbia) which he believed belonged to two species. The cluster of ten specimens from the Yukon in the Bold database confirms the presence of *Laotris* in North America (low resolution photographs of three of these specimens on BOLD show it has the wing venation and general habitus of *Laotris*). *Cerodontha luctuosa*, the host of *L. striatula*, is found in North America, as is *Chorebus cytherea* (Nixon, 1937) (Dacnusini), the commonest parasitoid of this fly in Europe (data in BOLD). It would thus not be surprising if *L. striatula* occurs in North America.

Acknowledgements

I am very grateful to David Gibbs, Malcolm Storey, Barry Warrington, Keith Bland & John Day who reared the wasps studied here. Mark Shaw (NMS) passed the *L. luzulae* specimens to me and has been an invaluable source of advice on the Braconidae for many years. I particularly thank Paul Hebert (University of Guelph) for sequencing these (and many other wasps) and Jayme Sones and her team at Guelph for all their technical assistance. Gavin Broad allowed me access to the main collection and unsorted material at the NHMUK and provided much helpful advice including commenting on a draft of this paper which was further improved by Mark Shaw and Javier Peris-Felipo's refereeing.

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JHR 95: 85–94 (2023)
doi: 10.3897/jhr.95.95646
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
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The International Society of Hymenopterists

First record of the genus *Sathon* Mason, 1981 (Hymenoptera, Braconidae, Microgastrinae) in China

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Academic editor: Jose Fernandez-Triana | Received 28 September 2022 | Accepted 13 December 2022 | Published 17 February 2023

https://zoobank.org/64EBC59C-DCAB-41FA-B34A-6E432820F4BF

Citation: Liu Z, Liu J-J, He J-H, Chen X-X (2023) First record of the genus *Sathon* Mason, 1981 (Hymenoptera, Braconidae, Microgastrinae) in China. Journal of Hymenoptera Research 95: 85–94. https://doi.org/10.3897/jhr.95.95646

Abstract

Sathon Mason, 1981 is reported for the first time from China through providing a diagnosis, description, and images of *Sathon falcatus* (Nees, 1834). The mitochondrial genome of *S. falcatus* was sequenced, annotated and analysed.

Keywords

China, Microgastrinae, mitogenomics, *Sathon*

Introduction

The genus *Sathon* was erected in the tribe Microgastrini by Mason (1981) based on some species from the *vitripennis*- and *falcatus*-groups of *Apanteles s.l.* (Nixon, 1965). Williams (1985) separated three species from *Sathon* Mason and erected a new genus, *Lathrapanteles* Williams, from the New World sooner after its determination. And later, Williams (1988) revised *Sathon* while reporting five new species from the New World. Austin & Dangerfield (1992) added two new species and three new combinations to this genus when reviewing species from the Australasian region. Recently, Fagan-Jeffries et al. (2019) reported one new species from Australia and Fernandez-Triana et al.

(2020) transferred six species from Ethiopian *Microgaster* Latreille to *Sathon* in sorting the world checklist of Microgastrinae parasitoid wasps.

So far, 23 species (Fernandez-Triana et al. 2020) have been described under this genus worldwide. It was strange that none had ever been reported from China before this study, not to speak of *Sathon falcatus* (Nees, 1834), typical representative of Williams's (1988) *S. falcatus*-group in the Old World which is distributed in 42 countries, including some neighboring countries of China, e.g., Japan, Korea, Mongolia, and Russia. We luckily found this species which represents a new genus record of China in Inner Mongolia during our ongoing revision of the Chinese Microgastrinae.

The status of the genus *Sathon* has been discussed by some taxonomists since it was established. Whitfield et al. (2002, 2009) and Arias-Penna et al. (2019) suggested that *Sathon* should likely be subsumed within *Glyptapanteles* Ashmead because they shared many characters. Plus, van Achterberg (2003) and Fernandez-Triana (2010) suggested that *Sathon* and *Glyptapanteles* should be part of an expanded *Protapanteles* Ashmead. It also has complicated relationships with the genera *Choeras* Mason and *Lathrapanteles* (Fagan-Jeffries et al, 2019; Austin and Dangerfield 1992; Fernandez-Triana et al. 2020).

Microgastrine is considered as the most species-rich subfamily of animals on Earth and has become a key group of organisms for studying parasitism, parasitoid genomics, and mating biology (Whitfield et al. 2018). Nuclear genes (Banks et al. 2006), mitochondrial COI, 16S, and 28S genes, along with morphological characters (Mardulyn et al. 1999; Whitfield et al. 2002) and anchored hybrid enrichment data (Parks et al. 2020) had been applied in earlier molecular studies. Here, we present the mitochondrial sequence of *S. falcatus*, which we hope it will be useful for subsequent phylogentic studies in the subfamily.

Material and methods

Collection, Identification and DNA extraction

This work is based on specimens in the collections of the Parasitic Hymenoptera Collection of Zhejiang University, Hangzhou, China. Materials were all collected by hand netting in Helan Mountain, Inner Mongolia (E105°49'–106°41', N38°19'–39°22'). Each dried specimen was tagged with a unique number.

Descriptions and measurements were made using a stereomicroscope (Zeiss Stereo Discovery V8). All photographs of the wasps were taken and processed using a digital camera KEYENCE VHX-2000C. The images were further processed using Adobe Photoshop CS6. Morphological terms for body structures and measurements follow Nixon (1965) and Mason (1981). The veins follow the modified Comstock-Needham system (van Achterberg 1993). The terminology of the cuticular sculpture follows Harris (1979).

Genomic DNA was extracted from the legs of a single specimen (No. 201006962) using a Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech,

China) following the manufacturer's protocols. Extracted genomic DNA were qualified by NanoPhotometer (IMPLEN, CA, USA) and Qubit 3.0 (Invitrogen, Life Technologies, Carlsbad, CA, USA) and a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All residual DNAs are archived (–30 °C) in the molecular laboratory of Hunan University of Arts and Science, Changde, China, and are available for further study upon request.

Genome sequencing, assembly and annotation

The extracted genomic DNA of the specimen was sheared into fragments of approximately 350 bp in length using the Ultrasonic Processor Covaris S220 (Covaris, Inc. MS, USA). High-throughput sequencing libraries were constructed using the Illumina TruSeq DNA PCR-Free HT Kit and sequenced using an Illumina Novaseq6000 with the strategy of producing 150 bp paired-ends by the Annoroad Gene Tech. (Beijing) Co., Ltd. Quality of raw sequencing reads was checked by FastQC version 0.11.3 (Andrews 2010), and low-quality reads and sites were filtered by Trimmomatic version 3.2.57 (Bolger et al. 2014).

The target mitochondrial reads were filtered out using BLAST (BLASTn with E value: 1×10^{-5}) against a reference data set containing Braconidae mitochondrial genomes via the FastqExtract script (Crampton Platt et al. 2015). The mitochondrial genome of *Sathon falcatus* was assembled by IDBA_UD version 1.1.3 (Peng et al. 2012) and SPAdes version 3.15.2 (Bankevich et al. 2012) with default parameters.

Annotation of the assembled genome was performed by using MITOS Web Server (Bernt et al. 2013). Start and stop codons of protein-coding genes (PCGs) were manually adjusted in Geneious Prime v11 by referencing to the published mito-genomes of Microgastrinae. Gene rearrangements were analyzed by comparing with the putative ancestral type of *Drosophila melanogaster* (Diptera: Drosophilidae).

Results

Taxonomy

Genus *Sathon* Mason, 1981

Sathon Mason, 1981: 78. Williams 1988: 540; Austin and Dangerfield 1992: 52; Fagan-Jeffries et al. 2019: 427. Fernandez-Triana et al. 2020: 945.

Type species. *Apanteles neomexicanus* Muesebeck, 1920, by original designation.

Diagnosis. Areolet of fore wing present or absent; metanotum with sublateral lobes slightly setose, exposing postero-lateral phragma of scutellum; propodeum with median carina present over most of length or almost completely absent, but marked by at least a trace of rugosity; tergite I somewhat narrow, length at least 3.0× longer than

apical width; tergite II subtriangular; hypopygium evenly sclerotized, without striae mid-ventrally; ovipositor sheaths at least half as long as hind tibia; often with large external genitalia in male.

Host. Bombycidae: *Bombyx mori* (L., 1758); Limacodidae: *Cheromettia lohor* (Moore, 1859), *C. sumatrensis* (Heylaerts, 1884); Noctuidae: *Actinotia polyodon* (Clerck, 1759), *Apamea lateritia* (Hufnagel, 1766), *Apamea monoglypha* (Hufnagel, 1766); Papilionidae: *Papilio zelicaon* Lucas, 1858; Pterophoridae: *Adaina microdactyla* (Hübner, 1813), *Emmelina monodactyla* (L., 1758); Psychidae: *Hyalarcta huebneri* (Westwood, 1854); *H. nigresens* (Doubleday, 1845), *Narycia* Stephens, 1836; Sesiidae: *Synanthedon tipuliformis* Clerck, 1759, *Zeiraphera griseana* (Hübner, 1799); Tortricidae: *Rhyacionia buoliana* (Denis & Schiffermüller, 1775) (Yu et al. 2016; Fernandez-Triana et al. 2020). This list of hosts records was compiled in a non-critical way by Yu et al. (2016) from the literature and it is very likely that several (perhaps many) records are inaccurate or erroneous.

Distribution. Worldwide.

Sathon falcatus (Nees, 1834)

Microgaster falcatus Nees von Esenbeck 1834: 175; type lost, Neotype designated by Wilkinson 1945: 113, ♀ – Germany: Zoologisches Museum, Hamburg University.
Microgaster equestris Haliday, 1834. Synonymized by Curtis 1837: 116.
Apanteles equestris; Hincks, 1944: 20.
Apanteles priapus Gautier & Cleu, 1927. Syn. by Wilkinson 1945: 133.
Apanteles gladiator Szepligeti, 1901. Syn. by Tobias 1971: 246.
Sathon falcatus: Mason 1981: 78; Williams 1988: 560.

Diagnosis. Body length 3.3–3.5 mm, fore wing length 3.6–3.8 mm. Antenna nearly 1.1× longer than body length, preapical segment of antenna 1.3× longer than wide. Scutellar sulcus curved, narrow with spare carinae in between. Propodeum 1.8× wider than long, not shiny, densely longitudinally rugose medially, with punctate-rugose aside, largely polished on anterior-medial part and posterior corners. Pterostigma 2.3× as long as its widest part. Tergite I slightly narrowing towards posterior margin, 2.2× longer than hind width, turned-over part as long as wide, shallowly and independently punctate, weakly rugulose-punctate laterally. Tergite II weakly striate laterally, polished medially, strongly curved apically. Ovipositor sheath 0.8× length of hind tibia, falcate.

Head. Transverse in dorsal view, 1.9× as wide as long, 1.1× wider than mesoscutum. Eyes 1.6× longer than temple dorsally. Gena slightly dull with poorly defined, shallow punctures, constricted behind eyes from dorsal view (Fig. 1e). Face (Fig. 1f) a little shiny with fine, sparse punctures with intervals 2–4 times of a puncture diameter, transverse, 0.8× as high as wide, sparsely pubescent. Ocelli big, posterior tangent to anterior ocellus just touching posterior pair of ocelli, distance between fore and a hind ocelli shorter than diameter of an hind ocellus, hind ocelli separated from one another

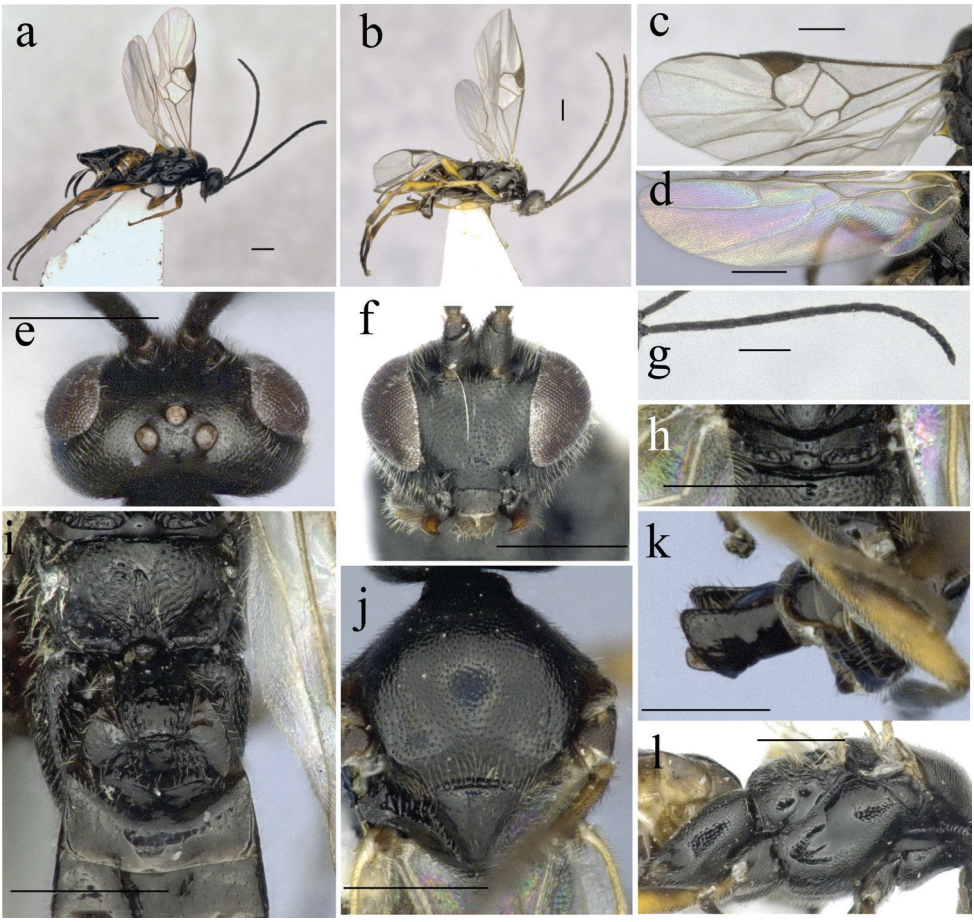

Figure 1. *Sathon falcatus* (Nees, 1834) (new record to China) **a** ♀, habitus, lateral view **b** ♂, habitus, lateral view **c** fore wing **d** hind wing **e** head, dorsal view **f** head, frontal view **g** antenna **h** metanotum **i** propodeum and terga I–III **j** mesosoma, dorsal view **k** male external genitalia **l** mesopleuron, lateral view. Scale line: 0.5 mm.

by 1.5× their own diameter and from eye by 1.4× their own diameter. Antenna nearly 1.1× longer than body length, preapical segment of antenna 1.3× longer than wide (Fig. 1g).

Mesosoma. Length:width:height = 29.0:17.5:20. Mesoscutum (Fig. 1j) shiny, no notaulic courses, punctures fine, intervals 2–3× of a puncture diameter, a little sparser posteriorly. Scutellar sulcus curved, narrow with spare carinae in between. Scutellum shiny, with sparse punctures, intervals 2–4× of a puncture diameter. Propodeum (Fig. 1i) 1.8× wider than long, not shiny, densely longitudinally rugose medially, with punctate-rugose aside, largely polished on anterior-medial part and posterior corners. Mesopleuron (Fig. 1l) highly shiny, largely polished, except anterior part shallowly punctate, intervals 2–3× of a puncture diameter.

***Legs*.** Hind coxa shallowly punctate dorsally. Inner spurs of hind tibia half-length of hind basitarsus, outer spur 2/5. Basitarsus of hind leg as long as tarsomeres 2–4.

***Wings*.** Pterostigma 2.3× as long as its widest part (Fig. 1c). Vein 1-R1 1.4× length of pterostigma, nearly 4.0× longer than its distance from apex of marginal cell. Vein r arising from apex of pterostigma, nearly perpendicular to and 0.8× as long as width of latter, r 1.2× longer than 2-SR, distinctly angled at meeting, 2-M 4/5 length of 2-SR. First discal cell of fore wing nearly 1.2× wider than high. Second submarginal cell of hind wing 1.5× wider than high, vein cu-a of hind wing a little incurved (Fig. 1d).

***Metasoma*.** 1.4× longer than mesosoma. Tergite I (Fig. 1i) slightly narrowing towards posterior margin, 2.2× longer than hind width, basal width 1.4× longer than apical width, basal 2/5 concave, turned-over part as long as wide, shallowly and independently punctate, weakly rugulose-punctate laterally, no longitudinal channel. Tergite II weakly striate laterally, polished medially, 1.9× wider than long in middle, strongly curved apically. Tergite III 1.6× longer than tergite II. Tergites posterior to tergite II polished, shiny, and sparsely pubescent. Hypopygium a little shorter than apex of metasoma. Ovipositor sheath 0.8× length of hind tibia, falcate.

***Colour*.** Black (Fig. 1a). Tegula brown. Palpi blackish brown and spurs pale yellow. Flagellum of antenna dark brown. Mandible yellowish brown. Legs mainly reddish yellow, except sometimes hind femur, apical fourth and all tarsi fulvous. Wing membrane hyaline, vein and pterostigma brown.

Male. Similar to female, except preapical segment of antenna much longer, 2.5× longer, colouration of legs lighter (Fig. 1b).

Hosts. Noctuidae: *Actinotia polyodon* (Clerck, 1759), *Apamea lateritia* (Hufnagel, 1766), *Apamea monoglypha* (Hufnagel, 1766); Pterophoridae: *Adaina microdactyla* (Hübner, 1813); Sesiidae: *Synanthedon tipuliformis* Clerck, 1759; *Zeiraphera griseana* (Hübner, 1799); Tortricidae: *Rhyacionia buoliana* (Denis & Schiffermüller, 1775) (Yu et al. 2016; Fernandez-Triana et al. 2020, see comments above).

Material examined. (ZJUH). 15♀♀5♂♂, Luanchaigou, Helan Mountain, Inner Mongolia, 26.VII.2010, Jie Zeng, Nos. 201006962, 201007079, 201006956, 201006892, 201006933, 201007110, 201006901, 201006900, 201007014, 201007015, 201007019, 201007020, 201007022, 201007013, 201007113, 201006895, 201006946, 201006913, 201007100, 201006910; 108♀♀29♂♂, Dayanggou, Helan Mountain, Inner Mongolia, 27.VII.2010, Hongfei Chai, Nos. 201007246, 201007253, 201007262, 201007316, 201007391, 201007216, 201007397, 201007399, 201007404, 201007401, 201007396, 201007395, 201007394, 201007380, 201007388, 201007402, 201007406, 201007400, 201007389, 201007390, 201007418, 201007414, 201007332, 201007331, 201007430, 201007429, 201007431, 201007433, 201007435, 201007255, 201007282, 201007434, 201007387, 201007386, 201007385, 201007341, 201007343, 201007359, 201007357, 201007377, 201007344, 201007345, 201007333, 201007348, 201007360, 201007371, 201007378, 201007213, 201007265, 201007252, 201007312, 201007314, 201007315, 201007352, 201007330, 201007327, 201007326, 201007325, 201007324, 201007322,

201007321, 201007318, 201007363, 201007317, 201007335, 201007307, 201007306, 201007303, 201007302, 201007301, 201007299, 201007297, 201007296, 201007293, 201007287, 201007283, 201007284, 201007278, 201007275, 201007272, 201007271, 201007270, 201007267, 201007264, 201007261, 201007257, 201007251, 201007249, 201007245, 201007244, 201007243, 201007242, 201007239, 201007235, 201007234, 201007229, 201007228, 201007227, 201007208, 201007205, 201007206, 201007209, 201007211, 201007212, 201007215, 201007392, 201007222, 201007204, 201007202, 201007201, 201007198, 201007200, 201007199, 201007196, 201007195, 201007194, 201007192, 201007191, 201007187, 201007186, 201007184, 201007185, 201007156, 201007160, 201007164, 201007162, 201007166, 201007169, 201007172, 201007176, 201007173, 201007174, 201007177, 201007179, 201007183, 201007420, 201007419; 1♀, Qiangganglingl, Helan Mountain, Inner Mongolia, 3.VIII.2010, Dingjie Zhang, No. 201006771; 1♀, Ganshuwan, Helan Mountain, Inner Mongolia, 9.VIII.2010, Yan Li, No. 201006663.

Distribution. China: Inner Mongolia; Afghanistan, Austria, Armenia, Azerbaijan, Belarus, Bosnia and Herzegovina, Croatia, Czech Republic, Denmark, Egypt, Estonia, Finland, France, Georgia, Germany, Hungary, Indonesia, Ireland, Italy, Japan, Kazakhstan, Kyrgyzstan, Korea, Latvia, Lithuania, Luxembourg, Macedonia, Mongolia, Montenegro, Netherlands, Poland, Romania, Russia, Serbia, Slovenia, Spain, Sweden, Switzerland, Tajikistan, Turkey, United Kingdom and Uzbekistan.

Notes. Characters of the examined specimens from China are mostly in agreement with the description in William (1988), except the legs are lighter (mainly reddish-yellow in female or yellow in male) than what he described (mainly rufo-fulvous in female). Characters combining large inflexible hypopygium, the length of ovipositor with its sheath and the typical large external genitalia in males (Fig.1k, 1.5× wider than width of hind femur), could be a useful way to distinguish *S. falcatus* from other related genera in China. We treated *Sathon* provisionally as a valid genus for the Chinese fauna, with only one distinguishable representative for now, though the size of genitalia varies in other *Sathon* species.

Genome characteristic analyses

A total of 5.76 Gb filtered clean data were produced. The imcomplete mitochondrial genome of *S. falcatus* is 14,492 bp in length (GenBank accession OP432054), containing 11 PCGs, 17 tRNA genes. The entire A+T content in the *S. falcatus* mitochondrial genome was 88%, which ranged from 75.60% (cox1) to 91.7% (atp8) with 39.7% of A, 7.1% of G, 48.3% of T, and 4.9% of C. It is common to find such relative high A+T proportion in the mitochondrial genome of Hymenoptera (Oliveira et al. 2008). The sequence of *COI* gene obtained closest match with a *Sathon* sp. sequence (Sample ID: CGTURK-1557, BIN ID: BOLD:ACE9685) by 99.07% and *Sathon falcatus* sequence (Sample ID: CGTURK-1532, BIN ID: BOLD:AAB3882) by 98.45% when blasted in the BOLD (Barcode of Life Database, http://www.boldsystems.org/), which confirmed the conspecificity of Chinese material with other *S. falcatus* specimens.

Figure 2. Gene rearrangement in the mitochondrial genome of *Sathon falcatus* (Nees, 1834).

Gene rearrangements were evident when compared with the ancestral type of *Drosophila melanogaster*: remote and local inversion occurred in *trnH* and *trnY* respectively, *trnK* and *trnD* were translocated, *cox3–nad4* were shuffled (Fig. 2). The rearrangements showed on mitogenomes evidenced the rapid evolution of this group compared to other lineages of Hymenoptera.

Acknowledgements

We thank the reviewers who critically reviewed the manuscript. Funding for this study was provided by the National Natural Science Foundation of China (32100351), Scientific Research Fund of Hunan Provincial Education Department (20K089) and Hunan Provincial Natural Science Foundation of China (2020JJ5392).

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JHR 95: 95–101 (2023)
doi: 10.3897/jhr.95.95964
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Trialling a convolution neural network for the identification of Braconidae in New Zealand

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Academic editor: J. Fernandez-Triana | Received 8 November 2022 | Accepted 18 January 2023 | Published 17 February 2023

https://zoobank.org/78CB3D2A-FB48-40B5-A232-9840782C49E3

Citation: Ward D, Martin B (2023) Trialling a convolution neural network for the identification of Braconidae in New Zealand. Journal of Hymenoptera Research 95: 95–101. https://doi.org/10.3897/jhr.95.95964

Abstract

Computer vision approaches, such as deep learning, potentially offer a range of benefits to entomology, particularly for the image-based identification of taxa. An experiment was conducted to gauge the ability of a convolution neural network (CNN) to identify genera of Braconidae from images of forewings. A deep learning CNN was trained via transfer learning from a small set of 488 images for 57 genera. Three-fold cross-validation achieved an accuracy of 96.7%, thus demonstrating that identification to genus using forewings is highly predictive. Further work is needed to increase both the coverage to species level and the number of images available.

Keywords

Braconidae, computer vision, diagnositics, identification, model

Introduction

Insect populations are challenging to study. One of the main problems is the identification of species, particularly in hyper diverse groups such as Hymenoptera, and because knowledge of biodiversity around the world is uneven (Amano and Sutherland 2013; Hoye et al. 2020). However, advances in computer vision approaches provide potential new solutions to this global challenge (Hoye et al. 2020; Greeff et al. 2022). Computer vision approaches, such as machine learning and deep learning, are currently

influencing a wide range of scientific disciplines but are only relatively recently being applied to entomology (Boer and Vos 2018; Marques et al. 2018; Hansen et al. 2019).

Recent studies on image-based insect identification are showing that deep learning models can extract features from images and learn to differentiate species to an accuracy approaching, or exceeding, human expertise (Valan et al. 2019; Hoye et al. 2020). For example, over half of British ground beetles (Carabidae) can be identified to species, and 74% to genus using convolutional neural networks trained on an image set of over 19,000 images (Hansen et al. 2019). Boer and Vos (2018) used over 10,000 images from AntWeb (www.antweb.org) (to classify ants at species level based on dorsal, head, and profile images. Accuracy of identification was between 62–92% for species and 79–95% for genus, depending on different configurations of the models. Marques et al. (2018) also examined the classification of ants, and achieved an accuracy of 80–90%, demonstrating that high confidence and robustness in ant genera identification can be achieved.

Further to identification and diagnostics, the use of images is also being combined with additional automation and/or robotics to undertake sampling in the field, routine laboratory sample processing, or extracting data from images (Ärje et al. 2020). For example, Bjerge et al. (2021) have developed an automated light trap to monitor moths and identify the species using computer vision-based tracking and deep learning. An automated field trial over 48 nights captured more than 250,000 images, an average of 5675 images per night, with a high validation score for the identification of the 8 most common moth species. Machine learning methods have been used to automate the extraction of data on insect herbivore damage from plant specimens in museums, including the ability to identify different types of herbivores (Meineke and Davies 2018).

In this paper, we test the ability of a convolutional neural network to classify genera of Braconidae that are present in New Zealand using images of the forewing.

Methods

Specimens

All specimens are from the New Zealand Arthropod Collection (**NZAC**), where the family Braconidae is well curated with almost all specimens (~18,000) sorted to at least genus level. However, relatively few endemic or native species have been described (Berry 2010).

Pinned specimens were selected that represent genera of Braconidae which have been recorded from New Zealand. This includes genera which are either endemic (restricted to New Zealand); native (in New Zealand but also naturally occur elsewhere); have been accidentally introduced through human trade; or intentionally introduced for biological control.

Taxa (and the number of images) are: *Aleoides* (10); *Alysia manducator* (Panzer, 1799) (10); *Apanteles* (12); *Aphaereta aotea* Hughes & Woolcock, 1976 (8); *Aphidius colemani* Viereck, 1912 (11); *Ascogaster elongata* Lyle, 1923 (10); *Asobara persimilis* (Papp, 1977) (10); *Aspicolpus* (10); *Aspilota parecur* Berry, 2007 (8); *Austrohormius* (10);

Bracon phylacteophagus Austin, 1989 (4); *Bracon variegator* Spinola, 1808 (10); *Caenophanes* sp5 (11); *Choeras helespas* Walker, 1996 (9); *Chorebus rodericki* Berry, 2007 (10); *Cotesia* (10); *Cryptoxilos thorpei* Shaw & Berry, 2005 (10); *Dacnusa areolaris* (Nees, 1811) (10); *Diaeretiella rapae* (McIntosh, 1855) (10); *Dinocampus coccinellae* (Schrank, 1802) (10); *Dinotrema longworthi* Berry, 2007 (10); *Diolcogaster* (10); *Dolichogenidea tasmanica* (Cameron, 1912) (10); *Doryctomorpha antipoda* Ashmead, 1900 (10); *Eadya daenerys* Ridenbaugh, 2018 (2); *Eubazus* (10); *Glyptapanteles* (10); *Habrobracon hebetor* (Say, 1836) (10); *Kauriphanes* (6); *Kiwigaster variabilis* Fernandez-Triana & Ward, 2011 (9); *Lysiphlebus testaceipes* (Cresson, 1880) (5); *Macrocentrus rubromaculata* (Cameron, 1901) (10); *Metaspathius* (7); *Meteorus pulchricornis* (Wesmael, 1835) (9); *Microctonus hyperodae* Loan, 1974 (9); *Microplitis* (10); *Monolexis fuscicornis* Förster, 1862 (3); *Neptihormius* (10); *Notogaster charlesi* Fernandez-Triana & Ward, 2020 (10); *Ontsira antica* (Wollaston, 1858) (10); *Opius* sp2 (10); *Pauesia nigrovaria* (Provancher, 1888) (8); Pholetesor (5); *Pronkia* sp4 (9); *Pseudosyngaster pallidus* (Gourlay, 1928) (10); *Rasivalva* (2); *Rhyssaloides* (9); *Sathon* sp1 (7); *Schauinslandia* (10); *Shireplitis bilboi* Fernandez-Triana & Ward, 2013 (2); *Shireplitis frodoi* Fernandez-Triana & Ward, 2013 (3); *Spathius exarator* (Linnaeus, 1758) (10); *Syntretus* (10); *Taphaeus* (10); *Therophilus* (5); *Trioxys* (10); *Venanides* (10); and *Xynobius* (10).

Some genera were not included because they are wingless, have very reduced wings, or there was an insufficient number of specimens.

An attempt was made to get 10 specimens from each genus. However, this was not always possible. The average number of forewings removed from a genus was 8.6 (range 5–12, median 10). To remove wings, a specimen was placed in a specimen manipulator and a micropin was used to gently move the tegula up and down until the forewing fell off. Wings were not 'pulled' because the membrane rips easily. Static electricity meant the wing stuck to the micropin and forceps, making it easy to put into a gelatin capsule. After all wings had been removed, wings were slide mounted with Euparal.

The specimen records, all images (zip folder), and one representative image of each genus are freely available via the datastore repository (https://doi.org/10.7931/xftx-6w25).

Imaging and image preparation

Images of the slide mounted wings were taken on a Nikon MZS25 scope with a Nikon DS-Ri2 camera (16.25 megapixels). There was no photo stacking. Images were cropped and edited using Adobe Photoshop (Fig.1A).

The following pre-processing corrections were applied to each image (Fig. 1B) so that the convolutional neural network focused on diagnostic features and not on irrelevant differences between the images (such as aspect ratios, colour balance, etc.): 1) colour converted to grayscale; 2) blurred to reduce image grain noise; 3) brightness and contrast standardised (to mean = 0.5, contrast range = -2 standard deviations to + 2 standard deviations, with the extreme values clipped); 4) aspect 'squashed' to be a square and down sampled to 299 by 299 pixels to match the network input filter size.

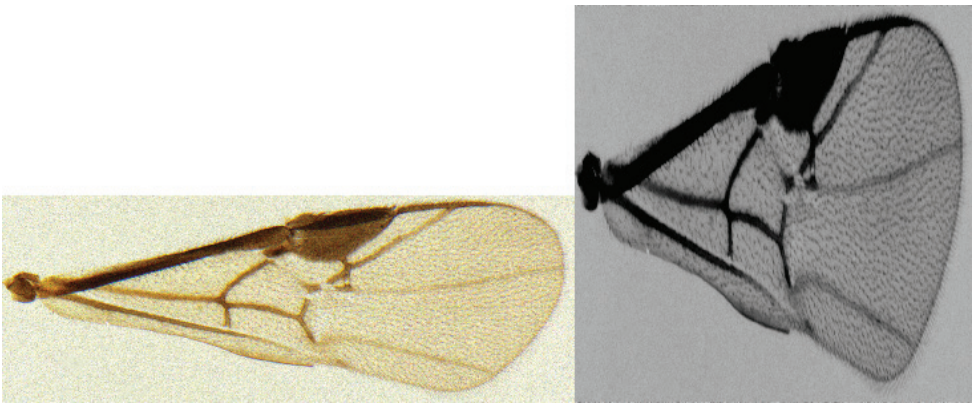

Figure 1. Examples of images: slide mounted forewing (left) and image with pre-processing corrections (right).

A few images were excluded from analyses as they had become ripped during the slide mounting process or were deemed poor quality (colouration, debris on wing) which was not spotted when wings were initially removed.

Model training and validation

Transfer learning was used to train an Xception network that had been initially trained on the Imagenet image set (www.image-net.org). The total number of images were split into three sets (folds) of 2/3 train, 1/3 test, via stratified round-robin cross-validation. The fully connected classification layers were trained for 200 epochs, followed by a further 200 epochs fine-tuning of all parameters. The learning rate was fixed at 0.0001 and the ADAM optimiser used to automatically adjust the update magnitude; this scheme resulted in a very smooth learning curve for this dataset that plateaued at around 200 epochs, reducing the need for validation sets to determine the optimal cut-off. Images were randomly augmented during training to reduce the chance of overfitting and to allow for variations in image conditions that may arise in future cases. Augmentation was conservative because the images were quite highly standardised. The augmentations used were (randomly shift the image up to 10% horizontally and vertically; randomly zoom the image up to +/-10%; randomly rotate the image up to +/-25 degrees).

Results and discussion

A total of 488 wings were used representing 57 genera. Results from cross-validation gave an overall accuracy of 96.7% (472/488; Table 1). Of the 16 misclassified images, 14 images had low confidence scores (<0.9), indicating the network struggled to classify them (Table 2), many of these are from the subfamily Microgastrinae.

Table 1. Accuracy of cross-validation runs on correct predictions to genus.

Cross-validation	Number of correct images / Total images	Percent accuracy
1	182/188	96.81%
2	152/156	97.44%
3	138/144	95.83%

Table 2. List of errors where the correctly identified image was incorrectly predicted. Scores represent the confidence of the model that the prediction is correct. Sorted by highest score.

Catalog number	Correct	Predicted	Confidence score
NZAC02012114	*Glyptapanteles*	*Dolichogenidea*	0.997
NZAC02011921	*Doryctomorpha*	*Caenophanes*	0.964
NZAC02012115	*Glyptapanteles*	*Sathon*	0.765
NZAC02011668	*Aphaereta*	*Asobara*	0.65
NZAC02012085	*Shireplitis*	*Venanides*	0.649
NZAC02012113	*Glyptapanteles*	*Dolichogenidea*	0.597
NZAC02012063	*Pholetesor*	*Sathon*	0.567
NZAC02012084	*Shireplitis*	*Venanides*	0.56
NZAC02012039	*Sathon*	*Glyptapanteles*	0.545
NZAC02011790	*Caenophanes*	*Doryctomorpha*	0.535
NZAC02011933	*Neptihormius*	*Metaspathius*	0.525
NZAC02012117	*Glyptapanteles*	*Dolichogenidea*	0.508
NZAC02011792	*Caenophanes*	*Doryctomorpha*	0.497
NZAC02012038	*Sathon*	*Shireplitis*	0.471
NZAC02012088	*Shireplitis*	*Venanides*	0.395
NZAC02011984	*Aleoides*	*Doryctomorpha*	0.293

This small experiment demonstrated that forewings appear to be highly predictive of genus level identifications. The model accuracy is particularly impressive given the very small number of images. Often hundreds or even thousands of images are needed to build these models. For example, Hansen et al. (2019) had a set of over 19,000 images for ground beetles (Carabidae), and Boer and Vos (2018) used over 10,000 images from AntWeb. We suggest our trial was successful because the forewing morphology (veins/cells) are already recognised as key diagnostic characters for Braconidae, and the images of a forewing are quite simple with considerably less 'noise' than dorsal and lateral habitus images of an insect body (Valan et al. 2019).

Two main questions need to be addressed in future work. Firstly, how well does only one species (or morphospecies) represent a genus. Several of the genera above are monotypic, and for some genera the forewing morphology will differ very little between species, but for genera with higher species diversity this condition is unlikely to hold. However, this was an initial trial of the technology, and as the number of species-level image sets increases then genus-level identification becomes less relevant. Secondly, how well will the model perform when additional species or genera are added. An increase in the number of 'classes' (taxa) will likely increase the morphological variability in the dataset, perhaps affecting model accuracy and consequently needing

more source images to overcome (Greener et al. 2021). A related issue is the level of image standardisation required. The images used in this study were all photographed and processed with the same equipment setup; adding subsequent imagery (either for the same taxa or novel ones) has the potential to cause the model to focus on spurious photographic differences during training. Similarly, the model has only been tested on images held out from the same set; how well it performs on other image sets (for the same taxa) needs to be tested to determine how well the model 'transfers' to novel image sets and whether further refinement of the training process is required, such as more aggressive image pre-processing and augmentation or the inclusion of more images.

Machine learning tools, particularly convolutional neural networks (CNNs), are fast becoming a valuable tool for the identification of insects (Valan et al. 2019; Hoye et al. 2020). Identifications are a vital part of making insects visible and accessible (Greeff et al. 2022). An increase in the number and level of taxa being identified offers many benefits, including accelerating the discovery and increasing the awareness of a greater proportion of biodiversity, providing informed information for applications such as other academic research, conservation, and biosecurity, and may free time for more research tasks.

At present, the major hurdle is the shortage of images (Valan et al. 2019; Greeff et al. 2022), particularly many images of the same species, rather than just one representative photo for a publication. Digitization efforts are underway in many countries that involve taking images of specimens, and large image libraries are available such as those on iNaturalist and for specific taxa (e.g., Antweb, www.antweb.org), however, these will not always cover, or be suitable for, every taxonomic group. Although the above model has been built for use in New Zealand, from a distinct set of genera of which several are endemic, the images from this project could be used for Braconidae in other countries or regions, albeit with very careful interpretation of the results. Consequently, it is vital that researchers facilitate sharing and exchange of their images (Valan et al. 2019), and that collaborative and user-friendly software be developed (Greeff et al. 2022).

Acknowledgements

Funded by the Ministry of Business, Innovation and Employment (MBIE) through the Strategic Science Investment Fund (SSIF) for Nationally Significant Collections and Databases (NSCDs) at Manaaki Whenua-Landcare Research (MWLR) via the Biota Portfolio (BIO) within the Research Priority Area (RPA) for Collections and Databases. Thanks to S. Malysheva for slide mounting the wings. Many thanks to the taxonomic experts who have identified specimens and worked on the New Zealand Braconid fauna over the years, especially to Sergey Belokobylkij, Donald Quicke, and Jose Fernandez-Triana.

Funded by the Ministry of Business, Innovation and Employment (MBIE) through the Strategic Science Investment Funding (SSIF) for Nationally Significant Collections and Databases.

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JHR 95: 103–112 (2023)
doi: 10.3897/jhr.95.97029
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Taking care of the enemy: egg predation by the Darwin wasp *Tromatobia* sp. (Ichneumonidae) on the cobweb spider *Chrysso compressa* (Araneae, Theridiidae)

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Academic editor: Gavin Broad | Received 1 November 2022 | Accepted 16 January 2023 | Published 17 February 2023

https://zoobank.org/582B83A0-E065-4B11-A519-EF02D797FBAC

Citation: Souza-Santiago BK, Messas YF, de Pádua DG, Santos AJ, Vasconcellos-Neto J (2023) Taking care of the enemy: egg predation by the Darwin wasp *Tromatobia* sp. (Ichneumonidae) on the cobweb spider *Chrysso compressa* (Araneae, Theridiidae). Journal of Hymenoptera Research 95: 103–112. https://doi.org/10.3897/jhr.95.97029

Abstract

Some wasp species use spiders as food resources, overcoming several anti-predator barriers that are exerted by spiders. *Tromatobia* ichneumonid wasps are spider egg predators that usually attack Araneidae species, although there are few records of predation on Clubionidae, Philodromidae, Linyphiidae, Tetragnathidae, and Theridiidae spiders. Here, we describe the interaction between *Tromatobia* sp. and *Chrysso compressa*, a subsocial theridiid spider that exhibits extended maternal care, in the Atlantic Forest of southeastern Brazil. We observed that the larva of *Tromatobia* sp. develop inside the egg sacs of *C. compressa*, preying on the entire egg mass and building cocoons that change the color and morphology of the egg sacs. Despite these structural modifications, we registered an adult female of *C. compressa* guarding and caring for the cocoons (attacked egg sac) of the predators as if they were offspring (non-attacked egg sac). To the best of our knowledge, this study represents the first record of *Tromatobia* preying on *Chrysso* eggs.

Keywords
egg sac, maternal care, Pimplinae, Serra do Japi

Introduction

Wasps are important natural enemies of spiders and adopt several foraging strategies to subdue their prey (Rayor 1996). For example, some wasp species act exclusively as koinobiont ectoparasitoids (polysphinctine wasps *sensu* Gauld & Dubois, 2006) or predators (spider-hunting wasps – Crabronidae, Pompilidae and Sphecidae; Mayr et al. 2020) of juvenile, sub-adult, or adult spiders. Some other wasp species are specialized in attacking and parasitizing individual eggs (e.g., *Baeus* Austin, 1985) or consuming part of the egg mass of spiders (e.g., *Tromatobia* Gauld et al., 2002). Consequently, spiders have evolved anti-predator mechanisms that prevent wasp attacks.

Spiders can minimize predation risk by avoiding detection, recognition, and access to predators by using several defensive cues and behaviors (Pekár 2013; Gawryszewski 2017). The anti-predator strategies of spiders include crypticity, mimicry, construction of three-dimensional webs (Blackledge et al. 2003) and refuges, presence of thick silk barriers in egg sacs, extended parental care, and, in some extreme cases, group living and sociality (Pekár 2013; Gawryszewski 2017). Understanding how wasps overcome these barriers and succeed in capturing spiders is an interdisciplinary research topic that combines studies on ecology, evolutionary behavior, physiology, and phylogeny.

Darwin wasps of the genus *Tromatobia* Foster, 1869 are specialized in attacking aerial-web building spiders (Fitton et al. 1987, 1988; Finch 2005; Yu et al. 2016; Broad et al. 2018), completing their larval life cycle inside spider egg sacs, and consuming part or the entire egg mass (Austin 1984, 1985; Villanueva-Bonilla et al. 2016). There is high interspecific variation in the number of eggs, from one to 14, that *Tromatobia* females can lay inside spider egg sacs (Nielsen 1923). This variation also occurs within the same species, as *Tromatobia blancoi* Gauld, 1991 can produce six-nine eggs per egg sac of the spider *Araneus thaddeus* (Hentz, 1847) (Jiménez 1987). Although rare, most reports of *Tromatobia*-spider interactions involve Araneidae species of the genera *Araneus*, *Araniella*, *Argiope*, *Cyclosa*, and *Zygiella* (e.g., Nielsen 1923; Jiménez 1987; Fitton et al. 1988; Quicke 1988; Oehlke and Sacher, 1991; Cortés et al. 2000; Sobczak 2012), indicating fine specialization for this family. However, there are a few records of *Tromatobia* attacking Clubionidae, Philodromidae, Linyphiidae, Tetragnathidae, and Theridiidae species (e.g., Austin 1985; Fitton et al. 1988; Oehlke and Sacher 1991; He et al. 1992). In fact, knowledge about the biology, ecology, and behavior of interactions with these minor hosts is scarce and requires further field observations and experimental studies.

Herein, we report the interaction between the egg predator wasp *Tromatobia* sp. (Ichneumonidae) and the cobweb spider *Chrysso compressa* (Keyserling, 1884) (Theridiidae) in the Brazilian Atlantic Forest, with notes on the behavior of the *C. compressa* guarding the predator's cocoon.

Materials and methods

Study species

Chrysso compressa belongs to a genus with 64 valid species distributed mainly in America and Asia (Levi 1957; World Spider Catalog 2022). Some of these species are known to exhibit subsocial behavior with extended maternal care (Miller and Agnarsson 2005; Yip and Rayor 2014). Such behavior involves a temporary period of coexistence of offspring in the maternal nest, when spiderlings cooperate in prey capture, feeding, and web maintenance before they disperse and live solitarily (Lubin and Bilde 2007). *Chrysso compressa* occurs in the Brazilian Atlantic Forest, where it constructs irregular webs on shrubby vegetation, and females care for their offspring until the juvenile stage (Santiago 2022). Adult spiders build refuges using two green leaves that are connected by silk, thereby forming a roof-like structure, which shelters the female and its offspring under the abaxial surface of the leaves (Fig. 1A, B). After dispersion from the nest, the juvenile spider moves to the adaxial surface of another leaf and takes refuge by connecting the side of edges of the leaf using silk threads (Fig. 1C, D).

Tromatobia sp.

The undetermined *Tromatobia* differs from the Costa Rican species (see Gauld et al. 1998), the South American *T. lineiger* Morley, 1914 (digital images analysed), and *T. huebrichi* (Brèthes, 1913) (see Porter 1979) by the combination of the following character states: pronotum without a small shelf-like projection; small ovipositor, 1.0–1.1 times as long as hind tibia; mesosoma reddish and metasoma blackish with thin white strips on the posterior margin of tergites. It was not possible to make a detailed comparison of most South American species (except *T. lineiger* and *T. huebrichi*) because the descriptions are old and succinct, based mainly on coloration. So we prefer to proceed with caution and leave it as an indeterminate species.

Study area

We conducted our study near the Base de Estudos de Ecologia e Educação Ambiental da Serra do Japi, Jundiaí, São Paulo, Brazil (1000 m above sea level; 23°13'53"S, 46°56'09"W), where there is a well-established and easily accessible population of *C. compressa* (Santiago 2022). The site is an environmentally protected area that constitutes one of the few remnants of the Atlantic Forest in southeastern Brazil. The reserve has a predominantly semi-deciduous mesophyll forest with distinct phytophysiognomies along an altitudinal gradient from 700 to 1300 m above sea level (Leitão-Filho 1992). The climate is CWA in Köppen's classification (Alvares et al. 2013), which is characterized by hot/rainy summers and cold/dry winters with average monthly temperatures ranging from 13.5 °C in July to 20.3 °C in January (Pinto 1992). Serra do Japi is a hotspot for spider-wasp interactions and is one of the most studied areas with respect to these interactions in the Neotropical region (Gonzaga et al. 2017).

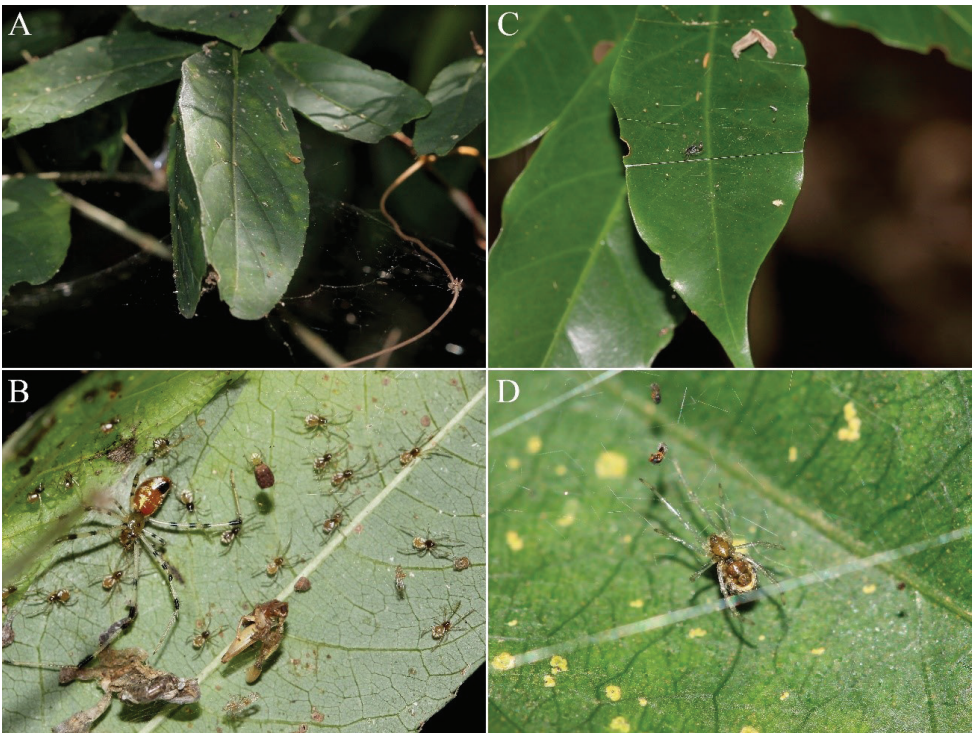

Figure 1. A upper view of the refuge of leaves constructed by subadult and adult individuals of *C. compressa* **B** adult female of *C. compressa* and the offspring (3° and 4° instar spiders) within the refuge under the abaxial surface of the leaves **C** refuge of juvenile spiders on the adaxial leaf surface **D** post-dispersion juvenile of *C. compressa* on its refugee. Photos: Brenda Santiago.

Field observations and data collection

One of us (first author BKSS) conducted monthly inspections from April 2021 to March 2022 to collect adult females and egg sacs of *C. compressa* on shrubby vegetation along forest edges and ecological trails in the study area. Each inspection consisted of visual searches with a sampling effort of four to five hours during the daytime (09:00 to 13:00). We maintained the female spiders we found and their respective egg sacs in the laboratory inside individual plastic pots containing pieces of cotton soaked in a liquid nutrient solution. We then recorded traits of non-attacked and attacked egg sacs (e.g., color, shape, number of eggs, and number of wasp cocoons), in addition to biological and behavioral data on maternal care performed by *C. compressa*. We also recorded under laboratory conditions the behaviors of one adult female spider guarding its own egg sac (hereafter "native") attacked by *Tromatobia* sp., and after we offered an attacked egg sac acquired from another spider in the field (hereafter "alien"). We fixed the adult wasps that emerged from the cocoons in 70% alcohol for subsequent identification. To obtain digital images of adult wasps and pupa, we used a Leica DMC4500 digital

camera attached to a Leica M205A stereomicroscope and stacked multiple layers using the software Leica Application Suite V4.10.0.

We deposited wasp voucher specimens in the Invertebrate Collection of Instituto Nacional de Pesquisas da Amazônia (curator J. A. Rafael) and spiders in the arachnid collection of the Taxonomic Collections of Universidade Federal de Minas Gerais (curator A. J. Santos).

Results

We collected 22 egg sacs of *C. compressa* (N = 6 in January, N = 10 in February, N = 4 in March, N = 1 in April, and N = 1 in May), of which five (22.7%) were attacked by *Tromatobia* sp. Healthy egg sacs harboured an average of 46 ± 21 eggs (N = 17), were light in color, round-shaped (diameter = 10.1 ± 3.7 mm, N = 17), and were usually located under the bodyguard of the adult female (Fig. 2A). The morphology of egg sacs that were attacked by *Tromatobia* sp. was found to be altered after the development of wasp larva; they were elongated and oval (average total length = 9.1 ± 1.2 mm and average total width = 2.5 ± 0.9 mm, N = 3), with the shape of cocoons adhered to each other longitudinally, on their longer sides. At this stage, the wasp cocoons, grey or brownish in color (Fig. 2B) became exposed and interspersed with a few silk remnants of the original egg sac (Fig. 2C). The attacked egg sacs that were collected in the present study contained two (N = 3) or three (N = 1) cocoons of *Tromatobia* sp. Adult wasps emerged through holes with approximately 2 mm in diameter located in the apical portion of the cocoon (Fig. 2D). In total, we obtained nine adult individuals of *Tromatobia* sp. (Fig. 2E), five females (average body length = 7.3 ± 0.9 mm) and four males (body length = 6.6 ± 0.8 mm).

We observed that the maternal care provided by *C. compressa* included the protection of egg sacs under the female body, between the forelegs and held by the pedipalps. We also registered the same behavior of females protecting the cocoons of *Tromatobia*, even in the case when predators had already fed on the entire egg mass (Fig. 2B). In addition, we observed that the spider we offered an alien egg sac adopted and attached it to the native egg sac. The spider bodyguarded both egg sacs containing wasp cocoons until an adult wasp emerged from the native egg sac. At this time, we removed the female spider and left the alien egg sac alone and undisturbed for 68 days; however, adult wasps did not emerge from this egg sac. Then we opened it carefully and found a cocoon with a dead male adult wasp almost fully-developed inside (Fig. 2F).

Discussion

To the best of our knowledge, this is the first report on an interaction between *Tromatobia* and *Chrysso*. Previous studies have indicated a strong affinity of *Tromatobia* for araneid orb-web spiders, with a few unusual records of attacks on other families

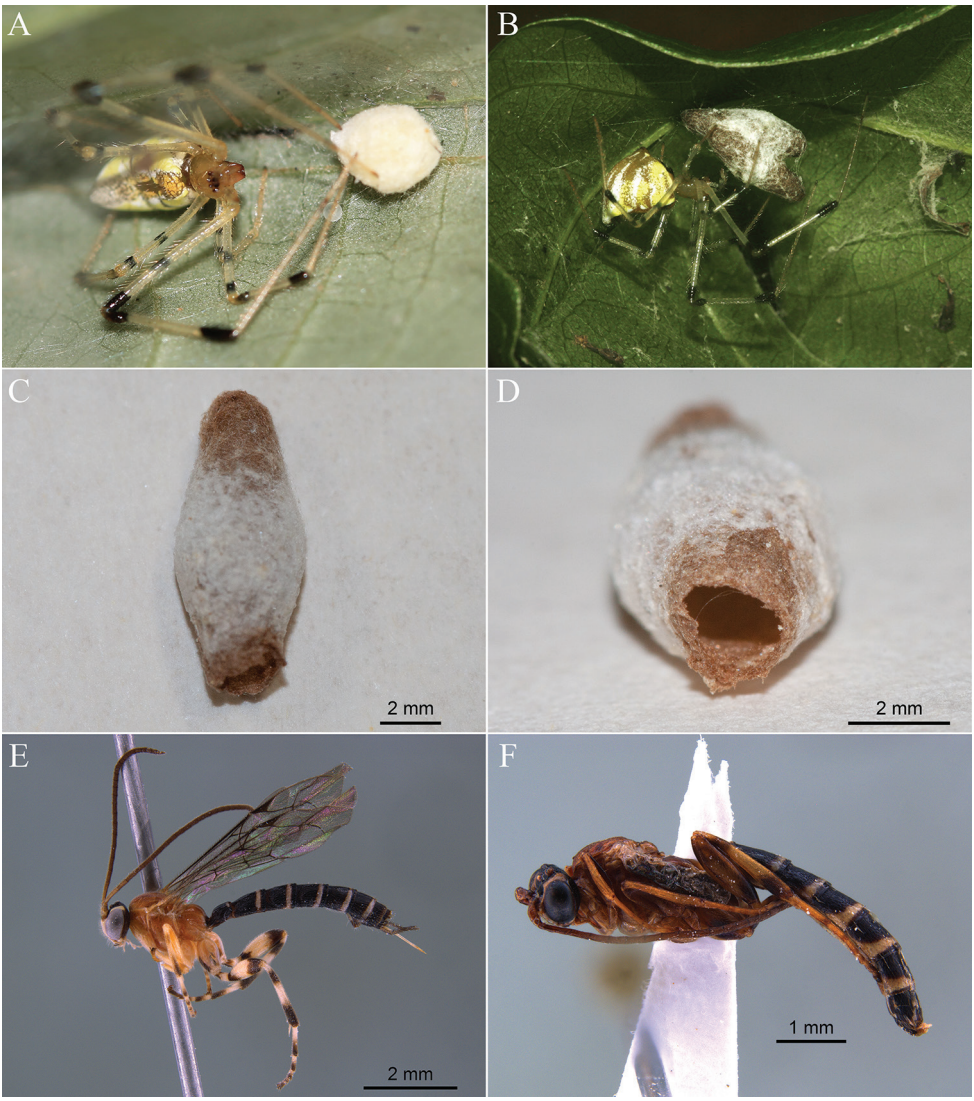

Figure 2. A adult female of *C. compressa* taking care of the egg sac **B** adult female of *C. compressa* taking care of cocoons of the egg predator wasp *Tromatobia* sp. **C** cocoon of the egg predator wasp *Tromatobia* sp. **D** cocoon hole through which adult wasps of *Tromatobia* sp emerge. **E** lateral view of an adult female of *Tromatobia* sp., and **F** lateral view of an adult wasp that did not emerge from the cocoon. Photos: Brenda Santiago and Diego Pádua.

including Theridiidae that construct three-dimensional webs. However, some records are rather poorly documented and potentially unreliable. Thus, our report is probably the best-documented record of *Tromatobia* parasitising non-araneid spiders. We showed that *Tromatobia* wasps could overcome anti-predator barriers and affect more than 20% of *C. compressa* egg sacs. Unlike the case of egg parasitoid species, in which

each wasp attacks an individual spider egg, *Tromatobia* sp. consumes the entire egg mass and affects the spiderling population to a greater extent. We recorded two to three cocoons per egg sac, similar to the observation made by Sobczak et al. (2012) for the sympatric orb-web spider *Araneus omnicolor* Keyserling 1893. However, other studies have reported instances where up to 14 individuals were observed per egg sac in *Tromatobia* species (e.g., Nielsen 1923; Jiménez 1987). Hence, is still unknown whether there is an optimal number of eggs and whether and how larvae compete for food resources.

Behavioral manipulations of hosts induced by parasitoid species have been well-studied in the last few years (Weinersmith 2019). In cases involving spiders and wasps, parasitoid species can alter host behavior by inducing the construction of modified webs that are used to protect the cocoon, thereby maximizing survival (Eberhard 2000, 2010; Gonzaga et al. 2017). However, *Tromatobia* sp. does not change the behavior of *C. compressa* but takes advantage of bodyguarding, a pre-existing behavior of maternal care. Although there is an evident morphological change in the egg sac, the wasp may use concealment mechanisms, such as chemical or tactile camouflage (Kaminski et al. 2020), to deceive adult spiders. We observed that the wasp of the alien egg sac that was adopted by *C. compressa* in the laboratory did not emerge from the cocoon after we removed the female spider. This anecdotal report suggests that the presence of adult females may be important for wasps. Hence, the effect of bodyguarding behavior on wasp survival should be further investigated in future studies.

Egg protection is crucial for the survival of spider progenies given the high diversity of selective pressures exerted by multiple predators and parasitoids (Bristowe 1971; Li et al. 1999; Yip and Rayor 2014). The behavior of many spider species that keep their egg sacs suspended, for example, is a strategy to avoid attack by wandering generalist predators, such as ants (Turnbull, 1973), but using this strategy can increase the conspicuousness of egg sacs to visually oriented predators (e.g., birds and wasps, Hieber 1992). Instead, egg sacs of *C. compressa* are usually under female guard within the refuge, and attacks may occur during foraging when females move to the web to catch prey. However, it is still unknown how *Tromatobia* sp. adults overcome the barriers of *C. compressa* in detecting, reaching, and successfully attacking egg sacs. In this way, further investigation should focus on identifying specific behaviors performed by the wasps and cues provided by spiders that facilitate egg predation.

Acknowledgements

This study was financially supported by the Instituto Nacional de Ciência e Tecnologia dos Hymenoptera Parasitoides da Região Sudeste Brasileira (HYMPAR/Sudeste – CNPq/FAPESP/CAPES) and CAPES, Finance Code 001 (Grant Number 88887.513500/2020-00 to BKSS and grant n° 88887.372005/2019-00 to DGP). We thank the Serra do Japi Foundation and the Postgraduate Program in Animal Biology of the University of Campinas for their structural and logistic support for this research, and the Invertebrate Collection of Instituto Nacional de Pesquisas da

Amazônia (INPA) for the use of imaging equipment. We thank Editage (www.editage.com) for the English language editing. We thank the reviewers Dr Keizo Takasuka, Dr Tamara Spasojevic, and the editor Dr Gavin Broad for the corrections and suggestions in the text. We also thank Dr Broad for sending the photographs of the holotype of *Tromatobia lineiger.*.

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JHR 95: 113–127 (2023)
doi: 10.3897/jhr.95.96903
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Key to the species of the genus *Subancistrocerus* de Saussure, 1855 (Hymenoptera, Vespidae, Eumeninae) from China with description of a new species

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Academic editor: Michael Ohl | Received 31 October 2022 | Accepted 16 January 2023 | Published 17 February 2023

https://zoobank.org/EC59F6B7-60A4-4D3E-B5DA-C0AEE2174300

Citation: Tan J-L, Wang M, Xu H, Tang Y, Liu Y (2023) Key to the species of the genus *Subancistrocerus* de Saussure, 1855 (Hymenoptera, Vespidae, Eumeninae) from China with description of a new species. Journal of Hymenoptera Research 95: 113–127. https://doi.org/10.3897/jhr.95.96903

Abstract

A newly discovered species, *Subancistrocerus clypeatus* **sp. nov.**, from China (Zhejiang) is described and illustrated. In addition, *Subancistrocerus kankauensis* (Schulthess-Rechberg) is redescribed and photographed after studying the type series. A key to the genus *Subancistrocerus* de Saussure, 1855 from China is presented. The distribution of this genus is briefly discussed.

Keywords

China, key, new species, redescription

Introduction

The small genus *Subancistrocerus* de Saussure, 1855, belonging to the subfamily Eumeninae (Hymenoptera: Vespidae), consists of 33 extant species and subspecies up to date, but mainly found in Oriental Region with 27 species and subspecies (Giordani Soika 1994; Gusenleitner 2000; Carpenter et al. 2010; Kumar 2013; Li and Chen 2014). Among them, most species are scattered in the Pacific Island countries such as Philippines, Indonesia, Malaysia, Palau etc. This genus can be easily recognized by the

characteristics as follows: small sized body, a pair of indistinct cephalic fovea, anterior face of pronotum with two separated foveae medially, a pair of prescutellar longitudinal grooves, tegula broad, smooth and shiny, tergum 1 (T1) wider than long in dorsal view with two transverse carinae close each other at crest of declivity. Giordani Soika (1994) included a key to the species of *Subancistrocerus* from the Oriental region and the juncture area with the Australian region in his revision together with a few line-drawings. However, the taxonomic research on the genus is quite complicated because of lacking figures and the limited illustrated characters. Schulthess-Rechberg (1934) reported the first species of *Subancistrocerus* named *S. kankauensis* from China (Taiwan) in the key with a limited description mostly about color pattern and without any figures. Although few species were reported mainly separated on basis of comparation with *S. kankauensis* (Giordani Soika 1994; Gusenleitner 2000; Li and Chen 2014), the illustration on *S. kankauensis* is still poor up to date. Yeh and Lu (2007) recorded the second species also from China (Taiwan) with a color photo of the habitus of *S. sichelii*. Kumar (2013) provided few pictures when reporting three species from India. Subsequently, Li and Chen (2014) recorded five species in China, however, only with pictures and descriptions on the two new species. Herein, we redescribe *S. kankauensis* (Schulthess-Rechberg, 1934) after checking the types preserved in SDEI (Müncheberg, Germany), and report a new species discovered from Zhejiang, China. A key to the species of this genus from China is provided. The distribution pattern of the genus is briefly discussed.

Materials and methods

Observations, descriptions and photographic images were made with an Opto-Edu A230903 stereomicroscope and a fluorescent lamp or with the Keyence VHX-5000 digital microscope and processed with Adobe Photoshop CS5, mostly to adjust the size and background. For the identification of the genus, Tan et al. (2018) and Giordani Soika (1994) were used. The research specimens are deposited in the collections of the College of Life Sciences, Northwest University, Xi'an, Shaanxi, China (**NWUX**) and the Senckenberg Deutsches Entomologisches Institut, Müncheberg (**SDEI**).

A key to the species of the genus *Subancistrocerus* de Saussure from China

1 Forewing without a brown cloudy patch along the costal margin; tergum 1 longer, at most 1.4× wider than long in dorsal view; black, with white or pale-yellow patches and bands. Male: Basal tarsomere of the mid-leg straight, cylindrical and long, almost 7× longer than wide. [widely spread in Oriental and present in Afrotropical region; China (Sichuan, Yunnan, Taiwan)] ..... ***S. sichelii* (de Saussure)**

– Forewing with a brown cloudy patch along the costal margin; black with predominantly brownish yellow patches and bands; tergum 1, about 1.5× wider

than long. Male: Basal tarsomere of the mid-leg of relatively short, arched and depressed, at most 5.0× longer than wide..... **2**

2 Tergum 2 densely punctate with large and coarse punctations; at least basally and laterally punctures almost as wide as those of the pronotum or meso-scutum. Male: The large antennal concavity begins in antennal article A10 or A11..... **3**

– Tergum 2 finely punctate with small and sparse punctures, much smaller than those on the pronotum or meso-scutum. Male: The large antennal concavity begins in A8. [distributed in Oriental region; China (Sichuan, Chongqing, Yunnan)] ..... ***S. camicrus*** **(Cameron)**

3 Apical band of T3 as wide as T1. Clypeus of female entirely yellow except margin, without black spots; apical margin weakly emarginate (nearly truncate). Male: Apical margin of clypeus truncate; only antennal articles A11 and A12 largely and very deeply concave. [China (Jiangxi, Taiwan)]..... ..... ***S. kankauensis*** **(Schulthess-Rechberg)**

– Apical yellow band of T3 much narrower than T1 or absent. Clypeus of female with a black spot medially, apical margin emarginate. Male: Apical margin of clypeus emarginated; antennal article begins in antennal article A10 or A11..... **4**

4 Female: Clypeus medially with wider and rounder longitudinal depression. Male: Antennal articles A10 to A12 concave, A13 gradually sharper apically, slightly curved finger shaped, about 2.1× as long as wide, with its apex reaching base of A10. Fore femur normal basally; basal tarsomere of midleg much shorter than following segments together, about 2.7× as long as wide. [China (Yunnan)] ..... ***S. jinghongensis*** **Li & Chen**

– Female: Clypeus medially with narrower longitudinal depression. Male: Antennal articles A11– A12 deeply concave; A13 roughly oval shaped, 1.7× as long as wide, with its apex reaching base of A11. Fore femur depressed basally; basal tarsomere of midleg about 5× as long as wide **5**

5 Apical yellow band of T1 gradually narrowed towards lateral sides; apical yellow band of T3 absent; pronotal spots relatively large, triangular and contiguous; clypeus of female wider than long, mainly yellow, with two roughly longitudinal carinae sub-medially, and with an irregular black spot medially; Male: Clypeus as wide as long; all tibiae black, at most with a whitish yellow elliptic spot. [China (Yunnan)] ..... ***S. compressus*** **Li & Chen**

– Apical yellow band of T1 parallel-sided, also laterally; a narrow apical yellow band of T3 present; pronotal spots irregular, small and separated; clypeus of female as wide as long, black and densely punctate laterally, with two sinuate carinae sub-medially, area between carinae yellow, smooth and shiny with a transversely black and weakly reticulate-punctate spot medially. Male: Clypeus about 1.1× longer than wide; all tibiae yellow, at most with a black spot. [China (Zhejiang)] ..... ***S. clypeatus*** **sp. nov.**

Table 1. List of *Subancistrocerus* spp. recorded from different countries.

Region	Country	Species name
Oriental	Philippines	*S. abdominalis* Giordani Soika, 1994; *S. angulatus* Giordani Soika, 1994; *S. bambogensis* Giordani Soika, 1981; *S. domesticus* (Williams, 1928); *S. similis negrosensis* Giordani Soika, 1994; *S. similis similis* Giordani Soika, 1994; *S. spinithorax* Giordani Soika, 1994
	Indonesia	*S. angulicollis* Giordani Soika, 1994; *S. clavicornis* (Smith, 1859); *S. imbecillus* (de Saussure, 1852); *S. obiensis* Giordani Soika, 1994; *S. thalassarctos* (Dalla Torre, 1889)
	Malaysia	*S. camicrus* (Cameron, 1904); *S. giordanii* Castro, 1993; *S. sichelii* (de Saussure, 1855)
	Borneo	*S. spinicollis* Giordani Soika, 1994
	Fed. States Micronesia	*S. yapensis* (Yasumatsu, 1945)
	China	*S. camicrus* (Cameron, 1904); *S. compressus* Li & Chen, 2014; *S. jinghongensis* Li & Chen, 2014; *S. kankauensis* (Schulthess-Rechberg, 1934); *S. sichelii* (de Saussure, 1855); *S.clypeatus* Tan, sp. nov.
	India	*S. camicrus* (Cameron, 1904); *S. reflexus* Giordani Soika, 1994; *S. sichelii* (de Saussure, 1855); *S. venkataramani* Kumar, 2013
	Chagos Archipelago	*S. sichelii* (de Saussure, 1855)
	Nepal	*S. camicrus* (Cameron, 1904); *S. sichelii* (de Saussure, 1855);
	Thailand	*S. camicrus* (Cameron, 1904); *S. reflexus* Giordani Soika, 1994; *S. sichelii* (de Saussure, 1855)
	Myanmar	*S. camicrus* (Cameron, 1904); *S. sichelī* (de Saussure, 1855)
	Laos	*S. camicrus* (Cameron, 1904); *S. indochinensis* Gusenleitner, 2000
	Bangladesh; Cambodia; Vietnam; Singapore; Sri Lanka	*S. sichelii* (de Saussure, 1855)
	Palau	*S. esakii* (Bequaert & Yasumatsu, 1939); *S. palauensis* (Bequaert & Yasumatsu, 1939)
Australian	Australia	*S. albocinctus* Giordani Soika, 1993; *S. monstricornis* (Giordani Soika, 1941)
	Papua New Guinea	*S. clavicornis* (Smith, 1859)
	Solomon Islands	*S. solomonis gizensis* Giordani Soika, 1981; *S. solomonis solomonis* Giordani Soika, 1981
Ethiopian	Kenya	*S. budongo* (Meade-Waldo, 1915); *S. burensis* (Giordani Soika, 1935)
	Mauritius; Seychelles	*S. sichelii* (de Saussure, 1855)
	Democratic Republic of Congo	*S. budongo* (Meade-Waldo, 1915); *S. massaicus massaicus* (Cameron, 1910)
	Uganda; Zimbabwe	*S. budongo* (Meade-Waldo, 1915)
	Burundi; Malawi; South Africa; Tanzania	*S. massaicus massaicus* (Cameron, 1910)
	Gabon	*S. massaicus occidentalis* Giordani Soika, 1989; *S. budongo* (Meade-Waldo, 1915)
	Burkina Faso; Ivory Coast; Senegal	*S. redemptus* Giordani Soika, 1965

Subancistrocerus kankauensis (Schulthess-Rechberg, 1934)

Figs 1–3

Odynerus kankauensis Schulthess-Rechberg, 1934: 69.
Nortonia kankauensis: Iwata 1939: 71–72 (ethology).
Ancistrocerus kankauensis: Giordani Soika 1941: 241 (in subgenus *Subancistrocerus*).
Subancistrocerus kankauensis: Giordani Soika 1981: 170, figs 1, 2; 1994: 14 (key), 44, figs 7, 16.

Material examined. "Syntypus", 3@, Kankau (Koshun), Formosa (CHINA: Taiwan), 22.IV.1912, VI.1912, IX.1912, H. Sauter", "DEI-GISHym, 16380", "*Odynerus kankauensis* Schulthess, 1934, Schulthess det."; "2$, Taihorin (CHINA: Taiwan), 11.VI.1912, H. Sauter", "*Odynerus kankauensis* Schulthess, 1934, Schulthess det."

Diagnosis. ♀**,** Length of body (up to apex of T2) 6.5 mm with forewing 5.5 mm long. Body black with yellow to brownish yellow parts as follows (Fig. 1A): mandible except a basal black triangle and its brown translucent apex, clypeus with a brown translucent apical margin and black lateral outlines, an inter-antennal spot, scape ventrally, a small spot on the apical ocular sinus, a spot behind the eye on upper surface of temple, a pair of irregular quadrilateral patches joined on pronotum dorsally, tegula except center and outline brown translucent, parategula, contiguous metanotal spots, propodeal valvula and apical lamella of sub marginal carina of propodeum, apical outside half portion of fore femora, apex of mid- and hind femora, tarsi and dorsal tibiae and of all legs, mid and hind basitarsomere, apical bands of terga 1–3, sternite 2 and S3 medially. Brown to darkish brown as follows: pedicel and flagellar segments ventrally, legs except yellow patches mentioned previously, fourth to remaining metasomal segments. Wings hyaline with veins and pterostigma dark brown, and with a brown cloudy patch along the costal margin; body with white to yellowish brown pubescence.

Redescription. ***Head.*** Frons deeply punctate and reticulate; temple relatively small and sparsely punctate. Clypeus as long as wide, with apical margin very weakly emarginate or nearly truncate between both very indistinct projecting teeth, which is 1/3 of the maximum width of the clypeus, moderately convex, with medial depression bounded laterally by two indistinct arched carinae. The basal half of the clypeus weakly punctate, while with apical half largely smooth (Fig. 2A, B). Antenna short and clavate (Fig. 1B), length of the first flagellar segment F1: width = 5.5: 4.1, F2 as wide as long, length of F3: width = 4.3: 4.8.

Mesosoma (Fig. 2C, D). Mesosoma 1.6× as long as wide and 1.5× as its high. Humeral corner of pronotum rounded; pronotum, mesoscutum, mesoscutellum densely punctate, punctures obviously larger and deeper than those on head; punctures on the yellow spots of metanotum sparser than of other parts. Lower part of the mesepisternum with punctures slightly smaller and less dense than those of the upper part. Dorsal face of the propodeum not well separated from lateral and posterior faces, coarsely reticulate, metapleuron impunctate and somewhat coriaceous with transverse wrinkles.

Metasoma (Fig. 2E–G). First tergum (T1) 1.56× wider than long. Surface between both transverse carinae of T1 smooth and shiny; horizontal surface of T1 behind the second carina to apical margin 2.1× as wide as long. Apical yellow band of T1 gradually narrowed towards lateral side. Terga 1–3 and S2–3 coarsely punctate with backwards open punctures, and with very narrow transparent lamellate apical margin, respectively; diameter of punctures less than distance between punctures. S3 with an arched carina laterally; fourth to remaining metasomal segments smooth basally and sparsely and finely punctate towards apical part.

Figure 1. Syntypes of *Subancistrocerus kankauensis* (Schulthess-Rechberg, 1934), habitus lateral view **A** female and its head frontal aspect **B** male.

Male. Similar to female except as follows (Fig. 3): clypeus yellow, smooth except very sparsely punctate basal half, medially convex with long pubescence, with ventral 1/3 portion strongly depressed, and with apical margin truncate and as wide as half of maximum width of clypeus (Fig. 3B); fore femora yellow. Antenna clavate with an apical cavity on the articles 11–12 (A11–A12) and last arti-

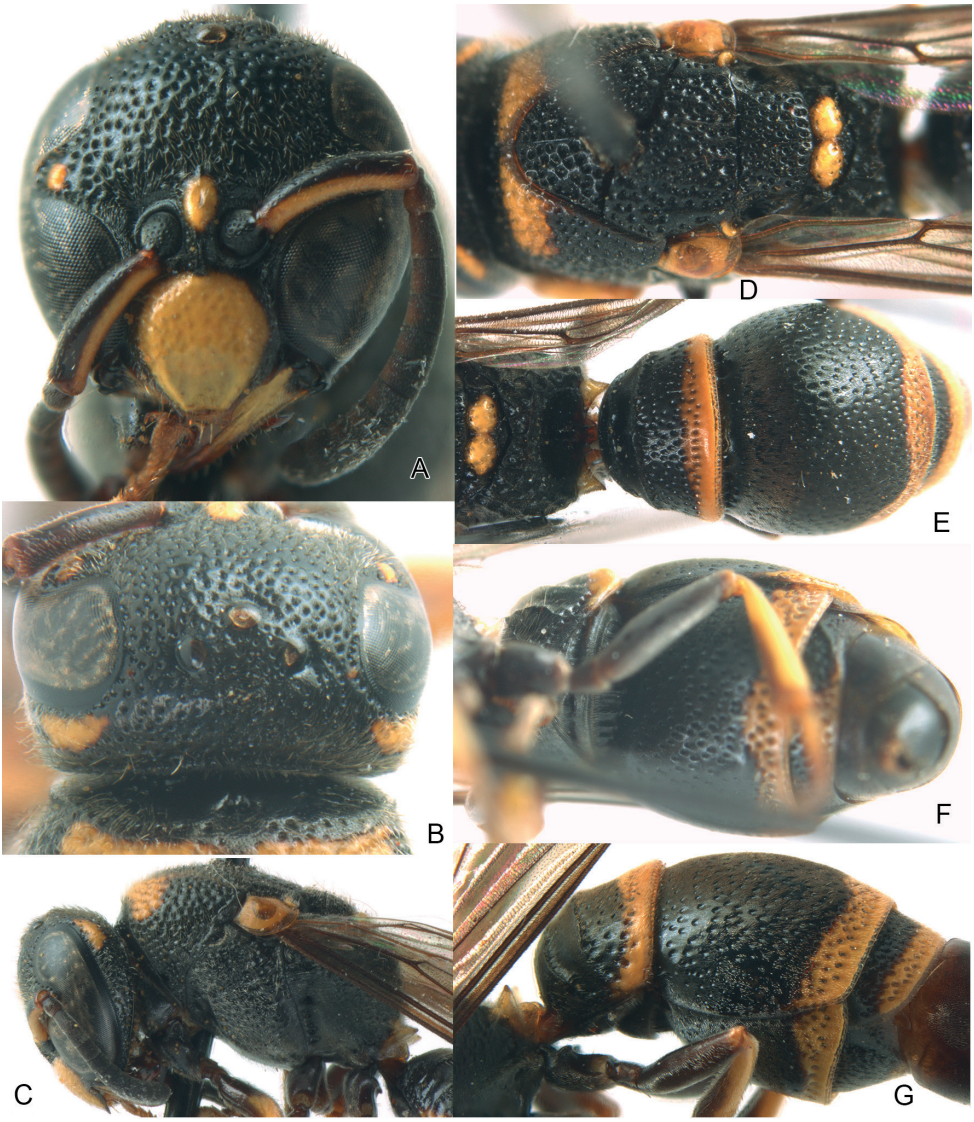

Figure 2. Syntypes of *Subancistrocerus kankauensis* (Schulthess-Rechberg, 1934), female **A** head, frontal aspect **B** ibid, dorsal aspect **C** head and mesosoma, lateral aspect **D** mesosoma, dorsal aspect **E** metasoma, dorsal aspect **F** ibid, ventral aspect **G** ibid, lateral aspect.

cle A13 flat, shaped as a corresponding cover reaching basis of A11, 1.5× as long as wide, rounded at apex, subparallel-sided bilaterally (Fig. 3D); A3–A9 laterally each with an indistinct or distinct tyloid (a carina-like prominence) without concavities ventrally. A3, A4 and A5 is 1.7×, 1.1× and 1.0× as long as wide. Basitarsomere of mid leg curved, 4.7× as long as wide,1.2× longer than the combine of tarsomeres 2–5 and pretarsus (Fig. 1B).

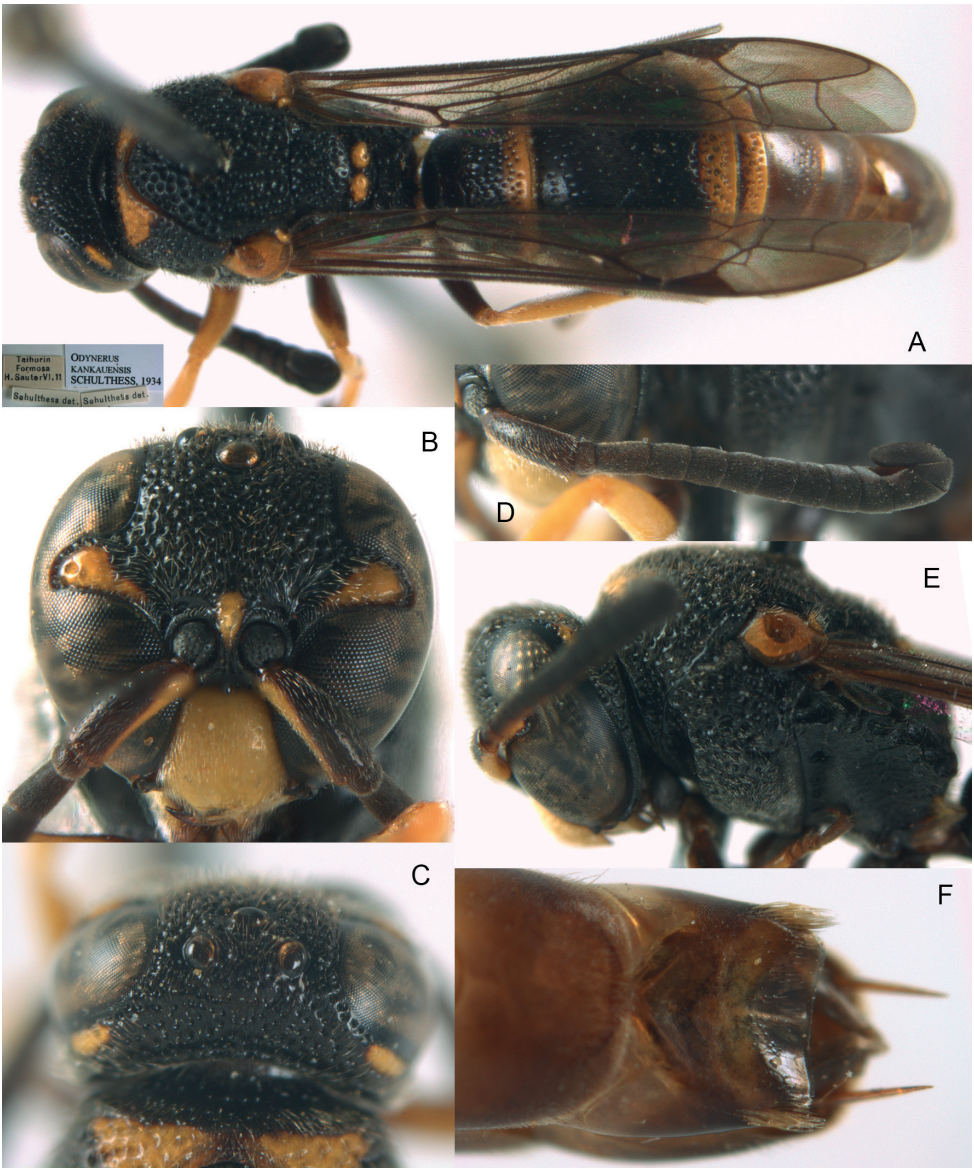

Figure 3. Syntypes of *Subancistrocerus kankauensis* (Schulthess-Rechberg, 1934), male **A** habitus, dorsal aspect **B** head, frontal aspect **C** ibid, dorsal aspect **D** antenna **E** head and mesosoma, lateral aspect **F** apex of metaosoma, ventral aspect.

***Subancistrocerus clypeatus* Tan, sp. nov.**
https://zoobank.org/EDB01089-3157-4AD6-AB60-0563A056D134
Figs 4–6

Material examined. ***Holotype***, 1♀, Shunxi, Qingliangfeng (30.13°N, 119.04°E), Zhejiang, China, 24.viii.2018, coll. Jiangli Tan. ***paratype***, 1♂, same data as holotype.

Diagnosis. The species is similar to two Chinese species *S. kankauensis* and *S. compressus* and to a species *S. indochinensis* from Laos mainly basis of the deep concavity of A11–A12 and A13 flat, reaching the basis of A11 and mid- basitarsomere curved, about 5.0× as long as wide, but can be separated from *S. kankauensis* and *S. compressus* by the characters indicated in the key. It also differs from *S. indochinensis* by the following characters: 1) in *S. clypeatus* sp. nov., clypeus of male slightly longer than wide (width: length = 2.0: 2.2) with apical emarginate (width: depth = 1.0: 0.2), but in *S. indochinensis*, clypeus as wide as long (width: length = 2.0: 2.0) with apical margin slightly emarginate (width: depth = 1.4: 0.2). 2) in *S. clypeatus* sp. nov., clypeus of female as wide as long (width: length = 2.0: 2.0) with apical slightly margin emarginate (width: depth = 1.4: 0.2), black laterally, yellow medially with a black transverse spot, while in *S. indochinensis*, the clypeus of female slightly wider than long (2.5: 2.3) with apical margin slightly emarginate (width: depth = 1.4: 0.2), black with a yellow transverse band basally and two yellow spots above the apical margin. 3) apical yellow band of T3 and S3 present in *S. clypeatus* sp. nov., but absent in *S. indochinensis*.

Description. ***Holotype***. ♀, Length of body (up to apex of T2) 6.3 mm with forewing 5.4 mm long.

Body black with yellow to brownish yellow maculation. Basal 2/5 of the mandible yellow except a small black triangle, and its distal 3/5 dark brown. Clypeus black laterally, yellow medially with a black transverse spot, and with its apical margin brown translucent. Other yellow or brownish yellow parts normally smooth or sparsely punctate as follows (Fig. 4A–D): an inter-antennal spot, scape ventrally, spot on ventral side of each flagellum, a small spot on the apical ocular sinus, a spot behind the eye on upper surface of temple, two small separated irregular spots on pronotum dorsally, tegula sub -core periphery, parategula, separated metanotal spots, propodeal valvula and apical lamella of submarginal carina of propodeum, dorso-apical fore femora, dorsal fore tibiae, wide apical bands of terga 1–2 and sternum 2, narrow band of T3, very small trace of S3 medially. Wings hyaline with veins and pterostigma dark brown, and with a brown cloudy patch along the costal margin; body with white to yellowish brown pubescence (Fig. 5A).

Head (Fig. 5B, C, E). Frons deeply punctate and reticulate; temple relatively small and sparsely punctate. Clypeus moderately convex, as wide as long with medial depression in shield shaped that bounded laterally by two indistinct sinuate carinae; apical margin emarginate (width: depth = 1.4: 0.2) between distinct projecting teeth, 0.3× of maximum width of the clypeus, laterally angulate; clypeus densely punctate laterally, weakly punctate to smooth at the medial depression portion, but densely punctate at black part. Antenna short and clavate, length of the first flagellar segment F1: width = 3.3: 2.5, F2 as wide as long, length of F3: width = 6: 7.

Mesosoma (Fig. 5D, F). Mesosoma 1.4× as long as wide and 1.6× as its high. Humeral corner of pronotum rounded; pronotum, mesoscutum, mesoscutellum densely punctate, punctures obviously larger and deeper than those on head. Mesepisternum densely reticulate-rugose, meshes on the upper part larger than on lower part. Propodeum densely reticulate-rugose dorsally and laterally; its posterior surface concaved, more or less shiny with fine, microscopic, diagonally striped (Fig. 4B); metapleuron impunctate and somewhat coriaceous with transverse wrinkles and densely white pubescence.

Figure 4. *Subancistrocerus clypeatus* Tan, sp. nov., holotype, ♀ **A** wing **B** propodeum posterior surface **C** fore tarsi **D** mid- and hind tibiae and pretarsus.

Metasoma (Fig. 5G–I). T1 about 1.8× wider than long. Surface between the two transverse carinae of T1 smooth and shiny; horizontal surface of T1 behind second carina to apical margin 2.5× as wide as long. T1 coarsely punctate behind second transverse carina, with backwards open punctures, and with very narrow transparent lamellate apical margin; diameter of punctures less than distance between punctures; apical yellow band of T1 evenly wide; S1 coriaceous without punctures. T2 punctate with punctures sparser medially than basally and subapically; S2 sparsely punctate with punctures relatively smaller than T2, diameter of punctures more than distance between punctures; T3 and S3 densely punctate similar to T1, lateral arched carina indistinct; T4 and S4 smooth basally and sparsely punctate distally, punctures gradually larger and deeper towards apex. Fifth to the remaining metasomal segments smooth basally and very finely punctate towards apex.

Figure 5. *Subancistrocerus clypeatus* Tan, sp. nov., female, holotype **A** forewing **B** head, frontal aspect **C** ibid, dorsal aspect **D** mesosoma, dorsal aspect **E** head, lateral aspect **F** mesosoma lateral aspect **G** metasoma, dorsal aspect **H** ibid, ventral aspect **I** ibid, lateral aspect.

Male (Fig. 6A): Similar to female except as follows: a large spot on ocular sinus; clypeus and mandible both entirely yellow with long pubescence; clypeus slightly longer than wide (length: width = 2.2: 2.0) without carinae, smooth except basal half finely

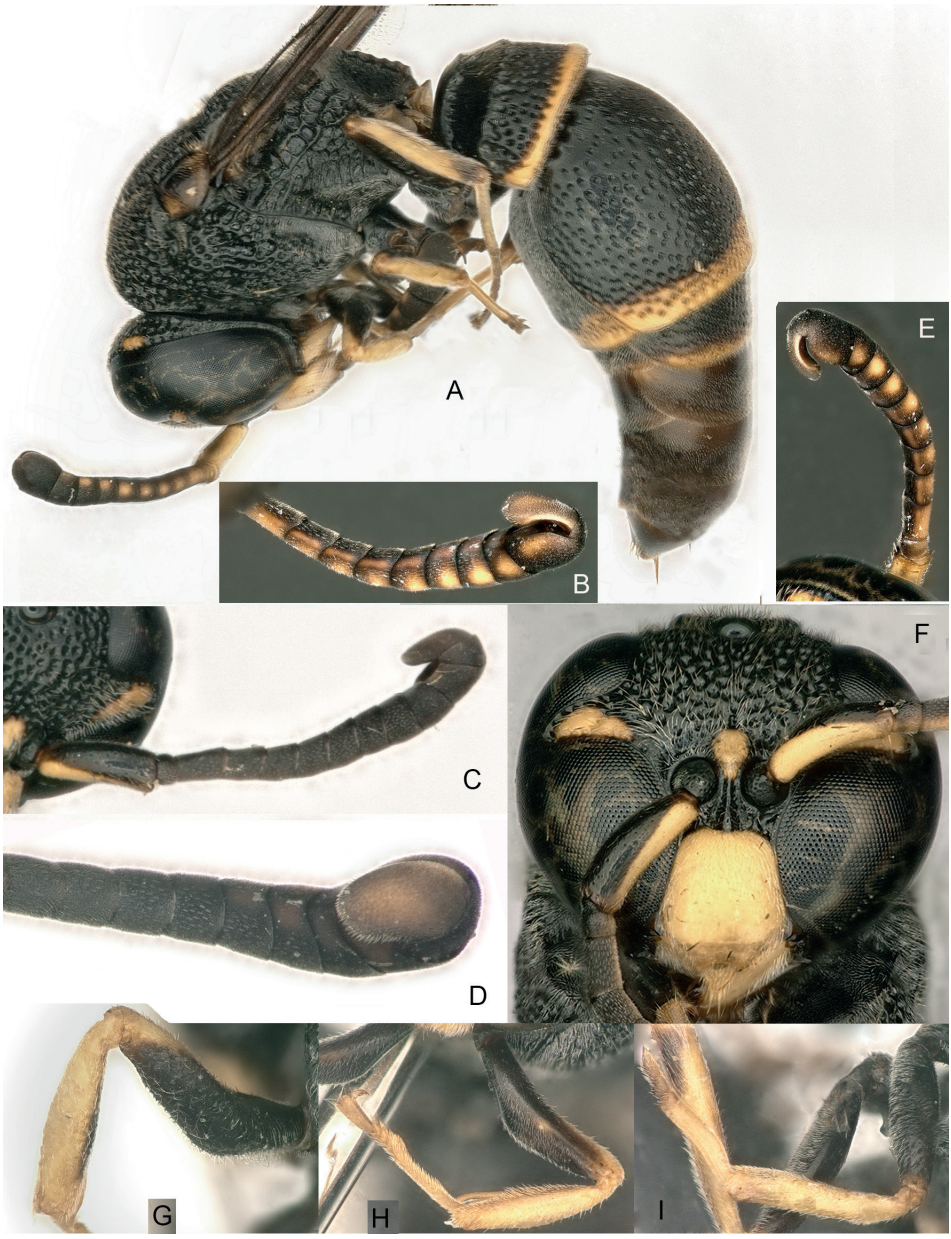

Figure 6. *Subancistrocerus clypeatus* Tan, sp. nov., male, paratype **A** habitus, lateral aspect **B, E** right antenna, ventral aspect, show the tyloids and yellow patches and deep concavity of A11–A12 **C** ibid, dorsal aspect **D** ibid, A4–A13 enlarged head, frontal aspect **F** head, frontal aspect **G** left fore-femora and tibia, frontal aspect **H** foreleg, caudal aspect **I** mid-leg without pretarsus, frontal aspect.

and sparsely punctate; medially convex with ventral 1/3 portion strongly depressed; apical margin emarginate (width: depth = 1.0: 0.2), 0.36× of maximum width of clypeus (Fig. 6F); dorsal apex of femora, tibiae except a dark brown patch on its ventral surface

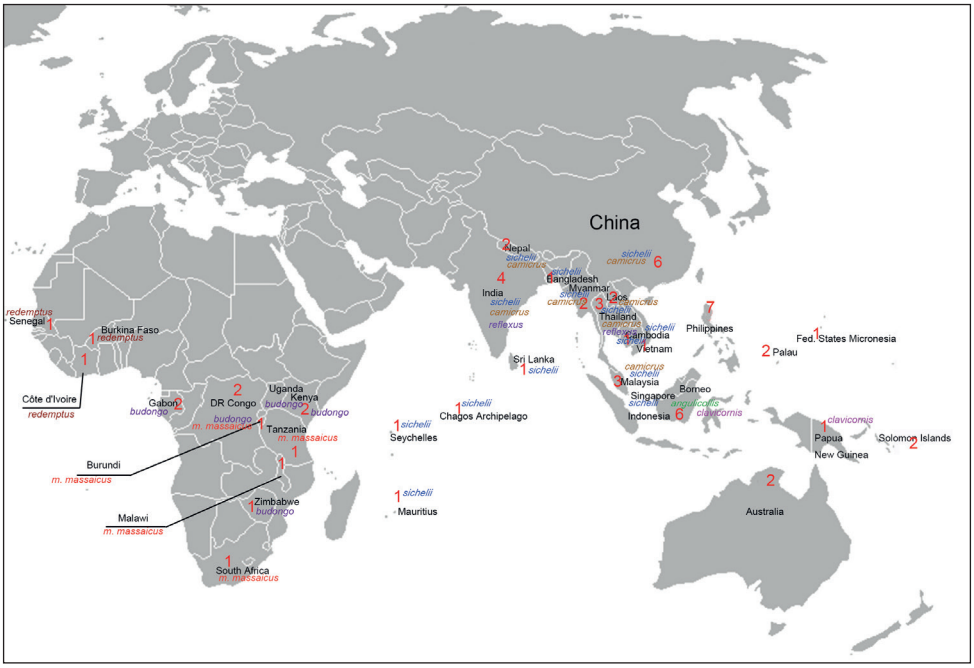

Figure 7. Distribution map of the species diversity on the genus *Subancistrocerus*. Note: the species that spread into at least two countries were inked in color; name of country inked black. Map download: http://ditu.ps123.net/world/314_3.html.

and basitarsomere of all legs yellow. Antennae clavate with an apical cavity on the article 11–12 (A11–A12); terminal article A13 flat, shaped as a corresponding cover reaching the basis of A11, 1.8× as long as wide, rounded apically; A3–A9 laterally each with an indistinct (A3) or a distinct tyloid (a carina-like prominence) without concavities ventrally (Fig. 6B–E). A3, A4 and A5 is 1.7×, 1.2× and 1.0× as long as wide. Basal half of fore femur ventrally compressed (Figs 6G–H); basitarsomere of mid leg curved, 5.0× as long as wide, 1.2× longer than the combine length of tarsomeres 2–5 and pretarsus (Fig. 6I).

Distribution. China (Zhejiang)

Etymology. The new species is named from Latin "clypeus, shield" referring to its shield shaped patch on the middle of female clypeus for the depression bordered by two longitudinal sinuate carinae.

Discussion

Since de Saussure (1855) coined for his division I of the subgenus *Ancistrocerus* Wesmael of the genus *Odynerus* Latreille and raised to the generic rank by Bequaert (1925), this widespread group received little attention. Within the genus, *S. sichelii* (de Saussure) was the most widely spread species followed by *S. camicrus* (Cameron), *S. massaicus massaicus* (Cameron 1910) and *S. budongo* (Meade-Waldo) in turn, most of the other species was en-

demic in one country. The distribution can be characterized as Palaeotropical, with China as the most speciose country after the Philippines (Fig. 7). Compared with its congeners, *S. clypeatus* Tan, sp. nov. is the most northern (30°N) species known so far. The same place, the first author has published another species viz. *Zethus velamellatus* Tan which is also the most northern Oriental distribution of the genus *Zethus* (Tan et al. 2018). Obviously, it is additional evidence supporting the boundaries of the Palaearctic-Oriental transitional zone as defined for mammals (between 33°N and 28°N; Hoffmann 2001).

Acknowledgements

We gratefully acknowledge Prof. Dr. Cornelis van Achterberg (Leiden, The Netherlands) and Prof. Dr. James M. Carpenter (New York, AMNH) for their excellent comments and great help. This research was funded by the National Natural Science Foundation of China (NSFC, No. 31872263, 31201732, 31572300) and GDAS Special Project of Science and Technology Development (No. 2020GDASYL-20200102021, 2020GDASYL-20200301003).

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JHR 95: 129–142 (2023)
doi: 10.3897/jhr.95.96365
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Quantitative morphology and mtDNA reveal that *Lasius maltaeus* is not endemic to the Maltese Islands (Hymenoptera, Formicidae)

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Academic editor: Francisco Hita Garcia | Received 15 October 2022 | Accepted 5 December 2022 | Published 17 February 2023

https://zoobank.org/9A24CFED-556E-422D-9570-389B4A5315CA

Citation: Menchetti M, Schifani E, Alicata A, Vila R (2023) Quantitative morphology and mtDNA reveal that *Lasius maltaeus* is not endemic to the Maltese Islands (Hymenoptera, Formicidae). Journal of Hymenoptera Research 95: 129–142. https://doi.org/10.3897/jhr.95.96365

Abstract

Lasius maltaeus Seifert, 2020 was recently described as a Maltese endemic ant based on quantitative morphology, after decades of uncertainties over the identity of the local population, which has a phenotype resembling *L. emarginatus* (Olivier, 1791). At the same time, Sicilian *L. emarginatus* populations were discovered to diverge in their mitochondrial DNA to a degree that suggested heterospecificity. Considering the biogeographic similarity of Malta and Sicily, with land bridges connecting them repeatedly until the last glacial maximum, we questioned the assumption that *L. maltaeus* was endemic to Malta. We integrated quantitative morphology and mtDNA in the study of the Maltese and southern Italian populations phenotypically close to *L. emarginatus*. We discovered that the range of *L. maltaeus* extends over most of Sicily, while the true *L. emarginatus* replace it in the north-eastern sector of the island, the nearby Aeolian Islands, and the Italian peninsula. The distributions of *L. emarginatus* and *L. maltaeus* in Sicily follow biogeographic patterns recalling the island's complex paleogeographic history. Further investigations should verify the existence of truly Maltese endemic ants, since the status of other allegedly endemic species is not strongly supported.

Keywords

ants, biogeography, Formicinae, Mediterranean islands, Sicily

* These authors contributed equally to this study.

Introduction

The ant genus *Lasius* Fabricius, 1804 is one of the most ecologically important ant genera in the Holarctic realm (Hölldobler and Wilson 1990; Seifert 2018, 2020). It currently counts 125 extant species (Bolton 2022), which belong to two major clades and at least 10 species groups, many of which independently evolved social parasitism (Maruyama et al. 2008; Boudinot et al. 2022).

The taxonomy of *Lasius* ants is considered among the most challenging among Holarctic ants (Seifert 2018). In particular, the former subgenus *Lasius* s. str., which is now known to represent two distinct lineages (the *brunneus* group and the *niger* group, see Boudinot et al. 2022), has witnessed significant taxonomic changes. It was once thought to count only seven species (Wilson 1955), but since then this number increased several times, especially in the last three decades (Van Loon et al. 1990; Seifert 1991; Seifert 1992, 2020; Schlick-Steiner et al. 2003; Borowiec and Salata 2013; Seifert and Galkowski 2016; Salata and Borowiec 2018; Schär et al. 2022).

The West-Palearctic *Lasius emarginatus* (Olivier, 1791) is an iconic European species characterized by a large geographic range and remarkable bicoloured appearance (Seifert 2018). However, at least four additional cryptic species with a superficially similar appearance were revealed during recent years (Seifert 2018, 2020): *L. tebessae* Seifert, 1992 from the Maghreb (Seifert 1992, 2020); *L. illyricus* Zimmermann, 1935 ranging from the Balkans to the Caspian sea, in a large area sympatric with *L. emarginatus* (Borowiec and Salata 2013; Schifani and Massa 2020; Seifert 2020); the Iranian *L. persicus* Seifert, 2020, partly sympatric with *L. illyricus* (Seifert 2020); and *L. maltaeus* Seifert, 2020 described from Malta.

The recent description of *L. maltaeus* came after a long debate on the identity of this Maltese morphotype. Baroni Urbani (1968) attributed the local bicolored *Lasius* ants with hairy scapes to *L. emarginatus*. However, Schembri and Collingwood (1981) considered the workers' head pilosity to be too dense, their frontal triangle too shiny and the scapi of males to be too hairy compared to samples of *L. emarginatus* from continental Europe, and instead proposed to classify the Maltese population as a reddish phenotype of *L. niger* (Linnaeus, 1758). Later on, the same authors changed their opinion, defining the Maltese ants as representatives of a morphospecies different from both *L. niger* and *L. emarginatus* (Schembri and Collingwood 1995). The issue remained uninvestigated until Seifert (2020) included Maltese specimens in a large West-Palearctic taxonomic revision based on quantitative morphology. He demonstrated their distinctiveness from both *L. niger* and *L. emarginatus*, and described the morphospecies as *L. maltaeus*, included within the *L. emarginatus* complex, emphasizing differences in head pilosity in agreement to the comments by Schembri and Collingwood (1981).

At the same time, an inventory of the Sicilian ant fauna highlighted a remarkable genetic distance for the mitochondrial COI of *L. emarginatus* samples from Sicily compared to those of the nearby Aeolian Islands and of peninsular Italy (Schär et al. 2020).

Similar results were also published from Corsica, yet the local population resembling *L. emarginatus* was attributed to *L. grandis* Forel, 1909 based on morphology (Blatrix et al. 2020; Seifert 2020). Sicily and the Maltese Islands are well-known for their strong biogeographic similarity (e.g., Thake 1985; Fattorini 2011; Salvi et al. 2014; Médail 2022), and were connected repeatedly by land bridges until the last glacial maximum (Foglini et al. 2016) (light blue line Fig. 1). These observations led us to investigate the relationships between the Sicilian populations historically attributed to *L. emarginatus* and *L. maltaeus*, questioning the endemic status of the latter.

Materials and methods

Ant specimens were manually collected and stored in 70–96% ethanol (Suppl. material 1: table S1, Fig. 1). Vouchers are deposited in the authors' personal collections (see Suppl. material 1: table S1):

MMBS M. Menchetti pers. coll., Barcelona, Spain;
ESPI E. Schifani pers. coll., Palermo, Italy;
AACI A. Alicata pers. coll., Catania, Italy;
BDEL R. Vila, Butterfly Diversity and Evolution Lab coll.

Morphological analysis

The morphological study was performed using stereoscopic microscopes at 45–80× magnification, in addition to photography-based morphometry. Species were identified using the keys provided by Seifert (2020).

Morphological measurements were obtained by taking pictures with a Carl Zeiss Stemi 2000-C stereomicroscope at magnification 2.25× equipped with a CMEX PRO-5 DC.5000p digital camera and ImageFocus 4 software (M. Menchetti) and at 5× magnification using Canon MP-E 65mm f/2.8 1–5× macro lens analysed with the software ImageJ (Schneider et al. 2012) (E. Schifani).

We measured a total of 22 workers of *L. maltaeus* from Malta and Italy (Sicily and Calabria) and 13 workers of *L. emarginatus* from Italy (Sicily, Aeolian Islands, Calabria and Emilia-Romagna). We recorded six characters, including one chaetotaxonomic and four morphometric characters needed to distinguish *L. emarginatus* from *L. maltaeus* according to Seifert (2020), and the cephalic size. The acronyms and character definitions follow Seifert (2020):

CL: maximum cephalic length in median line; the head must be carefully tilted to the position with the true maximum. Excavations of posterior head and/or clypeus reduce CL.
CW: maximum cephalic width; this is either across, behind, or before the eyes.

PoOc: postocular distance. Use a cross-scaled ocular micrometer and adjust the head to the measuring position of CL. Caudal measuring point: median occipital margin; frontal measuring point: median head at the level of the posterior eye margin. Note that many heads are asymmetric and average the left and right postocular distance.
MP6: length of the sixth (terminal) segment of maxillary palps.
nGen: with head in full face view, number of setae on head sides frontal of anterior eye margin (gena). The bilateral sum is halved.
CS: arithmetic mean of CL and CW as less variable indicator of body size.

For all specimens we also calculated ratios (CL/CW, PoOC/CL and MP6/CL) and the linear discriminant distinguishing *L. maltaeus* (D > 0) from *L. cinereus* Seifert, 1992, *L. emarginatus*, *L. illyricus* and *L. grandis* (D < 0) at step 22a of the dichotomous key by Seifert (2020): 45.42*PoOc/CL –0.183* CL/CW+55.63*MP6/CL +0.312*nGen –25.59. *Lasius emarginatus* was distinguished from the other above-mentioned species based on cuticle microsculpture, clypeal pubescence and setosity of the scapi following Seifert (2020). All measurements are presented in mm.

Genetic analysis

A total of seven *L. maltaeus* and three *L. emarginatus* specimens belonging to different nests were selected for the genetic analysis. A few legs per specimens were used. DNA-barcoding (mitochondrial gene cytochrome c oxidase I, COI, 658 bp) data was generated at two institutes: the Centre for Biodiversity Genomics, University of Guelph, Canada, using the primers LepF1 and LepR1 (deWaard et al. 2008); the Butterfly Diversity and Evolution Lab (BDEL), following the protocol by Schär et al. (2020) and using the primers LCO1490/HC02198 (Folmer et al. 1994). In the latter case, PCR products were visualized by gel electrophoresis and sent to Macrogen Europe for Sanger sequencing. Raw sequences were edited and aligned in Geneious Prime 2020.2.4 (Kearse et al. 2012). Chromatograms and sequences have been inspected for the presence of, respectively, double peaks and stop codons.

We also retrieved from GenBank a total of ten sequences of 658 bp identified as *L. emarginatus*: one from Schär et al. (2018) (accession number LT977448), four from Schär et al. (2020) (accession numbers MT606324, MT606325, MT606326, and MT606327) and five from Blatrix et al. (2020) (accession numbers MH138380, MH138381, MH138384, MH138385, and MH138386). The haplotype network was created using the program TCS 1.21 (Clement et al. 2000) and then graphically edited with tcsBU (dos Santos et al. 2016) and Adobe Illustrator CC 2019. All the newly generated sequences were submitted to GenBank (accession numbers OQ025622-OQ025631, see also the Suppl. material 1: table S1) and to BOLD (dataset DS-ANTLMAL, doi: http://dx.doi.org/10.5883/DS-ANTLMAL).

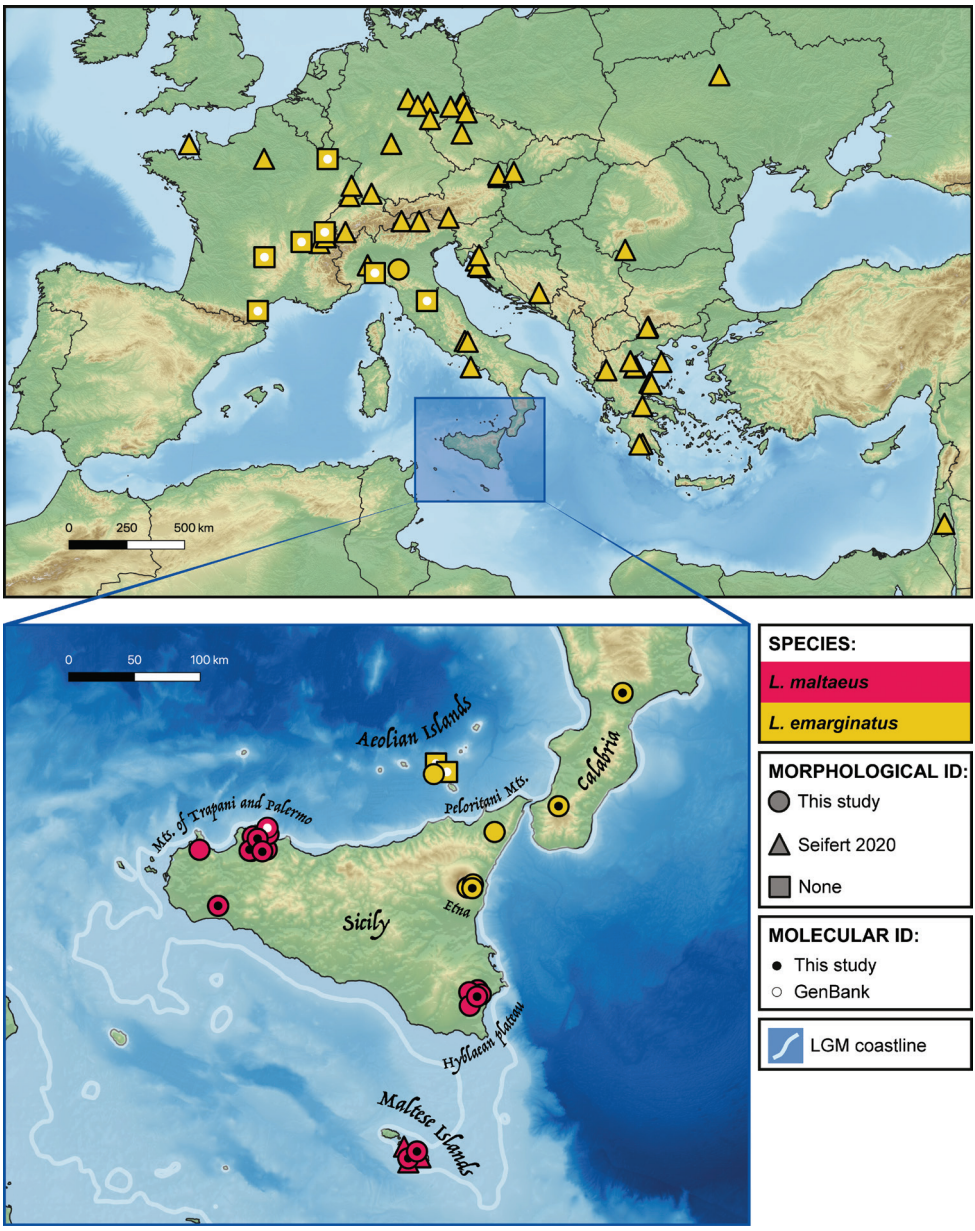

Figure 1. Distribution of the samples of *L. maltaeus* (red) and *L. emarginatus* (yellow) investigated in this study with quantitative morphology and/or molecular analysis. The shape of the points represents whether the samples were identified with the linear discriminant (circles: ID in this study; triangles: ID by Seifert 2020; squares: no linear discriminant was used). Inner circles indicate whether molecular data (COI) has been used (black circles: sequences generated in this study; white circles: sequences retrieved from GenBank). Light blues lines in the map below indicate the coastline during the Last Glacial Maximum (LGM). Main toponyms discussed in the text are highlighted in the map.

Results

Samples identified by quantitative morphology and/or molecular analysis as *L. maltaeus* and *L. emarginatus* were distributed allopatrically (Fig. 1). All samples from the Italian peninsula, the Aeolian Islands and from north-eastern Sicily (Peloritani mountains and Etna) belonged to *L. emarginatus*, while all samples from Malta and all other samples from Sicily (coming from the north-western and south-eastern sectors) were identified as *L. maltaeus* (Fig. 1).

Morphological analysis

Quantitative morphological data are summarized in Table 1 and raw data is available in the Suppl. material 1: table S2. All specimens were unambiguously classified as either *L. maltaeus* or *L. emarginatus* according to the linear discriminant scores even without considering nest means of multiple workers, while all individual characters overlapped between the two species (Table 1, Figs 2, 3). For what concerns *L. maltaeus*, our data slightly extend the minimum and maximum range of all investigated characters except for the maximum range of PoOC and CW.

Table 1. Summary of the morphological differences between *L. maltaeus* (n=22) and *L. emarginatus* (n=13) specimens measured in this study. All morphometric characters are reported in mm as mean ± standard deviation (minimum, maximum). The raw data is available in Suppl. material 1: table S2.

	L. maltaeus	***L. emarginatus***
CL	0.96 ± 0.07 (0.79, 1.11)	0.99 ± 0.05 (0.91, 1.05)
CW	0.9 ± 0.07 (0.73, 1.03)	0.93 ± 0.07 (0.82, 1.02)
PoOC	0.22 ± 0.02 (0.17, 0.25)	0.22 ± 0.02 (0.19, 0.25)
MP6	0.22 ± 0.02 (0.18, 0.26)	0.21 ± 0.01 (0.18, 0.23)
nGen	13.36 ± 2.45 (8, 18)	7.04 ± 1.83 (4, 10)
CS	0.93 ± 0.07 (0.76, 1.07)	0.96 ± 0.05 (0.87, 1.03)
CL/CW	1.07 ± 0.03 (1.01, 1.14)	1.07 ± 0.07 (1.01, 1.25)
PoOC/CL	0.23 ± 0.01 (0.20, 0.25)	0.22 ± 0.01 (0.21, 0.24)
MP6/CL	0.23 ± 0.02 (0.21, 0.26)	0.21 ± 0.01 (0.2, 0.22)
D	1.91 ± 0.83 (0.76, 3.92)	-1.35 ± 0.84 (-3.17, -0.17)

Figure 2. Frontal view of the head and lateral view of a *L. maltaeus* worker from Sicily (voucher ES16A036).

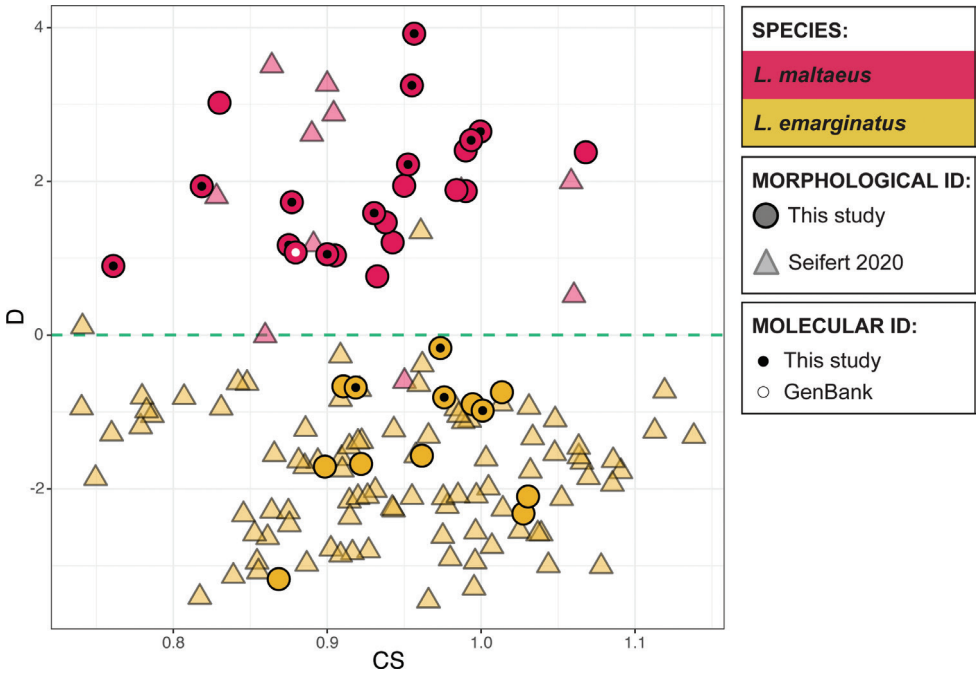

Figure 3. Morphological differences between *L. maltaeus* (red) and *L. emarginatus* (yellow) specimens according to cephalic size (CS) and the linear discriminant D from Seifert (2020) (green dashed line: < 0 *L. emarginatus*, > 0 *L. maltaeus*). Circles represent the specimens measured in this study, while triangles indicate the specimens from Seifert (2020). Sequenced specimens present an inner circle, which is black if generated in this study or white if obtained from GenBank (i.e. from Schär et al. 2020). Note that individual specimens may be misidentified by the discriminant scores, but not nest means of multiple workers according to the data provided by Seifert (2020).

Genetic analysis

We generated ten COI sequences with a length of 658 bp. The haplotype network analysis (Fig. 4) based on a total of 20 COI sequences (8 for *L. maltaeus* and 12 for *L. emarginatus*) show two distinct haplogroups that match the morphological species identification. Within the *L. maltaeus* haplogroup, the two *L. maltaeus* colonies analysed from Malta had distinct haplotypes, both shared with the Sicilian populations: one shares the haplotype with a colony from the Hyblean Plateau (voucher MM21B061a1 from Cavagrande del Cassibile, Siracusa) and the other with samples from the mountains in the area of Palermo (voucher MM18A053a1 from Monte Moarda and sequence MT606327 from Monte Pellegrino). A total of five haplotypes were found in *L. maltaeus*, with a maximum intraspecific mtDNA divergence of 0.6%, while in *L. emarginatus* we found six haplotypes and a maximum intraspecific mtDNA divergence of 1.1%. The minimum and maximum interspecific mtDNA divergence found were, respectively, 2.9% and 3.8%.

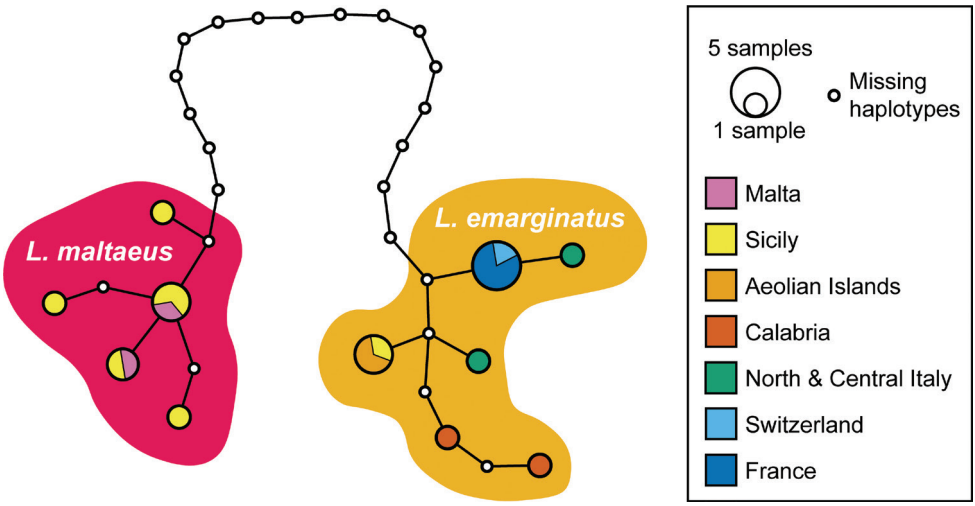

Figure 4. Haplotype network of *L. maltaeus* and *L. emarginatus* mitochondrial COI sequences. Colour and size of the circles indicate geographical origin and number of the samples, respectively.

Discussion

Quantitative morphology and mtDNA data agree and allow for the unambiguous distinction of *L. emarginatus* from *L. maltaeus*. Our results demonstrate that the range of the latter mostly resides in Sicily (where it shows the larger haplotype diversity), making it a Siculo-Maltese endemic rather than a Maltese endemic species. The long history of doubts over the identity of the Maltese populations, culminating with the description of *L. maltaeus* (Schembri and Collingwood 1981,1995; Seifert 2020), perhaps reflects the great interest of naturalists for the biodiversity of smaller islands, while the Sicilian populations were long overlooked. As a consequence of this discovery, at least two species of the *L. emarginatus* complex inhabit Italy, while further investigation should check for the possible presence of *L. illyricus* (Schifani 2022). As a result, *L. balearicus* Talavera, Espadaler & Vila, 2015 from Mallorca (Balearic Islands), *L. cypereus* Seifert, 2020 from Cyprus, and *L. kritikos* Seifert, 2020 and *L. tapinomoides* Salata & Borowiec, 2018 from Crete are the only *Lasius* species known to occur in a single island (Talavera et al. 2015; Salata and Borowiec 2018; Seifert 2020).

The vicariance between *L. emarginatus* and *L. maltaeus* in the broader context of the Siculo-Maltese archipelago and Italian peninsula follows a fascinating biogeographic pattern that reflects the complex paleogeographic history of the region. The region of Trapani and Palermo Mountains in north-western Sicily, and the Hyblaean plateau in south-eastern Sicily (recurrently linked to Malta by land bridges), represent the two most ancient sectors of the island to have emerged from the sea perhaps even before the upper Pliocene (Masini and Sarà 1998; Guarino and Pasta 2018). Both regions are recognized as well-defined biogeographic provinces hosting a significant number of endemic fauna and flora (Brullo et al. 1995, 2011; Guarino and Pasta 2018; Schifani

et al. 2020; Schmitt et al. 2021). On the other hand, the Etna and Peloritani regions of north-eastern Sicily, have a distinct geology and paleogeographic history and appear biogeographically more similar to the Italian peninsula or other Mediterranean regions: the Etna is fairly recent, emerging only about 570 thousand years ago, while the Peloritani alongside Calabria rotated counterclockwise from the Sardinian-Corsican microplate into their current position during the Alpine Orogeny (Stöck et al. 2008; Sciandrello et al. 2015; Scalercio et al. 2020; Schmitt et al. 2021).

The fauna and flora of north-western and south-eastern Sicily are more influenced by colonization from the Africa's Maghreb region compared to the north-east, which hosts more species from continental Europe (Masini and Sarà 1998; Stöck et al. 2008; Sciandrello et al. 2015; Alicata and Schifani 2019; Schifani et al. 2020, 2022a, b). The case of the green toads *Bufotes boulengeri siculus* (Stöck et al., 2008) and *Bufotes viridis balearicus* (Boettger, 1880) is particularly striking as the distribution of these two subspecies (the first of Maghrebian origin, the second one European) mirrors those of *L. maltaeus* and *L. emarginatus* respectively (Stöck et al. 2008; Dufresnes et al. 2019). Among other ants, the distribution of *Aphaenogaster trinacriae* Alicata & Schifani, 2019 resembles that of *L. maltaeus* within Sicily, while *Formica clara* Forel, 1886 and *Solenopsis orbula* Emery, 1875 may be restricted to the north-east of the island (Alicata and Schifani 2019; Schifani et al. 2021, 2022b). However, western and southern Sicily may also have acted as a refugium for relict European lineages, as recently shown among butterflies (Scalercio et al. 2020). Based on the available morphological and molecular data, it is possible that the sister species of *L. maltaeus* is the European *L. emarginatus*, but phylogenetic evidence for a broader number of species (e.g., including the Maghrebian *L. tebessae*) is needed to confirm this.

The ant faunas of Malta and Sicily share most species and the Siculo-Maltese archipelago may be considered as a single bioregion (Wang et al. 2022). The observation that the Maltese and Sicilian populations of *L. maltaeus* are not genetically differentiated and share two different COI haplotypes, suggest recent exchanges between the two (involving multiple queens), which is congruent with the hypothesis that the species exploited land bridges during the last glacial period. Beyond the case of *L. maltaeus*, our results question the existence of Maltese endemic ants. While *Lasius maltaeus* becomes the fourth Siculo-Maltese endemic species after *Aphaenogaster fiorii* Emery, 1915, *Temnothorax lagrecai* (Baroni Urbani, 1964), and *Temnothorax marae* Alicata, Prebus & Schifani, 2022 (Alicata and Schifani 2019; Schifani et al. 2022c), there are three remaining species which are currently considered Maltese endemics: *Aphaenogaster melitensis* Santschi, 1933, *Strongylognathus insularis* Baroni Urbani, 1968, and *Temnothorax splendidiceps* (Baroni Urbani, 1968) (Baroni Urbani 1968; Boer 2013). Each of them belongs to a taxonomically unresolved species group and their validity should be reassessed. In particular, the characters allegedly distinguishing *S. insularis* from its southern Italian counterpart *S. destefanii* Emery, 1915 were never quantified (Baroni Urbani 1968; Sanetra et al. 1999). A similar situation occurs with *T. splendidiceps* and the Sicilian endemic *T. laestrygon* (Santschi, 1931), with the difference that the species status of both is poorly supported because no character has been described to distinguish them from *T. exilis* in its current concept

(Baroni Urbani 1968; Salata et al. 2018). Finally, concerning *A. melitensis*, some morphological data are available, but the identity of the Sicilian morphotypes corresponding to the old *A. ionia* concept requires further investigation (authors' unpublished data) and the whole group awaits a comprehensive revision (Schifani et al. 2022d).

Estimated speciation times for ants (and especially Formicinae, see Schär et al. 2018, 2020) are on average longer than in many other insect groups and the short time of separation between Malta and Sicily suggests allopatric speciation of isolated Maltese populations to be unlikely.

Acknowledgements

We wish thank Antonio Adorno, Simone Costa, Emanuele Genduso, Roberto Ritrovato, Norian Saliba and Roberto Viviano for the specimens provided, and Cecília Corbella for help in the laboratory. We thank Sebastian Salata, Thiago Silva, one anonymous reviewer and the editor Francisco Hita-Garcia for their comments on an earlier version of this manuscript. Support for this research was provided by 'La Caixa' Foundation (ID 100010434) to Mattia Menchetti (grant LCF/BQ/DR20/11790020). Morphological data, specimen collection data and GenBank accession numbers are available in the Suppl. material 1. Sequences are also available in the BOLD dataset DS-ANTLMAL (doi: http://dx.doi.org/10.5883/DS-ANTLMAL).

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Supplementary material 1

The collecting data and voucher identifiers of the specimens

Authors: Mattia Menchetti, Enrico Schifani, Antonio Alicata, Roger Vila

Data type: morphological data, specimen collection data, GenBank accession numbers

Explanation note: The Suppl. material presents the collecting data and voucher identifiers of the specimens we analysed as well as the morphological data and the GenBank accession numbers of the sequences produced/analysed in this study.

Link: https://doi.org/10.3897/jhr.95.96365.suppl1

JHR 95: 143–198 (2023)
doi: 10.3897/jhr.95.96796
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Revision of the *leachella* group of *Megachile* subgenus *Eutricharaea* in the Western Palaearctic (Hymenoptera, Apoidea, Megachilidae): A renewed plea for DNA barcoding type material

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Academic editor: Jack Neff | Received 2 November 2022 | Accepted 26 December 2022 | Published 17 February 2023

https://zoobank.org/0AD4F90A-9A41-492D-84C3-C0AA1B8C275B

Citation: Praz CJ, Bénon D (2023) Revision of the *leachella* group of *Megachile* subgenus *Eutricharaea* in the Western Palaearctic (Hymenoptera, Apoidea, Megachilidae): A renewed plea for DNA barcoding type material. Journal of Hymenoptera Research 95: 143–198. https://doi.org/10.3897/jhr.95.96796

Abstract

The leafcutting bees of the *leachella* group of *Megachile* Latreille subgenus *Eutricharaea* Thomson are revised for the Western Palaearctic region using a combination of morphology and phylogenetic analyses of three genes (COI, LW-Rhodopsin, CAD). Although only seven species are recognized, much effort was needed to link delimitated taxonomic units to taxon names because of the difficulties in identifying type specimens. Numerous types were in a poor state of conservation, preventing straight-forward identification using morphology. This was in some cases aggravated by the fact that they often belonged to a sex that could not easily be identified; one type was a gynandromorph specimen whose identification is even more challenging. In several cases, the type locality was vague or unclear; in three cases, the type specimens originated from introduced populations for which the source of the introduction needed to be determined using DNA barcoding. In two cases, the type specimens consisted of several body parts not originating from a single individual but from two heterospecific specimens. We argue that this tedious nomenclatural work would have been greatly facilitated if a reference library of type specimens had been available. Our revision leads to the following taxonomic changes. *Megachile argentata* (Fabricius, 1793), described from northern Africa and with a convoluted taxonomic history, is demonstrated, based on morphometric analyses of its lectotype, to be conspecific with the species hitherto known as *M. pilidens* Alfken, 1924. After discussing and excluding several alternative options that would minimize nomenclatural changes, we place *M. pilidens* in synonymy with *M. argentata* (**syn. nov.**). Two new subspecies are described for morphologically slightly divergent insular populations, *M. leachella cretica* Praz, **ssp. nov.**

from Crete, and *M. leachella densipunctata* Praz, **ssp. nov.** from Cyprus. In addition, *M. albipila* Pérez, 1895 is treated as a subspecies of *M. leachella* Curtis, 1828 (**stat. nov.**). The following new synonymies are proposed: *M. compacta* Pérez, 1895 (not *M. compacta* Smith, 1879) and the replacement name *M. crassula* Pérez, 1896, *M. argyrea* Cockerell, 1931 and *Perezia maura* Ferton, 1914, are placed in synonymy with *M. argentata* (**syn. nov.**). *M. beaumonti* Benoist, 1951, is newly treated as a valid species (**stat. rev.**). *M. schmiedeknechti* Costa, 1884 is treated as a subspecies of *M. argentata* (**stat. nov.**), and *M. xanthopyga* Pérez, 1895 is placed in synonymy with *M. argentata schmiedeknechti* (**syn. nov.**). *M. bioculata* Pérez, 1902, *M. discriminata* Rebmann, 1968 and *M. ichnusae* Rebmann, 1968 are placed in synonymy with *M. leachella* (**syn. nov.**). *M. variscopa* Pérez, 1895, *M. timberlakei* Cockerell, 1920, *M. atratula* Rebmann, 1968, *M. striatella* Rebmann, 1968 and *M. sudai* Ikudome, 1999 are placed in synonymy with *M. pusilla* Pérez, 1894. Lectotypes are designated for *M. albipila*, *M. bioculata*, *M. compacta* Pérez, *M. pusilla, M. variscopa* and *M. xanthopyga*.

Keywords
DNA barcoding, morphometry, speciation, species delimitation, subspecies

Introduction

The subgenus *Eutricharaea* Thomson, 1872 of the genus *Megachile* Latreille, 1802 (Hymenoptera: Megachilidae) is taxonomically difficult: it includes numerous species with rather homogeneous morphology, leading to challenging identifications and intricate species boundaries. In addition, in the absence of proper examination of their type material, the status of several taxa remains unclear. The subgenus is native to the Eastern Hemisphere but has been introduced both accidentally (*Megachile apicalis* Spinola, 1808, *M. pusilla* Pérez, 1884, *M. concinna* Smith, 1879) and intentionally [*M. rotundata* (Fabricius, 1793)] into the Western Hemisphere. The latter species was introduced into North America and is reared commercially for the pollination of alfalfa (*Medicago* sp.) (Pitts-Singer and Cane 2011). In the Eastern Hemisphere, the subgenus is particularly diverse throughout the Palaearctic and in Africa. It also occurs in Southeast Asia and in Australia, although some Australian species included in this subgenus probably belong somewhere else (e. g., the species of the *M. chrysopyga* group; see Trunz et al. 2016). Many small leafcutting *Megachile* in the Eastern Hemisphere belong to this species-rich subgenus.

In the Palaearctic no comprehensive revision is available for this subgenus. Otto Rebmann described several species and presented identifications keys for some species groups (Rebmann 1967–1970), but he did so without a full revision of the type material. Species of *Eutricharaea* fall into several species groups segregated based on male genitalia, mainly the *rotundata* group [sometimes referred to as a distinct subgenus, *Neoeutricharaea* Rebmann, 1967; see Rebmann (1967a)] and the *leachella* group (Praz 2017), which include much of the diversity observed in the subgenus in the Palaearctic. In males, both groups can easily be separated based on the structure of the gonostylus (Praz 2017: figs 54, 56) as well as several other morphological criteria (see Praz 2017). Females are more difficult to separate; those of the *rotundata* group are mostly

characterized by the presence of a rounded, glabrous, impunctate and matt area on each side of the disc of T2, sometimes also on T3. This area is referred to as a "fovea", following Gonzalez et al. (2010). Species of the *leachella* group do not have a clearly delimited fovea on either T2 or T3, although the area of T2 where the fovea would be if present is very finely punctate and covered with short hairs, thus slightly different in its appearance from the rest of the tergal disc.

The *leachella* group includes two widely distributed species in the Palaearctic, *M. pilidens* Alfken, 1924 and *M. leachella* Curtis, 1828, which are the only species present in Central Europe (Amiet et al. 2004; Peeters et al. 2006; Scheuchl 2006). Both are easy to distinguish in the male sex, especially in the shape of S4, which has a small median tubercule covered by white hairs in *M. pilidens* and no tubercle but a patch of yellowish hairs in *M. leachella* (Amiet et al. 2004; Peeters et al. 2006; Scheuchl 2006). In addition, the *leachella* group includes the species allied to *M. concinna* (hereafter *concinna* complex), which are distributed from South Africa to Europe and Central Asia, and introduced into the Western Hemisphere (including South and North America) and probably into Japan, Hawaii and Australia (see note under *M. pusilla*). Species boundaries in the *concinna* complex are challenging and were examined by Soltani et al. (2017), who suggested the presence of four taxa in the Palaearctic, *M. anatolica* Rebmann, 1968, *M. leucostoma* Pérez, 1907, *M. pusilla* and *M. viridicollis* Morawitz, 1875. These taxa mostly represent geographic replacement "forms" with some evidence of phenetic intergradation along their contact zone. The arrangement of three forms around the Mediterranean Sea suggests a pattern of "speciation in a ring", where the two ends of the ring, *M. anatolica* and *M. pusilla*, coexist and maintain phenotypic and genetic integrity in sympatry over the region comprised between Greece and Italy; along the ring, there is apparent phenetic intergradation between *M. anatolica* and *M. leucostoma*, with transitional populations in the Levant (Soltani et al. 2017: fig. 6), and then possible intergradation between *M. leucostoma* and *M. pusilla* in northern Africa. *Megachile concinna* and *M. venusta* Smith, 1853 are considered to be Afrotropical species that are absent from the western Palaearctic (Soltani et al. 2017); *Megachile concinna* is introduced into the Caribbean, incidentally its type locality. Additional species in the *leachella* group include *M. walkeri* Dalla Torre, 1896, mainly distributed on the Arabian Peninsula, the northern African and Middle Eastern species *M. inexspectata* Rebmann, 1968 (not to be confused with *M. inexpectata* Pasteels, 1973 described from tropical Africa), and a species restricted to Sardinia, Corsica and Malta, *M. schmiedeknechti* Costa, 1884. Numerous additional names, many of which were proposed by Rebmann (1968), remain with an unclear taxonomic status (see Gonzalez et al. 2010 and Praz et al. 2021 for a treatment of some of these names). One name that has remained unclear for decades is *Apis argentata* Fabricius, 1793 (hereafter *M. argentata*), described from "Barbaria", the region in North Africa extending from Algeria to Libya. Much of the present work deals with settling the identity of this taxon, which has remained obscure for more than two centuries.

In the present study, we use a combination of genetic analyses and morphology to delimitate the species of the *leachella* group of the Western Palaearctic, and examine most type specimens to present a comprehensive revision of this challenging group of bees.

Methods

Molecular methods

The 658-bp fragment of the mitochondrial gene Cytochrome Oxidase I (DNA barcoding fragment; hereafter COI) was generated using the primers LepF and LepR or, if this primer pair did not yield amplicons or high-quality sequences, the alternate forward primer UAE3. Primer sequences and lab protocols are given in Trunz et al. (2016). All new DNA barcodes have been submitted to the Barcode of Life Data System (BOLD) platform (Ratnasingham and Hebert 2007) with process-ID numbers PAMEG016-22 to PAMEG058-22 (locality information is given in Suppl. material 1). In addition, we also sequenced the two nuclear genes CAD and LW-Rhodopsin (Soltani et al. 2017), given the known limitations of relying on a single mitochondrial genetic marker for species delimitation (Praz et al. 2019; Gueuning et al. 2020). We used the primers mentioned in Soltani et al. (2017) to amplify and sequences these two nuclear genes. Sequences of the nuclear genes have been submitted to Genbank with accession numbers OQ095208–OQ095231. Chromatograms were edited using Geneious 6.0.6 (Kearse et al. 2012) and the resulting sequences were aligned with Mafft (Katoh and Standley 2013). Single gene phylogenetic trees were reconstructed using maximum likelihood inference with RAxML 8.2.10 (Stamatakis 2014) with 1000 bootstrap replicates, applying a GTR + G model to a unique partition. For the two nuclear genes, the introns were removed; heterozygous specimens were excluded (see Soltani et al. 2017). The two nuclear genes were then concatenated and analyzed with RAxML, implementing a GTR + G model with two partitions (one by gene). Soltani et al. (2017) suggested introgression and allele sharing between some populations of *M. leachella* and *M. anatolica* for the gene LW-Rhodopsin. Single gene analyses for this gene suggested gene flow from *M. leachella* to *M. anatolica*. We therefore excluded LW-Rhodopsin sequences for *M. anatolica*, as did Soltani et al. (2017), in concatenated analyses (but not in single gene analyses). Genetic distances (presented only for COI) were computed using the Kimura 2-parameter (K2P) distance model in a test version of Paup 4.0 (Swofford 2002) kindly provided by D. Swofford.

Criteria used for species delimitation

We used our genetic analyses as a complement to morphology for species delimitation. We also used DNA barcodes for the identification of female specimens, which are often challenging to identify in the *leachella* group. For species delimitation, particular attention was given to the structure of male genitalia. The morphology of male sterna, the colour of front tarsi and the shape of a tooth on the gena just behind the base of the mandible were also important characters. In females, sculptural differences were primarily used for species delimitation, in particular the punctation of the terga (especially the disc of T4) and of the vertex, the length of the ocelloccipital distance and the shape of the apical clypeal margin. Vestiture colour was given low

priority for species delimitation given the known variation in this character, even if vestiture colour is useful for identification if geographic variation is considered. We recognize subspecies in a few cases for geographically well-separated, allopatric forms diverging from conspecific forms by either a single significant morphological feature, or by a small number of unsignificant features, taking into account the molecular results. A significant morphological feature corresponds to morphological differences typically observed between species in the subgenus *Eutricharaea*; examples include significant and discriminating differences in tergal punctation, the length of the ocelloccipital distance, or differences in the structure of the genital capsule. Unsignificant features include weak differences in punctation, differences in vestiture colour, or differences in integument colour. Taxa presenting a broad morphological cline (that is, intergradation of morphological features over a large geographic distance, typically over 100 km or so) were not separated as distinct subspecies. A rationale for recognizing subspecies is presented in each case. Overall, we favor a broad species concept, where geographically isolated forms are preferably treated as subspecies rather than split as distinct species.

Morphology

Morphology follows Michener (2007) and Praz (2017). The abbreviations T, S and OOD are used for metasomal terga, metasomal sterna and ocelloccipital distance, respectively. All pictures were taken using a Keyence VHX 1000 digital imaging system.

The females in the *leachella* group are notoriously difficult to identify, particularly in northwestern Africa, where four species occur, all of which exhibit snow white vestiture, unlike in southern Europe where vestiture colour can be used to separate at least *M. pilidens* from *M. leachella*, *M. anatolica* and *M. pusilla*. The lectotype of *M. argentata* (Figs 1–3) is a female specimen originating from northwestern Africa. To establish the identity of the lectotype of *M. argentata* with confidence, we first delimitated operational taxonomic units based on morphology (mostly male characters) and molecular results; second, we associated 29 female specimens to these taxonomic units from northwestern Africa using DNA barcodes; these barcoded females served as reference specimens for morphological examination. This approach suggested that the punctation of the vertex was a good discriminant character in northwestern Africa. We thus measured punctation in confidently identified specimens (mostly DNA barcoded specimens, otherwise morphologically typical specimens from localities with males and females caught together; Suppl. material 1) and in the lectotype of *M. argentata* to allow for statistically robust comparisons. To do so, we measured the size of the punctures in a designated area of the vertex on pictures taken using a Keyence VHX 1000 digital imaging system with the stacking option turned off. The specimens were placed in a way that the left ocellus, the right margin of the compound eye and the occipital ridge behind the lateral ocellus were in sharp focus, with the preoccipital ridge more or less horizontal on the picture. Pictures were processed with the software ImageJ 1.48 (Abràmoff et al. 2004). A picture of a microscope stage micrometer taken with the

Keyence imaging system applying the same setting was used; using that picture, the scale was set with the "set scale" command in ImageJ.

Once the scale was set, we first rotated, if necessary, each picture so that the two lateral ocelli would form a horizontal line. We measured the ocelloccipital distance (OOD), defined as the shortest distance between the posterior margin of the left ocellus (including the black, shiny, circular margin of the ocellus) and the occipital ridge (Fig. 1). We then drew a horizontal line tangential to the posterior margin of the left ocellus, and a vertical line tangential to the right border of the left eye (Fig. 1). We placed a rectangle with dimensions 1.1× OOD × 0.55× OOD so that its upper right corner was on the intersection of the two tangentials (Fig. 1). The area of each puncture within this rectangle was measured by drawing a circle or an ellipse on the puncture. A puncture along the margins of the rectangle was measured if more than half of its area was in the rectangle. For each puncture an estimate of the diameter was derived from its area (diameter equal to twice the square root of the area divided by pi), assuming that the puncture was a circle.

Material examined

Material from the following institutions has been examined. The type material has been examined by one of us (CP), and the distribution given for each species is based on material examined by CP.

AMHN American Museum of Natural History, New York, USA.
BMNH Natural History Museum, London, UK.
CPCN Collection of Christophe Praz, University of Neuchatel, Neuchatel, Switzerland.
CSE Collection of Christian Schmid-Egger, Berlin, Germany.
ETHZ Entomological Collection of ETH Zurich, Zurich, Switzerland.
MHNN Muséum d'Histoire Naturelle de Neuchâtel, Neuchâtel, Switzerland.
MNHN Muséum National d'Histoire Naturelle, Paris, France.
MZL Musée cantonal de zoologie, Lausanne, Switzerland.
NHMD Natural History Museum of Denmark, Copenhagen, Denmark.
NMB Naturhistorisches Museum, Basel, Switzerland.
NMBE Naturhistorisches Museum der Burgergemeinde Bern, Switzerland.
NMW Naturhistorisches Museum, Vienna, Austria.
OLML Oberösterreichisches Landesmuseum, Linz, Austria.
OUMNH University Museum of Natural History, Oxford, UK.
PCYU Collection of Laurence Packer, York University, Toronto, Canada.
SMFD Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt am Main, Germany.
SMNH Steinhardt Museum of Natural History, Tel Aviv University, Israel.
USNM Smithsonian institution, National Museum of National History, Washington, USA.
ZMHB Museum für Naturkunde, Berlin, Germany.

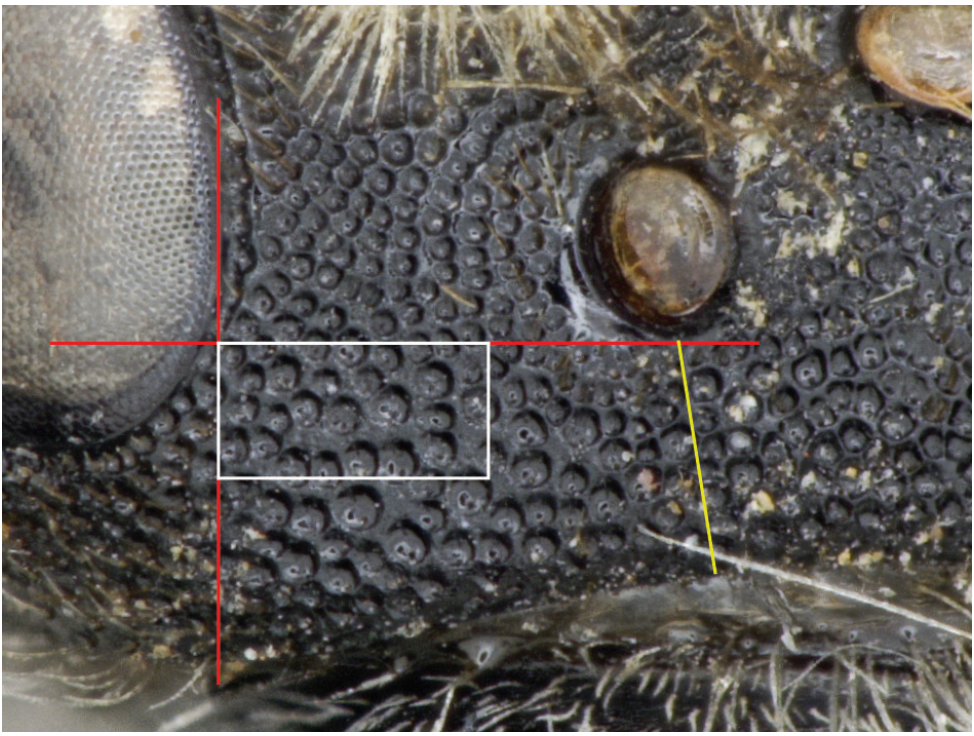


Figure 1. Vertex of the lectotype female of *Megachile argentata* (Fabricius, 1793), showing the ocelloccipital distance OOD (yellow line), two tangentials placed behind the lateral ocellus and along the inner margin of the compound eye (red lines), and the white rectangle with dimensions 1.1× OOD × 0.55× OOD within which the punctures were measured in our morphometric analyses.

Results

Lectotype of *Megachile argentata*

Identification of type specimens was performed after species delimitation had been done using combined molecular and morphological data (see below) but is presented first to settle the names of the different taxa before discussing their morphology and phylogenetic relationships.

Megachile argentata was described from "Barbaria", a region corresponding to the coastal area of northern Africa from Algeria to Libya. The specimens available to Fabricius were collected by R. L. Desfontaines, probably in modern Tunisia or eastern Algeria ["régences de Tunis et d'Alger" (Beylik of Tunis and Ottoman Algeria); Dureau de la Malle 1838]. Depending on the author, this name was used until 1968 for one of the species currently known as *M. leachella* or *M. pilidens*, or for both. In early works these two species were probably lumped under *M. argentata* (e. g. Schenck 1861; Morawitz 1875). In England or in Scandinavia, where *M. pilidens* does not occur, *M. argentata* has long been used as the valid name for *M. leachella* (e.g., Thomson 1872; Saunders 1896;

Perkins 1925); the type species of *Eutricharaea* (*M. argentata*), described in Thomson's second volume of "Hymenoptera scandinaviae", thus confidently refers to *M. leachella*.

Pérez (1879) was possibly the first author to be aware of the presence of two species closely related to *M. argentata*, which he referred to as *M. dorsalis* (= *M. leachella*; see Gogala 1998) and *M. argentata* (probably =*M. pilidens*). Unfortunately, he wrongly associated with the precisely diagnosed female of *M. dorsalis* a male of a species of the *rotundata* group with modified front tarsi; this male was redescribed later as *M. burdigalensis* Benoist, 1940. Due to this erroneous sex association, confusion has long persisted on the identity of *M. dorsalis* (e. g. van der Zanden 1996; Banaszak and Romasenko 1998), until Gogala (1998) examined the type material of *M. dorsalis* and of *M. burdigalensis*, confirmed that the lectotype female of *M. dorsalis* was conspecific with *M. leachella*, and described both sexes of *M. burdigalensis*. Friese (1899a) was also aware of the presence of two distinct species in this group, and in his key to the males he recognizes one species (*M. argentata*) with a yellow spot of hairs along the margin of S4 (cf. Fig. 53; probably *M. leachella*, contrary to Pérez's use of the name *M. argentata*) and one species with a small tubercule covered with white hairs (cf. Fig. 23; *M. pilidens*), which he refers to as *M. xanthopyga* Pérez, 1895, a species native, according to him, to Northern Africa, probably because *M. xanthopyga* was described with no locality information in Pérez's "Mellifères de Barbarie" [the bees of Barbaria]; *M. xanthopyga* was in fact described from Sardinia and has since then been placed in synonymy with *M. schmiedeknechti* (Benoist 1940).

Also aware of the presence of two distinct species in this group, Alfken (1924) treated *M. argentata* as the valid name for the species currently known as *M. leachella*; based on the male described by Pérez he considered *M. dorsalis* to be a species allied to *M. flabellipes* Pérez, 1895 in the *rotundata* group (thus likely *M. burdigalensis*); and he described *M. pilidens* for the second species. His differential diagnosis of both sexes of *M. argentata* and *M. pilidens* (Alfken 1924) clearly points to *M. leachella* and *M. pilidens* in their current usage. After this, *M. argentata* has mostly been used for *M. leachella* (e.g., Schmiedeknecht 1930; Erlandsson 1960) until 1967; Benoist (1940) however, used *M. argentata* for *M. pilidens* and "*argentata* var. *dorsalis*" for *M. leachella*.

Unfortunately, although Alfken clearly differentiated these two widespread European species, he did not examine the type specimen of *M. argentata*. Hurd (1967) examined four specimens preserved in the Fabricius collection in Copenhagen and designated a female specimen as the lectotype (Figs 1–3). He briefly describes this specimen and writes that it was not conspecific with the species known as *Megachile argentata* auct. (=*M. leachella*) in Europe, with no further details. Based on this description and presumably on additional notes sent by Hurd, but without examining the type, Rebmann (1967b) also stated that the type of *M. argentata* was not conspecific with the northern European taxon known as *M. argentata* auct. (=*M. leachella*), but that it was instead a member of the *rotundata* group. He thus resurrected the name *M. leachella* for the species so far referred to as *M. argentata* auct. and treated *M. argentata* as a *nomen dubium* in the *rotundata* group. This treatment was rejected by Warncke (1986), who argued "that the change of the name [of *M. argentata*] was fully unnecessary and that [Hurd's] lectotype designation was erroneous" since, according to him, the type material of

Figures 2, 3. Lectotype female of *Megachile argentata* **2** dorsal view **3** head in front view.

M. argentata should be deposited in the Defontaines collection in Paris, where it could not be located. He thus resurrected *M. argentata* as the valid name for *M. leachella*, a decision that was not followed (e.g., Westrich 1989; Gogala 1998; Banaszak and Romasenko 2001; Amiet et al. 2004; Scheuchl 2006). More recently, Schwarz and Gusenleitner (2011) examined the lectotype of *M. argentata* and published pictures and a redescription. They suggested that *M. argentata* was possibly conspecific with the species currently known as *M. pilidens* but refrained from formally placing *M. pilidens* in synonymy until more material from Northern Africa could be studied.

We have examined the lectotype female of *M. argentata* (Figs 1–3). This specimen perfectly agrees with the original description; we are also confident that it originates from northern Africa (see below). The lectotype undoubtedly belongs to the *leachella* group of species, based on the absence of a fovea laterally on T2, the presence of two spots of appressed, white hairs on the disc of T6 (Fig. 2), and the sculpture of the apical margin of the clypeus (Fig. 3). The latter two criteria exclude with certainty the taxon of the *concinna* complex present in northwestern Africa, *M. pusilla* (Soltani et al. 2017), which is smaller and characterized by reduced spots of appressed white hairs on the disc of T6 and the narrowly emarginate apical margin of the clypeus, with a comparatively wide impunctate premarginal area. The lectotype thus belongs to one of three species present in northwestern Africa, namely *M. pilidens*, *M. leachella* or *M. inexspectata*, which probably all occur in Algeria and Tunisia, the type locality of *M. argentata*.

The separation of the females of these three taxa can be difficult: unlike in central and northern Europe, the vestiture of both *M. leachella* and *M. pilidens* is snow-white in northern African populations, just as in *M. inexspectata*. Moreover, the differences in the punctation of the disc of the terga, especially T4, between *M. leachella* and *M. pilidens* appear to be less pronounced in northwestern Africa than in Europe, where this characteristic mostly (but not always) allows for the separation of both species (Amiet et al. 2004). The examination of 29 northwestern African females of the *M. leachella* group (excluding *M. pusilla*) identified using DNA barcodes revealed several morphological features allowing for a separation of the northwestern African females of *M. leachella*,

Table 1. Comparison of the females of *Megachile inexspectata*, *M. leachella albipila* and *M. argentata* in northwestern Africa.

Characters	*Megachile inexspectata*	*Megachile leachella albipila*	*Megachile argentata*
Vestiture			
Vestiture of dorsal side of scutum	Entirely consisting of white hairs; short scale hairs present over entire disc of scutum (Fig. 28)	As in *M. inexspectata*	scutum covered with intermixed light and dark hairs; short scale-like hairs mosty present anteriorly and posteriorly, less abundant on central parts of disc of scutum
Lateral hairs on terga	Dark, erect hairs restricted to lateral parts of T6, sometimes T5 (Fig. 25)	As in *M. inexspectata* (Fig. 34)	Dark, erect hairs present laterally on T3-T6 (Fig. 15)
Light hairs on disc of T6	Sometimes forming one large spot of hairs (Fig. 25)	Mostly forming two separated spots of hairs	always forming two well-separated spots of hairs (Fig. 15)
Sculpture			
Punctation of vertex	Dense, punctures small (diameter on average 40 µm) (Fig. 27)	As in *M. inexspectata*, puncture diameters 35–40 µm, punctation slightly less dense, often with shagreened interspaces (Fig. 41)	Punctures larger (on average 50 µm), punctation less dense, interspaces up to one puncture diameter, surface of interspaces smooth (Figs 1, 20)
Punctation of disc of T4	Dense, interspaces mostly smaller than one puncture diameter (Fig. 26)	Mostly sparse, interspaces mostly larger than one puncture diameter (Fig. 34)	Dense, interspaces smaller than one puncture diameter (Fig. 19)

M. pilidens and *M. inexspectata* (Table 1). A separation of *M. pilidens* is mostly possible, but the separation of the other two species is sometimes difficult. Based on these features (Table 1), the lectotype of *M. argentata* clearly agrees with *M. pilidens*. The punctation is dense on the disc of T4 (Fig. 2); there are numerous erect, dark setae laterally on T4 and T5 (a condition not observed frequently in *M. inexspectata* and *M. leachella* in northwestern Africa). The vestiture is snow white in northwestern African populations of *M. pilidens*, and nowhere else, strongly suggesting that the lectotype indeed originates from "Barbaria". Fabricius's original description mentions that the head and the thorax have "ash-coloured" vestiture, which corresponds better to the northern African populations of *M. pilidens*, than to those of *M. leachella* (Table 1). The description also mentions that the top of the metasoma is black, the margin of the segments white; this possibly refers to the short, dark vestiture on the disc of the terga, which is more extensively developed in *M. pilidens* than in *M. leachella* in northwestern Africa.

This identification is confirmed by our measurements of the OOD and of the size of the punctures on the vertex (Figs 1, 4): OOD-values were significantly different across the three species (Fig. 4) (ANOVA, $F=12.03$, $df=2, 24$, $P<0.001$); Tukey's post hoc test suggests that the OOD values were significantly larger in *M. pilidens* than in the other two species ($P<0.026$ in both cases), which did not significantly differ ($P=0.66$). The OOD value for the lectotype of *M. argentata* was 361 µm, in the range of the values measured for *M. pilidens*, but not for the other two species (Fig. 4). Lastly, the average diameters of the punctures on the vertex were significantly different across the three species (ANOVA, $F=71.76$, $df=2, 24$, $P<0.001$): Tukey's post hoc test suggests that all three species have significantly different average puncture diameters (*M. leachella* versus *M. inexspectata*: $P=0.043$; other comparisons $P<0.001$). With an average puncture diameter of 50.8 µm, the lectotype of *M. argentata* was again in the range of values measured for *M. pilidens*, but not for the other two species. Combining

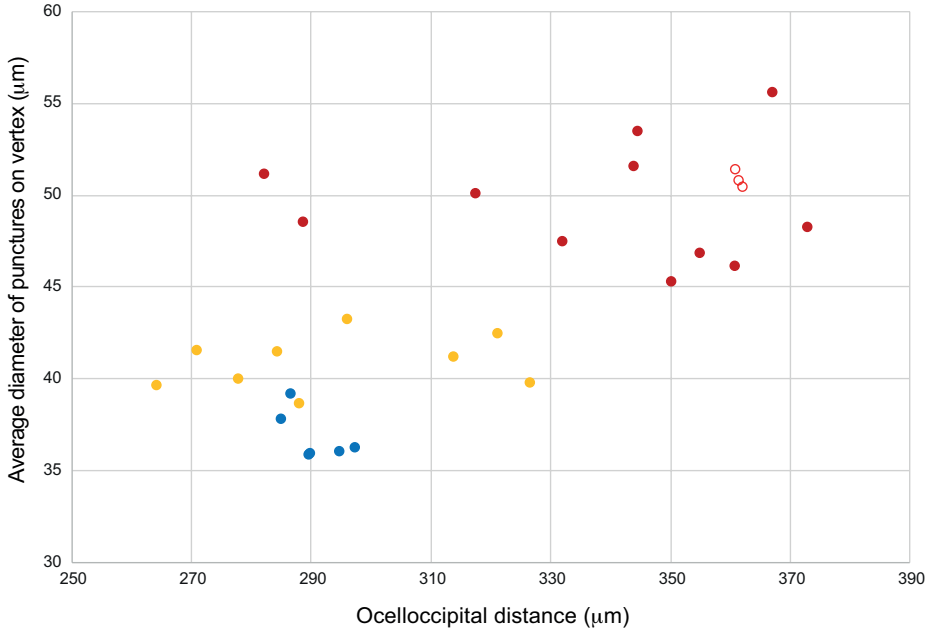


Figure 4. Results of the morphometric analyses of female specimens of *Megachile argentata* (red circles), *M. inexspectata* (yellow) and *M. leachella* (blue); three measurements of the lectotype of *M. argentata* are shown as open red circles; relationship between the ocelloccipital distance and the average diameter of the punctures included in the white rectangle of Fig. 1.

the OOD-values with the average diameter of the punctures on the vertex allowed for an unambiguous separation of *M. pilidens* and the other two species, which were more difficult to separate; three replicate measurements of the lectotype of *M. argentata* clearly clustered within *M. pilidens* (Fig. 4).

In summary, the identity of the lectotype designated by Hurd (1967) is clearly established as *M. pilidens*; it is likely that this specimen originates from northwestern Africa, since it has snow white vestiture; and Fabricius' original description, although vague, better corresponds to *M. pilidens* than to any other species of the *leachella* group. The synonymy of *M. pilidens* with *M. argentata* represents a nomenclatural change for a widely distributed species. We briefly discuss here alternative options regarding the treatment of this name.

The first option would be to follow Warncke's (1986) view that the lectotype was not part of the type series and more generally that the identity of *Megachile argentata* auct. should not have been changed, as has been done for other names by Linnaeus [e.g., *M. centuncularis* (Linnaeus, 1758), where a putative syntype was in fact *M. ligniseca* (Kirby, 1802)], or by Fabricius. A notorious example is *M. rotundata*: the lectotype, which was in agreement with the original description, was in fact a male of *M. centuncularis*. Roberts (1974) proposed to suppress this lectotype and to designate a neotype corresponding to *M. rotundata* auct. Although *M. rotundata* auct. is probably not native to Denmark (Erlandsson 1960; Holm 1982), the type locality

of *M. rotundata*, the proposal was accepted because of the economic importance of *M. rotundata* and the large number of scientific papers using this name (Roberts 1978). In the case of *M. argentata*, discarding the current lectotype does not appear appropriate, for the following reasons. First, we do not agree with Warncke (1986) that the lectotype designation was erroneous, for reasons explained above. Second, it would not be correct to state that the lectotype of *M. argentata* cannot be identified using morphology, as demonstrated above. Third, *M. argentata* has been used as a valid name until 1967 (although mostly for *M. leachella*, but also for *M. pilidens*), and in fact in many museums, specimens of *M. pilidens* and *M. leachella* are still mixed under *M. argentata.* Fourth, and most importantly, according to our species delimitation hypothesis (see below), another name has priority over *M. pilidens*: *M. schmiedeknechti*, a name currently in usage (e. g., Rebmann 1968; Rasmont et al. 1995; Nieto et al. 2014; Balzan et al. 2016; Cassar and Mifsud 2020). Therefore, neither a protection of *M. pilidens* nor of *M. schmiedeknechti* appear to make sense, since either approach would result in a major nomenclatural change. Furthermore, a reversal to the pre-1967 situation with *M. argentata* being the name of *M. leachella* would also constitute a major nomenclatural change since all current works use the latter name.

Two additional aspects need to be discussed. First, given that the lectotype of *M. argentata* originates from an undersampled geographic region (Algeria and Tunisa), could it belong to an additional, hitherto unknown taxon? We consider this possibility as very unlikely. In contrast to other groups of bees, such as osmiine (e.g., Müller 2012, 2022) or *Andrena* species (e.g., Wood 2021; Wood et al. 2021; Praz et al. 2022), most species of *Megachile* have broad distributions. We have examined hundreds of *Eutricharaea* from Morocco and Tunisia (admittedly fewer from Algeria); while northwestern Africa still hosts numerous unclear *Eutricharaea* taxa, these all belong to the more diverse *rotundata* group. The chance that an unknown species of the *leachella* group still exists in northwestern Africa is therefore considered to be small. The numerous barcoded specimens presented here also minimize the chance that additional cryptic diversity is found in this group in northwestern Africa.

Second, there are minimal morphological differences between northwestern African populations of "*M. pilidens*" (in the following paragraph "*argentata*"), and other western Palaearctic populations of this taxon ("*pilidens*"): vestiture is snow white in "*argentata*" (Fig. 15), except the dark vestiture on the thorax; it is yellowish brown in "*pilidens*" (Fig. 16), including on the thorax; in addition, the fringe of hairs on the margin of T5 in the male is continuous in "*argentata*" while it is reduced in other populations (except in populations of the taxon currently named *M. schmiedeknechti*). On the Island of Pantelleria, located between Tunisia and Sicily, the white form ("*argentata*") occurs, while on Sicily the regular-looking "*pilidens*" occur; in Malta, populations are morphologically divergent and have been attributed to *schmiedeknechti* (see below). Given these differences, could future studies suggest that "*argentata*" and "*pilidens*" represent two closely related, distinct, allopatric species? We argue that these two forms are conspecific, for the following reasons. First, we do not consider the minimal morphological differences between them as indicative of distinct species,

based on comparisons with other species of the *leachella* group. Variation in vestiture colour is for example larger in *M. leachella* than in *M. argentata/pilidens*. Second, our genetic data, including both mitochondrial and nuclear markers, also strongly suggest that "*argentata*" and "*pilidens*" belong to one unique evolutionary lineage; there were minimal differences between both, although these differences were considerably smaller than within-species differences in *M. leachella*. Third, a few specimens of *"pilidens"* examined from southern Spain have the vestiture lighter than in the rest of continental Europe, bridging the small morphological gap between "*pilidens*" and "*argentata*".

Based on the evidence assembled here, we thus place *M. pilidens* in synonymy with *M. argentata* (syn. nov.).

Holotypes of *Megachile inexspectata* and *M. striatella*

Megachile inexspectata was described from a single male specimen collected in Mut, Turkey in 1965 by Maximilian Schwarz. The holotype is in poor condition, probably because it has been relaxed for the preparation of the genitalia. It is labeled as follows (here and throughout the paper, the order of labels starts with the label closest to the body): 1. [a cardboard piece to which the genitalia and a T7 are glued]; 2. "Türkei Mut 12.VI.1965 leg. M. Schwarz". 3. [A yellow label with number 27, handwritten]. 4. "*inexspectata* n. sp. ♂ det. Dr Rebmann 1966". 5. "Typus" [printed on red paper, with SMFH 1866, handwritten on the reverse side]. 6. Senckenberg-Museum Frankfurt/Main. 7. "*Megachile walkeri* ♂? D. B. Baker det. 1990". The examination of the holotype reveals the following issue. The nearly entire metasoma, including T7, is still attached to the pinned specimen. The genitalia have been extracted and are glued on a piece of cardboard attached to the same pin (Fig. 31); an additional T7 is glued to the piece of cardboard alongside the genitalia. Body and genitalia do not appear to be conspecific: the gonostylus is like that of *M. walkeri*, as indicated in the original description of *M. inexspectata* (Rebmann 1968: fig. 11), thus with a short, blunt preapical process (Figs 30, 31). Only two Palaearctic species of *Eutricharaea* species have such a gonostylus: *Megachile walkeri* and the species hitherto referred to as *M. inexspectata*. The pinned specimen, however, does not belong to either species, as there is a pointed tooth behind the mandibular base (cf. Fig. 12), which suggests that it belongs either to *M. leachella* or to a species of the *concinna* complex. Both *M. walkeri* and *M. inexspectata* have a blunt, truncate tooth behind the mandibular base, as in *M. pilidens*.

The holotype of *M. striatella* Rebmann, 1968, also in poor condition, is labeled as follows: 1. a cardboard with the genitalia glued; 2. "El Kantara [Algeria], 7. Juli 1904, Dr. Gulde". 3. Typus [printed on red paper]; SMF H 1593 [written on reverse side of label]. 4. *Megachile concinna* D. B. Baker det. 1990. 5. Senckenberg Museum Frankfurt am Main. Examination of this specimen reveals that the body of the holotype is most probably *M. inexspectata*, as indicated by the lack of tooth behind the mandibular base and the conspicuous patch of yellow hairs medially on S4 (cf. Fig. 29); T7 is missing and not found alongside the genitalia. The latter, glued on a piece of paper, are like in the *concinna* complex (cf. Fig. 14, 66), with a simple gonostylus. Since all

members of the *concinna* complex have a distinct tooth behind the mandibular base, we conclude that the body of the holotype is not conspecific with the genitalia. Male paratypes clearly belong to the *concinna* complex, probably to *M. pusilla*, the only member of the *concinna* complex so far known in Algeria (Soltani et al. 2017).

Rebmann relaxed his specimens during 24 hours for the preparation of the hidden sterna (Rebmann 1968); during this process, the locality labels have to be removed. We hypothesize that the body of *M. inexspectata* and of *M. striatella* have been associated with the wrong genitalia during preparation. Following this hypothesis, the genitalia and T7 glued on a piece of cardboard beneath the holotype of *M. inexspectata* probably belong to the pinned body of the holotype of *M. striatella*; and the genitalia of the holotype of *M. striatella* probably belong to the pinned specimen of the holotype of *M. inexspectata*. This hypothesis is further substantiated by the following fact: we examined numerous bees collected by M. Schwarz during his 1965 trip to Turkey; all of them were pinned using entomological pins with a glass head (hereafter "new pin"), a type of pin little used, possibly not at all, at the beginning of the century, when the type series of *M. striatella* was collected in Algeria. All paratypes of *M. striatella* have another type of pin ("old pin"), where the head is made of a small piece of curled metal. The body of the holotype of "*M. striatella*", although supposedly collected in 1904, is pinned with a pin of the new type, just like all the specimens collected by M. Schwarz in Turkey, while the body of the holotype of "*M. inexspectata*" is pinned with an old pin. The most probable hypothesis is therefore that the genitalia and body of both holotypes of *M. striatella* and *M. inexspectata* have been mixed during relaxation. All dissected specimens in the Rebmann collection have been examined, and no additional inconsistencies (in particular, a missing T7) have been found.

Assuming that our hypothesis is correct, we are left with the following two possibilities to resolve this confused taxonomic situation. The first possibility would be to submit a request to the International Commission on Zoological Nomenclature to discard current holotypes and to designate neotypes for both taxa. The second possibility is to invoke article 73.1.5 of the code, which states that "if a subsequent author finds that a holotype which consists of a set of components (e.g. disarticulated body parts) is not derived from an individual animal, the extraneous components may, by appropriate citation, be excluded from the holotype". Following this last approach, we assume that the genitalia (but not the body) of the specimens originate from the correct location (Mut, Turkey for the genitalia of the holotype of *M. inexspectata* and Kantara, Algeria for the genitalia of the holotype of *M. striatella*), exclude the body of these two specimens from the holotype, and declare that only the genitalia serve as name-bearing types for these two taxa. Based on current knowledge of this group of bees in Turkey and Algeria, only *M. inexspectata* (see Norfolk and Dathe 2019; Boustani et al. 2021; Praz et al. 2021) has the genitalia of the "*walkeri*" type in Turkey; and only *M. pusilla* has a simple gonostylus in Algeria. We therefore place *M. striatella* in synonymy with *M. pusilla* (syn. nov). The cardboard with the genitalia (Fig. 31) and T7 of *M. inexspectata* has been placed onto a separate pin, together with all the labels (see above). An eighth label has been added, handwritten on red paper: "Holotypus *Megachile inexspectata*

Rebmann C. Praz 15.09.2021". The body of the specimen is kept next to this holotype, with the following two new labels. 1. "*Megachile* cf. *pusilla* det C. Praz 2021". 2. (handwritten on white paper) "Specimen originally associated with the holotype genitalia of *M. inexspectata* Rebmann. Of no type value. C. Praz 2021". In addition, one male specimen of *M. inexspectata* with the following label information: 1. "Jordan W, 30kim W Tafila, 2.5.1996 leg. Marek Halada" and 2."*Megachile inexspectata* Rebmann det. C. Praz 2021" is deposited next to the holotype (genitalia). Under *M. striatella*, the cardboard with the genitalia has been placed onto a separate pin with the label "Holotypus *Megachile striatella* Rebmann C. Praz 15.09.2021". The body of the specimen is kept next to this holotype, with the following labels: 1. "*Megachile* cf. *inexspectata* det C. Praz 2021". 2. (handwritten on white paper) "Specimen originally associated with the holotype genitalia of *M. striatella* Rebmann. Of no type value. C. Praz 2021".

Phylogenetic analyses

COI

The labelling of the taxa in the tree is done in anticipation of our final delimitation and taxonomic decisions. For clarity, specimens of *M. argentata* from outside of northwestern Africa are labeled as "*pilidens*", those from Sardinia and Malta as *M. schmiedeknechti*. The phylogenetic tree (Fig. 5) inferred using the mitochondrial gene COI recovered all species as monophyletic group, except for *M. viridicollis*, which formed a grade from which *M. anatolica* arose. Support was maximal (Bootstrap support of 100%) for all species except for *M. leachella* (67%) and *M. anatolica* (73%).

Within-species distances in the taxa of the *concinna* complex have been presented elsewhere (Soltani et al. 2017) and are not discussed here. Within-species distances were small (less than 0.2%) in *M. walkeri*. In *M. inexspectata*, there were two clades, one including specimens from Israel, and one with northwestern African specimens. Genetic distances within these clades were low (0–0.6%), and the average distance between these clades was 3.4% (minimum 2.9, maximum 4.0%).

By contrast, within-species genetic distances were considerable in *M. leachella*. First, two specimens from Crete (treated here as a new subspecies, *M. leachella cretica* ssp. nov.) were divergent and formed a well-supported clade that was sister to all other specimens of *M. leachella* (Fig. 5). Genetic distances between this clade and other specimens of *M. leachella* were on average 5.3% (minimum 4.4, maximum 6.1%). Within the rest of *M. leachella*, there was weak structuring, with one clade composed of specimens from Iran, Kyrgyzstan, Israel and Greece, and one clade of all specimens from northwestern Africa (delineated here as the subspecies *M. leachella albipila* Pérez, 1895), all specimens from the Sardinia, and two specimens from Cyprus (delineated here as the subspecies *M. leachella densipunctata* ssp. nov.); several specimens from different localities (Italy, Austria, the UK, Spain, etc) formed a grade outside of these two clades. The average genetic distance among all specimens of *leachella* (except for the specimens from Crete) was 0.9% (minimum 0, maximum 2.5%). The two individuals

Figure 5. Best tree found in maximum likelihood analyses of sequence data of the mitochondrial gene COI showing the phylogenetic relationships among the Palaearctic species of the *leachella* group of *Megachile* (*Eutricharaea*). Bootstrap support values are based on 1000 bootstrap replicates.

from Cyprus (belonging to the subspecies *M. leachella densipunctata* ssp. nov.) were separated from other specimens of *M. leachella* (excluding those from Crete) by an average distance of 1.3% (minimum 0.8, maximum 2.5%).

Within *M. argentata*, within-species genetic distances were small (max: 0.5%). Three specimens from northwestern Africa were weakly divergent from all other specimens (average genetic distances 0.2%; minimum 0.2, maximum 0.5%). Two specimens from Sardinia, attributed to the subspecies *M. argentata schmiedeknechti*, were only weakly separated from all other specimens (average genetic distances 0.2%; minimum 0.2, maximum 0.5%). One specimen from Malta, attributed to *M. argentata schmiedeknechti*, had identical sequence with several specimens from central Europe (Fig. 5).

Nuclear genes

Single genes phylogenies based on opsin and CAD (data not shown) were little resolved, but all species were recovered as monophyletic groups, except as follows: *Megachile pusilla* did not form a monophyletic group in analyses of opsin, and *M. leucostoma* in analyses of CAD; in analyses of opsin, some populations of *M. leachella* shared an allele with *M. anatolica* and *M. viridicollis* (see Soltani et al. 2017). In analyses of the concatenated, two-genes dataset (Fig. 6), all species formed monophyletic groups, except *M. leucostoma*, which formed an unresolved polytomy with one specimen of *M. viridicollis* and a clade containing all specimens of *M. anatolica*. Within *M. leachella*, two specimens from Cyprus (*M. leachella densipunctata* ssp. nov.) formed a clade that was sister to a clade containing all other specimens. There were two clades within the latter clade, one including specimens from Kyrgyzstan, Greece and Israel; and one including all other specimens. Unlike in COI-based phylogenies, the only sequenced specimen from Crete (*M. leachella cretica*) was not strongly divergent from *M. leachella sensu lato*. This specimen was more closely related to western European and western Mediterranean populations, than to the nearby southeastern European populations, in agreement with some morphological characters (see below).

Within *M. argentata*, the only sequenced specimen from northwestern Africa was sister to all other specimens; both specimens from Sardinia (*M. argentata schmiedeknechti*) were weakly divergent from two specimens from Greece and Switzerland.

Species delimitation

Megachile argentata

This species is sculpturally very uniform throughout its range. The colour of the vestiture is however variable geographically. As discussed above, the populations from northwestern Africa (Fig. 15) have lighter vestiture than the continental populations (Fig. 16); similarly light-coloured populations are also found on the Island of Pantelleria. We do not recognize different subspecies for continental ("*pilidens*") and northwestern African ("*argentata*") populations because the morphological differences are minor, and because southern Iberian specimens appear to be intermediate.

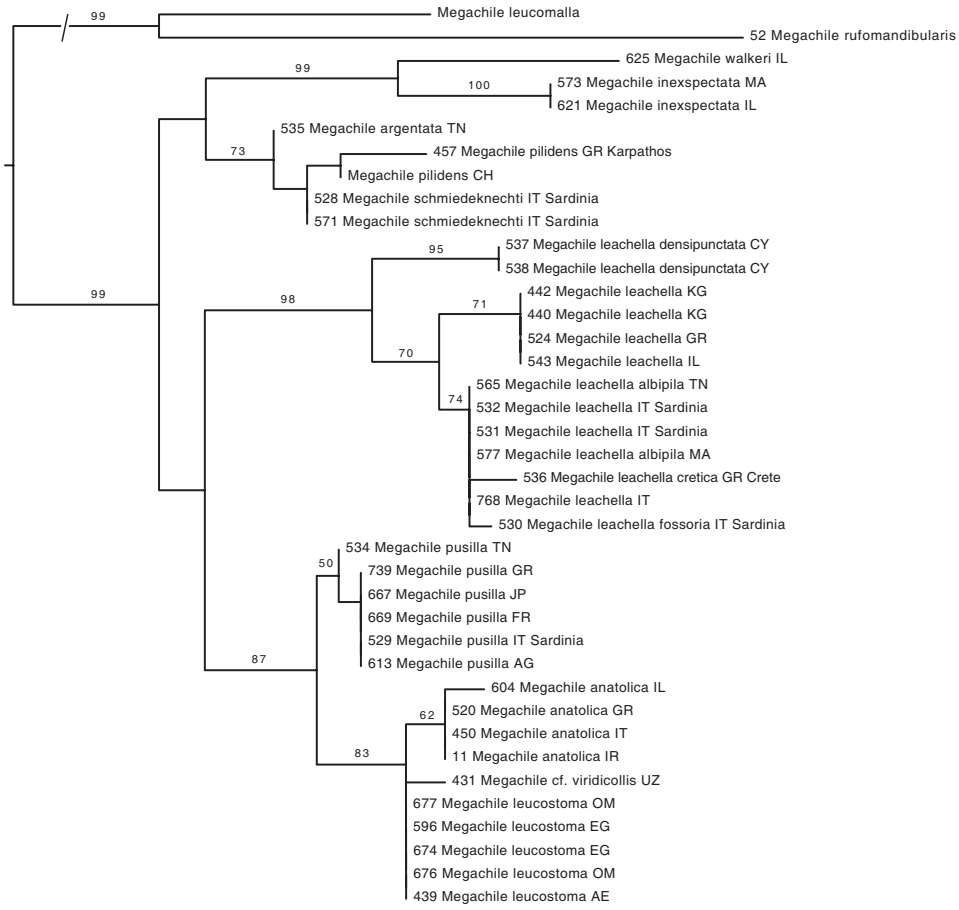


Figure 6. Best tree found in maximum likelihood analyses of sequence data of the two nuclear genes lw rhodopsin and CAD showing the phylogenetic relationships among the Palaearctic species of the *leachella* group of *Megachile* (*Eutricharaea*). Bootstrap support values are based on 1000 bootstrap replicates. The oblique line along the branch joining the outgroup taxa and the ingroup indicates that this branch has been shortened for better graphic representation.

The vestiture is red-orange on the Islands of Malta (Fig. 17), and yellow-orange on Corsica and Sardinia (Fig. 18); this insular form has so far been referred to as *M. schmiedeknechti*, described from Sardinia. van der Zanden (1983: 138) stated that the form found in Malta was not conspecific with *M. schmiedeknechti* due to subtle differences in punctation, and treated the Maltese form as *M. xanthopyga*, probably assuming that *M. xanthopyga* had been described from northern Africa (see above). Our genetic data does not support the recognition of *M. schmiedeknechti* as a distinct species. Rebmann (1968) indicated that *M. schmiedeknechti* and *M. pilidens* differed in the structure of S5 and S6; we have dissected and examined several males of both taxa and did not find any consistent difference, neither could we confirm the characters presented by Rebmann (1968). Moreover, Rebmann (1968) mentioned the presence

of *M. pilidens* on the Island of Sardinia; we have not been able to locate specimens to verify this hypothesis, which probably relies on misidentified specimens. Benoist (1940: 65) mentions that *M. schmiedeknechti* occurs in Southern France [Var: Callian]. A specimen from Callian, Var, leg. Berland 1924, is preserved in MNHN under *M. schmiedeknechti*; although it bears the identification label "*M. schmiedeknechti* det. van der Zanden", it is a female specimen of *M. melanopyga*. It is probable that Benoist's record of *M. schmiedeknechti* from Callian is based on this specimen. Mentions of *M. schmiedeknechti* (or from *M. xanthopyga*) from northern Africa (e.g., Friese 1899a; Benoist 1940) probably refer to the erroneously assumed type locality of *M. xanthopyga*.

Contrasting the current view, we propose to treat *M. schmiedeknechti* as a subspecies of *M. argentata*, for the following reasons. First, only vestiture colour appears to separate it from continental (including northwestern African) populations, and no sculptural difference, except that tergal punctation in both sexes is finer and denser in *M. schmiedeknechti*, especially in the female; as discussed above, vestiture colour is variable in the *leachella* group and in bees in general. Second, DNA barcodes are shared or very similar between continental and insular forms (Fig. 5), unlike in other taxa restricted to Corsica and Sardinia, such as *Bombus* (*terrestris*) *xanthopus* (Kriechbaumer, 1870) (see note under table 4 in Williams et al. 2012; Williams 2021) or *Andrena antonellae* Praz & Genoud, 2022 (Praz et al. 2022). Third, in contrast to Sardinia and Corsica, the Island of Malta was connected to the Italian mainland at the end of the last glaciation (Furlani et al. 2013), suggesting that Maltese populations of *M. schmiedeknechti* may not have been isolated from continental ones for more than one glaciation cycle. Lastly, a form of *M. argentata* with partly orange scopa is known from the Island of Karpathos (Greece), indicating that the orange vestiture is not unique to *M. schmiedeknechti.* The following hypothesis can be formulated to explain the presence of this colour form of *M. argentata* on the Islands of Corsica, Sardinia and Malta. This colour morph may have been more widely distributed during or just after the end of the last Glaciation, explaining why it is currently found on distantly located Islands. After the end of the last glaciation, this orange form may have been replaced by the regular looking form currently present everywhere in Europe, possibly from an eastern refugium, except on these three Islands. This scenario could also explain why a slightly orange-coloured form is present on the Island of Karpathos, located over 1000 km from Malta. An alternative hypothesis is that the populations of *M. argentata schmiedeknechti* in Malta has independently converged to the orange colour observed on Sardinia and Corsica. Yet another possibility is that *M. argentata* colonized Malta or either Corsica or Sardinia from continental populations long ago; divergence and isolation may have resulted in the appearance of the orange vestiture condition, and then one event of long-distance dispersal could have enabled exchanges between Malta and either Corsica or Sardinia. Since *M. argentata* only rarely nests in dead wood, we consider this hypothesis as unlikely; a long-distance dispersal event between either Corsica or Sardinia and Malta, appears far less likely than dispersal between southern Italy and Malta.

We recognize *M. schmiedeknechti* as a valid subspecies of *M. argentata* and propose the following new combination: *Megachile argentata schmiedkechti*, stat. nov. This subspecies is morphologically well characterized, geographically well-delimitated and no intermediate form is known to exist (except for the populations found

on the Island of Karpathos, which are not located near the contact zone between *M. argentata schmiedeknechti* and the continental form). The main argument for treating this insular form as a subspecies and not simply as a geographic form is the abrupt contact zone with no transitional populations between Malta and the Italian mainland. Such an abrupt transition may be indicative of some reproduction interference between both forms, and suggests that the recognition of *schmiedeknechti* as a distinct conservation unit is meaningful. We do not recognize a distinct subspecies for the populations of Karpathos because the phenotypic differentiation of these populations with respect to those found in mainland Greece is weak. Our treatment of the Maltese populations as belonging to that subspecies has to be considered as tentative. Future work including more in-depth genetic analyses should further examine the relationships between mainland European, insular and northern African populations of *M. argentata*.

Megachile inexspectata

This taxon forms two putatively allopatric populations, one from the Sinai Peninsula to southern Turkey (eastern populations), the other in northwestern Africa (western populations). The mitochondrial genetic divergences between these two populations were considerable (3.4% in COI analyses; Fig. 5), but morphological differentiation or nuclear genetic differences (Fig. 6) were weak. The only character that separates these two populations is the white vestiture on the disc of T6: in eastern populations, this vestiture consistently forms a single spot (Fig. 25), while in western populations, two spots are often (but not always) separated by a thin line of black hairs, as in *M. leachella* (cf. Figs 32, 33, 36). We consider these genetic and morphological differences to be weak and do not recognize distinct subspecies within *M. inexspectata*.

Megachile leachella

Unlike *M. argentata*, *M. leachella* exhibits considerable morphological variation over its wide range, including variation in vestiture colour, in sculpture and in the structure of the genital capsule. There was also considerably more genetic structuring in *M. leachella* than in *M. argentata* (Figs 5, 6). Both taxa have comparable ranges, although the distribution of *M. leachella* expands further north than that of *M. argentata*. One explanation for this elevated variation within *M. leachella* is the association of this species with sandy habitats, which are not continuously distributed in central and southern Europe. Because some of the morphological variation in *M. leachella* corresponds to geographically isolated forms, we propose the recognition of several subspecies. Some of these forms exhibit pronounced morphological or genetic differences and could well be treated as distinct species. Since none of these delineated forms co-exists in sympatry with the regular-looking *M. leachella*, and since there is a continuum from weakly to strongly differentiated forms, we prefer a broad species-concept and the recognition of one widely distributed species with the most conspicuous forms split as subspecies.

In continental Europe and in the UK, phenotypic variation is essentially restricted to vestiture colour in the female sex (Schwarz and Gusenleitner 2012). In the UK, *M.*

leachella has a bright yellowish vestiture (Fig. 32), a condition not observed in continental Europe, where a grade is observed from snow-white vestiture in southern Europe (cf. Fig. 33) to gradually darker, grey-brown vestiture in central and northern Europe. The punctation of the vertex appears to follow a similar pattern: in southern Europe, the punctation is fine and dense (e.g., cf. Figs 41, 43); it becomes coarser towards the north and in the UK (Fig. 40). Since this pattern in vestiture colour and punctation does not allow for the delineation of clearly delimitated taxa, we do not recognize subspecies in continental Europe.

The populations from northwestern Africa (from Tunisia to Morocco) exhibit a striking difference in the structure of the genital capsule: the gonostylus has a short preapical process (Fig. 55), much shorter than anywhere else (Figs 54, 56). For this reason, the northwestern African populations are treated as a distinct subspecies, *Megachile leachella albipila* stat. nov. We do not recognize this taxon as a distinct species, for the following reasons. First, there is variation in this genital character, as the length of the preapical process of the gonostylus in northwestern African populations and in other populations of *M. leachella* is variable (e.g., Figs 54, 56), although the process is never as long in northwestern African as elsewhere. Second, *M. leachella leachella* appears to be replaced by *M. leachella albipila* in northwestern Africa; if both taxa were demonstrated to maintain distinctiveness in sympatry, they should be recognized as distinct taxa, but such evidence is currently lacking. Third, splitting *M. leachella albipila* as a distinct species would render *M. leachella* paraphyletic according to our genetic analyses (Figs 5, 6); while paraphyletic species are theoretically possible, we favor the delineation of monophyletic species. Fourth, our genetic analyses including both mitochondrial and nuclear genes suggest little differentiation between *M. leachella albipila* and central European *M. leachella*; the other subspecies of *M. leachella* are genetically more divergent from nominal populations of *M. leachella* (Figs 5, 6). As a final note, the following hypothesis for the divergent genital structure of *M. leachella albipila* can be formulated: it is possible that the northwestern African population of *M. leachella* are the outcome of past introgression with *M. pusilla*, given that the genital structure of *M. leachella albipila* (Fig. 55) is intermediate between that of *M. leachella leachella* (Figs 54, 56) and *M. pusilla* (Fig. 66). Our genetic data, however, indicates no signature of possible introgression between *M. leachella* and *M. pusilla*.

On the Islands of Crete, a distinct form is found, which is morphologically slightly divergent but genetically strongly divergent from all other populations of *M. leachella* (genetic distances in COI on average 5.3%). Morphological differences include the presence of numerous dark hairs laterally on the terga, smaller spots of white hairs on the disc of T6 (Fig. 35), the dark vestiture on the scutum, and some differences in the punctation (Figs 38, 42) (see below, taxonomic part). The punctation of the vertex is coarse (Fig. 42) as in northern European populations (Fig. 40), but unlike in southeastern Europe (cf. Figs 41, 43). The phylogeny based on nuclear genes also suggests a closer relationship with the central European populations than with the southeastern European ones. We propose to delineate the Crete populations as a new subspecies, *M. leachella cretica* ssp. nov. This taxon could be recognized as a distinct species based on COI-phylogenies and genetic distances. Nuclear genetic data however suggested a close relationship with other populations of *M. leachella sensu lato*, as does morphology. For this reason, this taxon is recognized as a subspecies and not as a distinct species.

On the Island of Cyprus, the populations of *M. leachella* are morphologically strongly divergent from those on continental Europe. The punctation of the terga in the female sex is fine and dense (Figs 36, 39), as in *M. argentata* (Figs 15–19) or *M. inexspectata* (Figs 25, 26) but strongly different from most other populations of *M. leachella* (Figs 32–34, 37), except for *M. leachella cretica* ssp. nov. (Figs 35, 38), which is intermediate between regular *M. leachella* and *M. leachella densipunctata* ssp. nov. Some specimens from the Levant also have comparatively dense tergal punctation and are intermediate between *M. leachella* and *M. leachella densipunctata* ssp. nov. In addition, the base of the terga is strongly impressed in *M. leachella densipunctata* (Figs 36, 39, 47), a condition not observed in other populations of *M. leachella*. The mitochondrial genetic analyses suggested only small divergences (on average 1.32%; Fig. 5) from continental populations of *M. leachella*. By contrast, the nuclear genetic data (Fig. 6) suggested considerable divergences from other populations of *M. leachella*, in agreement with the pronounced morphological differences. Thus as in *M. leachella cretica* ssp. nov., genetic results based on the mitochondrial gene were less in agreement with morphology than those based on nuclear genes; possibly the mitochondrial genome is more prone to repeated selective sweeps (e.g., Jiggins 2003; Bazin et al. 2006) or introgression events (Nicholls et al. 2012; Klopfstein et al. 2016), compared to nuclear markers (Gueuning et al. 2020). We delineate the Cypriote populations as a new subspecies, *M. leachella densipunctata* ssp. nov. This taxon could be recognized as a distinct species based on a pure morphological concept. The absence of differences in male genitalia and hidden sterna, the high variability in punctation characters in *M. leachella sensu lato*, the comparatively low levels of genetic differentiation, and the fact that this subspecies is geographically well-isolated and does not co-occur with the other subspecies of *M. leachella* lead us to treat this taxon as a subspecies.

Lastly, the populations from Sardinia and to some extend Corsica exhibit weak morphological differentiation compared to continental European populations. In particular, in males the front tarsi are yellowish white (Fig. 50) and the last antennal segment slightly enlarged in Sardinian populations; in addition, the ventral surface of the second tarsal segment has a distinct dark maculation, approaching the condition observed in numerous species of *Megachile* with modified and enlarged front tarsi; elsewhere, the front tarsi are predominantly dark brown (there is considerable variation in this character; Figs 48, 49, 51, 52) and no distinct dark maculation is observed under the second segment; and the last antennal segment is usually not larger than the next to last. Females from Sardinian populations have the vestiture snow white (Fig. 33), as in southern Europe populations, but there are numerous dark hairs laterally on T4-T6; such dark hairs are mostly missing in southern European populations (but present for example in Crete: Fig. 35). Corsican populations appear to be intermediate between Sardinian and southern European populations: the male front tarsi are intermediate between the condition observed in Sardinia and that in European populations; no dark maculation is visible under the second tarsal segment. The female often has dark lateral hairs on T4-T6, as in Sardinian populations. The distinctiveness of the male from Sardinian populations has been noted before: Rebmann (1968) described *M. ichnusae* Rebmann, 1968 from two males collected in Sardinia (the female described as *M. ichnusae* belongs to *M. fertoni* Pérez,

1896, a member of the *rotundata* group of species). *Megachile argentata* var. *fossoria* Ferton, 1909 has been described from Corsican populations. Given that the morphological differences between the Sardinian populations and other populations are unsignificant (although constant), given the intermediate nature of the Corsican populations, and given the weak genetic differentiation between Sardinian and other populations, we do not recognize a distinct subspecies for the Corso-Sardinian populations of *M. leachella*. Consequently, we place *M. ichnusae* in synonymy with *M. leachella* (syn. nov).

Lastly, we have examined several females of *M. leachella* from Lesvos, in which the ocelloccipital distance was particularly long, approaching the condition observed in *M. anatolica* (cf. Fig. 9). In these specimens the clypeus apical margin was also atypical for *M. leachella*. Whether these specimens are aberrant specimens of *M. leachella*, or indicate a somehow introgressed population of *M. leachella* with *M. anatolica*, is not clear and requires additional research.

Systematic part

Western Palaearctic species

***Megachile anatolica* Rebmann, 1968**
Figs 7–14

Megachile anatolica Rebmann, 1968: 37, ♂ nec ♀, "Mut [Turkey, approx. 36.64°N, 33.44°E]". Holotype ♂ (SMFD).

Material examined. Type material. ***Holotype*** ♂ (SMFD) of *M. anatolica* (Fig. 11); one paratype ♀ (SMFD) examined is probably a female of *M. inexspectata*.

Other material. 104 specimens from the following countries: Croatia, Cyprus, Greece, Iran, Israel, Italy, Jordan, Lebanon, Turkey (Suppl. material 1).

Distribution. From Italy eastwards through the Levant including Lebanon, Turkey, Israel northwest of the Dead Sea, Iran; distribution in Central Asia remains to be established due to unclear relationship with *M. viridicollis*.

Geographic variation. The species varies in the colour of the vestiture as well as in body size and in the length of the OOD. In Italy, Greece, Cyprus and western Turkey, the scopa is white (black on S6) and the OOD is large (Fig. 9). In the Levant the species presumably intergrades with *M. leucostoma*; populations in northern Israel and northern Jordan have the scopa nearly entirely orange, as observed in eastern populations of *M. leucostoma* (see Praz et al. 2021). Moreover, the length of the OOD shows clinal variation from northern Israel (condition as in typical *M. anatolica*) to Southern Israel (condition as in typical *M. leucostoma*); specimens from Central Israel are intermediate (Soltani et al. 2017: Fig. 6). In Iran, the scopa is white (black on S6), but specimens are smaller and the OOD short, as in *M. leachella* or *M. pusilla*. While in some Iranian populations the clypeus margin is denticulate, as in typical *M. anatolica* (Fig. 10), in other populations (e.g., in the region of Teheran), the clypeus is as in *M. pusilla*

Figures 7–14. *Megachile anatolica* **7** female metasoma **8** female metasomal tergum 4 **9** female vertex **10** apex of female clypeus **11** dorsal view of head of holotype male **12** front view of male head with distinct tooth behind mandibular base **13** male metasomal sterna 3–5 **14** male genitalia.

(cf. Fig. 64), making these populations superficially identical to *M. pusilla*, albeit genetically closer to *M. anatolica.* Whether the variation observed in Iran is the result of introgression with *M. leucostoma*, which has not been reported in Iran but is present on the Arabian Peninsula, e.g., in the United Arab Emirates, remains to be established.

Note. The relationship between *M. anatolica* and the Central Asian species *M. viridicollis* is not clear; these two taxa may eventually be treated as conspecific, in which case *M. anatolica* would be placed in synonymy with *M. viridicollis*; see under *M. viridicollis*.

Megachile argentata **(Fabricius, 1793)**

Figs 1–3, 15–24

Megachile argentata argentata **(Fabricius, 1793)**

Apis argentata Fabricius, 1793: 336 [sex not indicated], "in Barbaria" [Algeria or Tunisia]. Lectotype ♀ (NHMD), by designation of Hurd (1967).

Megachile compacta Pérez, 1895: 24, ♀, [Algeria]. Preoccupied, not *Megachile compacta* Smith, 1879. Lectotype ♀ (MNHN), by present designation (see below). New Synonymy.

Megachile crassula Pérez, 1896: 1. *Nomen novum* for *M. compacta* Pérez. New synonymy.

Perezia maura Ferton, 1914: 233, ♀ [gynandromorph specimen], "Cimetière de Djidjelli" [Jijel, Algeria]. Holotype intersex (MNHN). Preoccupied, not *Megachile maura* Cresson, 1865. Synonymy with *M. centuncularis* in Pasteels (1969: 248). Synonymy with *M. leachella* in van der Zanden (1988: 56). New synonymy.

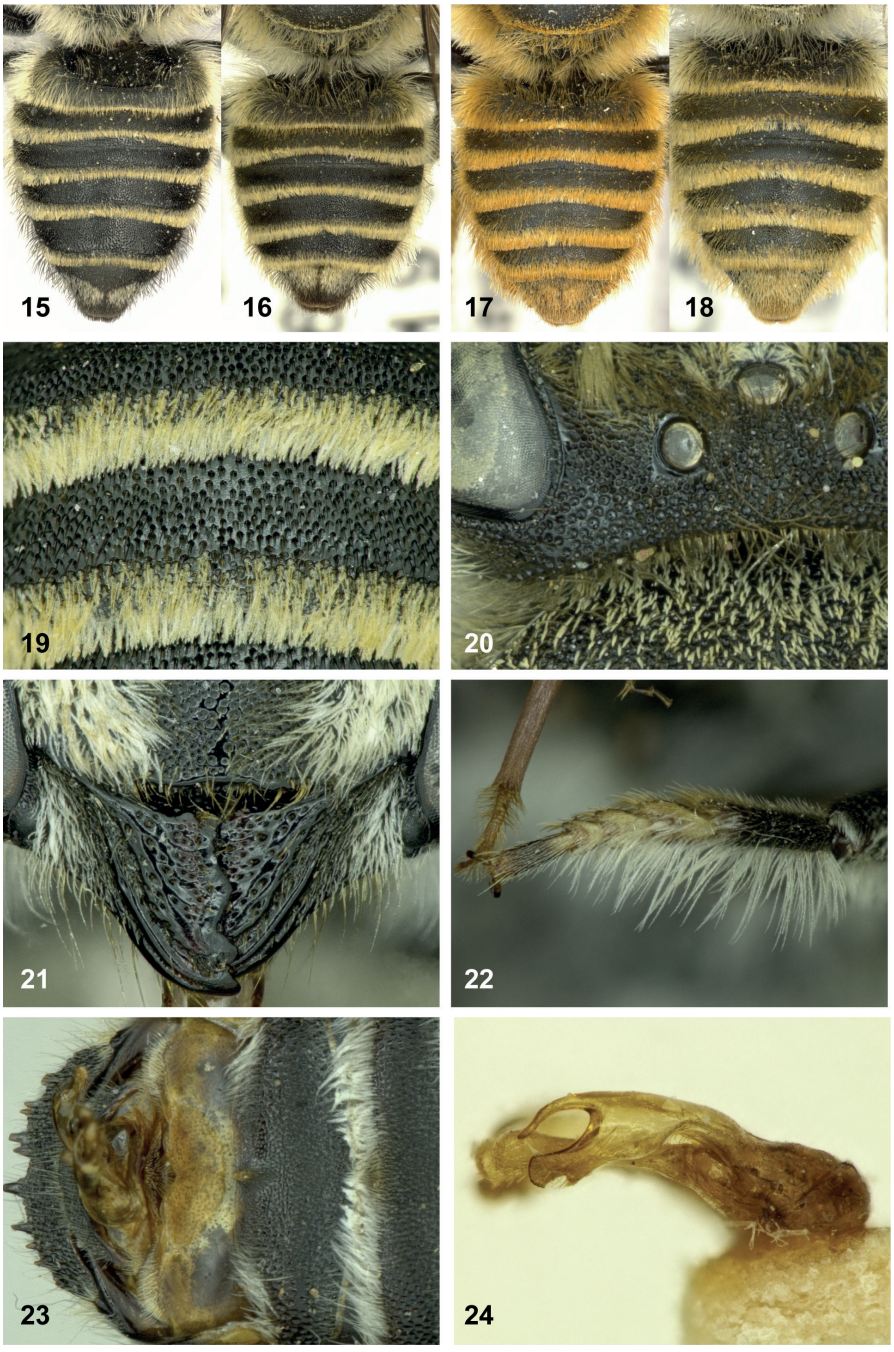
Megachile pilidens Alfken, 1924: 88, ♀ ♂, "Triest [Trieste, Italy]". Lectotype ♀ (ZMHB), by designation of Tkalců 1967: 100. New synonymy.

Megachile argyrea Cockerell, 1931: 275, ♀ ♂, "Asni [Morocco]". Holotype ♀ (USNM), paratypes ♀ ♂ (BMNH). New synonymy. Synonymy with *M. pilidens* in Hedicke, 1933: 42.

Material examined. Type material. ***Lectotype*** ♀ of *M. argentata* (NHMD) (Figs 1–3; see above, identity of type specimens).

Lectotype ♀ of *M. crassula* (MNHN), by present designation, a female in good condition labeled as follows: 1. "Algeria" [printed]; 2. *crassula* JP [handwritten, presumably by Pérez]; 3. "Museum Paris Coll. J. Pérez" [printed on blue paper]; 4. "Holotype *M. crassula* des. Baker 1991" [printed and handwritten on red paper]; 5. "Lectotype *M. crassula* des. C. Praz 2022". Although the original description does not mention the number of specimens, we prefer to designate this specimen as the lectotype.

Holotype [gynandromorph] of *Perezia maura* (MNHN), labeled as follows: 1. "Djidjelli 17-8/13 p. 315" [handwritten by Ferton]; 2. "*Perezia maura* ♀ Fert" [handwritten by Ferton]; 3. Holotype [printed, red]; 4. Muséum Paris Ch. Ferton 1902 [printed, blue paper]; 5. *Megachile pilidens* Alfken [symbol for gynandromorph] det. G.v.d. Zanden 1986 ; 6. "*Perezia maura* Ferton = gynandromorph de



Figures 15–24. *Megachile argentata* **15** female metasoma of northwestern African populations, *M. argentata argentata* **16** female metasoma of European, Levant, Turkey and Central Asian populations, *M. argentata argentata* **17** female metasoma of Malta populations, *M. argentata schmiedeknechti* **18** female metasoma of Corsican and Sardinian populations, *M. argentata schmiedeknechti* **19** female metasomal tergum 4 **20** female vertex **21** apex of female clypeus **22** male front tarsi **23** male metasomal sterna 3–5 **24** male genitalia.

Megachile centuncularis J. Pasteels det., 1969"; 7. *Megachile argentata* intersex det C. Praz 2022. It is unclear why van der Zanden (1988) placed this taxon in synonymy with *M. leachella* since the label on the specimen suggests he correctly identified the holotype as *M. pilidens*.

Lectotype ♀ of *M. pilidens* (ZMHB). A female without metasoma, but clearly identified as *M. argentata* (see Tkalců 1967).

Paratypes ♀ and ♂ of *M. argyrea* (BMNH), all in good condition and clearly corresponding to *M. argentata*. We did not examine the holotype ♀ of *M. argyrea*, but pictures are available at https://collections.nmnh.si.edu/search/ento/; based on these pictures, the holotype agrees with *M. argentata* in the dark vestiture of the lateral parts of T5 (absent in *M. leachella albipila* and *M. inexspectata*). Punctation of the vertex is not visible but the dense punctation of the terga appears to exclude *M. leachella albipila*. This character and the identity of examined male and female paratypes (BMNH) collected in the same site confirm synonymy with *M. argentata.*

Note. *Megachile beaumonti* Benoist, 1951, is currently treated as a synonym of *M. crassula* (van der Zanden 1990: 53). We have examined the holotype ♀ of this taxon (MZL); *Megachile beaumonti* is not conspecific with *M. argentata*, but is a valid species of the *rotundata* group (stat. rev.).

Other material. 216 specimens from the following countries: Croatia, France, Germany, Greece, Iran, Italy, Kyrgyzstan, Lebanon, Morocco, Northern Macedonia, Portugal, Russia, Serbia, Spain, Switzerland, Tunisia, Turkey, Ukraine (Suppl. material 1).

Distribution. Widespread and abundant in southern Europe; in expansion in central and northern Europe, e.g., in northern Switzerland (C. Praz et al., in prep.), the Netherlands (Peeters et al. 2006) and Germany (LUBW 2007; Schweitzer and Theunert 2019). Present in the Levant (Turkey, Lebanon), but so far its presence has not been confirmed in Israel and Egypt (Praz et al. 2021). We have examined specimens from Rhodos, Lesvos, Samos, Karpathos, Patmos, Chios, but neither from Cyprus [the record of *M. pilidens* by Varnava et al. (2020) is probably an identification error and likely refers to *M. leachella densipunctata* ssp. nov.] nor from Crete. Further east, the species is present in Iran, Armenia and Kyrgyzstan.

Geographic variation. Geographic variation has been discussed above in detail. The northwestern African populations, as well as populations from the Island of Pantelleria, have overall snow-white vestiture in the female sex (Fig. 15) and dark vestiture on the mesonotum and vertex, and in the male sex a continuous fringe of hairs on the margin of T6. On the Island of Karpathos, the female scopa is orange on S5 and S6, approaching the condition observed in *M. argentata schmiedeknechti*.

Megachile argentata schmiedeknechti Costa, 1884

Megachile Schmiedeknechti Costa, 1884: 169, ♀ ♂, [Italy, Sardinia].

Megachile xanthopyga Pérez, 1895: 25, ♀ ♂, "Sassari [Italy, Sardinia]". Lectotype ♀ (MNHN), by present designation (see below). Paralectotypes ♀ (MNHN), by present designation.

Type material. ***Lectotype*** ♀ (MNHN) of *M. xanthopyga*, a female in good condition labeled as follows: 1. "MNHN Paris EY33616" [printed, with QR-code]; 2. [blue circular disc]; 3. "Sassari" [handwritten, presumably by Pérez]; 4. "Museum Paris Coll. Pérez 1915" [printed]; 5. "Lectotype *Megachile xanthopyga* des. Baker 1991" [printed and handwritten on red paper]; 6. "*Megachile schmiedeknechti* det. van der Zanden 1995" [printed]. This lectotype designation has not been published and is accepted here. Two additional females from Sassari, each with a label "paralectotype *M. xanthopyga* des. Baker 1991" are designated as paralectotypes. The type locality of *M. xanthopyga* is not given in the original description. In his handwritten catalogue (available at https://science.mnhn.fr/catalogue/ey-bib-perez1), Pérez indicates under *M. xanthopyga* "Bonifacio, Sassari, ♀ mai-août, ♂ mai-juillet".

Note. We did not examine type specimens of *M. schmiedeknechti* and do not know whether syntypes exist. The identity of the species is clear from the original description. The date of the original description of *M. schmiedeknechti* is unclear; its first, very brief description in Latin was published in the "Rendiconto dell'Accademia delle Scienze fisiche e matematiche, fascicolo 12, anno XXIII, dicembre 1884"; it is unclear if this volume was printed in 1884. A longer description, including a description in Italian, was published in 1885 (Costa 1885a) in a separate book ("Tipografia della Reale Accademia delle Scienze Fis. e Mat., Napoli"). A third description, only in Latin, in the "Bulletino della Società Entomologica Italiana" (Costa 1885b).

Other material. 18 specimens from France (Corsica), Italy (Sardinia) and Malta (Suppl. material 1).

Distribution. Restricted to Malta, Corsica, and Sardinia.

Geographic variation. The Maltese populations have the vestiture bright red orange (Fig. 17), while the Corso-Sardinian populations yellowish orange (Fig. 18).

Note. As noted above, it is possible that the superficially similar appearance of the Maltese and Corso-Sardinian populations is the result of independent convergent evolution. Our genetic analyses did not suggest a close relationship between these populations (although no nuclear sequence data was available for the Maltese populations). Awaiting additional genetic results, we continue to refer the Maltese populations as *M. argentata schmiedeknechti*.

Megachile inexspectata Rebmann, 1968

Figs 25–31

Megachile inexspectata Rebmann, 1968: 43, ♂, "Mut [Turkey, approx. 36.64°N, 33.44°E]". Holotype ♂ (restricted to genitalia) (SMFD).

Material examined. Type material. ***Holotype*** ♂ of *M. inexspectata* (SMFD) (Fig. 31; see above).

Other material. 63 specimens from the following countries: Cyprus, Greece (Rhodes), Israel, Jordan, Lebanon, Morocco, Turkey (Suppl. material 1).

Distribution. Morocco, Egypt (Sinai), Israel, Lebanon, Cyprus, Rhodes, Turkey.

Geographic variation. See above, species delimitation.

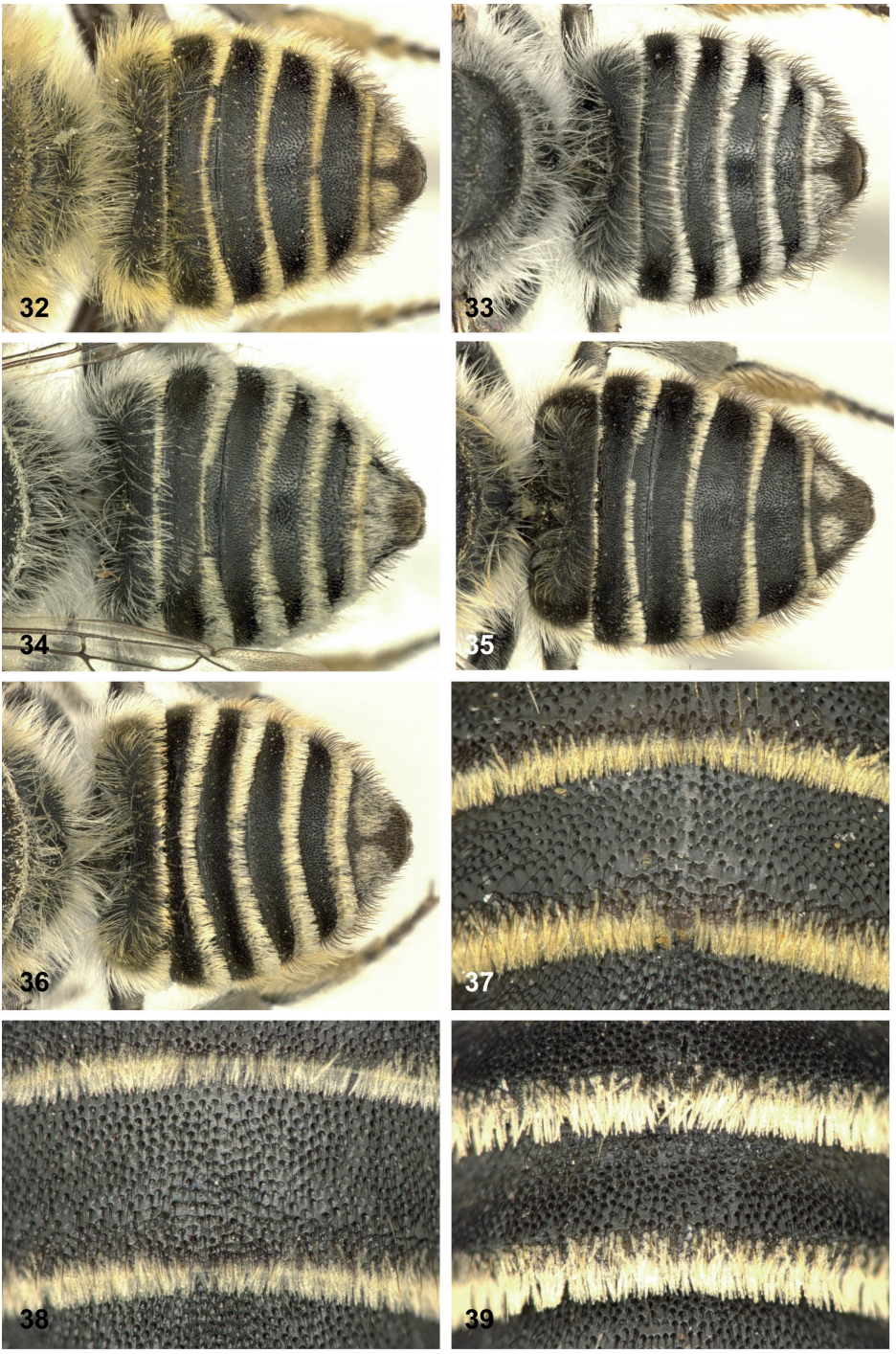
Figures 25–31. *Megachile inexspectata* **25** female metasoma **26** female metasomal tergum 4 **27** female vertex **28** female scutum **29** male metasomal sterna 3 and 4 **30** male genitalia **31** male genitalia, holotype of *M. inexspectata*.

Megachile leachella **Curtis, 1828**

Figs 32–56

Megachile leachella albipila **Pérez, 1895, stat. nov.**

Megachile albipila Pérez, 1895: 23, ♀ ♂, "Alger [Algeria]". Lectotype ♂ (MNHN); paralectotype ♀ (MNHN), by present designation.



Figures 32–39. *Megachile leachella* **32–36** female metasoma **32** UK, *M. leachella leachella* **33** Corsica and Sardinia, *M. leachella leachella* **34** northwestern Africa, *M. leachella albipila* **35** Crete, *M. leachella cretica* ssp. nov. **36** Cyprus, *M. leachella densipunctata* ssp. nov. **37–39** female metasomal tergum 4 **37** UK, *M. leachella leachella* **38** Crete, *M. leachella cretica* ssp. nov. **39** Cyprus, *M. leachella densipunctata* ssp. nov.

Material examined. Type material. ***Lectotype*** ♂ (MNHN) of *M. albipila*, a male in good condition, the tip of the abdomen and the extracted genitalia are placed in a transparent vial preserved under the specimen. This lectotype is labeled as follows: 1. [red, circular disc]; 2. "Alger" [handwritten, handwriting of Pérez]; 3. "Museum Paris, Coll. J. Pérez 1915 [printed on blue paper]. 4. "Paralectotype *M. albipila* Pérez 1896 D. B. Baker des. 1991" [printed and handwritten on blue paper]. 5. "Lectotype *Megachile albipila* des. C. Praz 2022" [printed and handwritten on red paper]. Paralectotype ♀ (MNHN), also from Alger, labelled as follows: 1. [a green circular disc]; 2. "Alger [handwritten, handwriting of Pérez]; 3. "hôte de [host of] *Coelioxys afra"* [handwritten]. 4. "Museum Paris, Coll. J. Pérez [printed on blue paper]. 5. Lectotype *Megachile albipila* Pérez 1896 D. B. Baker des. 1991" [printed and handwritten on red paper]. 6. "Paralectotype *Megachile albipila* des. C. Praz 2022" [printed and handwritten on red paper]. D. Baker's lectotype designation has not been published and is not accepted here; the male specimen, designated here as the lectotype, corresponds to the taxon delineated here as a subspecies of *M. leachella*; in particular, the short preapical process of the gonostylus is clearly visible. The female specimen, however, may belong to either *M. inexspectata* or to *M. leachella albipila*. Based on the punctation of the tergal discs, it rather belongs to *M. inexspectata*, although this identification is tentative given the difficulties in separating females of these two species. For this reason, the male specimen is designated as the lectotype.

Other material. 44 specimens from Algeria, Morocco and Tunisia (Suppl. material 1).

Distribution. Morocco, Algeria and Tunisia. All males examined from these three countries had the preapical process of the gonostylus short (Fig. 55), the feature that characterizes this subspecies. We conclude that the nominal subspecies *leachella* s. str. is absent from northwestern Africa.

Megachile leachella cretica Praz, new subspecies

https://zoobank.org/60AB1119-111D-4AEB-A51F-CBE81F8ECF11

Type material. ***Holotype*** ♀ (Fig. 44), Greece • Kreta [Crete], Ackerbrachen N Kournas See; 30m a.s.l; 35°20.239'N, 24°16.766'E; 4.vi.2012; V. Mauss leg.; (CPCN).

Paratypes (Suppl. material 1): 16♀ 6 ♂. Greece • ♀; Anatoli [Crete]; 11.vi.2005; Le Goff leg.; G. Le Goff Coll. • ♀; Crete Armeni (N. Rethimnis); 27.vii.2007; Le Goff leg.; Unique identifier: GBIFCH00265014; CPCN • ♀; Crete, Paleochora Camping; 0m a.s.l.; 20.x.2011; A. Müller leg.; Unique identifier: GBIFCH00264819; CPCN • ♀; Crete, Paleochora Camping; 0m a.s.l.; 13.x.2011; A. Müller leg.; Unique identifier: GBIFCH00265010; CPCN • ♀; Crete, Paleochora Camping; 0m a.s.l.; 20.x.2011; A. Müller leg.; Unique identifier: GBIFCH00265011; DNA extraction number 536; BOLD: 14514-C03; CPCN • ♀; Crete, Paleochora Camping; 0m a.s.l.; 13.x.2011; A. Müller leg.; Unique identifier: GBIFCH00265012; CPCN • ♀; Crete, Paleochora Camping; 0m a.s.l.; 20.x.2011; A. Müller leg.; A. Müller Coll. • ♀; Crete, Paleochora Camping; 0m a.s.l.; 13.x.2011; A. Müller leg.; A. Müller Coll. • ♀; Crete, Sougia, Tamarix; 0m a.s.l.; 14.x.2011; A. Müller leg.; Unique identifier: GBIFCH00264820; CPCN • ♀; Kreta Spili; 6.x.1993; F. Amiet leg.; Unique identifier: GBIFCH00265047; CPCN • ♀, 2♂; Kreta [Crete], Agia

Figures 40–45. *Megachile leachella* **40–43** female vertex **40** UK, *M. leachella leachella* **41** northwestern Africa, *M. leachella albipila* **42** Crete, *M. leachella cretica* ssp. nov. **43** Cyprus, *M. leachella densipunctata* ssp. nov. **44** holotype female of *M. leachella cretica* ssp. nov. **45** holotype female of *M. leachella densipunctata* ssp. nov.

Galini; 5.x.1993; F. Amiet leg.; F. Amiet Coll. (NMBE) • ♂; Kreta [Crete], Berghang W von Amoudhari; 35°11.926'N, 24°04.396'E; 800–1100 m a.s.l.; 8.vi.2012; V. Mauss leg.; Unique identifier: GBIFCH00265018; CPCN • ♂; Kreta [Crete], S Stomio Phrygeana an Küste; 35°19.35'N, 23°33.04'E; 10 m a.s.l.; 10.vi.2002; A.W. Ebmer leg.; Unique identifier: GBIFCH00265016; CPCN • ♀; Kreta [Crete], S. Kallikratis Kulturland, obere Olea-Zone; 35°14.34'N, 24°15.38'E; 700 m a.s.l.; 6.vi.2002; A.W. Ebmer leg.; Unique identifier: GBIFCH00265013; CPCN • 2♀, ♂; Kreta [Crete], Spili; 6.x.1993; F. Amiet leg.; F. Amiet

Coll. (NMBE) • ♀; Kreta [Crete], Vai 10m Phoenix th-Zone, an Thymus; 35°15.08'N, 26°15.39'E; 25.iv.2001; A.W. Ebmer leg.; Unique identifier: GBIFCH00265015; CPCN • ♂; Kreta [Crete], W Levka Ori N Vigia, Felssteppe; 35°21.50'N, 23°49.13'E; 750–800 m a.s.l.; 7.vi.2002; A.W. Ebmer leg.; Unique identifier: GBIFCH00265017; CPCN • ♀; Kreta [Crete], Umg. Von Loutro; 35°11.926'N, 24°04.396'E; 10 m a.s.l.; 7.vi.2012; V. Mauss leg.; V. Mauss Coll. • 2 ♂; Graecia Kriti; Iraklio Rodià; 12.vi.1996; leg. Scaramozzino; Max. Schwarz Coll. (OLML) • ♂; Graecia Kriti; Iraklio Matala; 15–17.vi.1996; leg. Scaramozzino; Max. Schwarz Coll. (OLML) • ♀; Kreta; Matala, Korno-Beach, Sand; 21.10.1996; leg. Stefan Tischendorf; S. Tischendorf Collection.

Distribution. Restricted to the Island of Crete, Greece.

Description. Female: highly similar to southern populations of the nominal subspecies, differs as follows: apical tergal fringes snow white (Figs 35, 44), as in most southern European populations of the nominal subspecies; spots of white hairs of disc of T6 reduced to two well-separated spots (Fig. 35), a condition otherwise not frequently observed in *M. leachella*. Terga 2–6 laterally with numerous erect dark hairs; such dark hairs are present in the UK, in Central Europe and on Sardinia and Corsica, but not elsewhere (e.g., in Greece, Turkey), where dark hairs are restricted to T5-6. Vertex and scutum covered with black hairs intermixed with light hairs. Vertex punctation particularly coarse and sparse (Fig. 42), similar to or even coarser than in the UK (Fig. 40) or central European populations, but unlike in southern Europe, where the vertex punctation is comparatively small and dense (cf. Figs 41, 43). Punctation on disc of T4 comparatively dense (Fig. 38), on average denser than in other populations (Fig. 37), except in *M. leachella densipunctata* ssp. nov. from Cyprus (Fig. 39). There is however considerable variation in this character in *M. leachella sensu lato* and the condition observed in Crete is within the observed range of variation in the species. *Megachile leachella cretica* ssp. nov. shows a combination of characters present in southern European (in particular the snow white vestiture) and central or northern European populations (dark hairs laterally on the terga, coarse punctation on the vertex).

Male: nearly identical to the nominal subspecies (Fig. 46); the front tarsal segments 2–4 are more consistently orange, approaching the condition observed in *M. leachella densipunctata* ssp. nov. (cf. Fig. 52) (usually at least partly dark brown in other populations of *M. leachella*; Figs 48, 49, 51); the preapical process of the gonostylus (Fig. 56) appears to be slightly shorter on average than in the nominal subspecies (Fig. 54), but only few males were available for study.

Etymology. The subspecies epithet refers to the geographic distribution of this taxon, which is probably restricted to the Island of Crete.

Megachile leachella densipunctata Praz, new subspecies

https://zoobank.org/5F018807-57B4-4129-8C37-D4D76A9CA9B6

Type material. ***Holotype*** ♀ (Fig. 45), Cyprus • 8 km N Pafos, Mavrokolympos Res.; 34.85°N, 32.40°E; 20.vi.2013; Schmid-Egger leg.; (CPCN).

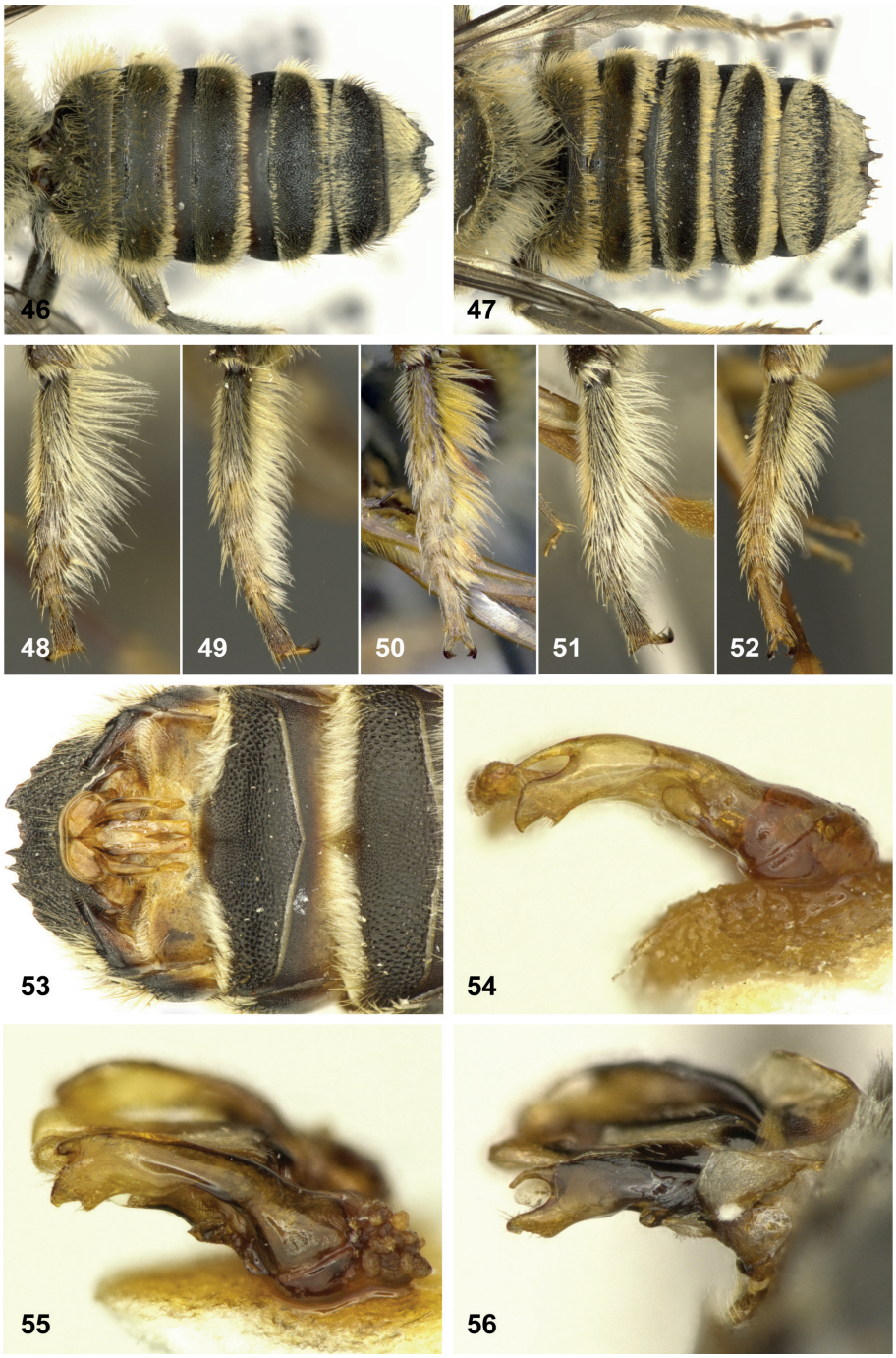
Paratypes (Suppl. material 1): 27♀ 19 ♂, Cyprus • ♂; 15 km SE Pafos, Kouklia; 34.72°N, 32.55°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265023; CPCN • ♂; 15 km SE Pafos, Kouklia; 34.72°N, 32.55°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265025; CPCN • ♂; 15 km SE Pafos, Kouklia; 34.72°N, 32.55°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265026; CPCN • ♂; 15 km SE Pafos, Kouklia; 34.72°N, 32.55°E; 20.vi.2013; Schmid-Egger leg.; A. Müller Coll. • 2♀ 2♂; 15 km SE Pafos, Kouklia; 34.72°N, 32.55°E; 20.vi.2013; C. Schmid-Egger leg.; C. Schmid-Egger Coll. • ♀; 1 kmSE Pano Panagia, Quercus infectoria-Zone; 34.54(.32)°N, 32.37(.34)°E; 800 m a.s.l.; 6.vi.2013; A. W. Ebmer leg.; A. W. Ebmer Coll. • ♀; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265019; CPCN • ♀; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265020; CPCN • ♀; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265021; CPCN • ♀; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265022; CPCN • ♀; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; A. Müller Coll. • ♀; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; OLML • ♂; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265027; CPCN • ♂; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; Schmid-Egger leg.; OLML • 6♀ 2♂; 20 km NNW Pafos, Lara Beach; 34.94°N, 32.31°E; 20.vi.2013; C. Schmid-Egger leg.; C. Schmid-Egger Coll. • ♀; 6 km W Polis, botanical Garden; 35.03°N, 32.37°E; 20.vi.2013; C. Schmid-Egger leg.; C. Schmid-Egger Coll. • ♂; 8 km N Pafos, Mavrokolympos Res.; 34.85°N, 32.40°E; 20.vi.2013; Schmid-Egger leg.; Unique identifier: GBIFCH00265028; CPCN • 2♀ 1♂; 8 km N Pafos, Mavrokolympos Res.; 34.85°N, 32.40°E; 20.vi.2013; C. Schmid-Egger leg.; C. Schmid-Egger Coll. • ♂; ca 5 km N Lemithou Pinus-Zone; 34°58.08'N, 32°48.27'E; 1170 m a.s.l.; 15.vi.2013; A.W. Ebmer leg.; Unique identifier: GBIFCH00265035; CPCN • 2♀; ca 5 km N Lemithou Pinus-Zone; 34°58.08'N, 32°48.27'E; 1170 m a.s.l.; 15.vi.2013; A. W. Ebmer leg.; A. W. Ebmer Coll. • ♀; ca 5 km N Lemithou Pinus-Zone,; 34°58.08'N, 32°48.27'E; 1170 m a.s.l.; 15.vi.2013; A.W. Ebmer leg.; Unique identifier: GBIFCH00265030; CPCN • 3♂; Moni Troodotissa Umg. P. brutia/Qu. alnifolia/Arbustus andrachne-Zone; 1350 m a.s.l.; 16.vi.2013; A. W. Ebmer leg.; A. W. Ebmer Coll. • ♀; NE Nata, Ufer des Xerós/Obstgarten; 34°47.17'N, 32°35.38'E; 130 m a.s.l.; 7.vi.2013; A.W. Ebmer leg.; Unique identifier: GBIFCH00265033; CPCN • 2♀; S Kakopetria, E Platania, P. Brutia/Qu. alnifolia/A. andrachne; 34°56.54'N, 32°55.59'E; 1200 m a.s.l.; 12.vi.2013; A. W. Ebmer leg.; A. W. Ebmer Coll. • ♀; S Kakopetria, E Platania, P. Brutia/Qu. alnifolia/A.andrachne; 34°56.54'N, 32°55.59'E; 130 m a.s.l.; 12.vi.2013; A.W. Ebmer leg.; Unique identifier: GBIFCH00265031; CPCN • ♀; Strasse Prodromos>Troodos, an *Lotus corniculatus*; 34°56.55'N, 32°51.01'E; 1550 m a.s.l.; 16.vi.2013; A.W. Ebmer leg.; Unique identifier: GBIFCH00265032; CPCN • ♂; Troodos Mt. Olympos; 34.93°N, 32.86°E;

1900 m a.s.l.; 20.vi.2013; C. Schmid-Egger leg.; C. Schmid-Egger Coll. • ♂; W Polis, ca. 3 kmW Neo Chorio, Ag. Minas ruderal/Feuchstelle; 35°01(.23)'N, 32°20(.36)'E; 240 m a.s.l.; 5.vi.2013; A.W. Ebmer leg.; Unique identifier: GBIFCH00265034; CPCN • ♀; 2011; Sedivy & Müller leg.; Unique identifier: GBIFCH00265024; DNA extraction number 538; BOLD 14514D09; CPCN • ♂; 2011; Sedivy & Müller leg.; Unique identifier: GBIFCH00265036; DNA extraction number 537; CPCN • ♀; Limassol Cherkes Chiftlik; Site 1 36SVD9916334336; 1.ix.2017; A. Varnava leg.; Unique identifier: AV-17-01331; A. Varnava Collection • ♀; Limassol Cherkes Chiftlik; Site 1 36SVD9916334336; 15.vii.2016; A. Varnava leg.; Unique identifier: AV-16-00324; CPCN • ♂; Limassol Cherkes Chiftlik; Site 3 36SVD99003334989; 21.ix.2017; A. Varnava leg.; Unique identifier: AV-17-01382; A. Varnava Collection • ♂ SBA Akrotiri, Site 1 36SVD9735128734; 1.ix.2017; A. Varnava leg.; Unique identifier: AV-17-01296; A. Varnava Collection • ♂ SBA Akrotiri, Site 1 36SVD9735128734; 1.ix.2017; A. Varnava leg.; Unique identifier: AV-17-01299; CPCN • ♂; Limassol Cherkes Chiftlik; Site 1 36SVD9916334336; 1.ix.2017; A. Varnava leg.; Unique identifier: AV-17-01335; A. Varnava Collection • ♂; Limassol Cherkes Chiftlik; Site 1 36SVD9916334336; 1.ix.2017; A. Varnava leg.; Unique identifier: AV-17-01330; CPCN • ♀; [Cyprus] Unique identifier: AV-16-01878; A. Varnava Collection • ♀; [Cyprus] Unique identifier: AV-16-02182; A. Varnava Collection • ♀; Chlorana (pan trap); 34.793°N, 32.408°E; 18.10.2021; leg. V. Soon; Natural History Museum of the University of Tartu • ♀; Akrotiri; 34.6346°N, 32.9198°E; 16.10.2021; leg. V. Soon; Natural History Museum of the University of Tartu.

Distribution. Restricted to Cyprus.

Description. Female: similar to southern populations of the nominal subspecies, differs as follows: apical tergal fringes yellowish white (Figs 36, 45), not snow white as in most southern European populations (e.g., Greece, Turkey) of the nominal subspecies; spots of white hairs of disc of T6 large (Fig. 36), unlike in *M. leachella cretica* ssp. nov., but as in southern European populations; Terga 3-6 laterally with dark hairs (Fig. 36), approaching the condition observed in *M. leachella cretica* ssp. nov. Fig. 35) but unlike in southern European populations of the nominal subspecies. Vertex punctation small and dense (Fig. 43), as in southern European populations, but unlike in *M. leachella cretica* ssp. nov. The most striking feature characterizing *M. leachella densipunctata* ssp. nov. is the particularly dense punctation of the terga, especially the disc of T4 (Fig. 39); moreover, the terga are conspicuously impressed basally (Figs 36, 39). Such dense punctation and impressed basis are otherwise not observed in *M. leachella sensu lato*; specimens from Israel also have comparatively dense tergal punctation, approaching the condition observed in *M. leachella densipunctata* ssp. nov.

Male: nearly identical to the nominal subspecies, differs as follows: light vestiture of terga particularly developed, forming two conspicuous bands of hairs, one basally and one apically (Fig. 47); in particular the disc of T5 is nearly entirely covered with light hairs, unlike in most other populations of *M. leachella sensu lato*; specimens from Israel have similar tergal vestiture. Terga basally strongly impressed, so that disc is concave basally and strongly convex apically (Fig. 47); in all other populations of



Figures 46–56. *Megachile leachella* **46, 47** male metasoma **46** Switzerland, *M. leachella leachella* **47** Cyprus, *M. leachella densipunctata* ssp. nov. **48–52** male front tarsi **48** UK, *M. leachella leachella* **49** Switzerland, *M. leachella leachella* **50** Corsica and Sardinia, *M. leachella leachella* **51** Crete, *M. leachella cretica* ssp. nov. **52** Cyprus, *M. leachella densipunctata* ssp. nov. **53** male sterna 4–6 **54–56** male genitalia **54** UK, *M. leachella leachella* **55** northwestern Africa, *M. leachella albipila* **56** Crete, *M. leachella cretica* ssp. nov.

M. leachella, disc nearly flat. Front tarsal segments 2–4 consistently orange (Fig. 52) (usually at least partly dark brown in other populations of *M. leachella*; Figs 48, 49, 51). Genitalia as in the nominal subspecies.

Etymology. The subspecies epithet refers to the particularly dense punctation of the terga of the female.

Megachile leachella leachella Curtis, 1928

Megachile leachella Curtis, 1828: [explanation to Plate 218], [sex not indicated], "[England]".

Megachile dorsalis Pérez 1879: 223, ♀ nec ♂, "Bordeaux; environs de l'étang de Cazaux; Arcachon; Royan [France]". Lectotype ♀, by designation of van der Zanden (1996: 886) (MNHN).

Megachile bioculata Pérez, 1902: 119, ♀ [erroneously indicated as ♂], "Catalogne [Spain: Catalonia]". Lectotype ♀, by present designation (see below) (MNHN).

Megachile argentata var. *fossoria* Ferton, 1909: 550, [sex not indicated], "Propriano [France, Corsica]". Lectotype ♀, by designation of Schwarz & Gusenleitner (2011: 258) (MNHN); paralectotypes ♀ ♂ (MNHN).

Megachile ichnusae Rebmann, 1968: 31, ♂ nec ♀, "Sardinien, Siniscola" [Italy, Sardinia, approx. 40.58 N, 9.70 E]. Holotype ♂, (SMFD). New synonymy.

Megachile discriminata Rebmann, 1968: 34, ♂, "Turkestan, 189., Golodnaja Step. [Uzbekistan]". Holotype ♂ (ZMHB); paratype ♂ (SMFD). New synonymy.

Megachile leachella maadiensis van der Zanden, 1986: 67, ♂, "[no locality given: Egypt]". Details on type material or type locality not given.

Material examined. Type material. ***Lectotype*** ♀ (MNHN) of *M. dorsalis* (see also Gogala 1998; Schwarz and Gusenleitner 2011).

Lectotype ♀ (MNHN) of *M. bioculata*, a female specimen labeled as follows: 1. [red, circular disc]; 2. "Barcelone" [handwritten, handwriting of Pérez]; 3. "*bioculata* JP" [handwritten, handwriting probably of Pérez]; 4. "Muséum Paris Coll. J. Pérez 1915" [printed]; 5. Lectotype *Megachile bioculata* Pérez des. C. Praz 2022" [printed and handwritten on red paper]. 6. MNHN Pérez EY2292 [catalogue of type MNHN]. 7. *Megachile leachella* det. C. Praz 2022 [printed and handwritten].

Lectotype ♀ (MNHN) of *M. argentata* var. *fossoria* (see also Schwarz and Gusenleitner 2011).

Holotype ♂ (SMFD) of *M. ichnusae* (SMFD). The female paratype is a female of *M. fertoni*.

Holotype ♂ (ZMHB) and paratype ♂ (SMFD) of *M. discriminata*.

Other material. 174 specimens from the following countries: Croatia, Egypt, UK, France, Greece, Iran, Israel, Italy, Kyrgyzstan, Montenegro, North Macedonia, Portugal, Spain, Switzerland, Syria, Turkey, Uzbekistan (Suppl. material 1).

Distribution. Widely distributed in Europe from Portugal, Spain, France, north to the UK, Scandinavia, northern, central, southern and eastern Europe, Levant (Turkey, Leba-

non, Egypt, Israel), Iran, central Asia. We have examined specimens of the nominal subspecies from Chios, Lesvos, Rhodos and Santorini, but from no other Aegean Islands (see above for the populations of Crete and Cyprus). Highly similar (>98% similarity) DNA barcodes from unidentified specimens from the Beijing Region (Genbank accession number KC560312; Chesters et al. 2013) strongly suggests the presence of this species in China.

Geographic variation. See above (species delimitation).

Megachile leucostoma Pérez, 1907

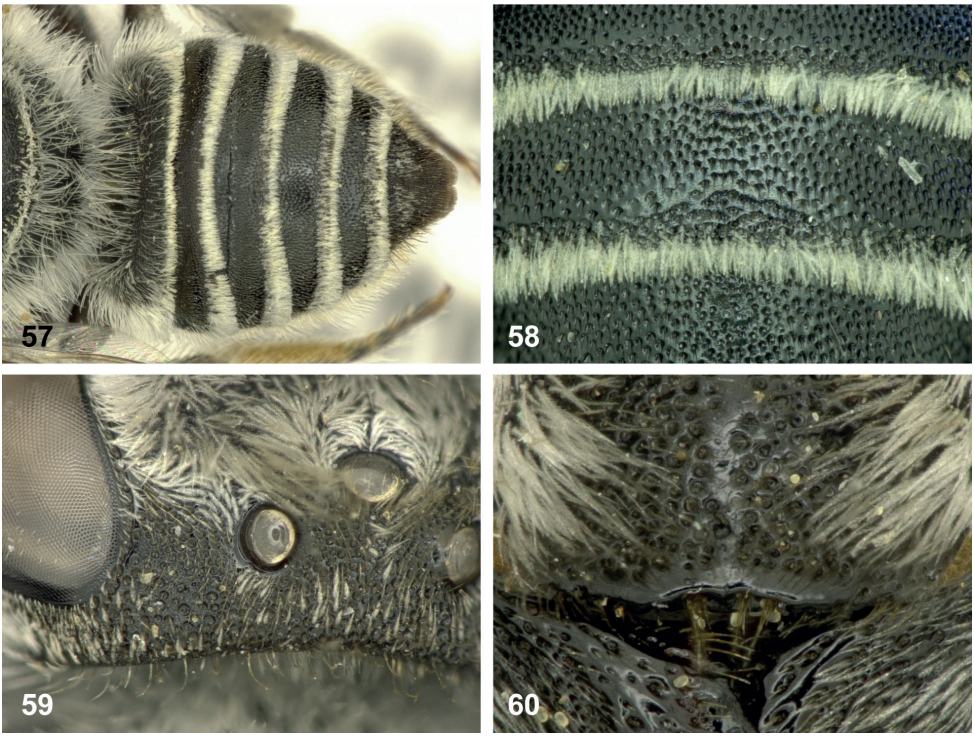
Figs 57–60

Megachile leucostoma Pérez, 1907: 489, ♂, "Dibba [Oman]". Holotype ♂ (MNHN).

Megachile submucida Alfken, 1926: 126, ♀, ♂ partim, "Kingi [Maryut, sw von Alexandria; Egypt]". Lectotype ♀, by designation of van der Zanden (1986: 66) (SMFD). Synonymy in Praz et al. (2021).

Megachile microxantha Cockerell, 1937: 205, ♂, "Aden [Yemen]". Holotype ♂ (BMNH). Synonymy in Praz et al. (2021).

Megachile privigna Rebmann, 1968: 40, ♂, "Fayed [Egypt]". Holotype ♂ (SMFD). Synonymy in Praz et al. (2021).



Figures 57–60. *Megachile leucostoma* **57** female metasoma **58** female metasomal tergum 4 **59** female vertex **60** apex of female clypeus.

Examined material and distribution. See Praz et al. (2021).

Geographic variation. In Israel, Jordan and Oman, the scopa is nearly entirely orange (see above under *M. anatolica*); in the UAE, the scopa is mostly white, orange on S6. In Egypt, the scopa is usually dark on S6, white on S2–S5, but often slightly orange on S5. Whether the white scopa, as observed in Egypt, is the result of introgression with *M. pusilla*, remains unknown. It is also possible that the orange scopa is the result of introgression with the taxon referred to as *M. venusta* from Africa, which nearly always has orange scopa. Given its wide distribution in Egypt and in the Arabian Peninsula, *M. leucostoma* may in fact be present in the Afrotropical region, and could be conspecific with an African taxon, for example *M. modestissima* Dalla Torre, 1896 (a replacement name for *M. modesta* Smith, 1879, presumably described from the Karthoum area; see below). *Megachile modestissima* is currently placed in synonymy with *M. venusta*.

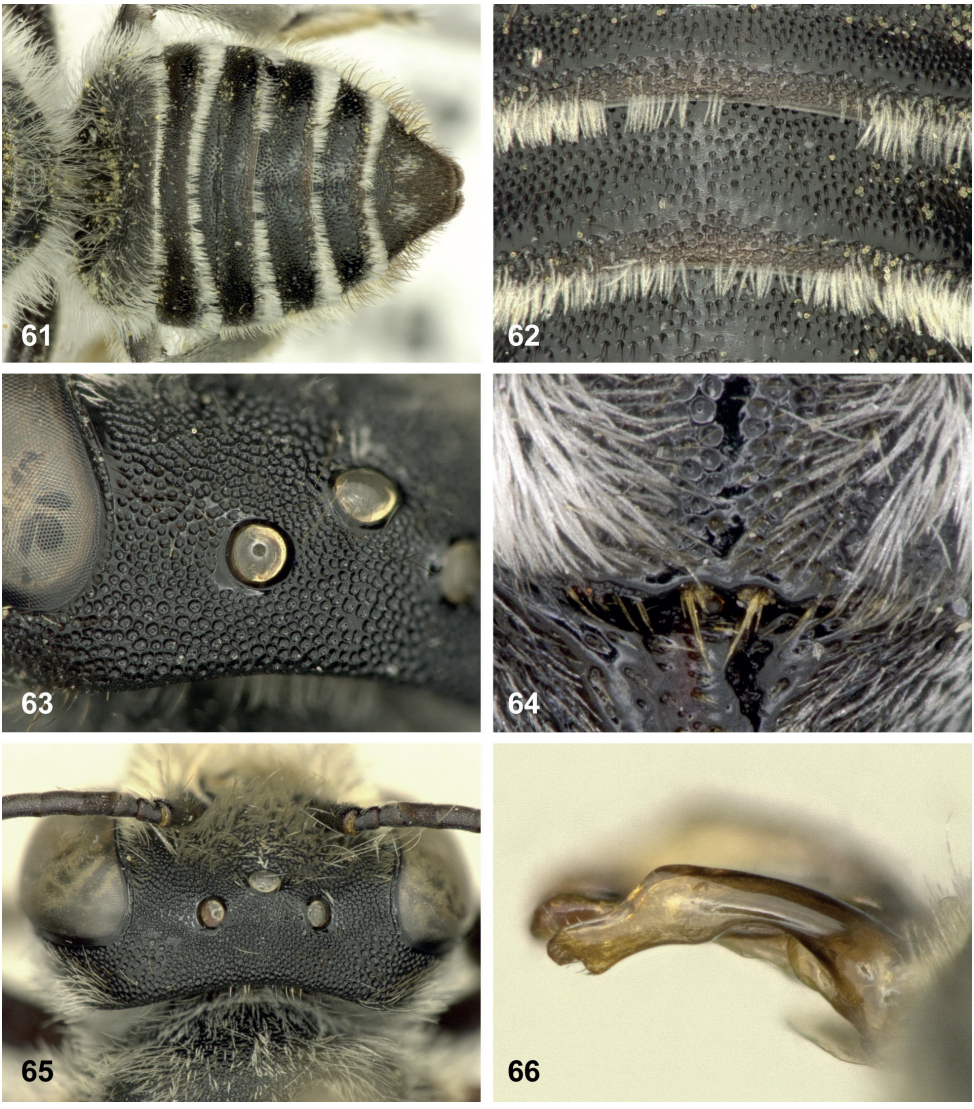
Megachile pusilla Pérez, 1884

Figs 61–66

Megachile pusilla Pérez, 1884: 263, ♀ ♂, "Portugal". Lectotype ♀, by present designation (MNHN); paralectotypes ♀ (MNHN), by present designation.
Megachile variscopa Pérez, 1895: 24, ♀, "Bône" [Annaba, Algeria]. Lectotype ♀, by present designation. New synonymy.
Megachile timberlakei Cockerell, 1920: 119, ♂ ♀, "Kaimulai [sic]|, Oahu" [Kaimuki, Honolulu, Hawai, USA; introduced]. Holotype ♂ (USNM). New synonymy.
Megachile atratula Rebmann, 1968: 38, ♂ ♀, "Rapallo" [Italy]. Holotype ♂ (SMFD?), paratypes ♂ ♀ (SMFD). New synonymy.
Megachile striatella Rebmann, 1968: 41, ♂ ♀, "El Kantara" [Algeria]. Holotype ♂ (restricted to genitalia) (SMFD), paratypes ♂ ♀ (SMFD). New synonymy.
Megachile sudai Ikudome, 1999: 3, ♀, [Okinawa, Japan; introduced]. New synonymy.

Material examined. Type material. ***Lectotype*** ♀ (MNHN) of *M. pusilla*, a well-preserved female, by present designation. The specimen is labeled as follows: 1. "Portug [handwritten, handwriting of Pérez; = Portugal]. 2. "Muséum Paris Coll. J. Pérez 1915" [printed]. 3. Lectotypus *Megachile pusilla* ♀ Pérez design. Malisheva 1989 [printed and handwritten on red paper] ; 4. Museum Paris EY0000002295. Two additional females labelled as follows are designated as paralectotypes. 1. "Portug" [handwritten, handwriting of Pérez; = Portugal]; 2. "Muséum Paris Coll. J. Pérez 1915" [printed]. 3. Paralectotypus *Megachile pusilla* ♀ Pérez des. C. Praz 2022 [printed and handwritten on red paper] ; 4. Museum Paris EY0000002296; and 1. "Portugal" [printed]; 2. "Muséum Paris Coll. J. Pérez 1915" [printed]. 3. Paralectotypus *Megachile pusilla* ♀ Pérez des. C. Praz 2022 [printed and handwritten on red paper] ; 4. Museum Paris EY0000002297.

Lectotype ♀ (MNHN) of *M. variscopa*, a well-preserved female labeled as follows: 1. "Bône" [handwritten, possibly by Pérez]; 2. "Muséum Paris Coll. J. Pérez 1915"



Figures 61–66. *Megachile pusilla* **61** female metasoma **62** female metasomal tergum 4 **63** female vertex **64** apex of female clypeus **65** dorsal view of male head **66** male genitalia.

[printed on blue paper]; 3. "Lectotype *M. variscopa* Pérez des. van der Zanden 1989" [printed and handwritten on red paper]. 4. "*Megachile albohirta* det. van der Zanden 1994"; 5. "*Megachile pusilla* det. C. Praz 2022" [printed and handwritten]. *Megachile albohirta* (Brullé, 1839) was considered to be conspecific with *M. concinna* by Tkalcu (1993), explaining van der Zanden's identification as *M. albohirta* (see Praz 2017). One additional female from Bône is designated as a paralectotype. It is labeled as follows. 1. "Bône" [handwritten, possibly by Pérez]; 2. "Muséum Paris Coll. J. Pérez 1915" [printed on blue paper]; 3. "Paralectotype *M. variscopa* Pérez des. C. Praz 2022"

[printed and handwritten on red paper]; 4. "*Megachile pusilla* det. C. Praz 2022" [printed and handwritten].

Paratypes ♂ ♀ (SMFD) of *M. atratula.* The holotype, indicated to be in Rebmann's collection (SMFD) could not be located.

Holotype ♂ (SMFD) of *M. striatella* (see above). ***Paratypes*** ♀ ♂ of *M. striatella* (SMFD).

Notes: we did not examine the holotype of *M. timberlakei*, but pictures are available on the online catalogue of USNM (https://collections.nmnh.si.edu; catalogue entry number 536683). The simple gonostylus, typical of the *concinna* complex is visible. In addition, two DNA barcodes for specimens collected in Hawaii and identified as *M. timberlakei* are 100% identical with *M. pusilla*.

We did not examine the type material of *M. sudai*, but examined and sequenced specimens from Okinawa kindly sent by H. Nagase; these specimens perfectly agree with *M. pusilla*; see Nagase (2016).

Other material. 54 specimens from the following countries: Argentina (introduced), France, Greece, Greece (Crete), Italy, Japan (introduced) Malta, Morocco, Spain, Tunisia, USA (introduced) (Suppl. material 1). *Megachile timberlakei* was mentioned from the Galapagos Islands (Rasmussen et al. 2012); identity of these specimens should be checked using DNA barcodes given the challenging identifications in this group; they likely belong either to *M. pusilla* or to *M. concinna*.

Distribution. See Soltani et al. 2017: fig. 3. Northwestern Africa (Tunisia, Algeria, Morocco), southern Europe (Portugal, Spain, France including Corsica, Italy including Sicily and Sardinia, Slovenia, Greece including Crete). Presumed introduced in Madeira (Kratochwil et al. 2018). Introduced in southern America, northern America, Japan, Hawaii, and probably Australia (see note below).

Geographic variation. Specimens from Algeria and Tunisia have the scopa often partly orange on S5; whether this condition results from introgression with *M. leucostoma* remains to be established.

Note. A published barcode generated from a specimen collected near Perth, Western Australia (BOLD accession number MSAPB1368-19) is 100% identical to sequences of *M. pusilla*, suggesting that *M. pusilla* has also been introduced into Australia. This specimen is identified as *M. obtusa* Smith, 1853. We examined a picture of the holotype of *M. obtusa* (OUMNH); this species has modified front tarsi and does not belong to the same species group as *M. pusilla.*

Megachile walkeri Dalla Torre, 1896

Megachile fulvescens Walker, 1871: 47, ♀ ♂, "Harkeko; Wâdy Gennèh; Wâdy Ferran; Mount Sinai [Arkiko, Eritrea; ?; Wadi Feiran, Sinai, Egypt; Sinai, Egypt]". Preoccupied, not *M. fulvescens* Smith, 1853.

Megachile walkeri Dalla Torre, 1896: 452. Replacement name for *M. fulvescens* Walker, 1871.

Megachile argentata var. *moricei* Friese, 1899b: 334, ♀ ♂, "Elephantinen; Philae; Ober-Aegypten-Assuan [Egypt, Elephantine and Philae Island; Aswan].
Megachile blanda Rebmann, 1968: 44, ♂, "Luxor [Egypt]". Holotype ♂, SMFD. Preoccupied, not *M. blanda* Mitchell 1930. Synonymy in Praz et al. (2021: 307).

Examined material and distribution. See Praz et al. (2021).

Additional, extralimital species

Megachile concinna Smith, 1879

Megachile concinna Smith, 1879: 79, ♀, "St. Domingo [Dominican Republic; introduced]". Syntype (or holotype) ♀ (BMNH).
Megachile multidens Fox, 1891: 345, ♀ ♂, "Kingston [Jamaica; introduced]".

Material examined. Type material. ***Syntype*** (or holotype) ♀ of *M. concinna* (BMNH). The original description does not indicate the number of specimens, it is therefore not clear whether this specimen is the holotype (by monotypy) or a syntype. This female specimen agrees with the original description; it is slightly larger than other members of the *concinna* complex, as indicated by F. Smith, who gave its length as 4 lines (=approx. 8.4 mm), while the length given for *M. venusta* is 3 ¼ lines (7.8 mm); also as indicated in the original description, there are two spots of white hairs on the disc of T6, unlike in *M. venusta*.

Note: It is likely that *M. derelictula* Cockerell, 1937, described from Barbados, is also a synonym of *M. concinna*.

Other material. 13 specimens from the following countries: Benin, Cape Verde, Dominican Republic (introduced), French Guyana (introduced), Kenya, Republic of Trinidad and Tobago (introduced) (Suppl. material 1).

Note. This species is difficult to separate from other members of the *concinna* complex. The genetic analyses of Soltani et al. (2017) suggested that *M. concinna* and *M. venusta* are distinct and both widely distributed in Africa, although only few specimens have been analyzed; these authors also reported specimens of both species in sympatry in one site in Kenya (Suppl. material 1). For these reasons, these two species are treated as distinct. Compared to most other members of the *concinna* complex, *M. concinna* is on average slightly larger. The female has the scopa white (dark on S6), contrary to *M. venusta* which mostly has the scopa orange; the punctation on the terga appears to be slightly denser in *M. concinna* compared to other members of the complex; and the disc of T6 has two distinct spots of hairs, unlike in *M. venusta* (Soltani et al. 2017: table S3). Based on the few specimens examined, the male of *M. concinna* can not be separated from the other species of the *concinna* complex. The identity of this species is based on DNA barcoded specimens from the Dominican Republic (the type locality of *M. concinna*), assuming that only one species of the *concinna* complex is present there.

Megachile venusta **Smith, 1853**

Megachile venusta Smith, 1853: 159, ♀, "Port Natal; Cape of Good Hope [Durban; Cape of Good Hope; South Africa]". Syntype ♀ (BMNH).

Megachile modesta Smith, 1879: 63, ♀, "White Nile. Collected by Consul Petherick [following Baker 2002: 22, the type locality is likely Khartoum, where John Petherick was the British Vice-Consul]". Syntype ♀ (BMNH). Preoccupied, not *M. modesta* Smith, 1862.

Megachile modestissima Dalla Torre, 1896: 439. Replacement name for *M. modesta* Smith, 1879.

Material examined. Type material. ***Syntype*** ♀ of *M. venusta* (BMNH).

Syntype ♀ of *M. modesta* Smith, 1879 (BMNH).

Other material. 11 specimens from the following countries: Central African Republic, Kenya, Senegal, South Africa (Suppl. material 1).

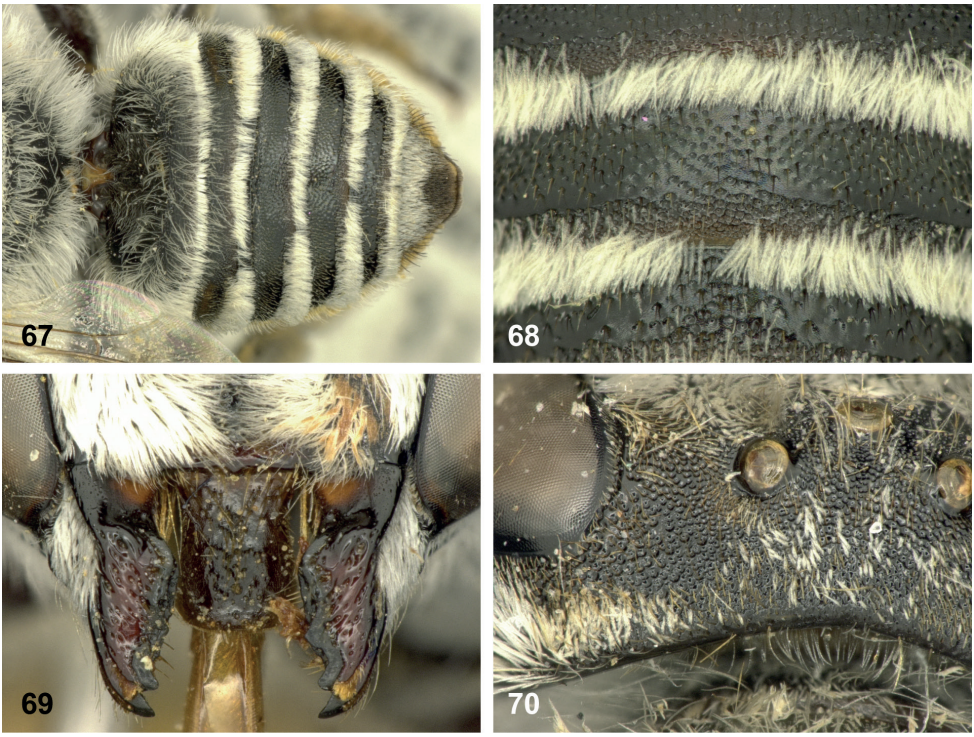
Note. The identity of this species is unclear and requires additional work; for this reason, the list of synonyms given above is incomplete. In the phylogenetic analyses of Soltani et al. (2017), two main clades were recovered, referred to as *M. venusta* clade 1 and *M. venusta* clade 2. Both contained specimens agreeing with members of the *concinna* complex; females of both clades are mostly characterized by the partly orange scopa (one barcoded female from Senegal had white scopa, black on S6, as in *M. concinna*). It is unclear whether these two clades are conspecific. Clade 2 was restricted to South Africa, while clade 1 contained specimens from Kenya, South Africa, the Central African Republic and Senegal. The name *M. modestissima* may apply to clade 1, *M. venusta* to clade 2, if these two clades are demonstrated to represent two distinct species. Additional work is needed to properly delineate species, to obtain data on their distribution, and to properly identify the relevant type material.

Megachile viridicollis **Morawitz, 1875**

Figs 67–70

Megachile viridicollis Morawitz, 1875: 117, ♂, "поймать только раз в степи Кизиль кумъ 15 мая у восточной окрайны горы Каракъ [Caught only once in the steppe of Kyzyl Kum, the 15. May, at the eastern edge of the mountain Karak; Kyzyl Kum does not refer to the Kyzyl Kum Desert, but probably to the locality Қызылқұм, approx. 41.911N 67.988E, Kazakhstan]".

Material examined. Type material. We were not able to examine the type material of *M. viridicollis*. The placement of this species into the *concinna* complex is based on the original description, which mentions that the species is highly similar to "*M. argentata*" (either *M. pilidens* or *M. leachella*), but markedly larger, and with a conspicuous tooth at the base of the mandible. We interpret this tooth as the tooth present just behind the



Figures 67–70. *Megachile viridicollis* **67** female metasoma **68** female metasomal tergum 4 **69** apex of female clypeus **70** female vertex.

base of the mandible (as in Fig. 12). We also examined one male specimen identified as *M. viridicollis* from "Baigakum bei Djulek Turkest. [Kazakhstan: Baygekum, Zholek; approx. 44.314 N 66.475 E]", identified by L. Wollmann (who possibly had access to material identified as *M. viridicollis* by Morawitz) and perfectly agreeing with the original description of *M. viridicollis*. Baygekum is located approximately 200 km NE of the type locality of *M. viridicollis*.

Other material. Eight specimens from Kazakhstan and Uzbekistan (Suppl. material 1).

Description. The following description is based on one male specimen from Baygekum and several female specimens from Gazli, Uzbekistan, presumed to be conspecific.

Male: Member of the *concinna* complex, as determined by the presence of the tooth behind the mandibular base and the apically simple genitalia. OOD as in European populations of *M. anatolica* (as in Fig. 11), thus nearly as long as interocellar distance. Vestiture particularly long and dense, in particular tergal fasciae thicker and longer. Larger than all other species of the *concinna*-complex (body length 10 mm).

Female: similar to *M. anatolica*, differs from that species in the following characteristics: larger (body length 11 mm). Vertex laterally covered with short, brownish hairs, so that integument is not visible under vestiture unless the hairs are removed (Fig. 70); punctation dense and fine (Fig. 70). Clypeus apically without teeth, broadly emarginated (as in *M. pilidens*) but with a conspicuous, rounded, thickened margin,

medially with a small tooth (Fig. 69). Metasoma with snow white vestiture forming particularly dense tergal fasciae and entirely covering the base of T6. Punctation of disc of T4 sparse (Fig. 68), similar to European populations of *M. anatolica* (Fig. 8).

Distribution. So far known only from few specimens from Kazakhstan and Uzbekistan.

Geographic variation and note. The identity of this taxon remains unclear, as very little material has been studied. Soltani et al. (2017) presented sequence data for two populations, one based on several male specimens from the region of Khiva, Uzbekistan (41.33N, 60.35E and 41.36N, 60.35E), and one based on several female specimens from Gazli, Uzbekistan (40.383N, 63.100E); these two populations were genetically strongly divergent (4.1 %). The male specimens from the Khiva region are markedly smaller than the male examined from Baygekum (see above), while the females from Gazli are particularly large for the *concinna* complex. It is possible that the populations from Khiva represent transitional populations to *M. anatolica*. Additional research including more material from Central Asia is needed to better delineate this species. *Megachile viridicollis* and *M. anatolica* are genetically closely related, and both may be treated as conspecific, in which case the name *M. viridicollis* would have priority. The striking differences in female morphology lead us to treat both taxa are distinct species.

Identification key for the species of the *leachella* group in the Western Palaearctic. Extralimital species (*M. concinna*, *M. venusta* and *M. viridicollis*) are excluded

Males

1 S4 medially with a small tubercule covered with yellowish to greyish hairs (Fig. 23). Front tarsal segments 2–4 whitish-brown (Fig. 22). Gena without tooth behind mandibular base. Gonostylus bifid, with a long and slender preapical process (Fig. 24) (Palaearctic, from Morocco to Central Asia) ..... ***Megachile argentata* (Fabricius)**

– S4 medially without tubercule, with a small (Figs 13, 53) or large patch of yellowish hairs (Fig. 29). Front tarsal segments mostly dark brown (Figs 48, 49, 51, 52) (except in Sardinian populations of *M. leachella*; Fig. 50). Gena with or without tooth behind mandibular base. Gonostylus with or without preapical process **2**

2 S4 medially with particularly dense patch of yellowish hairs (Fig. 29). Gena without tooth or only with short, truncate tooth behind mandibular base. Gonostylus bifid with a short, broad and strongly curved preapical process, process only 1.5 times as long as wide (Figs 30, 31). **3**

– S4 with a small spot of yellowish hairs medially (Fig. 13). Gena with tooth behind mandibular base (Fig. 12) (best visible in front view, especially if mandibles are closed). Gonostylus either apically bifid, with short or long preapical process, but process of different shape (Figs 54–56), or simple (Figs 14, 66)..... **4**

3 Integument of T1-T2 predominantly orange (Arabian Peninsula, Egypt including Sinai Peninsula, Israel, Iran) ..... ***M. walkeri* Dalla Torre**
– Integument of T1-T2 predominantly dark brown (Northern Africa, Israel, Sinai Peninsula, Lebanon, Turkey, Cyprus, Rhodos)..... ***M. inexspectata* Rebmann**
4 Gonostylus apically bifid, with short or long preapical process (Figs 54–56). Often slightly larger, body length 8–9mm ..... ***Megachile leachella* Curtis**
– Gonostylus apically simple (Figs 14, 66). Often slightly smaller, body length 7mm (except *M. anatolica*, which can be distinguished by the longer ocelloccipital distance) (*M. concinna* complex) **5**

The separation of the following species is difficult in the male sex

5 Vertex mostly longer, ocelloccipital distance subequal to interocellar distance (Fig. 11) (the ocelloccipital distance becomes gradually shorter towards the East, and in Iran, the condition is identical with the following species). Body size approximately 8–9 mm. (Italy, southeastern Europe, Turkey, Northern Israel, Syria, Lebanon, Iran) ..... ***M. anatolica* Rebmann**
– Vertex shorter, ocelloccipital distance shorter than interocellar distance (Fig. 65). Body size approximately 7–8 mm. (Southern Europe, Northern Africa, presumed absent from Northern Israel, Turkey, Lebanon)..... **6**
6 Southern Europe, northwestern Africa (identity of populations located between Egypt and Tunisia unclear) ..... ***M. pusilla* Pérez**
– Arabian Peninsula, southern Israel, Egypt ..... ***M. leucostoma* Pérez**

Females

1 Integument of T1 and T2 predominantly brown-orange (Praz et al. 2021: fig. 8). Ventral surface of trochanters and femora 2 and 3 covered with short, modified, capitate hairs (Praz et al. 2021: cf. fig. 3)..... ***M. walkeri* (Dalla Torre)**
– Integument of T1 and T2 dark brown. Ventral surface of trochanters and femora 2 and 3 without short, modified hairs **2**
2 Vertex mostly longer, ocelloccipital distance approximately equal to 1.5–1.8 x diameter of lateral ocellus (Fig. 9; this character gets gradually less pronounced towards the east; in Iran the condition is as in the following species). Body size approximately 8–9 mm. Apical clypeal margin with 5 comparatively large blunt teeth (Fig. 10). Disc of T6 with two small, separated spots of appressed white hairs (Fig. 7). (Southeastern Europe, Turkey, Northern Israel, Syria, Lebanon, Iran) ..... ***M. anatolica* Rebmann**
– Vertex shorter, ocelloccipital distance approximately subequal to diameter of lateral ocellus (e.g. Fig. 20). Apical clypeal margin straight without rounded teeth, or with fewer teeth, or with 5 less conspicuous teeth (Figs 21, 60, 64). Disc of T6 with vestiture variable, but if body size above 8 mm, then disc of

T6 usually with large sports of appressed white hairs (Figs 15–18, 25, 32, 33, 34, 36) **3**

3 Apical clypeal margin with emargination narrow, often with only three little-visible, rounded teeth (Figs 60, 64). Disc of T6 either without appressed white hairs, or with two small, well-separated spots (Figs 57, 61). (*M. concinna complex*, in part) **4**

– Apical clypeal margin with emargination broad, either straight with little visible rounded teeth, or with five very small teeth (Fig. 21). Disc of T6 with extensive appressed, white vestiture (Figs 15–18, 25, 32–36) **5**

4 Scopa usually white, black on S6, sometimes with isolated orange hairs on S5. Southern Europe, northwestern Africa (identity of populations comprised between Egypt and Tunisia unclear) ..... ***M. pusilla*** **Pérez**

– Scopa often extensively orange, sometimes entirely orange, although also white (dark on S6) in Egypt. Arabian Peninsula, southern Israel, Egypt ..... ***M. leucostoma*** **Pérez**

5 Disc of T4 with comparatively sparse punctation (there is much variation in this character; a separation from *M. argentata* is not always straightforward, especially in regions where the vestiture colour of *M. leachella* is not snow white and does not offer additional discriminating characters with *M. argentata*), interspaces on average as large or larger than puncture diameters (Fig. 37) (except in Crete and Cyprus, ssp. *cretica* ssp. nov., Fig. 38, and *densipunctata* ssp. nov. Fig. 39). Vestiture brown in Northern Europe (Fig. 32), becoming progressively lighter towards southern Europe and northern Africa, where it is snow-white (Fig. 34). In northwestern Africa, see additional characters in Table 1 ..... ***M. leachella*** **Curtis**

– Disc of T4 more densely punctured (Figs 19, 26), interspaces on average smaller than puncture diameters. Vestiture brown or snow white. **6**

6 Vestiture snow white (Fig. 25). Disc of T6 with extended area covered with white, appressed hairs, white hairs often not forming two separated spots (Fig. 25). Mesonotum densely covered by scale-like white hairs (Fig. 28). Terga 4 and 5 laterally mostly without erect dark hairs. Punctation of vertex comparatively fine and dense, puncture diameters on average less than 45 µm (Fig. 27). Ocelloccipital distance often shorter than 300 µm..... ***M. inexspectata*** **Rebmann**

– Vestiture grey-brown (Fig. 16), yellow-orange in Corsica and Sardinia (Fig. 18), red-orange in Malta (Fig. 17), snow white in northwestern Africa (Fig. 15). Disc of T6 with two of white hairs clearly separated by dark hairs (Figs 15–18). Mesonotum usually with scale-like hairs restricted to anterior and posterior parts. Terga 4 and 5 laterally mostly with numerous erect dark hairs. Punctation of vertex more coarse, punctures on average above 45 µm diameter (Fig. 20). Ocelloccipital distance mostly longer than 300 µm..... ***M. argentata*** **(Fabricius)**

Discussion

This taxonomic revision of a small group of widely distributed leafcutting bee taxa exemplifies one of the problems associated with taxonomic revisions: the identification of type material. This task was complicated by: i. poorly preserved types, where the morphological characters were difficult to examine (e.g., many holotypes of species described by O. Rebmann); ii. type specimens belonging to a sex that was not easily identified (e.g., *M. argentata*), or consisting of a gynandromorph individual (*Perezia maura*); iii. type specimens consisting of several body parts not originating from a single individual (*M. inexspectata*, *M. striatella*); iv. type specimens originating from introduced populations, where the source of the introduced population was initially unknown (*M. timberlakei* and *M. sudai*; *M. concinna*); and v. type specimens whose type locality is unclear (e.g., *M. modesta* Smith, 1879: type locality "White Nile", a nearly 4000-km river segment that flows from Uganda to Sudan). DNA barcoding was pivotal for solving most of these issues, especially for confidently establishing the identity of introduced populations of *M. pusilla* and *M. concinna.* However, what should have been a simple taxonomic revision of seven rather well-characterized species turned into a tedious study. This case exemplifies a major paradox in taxonomy, as our study would have been completed at least twice as quickly if no previous taxonomic work had been conducted at all, enabling us to simply describe the species as new.

One approach would have strongly accelerated the process: the assembly of a DNA barcode reference library for the most problematic type specimens. We could have focused on the scientifically interesting part of a taxonomic revision – species delimitation – and the fastidious nomenclatural translation of species delimitation into names would have been more straightforward. Laboratory protocols are available to obtain full DNA barcodes from 19th century specimens (Strutzenberg et al. 2012; Prosser et al. 2015; Hausmann et al. 2016); this approach has not been tested for Hymenoptera although NGS sequencing of old museum specimens using a single leg has been conducted (Blaimer et al. 2016). We nevertheless argue against the systematic barcoding of all types, since our finding would certainly not apply to all bee genera or bee faunas. In some specific cases, however, we feel that the barcoding of type material would dramatically speed up taxonomic revisions: revising the Afrotropical fauna of *Megachile*, for example, would take decades at the current pace given the numerous unclear names (see note above under *M. venusta*) and the scarcity of recent material. Many species are probably widely distributed and geographically variable, as shown here for *M. venusta.* The type material of species described by T. D. A. Cockerell and J. J. Pasteels, representing a significant proportion of the Afrotropical *Megachile* fauna, are likely to yield usable DNA. Given the unprecedented rate of biodiversity decline and the implications of taxonomic impediments for bee conservation (Praz et al. 2022), we argue that any methodological innovation that speeds up taxonomic revisions is worth consideration. DNA-barcoded type material represents a thus-far little used application of DNA barcoding to speed up taxonomy and to alleviate one of the inevitable impediments of taxonomy – the problem of unclear names and type specimens.

Acknowledgements

This study was only possible with the continuous support of Maximilian Schwarz, to whom CP expresses his gratitude. We thank all people who kindly made material available for study, especially Felix Amiet, Achik Dorchin, P. Andreas Ebmer, Alireza Monfared, Andreas Müller, Christian Schmid-Egger, Maximilian Schwarz, Thomas Wood, and the people mentioned in Suppl. material 1. Laurence Packer and Sam Droege kindly made unpublished DNA barcodes available. We thank Jessica Litman, Thomas Wood, Matthieu Aubert and Andreas Müller for numerous discussions on the taxonomy of this group of bees. Three anonymous reviewers proposed numerous changes, which improved the manuscript. Doug Yanega discussed issues related to the interpretation of the code. We thank the Museum of Natural History of Neuchatel (Jessica Litman) for allowing us to use their Keyence VHX 1000 digital imaging system. We also thank the curators of OUMNH (James Hogan), MNHN (Agnièle Tourey-Alby), SMFD (Jennifer Steppler), OLML (Esther Ockermüller, Fritz Gusenleitner & Martin Schwarz), NMBE (Hannes Baur) for their help accessing their collections.

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Supplementary material 1

List of examined specimens, with BOLD and genbank accession numbers

Authors: Christophe J. Praz, Dimitri Bénon

Data type: Occurences

Link: https://doi.org/10.3897/jhr.95.96796.suppl1

JHR 95: 199–213 (2023)
doi: 10.3897/jhr.95.87165
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Genetic evidence for parthenogenesis in the small carpenter bee *Ceratina dallatoreana* (Apidae, Ceratinini) in its native distribution range

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Academic editor: Christopher K. Starr | Received 31 May 2022 | Accepted 8 January 2023 | Published 17 February 2023

https://zoobank.org/0E8335A9-6BD9-4452-BDFE-E9326901A6C9

Citation: Mikát M, Straka J (2023) Genetic evidence for parthenogenesis in the small carpenter bee *Ceratina dallatoreana* (Apidae, Ceratinini) in its native distribution range. Journal of Hymenoptera Research 95: 199–213. https://doi.org/10.3897/jhr.95.87165

Abstract

Arrhenotoky is the typical mode of reproduction in Hymenoptera. Diploid females develop from fertilized eggs, whereas haploid males originate from unfertilized eggs. However, some taxa of Hymenoptera have evolved thelytoky, in which diploid females originate parthenogenetically from unfertilized diploid eggs. In contrast to some other hymenopteran lineages, like ants and parasitic wasps, thelytoky is generally very rare in bees.

Here, we evaluated the frequency of thelytoky in the small carpenter bee *Ceratina dallatoreana*, which was previously assumed to be thelytokous. By comparing genotypes of microsatellite loci between mothers and their offspring, we found that all female offspring were genetically identical to their mothers. We conclude that parthenogenesis is the prevailing and perhaps obligate mode of reproduction in *C. dallatoreana*. We also classify the cytological mode of this parthenogenesis as apomixis, or automictic parthenogenesis with central fusion and extremely reduced or non-existing recombination, because offspring showed no decrease of heterozygosity.

Because sociality is influenced by relatedness and *Ceratina* are ancestrally facultatively social, the high relatedness afforded by parthenogenesis should associate with social living in the nest. In accordance with previous work, however, we found no social nests of *C. dallatoreana*.

Keywords

Apidae, heterozygosity, relatedness, sociality, thelytoky, Xylocopinae

Introduction

Sexual reproduction predominates in animals (Simon et al. 2003; Kooi et al. 2017). However, parthenogenesis (embryo development without fertilization) has evolved in many lineages (Normark 2003; Engelstädter 2008; Neiman and Schwander 2011; Thierry 2013; Gokhman and Kuznetsova 2018). Obligately parthenogenetic lineages are usually recent (Neiman et al. 2009; Fujita et al. 2020), and though obligate parthenogenesis can be successful in the short-term (microevolutionary scale), sexual reproduction is more successful in the long-term (macroevolutionary scale) (Neiman and Schwander 2011; Thierry 2013). The frequency of parthenogenesis varies across insect orders, but it is especially common in stick insects and mayflies (Tvedte et al. 2019; Liegeois et al. 2021).

Different types of parthenogenesis most likely evolved by different mechanisms, with each type showing characteristic modes of inheritance (Engelstädter 2008). Only females are present in obligately parthenogenetic populations. More commonly, however, parthenogenesis is facultative, co-occurring with sexual reproduction and both sexes present in the population (Normark 2003; Liegeois et al. 2021)

Cytological mechanisms of parthenogenesis influence the genetic diversity and heterozygosity in the population (Pearcy et al. 2006; Engelstädter 2017; Hörandl et al. 2020). The presence or absence of meiosis defines two general processes: 1) mitotic parthenogenesis in the absence of meiosis, and 2) meiotic parthenogenesis, in which meiosis is present but diploidy is restored by several different mechanisms (Stenberg and Saura 2009). If mitotic parthenogenesis persists in a population, heterozygosity will increase, because the meiotic recombination leading to the loss of alleles is absent (Schwander and Crespi 2009; Tsutsui et al. 2014; Tvedte et al. 2019). On the other hand, meiotic parthenogenesis should lead to decreased heterozygosity, because diploidy is restored by endomitosis or fusion of the products of meiosis. Therefore, the frequency of heterozygotes in offspring may be the same or smaller than that of a parent in each locus. Typically, automictic parthenogenesis with terminal fusion (fusion of sister pronuclei) leads to a rapid decrease in heterozygosity (Engelstädter 2017; Alavi et al. 2018). However, heterozygosity can be retained in the case of meiotic parthenogenesis with central fusion if crossing over is absent during meiosis (Stenberg and Saura 2009; Engelstädter 2017). In these cases, the different products of meiosis I merge, with complementary halves of the mother's genetic information. Therefore, the heterozygosity in populations with this type of meiotic parthenogenesis increases similarly as in populations with mitotic parthenogenesis, despite differences in the cytological mechanisms of parthenogenesis (Engelstädter 2017).

A haplodiploid sex determination system is widespread among Hymenoptera (Normark 2003; Kooi et al. 2017). Males originate from unfertilized eggs, and are therefore haploid. Females develop from fertilized eggs and are therefore diploid (Gerber and Klostermeyer 1970; Mueller 1991; Stubblefield and Seger 1994). The sex of offspring depends on whether the (mated) mother fertilizes an egg (Gerber and Klostermeyer 1970; Stubblefield and Seger 1994). Unmated females can produce only male offspring arrenotokously (Shukla et al. 2013).

Thelytokous reproduction has evolved repeatedly in Hymenoptera (Vorburger 2014; Kooi et al. 2017), such as sawflies and parasitic Hymenoptera (especially Chalcidoidea, Cynipoidea and Ichneumonoidea). It is found less frequently in aculeate Hymenoptera (Kooi et al. 2017). The best evidence for thelytoky in aculeate Hymenoptera is from social species (Wenseleers and Van Oystaeyen 2011; Goudie and Oldroyd 2018). It evolved repeatedly in ants and has been documented in at least 50 species to date (Heinze 2008; Rabeling and Kronauer 2013; Goudie and Oldroyd 2018). In bees, facultative thelytoky is known in *Apis mellifera capensis*, in which workers lay thelytokous eggs, usually not in the nest where they originated (Goudie and Oldroyd 2014, 2018). However, thelytoky is rare in solitary nesting and weakly social Hymenoptera, with few exceptions (Kooi et al. 2017). Based on the sex ratio in populations of *Ceratina* bees (Apidae: Xylocopinae), thelytoky is predicted to occur in several species, including *C. dallatoreana* (Daly 1966; Snelling 2003). Males are extremely rare in *C. dallatoreana* (Daly 1966, 1983), leading to the hypothesis that this species reproduces by thelytokous parthenogenesis. Here, we test this hypothesis.

C. dallatoreana females nest in broken dead stems with pith, constructing a linear series of cells (Daly 1966). Although facultative sociality is common in this genus (Sakagami and Maeta 1977; Rehan et al. 2009; Groom and Rehan 2018), social nests have not been detected in this species to date (Daly 1966; Mikát et al. 2022). This species is endemic to the Mediterranean region and Central Asia (Terzo 1998; Terzo and Rasmont 2004; Fig. 1), and has been introduced into California, USA (Daly 1966). We used microsatellite genetic markers to examine the frequency of parthenogenesis in different populations across the native range of *C. dallatoreana* and to assess if the allele frequency aligns with Hardy-Weinberg equilibrium. Moreover, we attempt to infer the mode of parthenogenesis from the pattern heterozygosity inheritance.

Figure 1. Western Palearctic Region, showing the native range of *Ceratina dallatoreana*, based on Terzo and Rasmont (2004, 2011) and new localities from this study. Red – range of *C. dallatoreana*. Black triangles – sources of samples for this study.

Methods

We collected nests of *C. dallatoreana* in several locations across its native area of distribution, in Cyprus (2018, 2019), Italy (Puglia and Lazio regions, 2013 and 2017), Greece (Crete 2018, 2020), Albania (2018) and Tajikistan (2019) (Fig. 1). Coordinates of collection locations are shown in Suppl. material 1. Additionally, we analyzed females collected in Georgia (2013–2014) and North Macedonia (2014). We collected these females outside of their nests with nets or pan traps.

Nests were collected from natural nesting opportunities, as well as in stems broken or cut by human management. The most common nesting substrates were *Rubus* spp. and *Foeniculum vulgare*. In Cyprus and Crete we cut stems of these plants to increase nest density for ease of sampling several months later. To ensure that all inhabitants were inside the nest, nests were collected in the evening after 18:00 local time. Nests were opened lengthwise with garden clippers, and the number of adults, number of juveniles, and the stages of juveniles were noted. All individuals were preserved in 96% ethanol for further analysis.

Extraction of DNA

We isolated DNA using the Chelex protocol (Coombs et al. 1999). DNA was usually isolated from part of an individual (one or two legs from adults or pupae, part of the body from most larvae), but whole eggs and whole bodies of small larvae were also used. Samples were transferred to microcentrifuge tubes and dried for at least three hours. Later, we added 8 µl of proteinase K and 50 µl of 10% Chelex solution. This mixture was vortexed and inserted into a thermo cycler. The mixture was heated to 55 °C for 50 min and 97 °C for 8 min then cooled. The mixture was then vortexed and inserted into a centrifuge. After this 30 µl of supernatant were transferred to a well in the PCR plate.

Optimization of multiplex

We selected 12 female *C. dallatoreana* (9 from Cyprus and 3 from Tajikistan) for testing of microsatellite loci. We used microsatellite primers developed for *C. nigrolabiata* (Mikát et al. 2019). Fourteen microsatellite loci were arranged in two multiplexes. The first multiplex was previously applied to *C. nigrolabiata*, *C. chalybea* and *C. cyanea* (Mikát et al. 2019). The second multiplex contained six loci. Four were different from loci in the first multiplex (17 and 36 marked by 6FAM, 9 marked by VIC and 7 marked by PET), and two loci (12 and 51) were shared with the first multiplex but marked by a different color.

We evaluated results of amplification and obtained four possibilities for each locus: a) a locus was successfully amplified in all cases and was polymorphic (eight loci). b) a locus was successfully amplified in some cases and was polymorphic (two loci), c) a locus was amplified in all cases but was not polymorphic (three loci), or d) amplification of locus failed (one locus, Suppl. material 2: table S1).

Polymorphic and reliable loci were loci numbers 30, 23, 8, 67, 17, 36, 9, and 12 (Suppl. material 2: table S1), locus numbers corresponding to *C. nigrolabiata* (Mikát et al. 2019). However, we excluded locus 30 for overlap with same color-marked loci and locus 8 for interaction of primers with primers for another locus. Six microsatellite loci were thus retained for final analysis (Suppl. material 2: tables S1, S2).

PCR and Fragmentation analysis

We used Type-it Multiplex PCR Master Mix (Quiagen) according to the manufacturer's protocol. Primers of six microsatellite loci were use in a concentration of 0.05 μmol/l. We used these PCR conditions: 95 °C for 15 min; 30 cycles of 94 °C for 30 sec, 60 °C for 90 sec, 72 °C for 60 sec; and finally 60 °C for 30 min. After PCR, we mixed 0.8 μl of PCR product with 8.8 μl of formamide and 0.4 μl of marker Liz 500 Size scanner (Applied Biosystems). We heated the mixture to 95 °C for 5 min and then cooled it to 12 °C. Fragmentation analysis was performed on a 16-capillary sequencer at the Laboratory of DNA Sequencing at the Biological section of Faculty of Science, Charles University, Prague. Identification of alleles was performed in Gene Marker (Soft Genetics) software.

Analysis of ploidy and heterozygosity

We included mothers from nests and additional individuals in this analysis. We did not include offspring, as they had the same genotypes as their mothers. For each locus, we checked if an individual had one allele (homozygote) or two alleles (heterozygote). Individuals were considered diploid when heterozygous at least one locus. Individuals with only one allele at each locus were considered haploid. We analyzed 132 females (30 from Crete, 64 from Cyprus, 11 from Georgia, 12 from Italy, 9 from Tajikistan, 3 from Albania, and 3 from North Macedonia). We also analyzed one gynandromorph (individual with female head morphology and male abdomen morphology) from Tajikistan.

Analysis of diversity of multilocus genotypes

We counted multilocus genotypes for different localities. As one locality we defined an area where collected samples are at most ten kilometers from each other. In this analysis, we included adult females. We present only data from localities where at least three adult females were genotyped.

Analysis of deficit or surplus of heterozygotes

We used adult females for this analysis. We samples from two populations: Lefkara village, Cyprus (n = 50), and Georgioupoli village, Crete (n = 26). All individuals were collected at most 10 kilometers from each other in the same population. We calculated observed and expected heterozygosity using software Genepop, version 4.7.5. (Rousset 2020). Finally, we tested the possible deviation from Hardy-Weinberg equilibrium and

heterozygote excess (proportion of heterozygotes higher than in populations in Hardy-Weinberg equilibrium) or heterozygote deficiency (proportion of heterozygotes lower than in population in Hardy-Weinberg equilibrium), also using Genopop.

Analysis of parthenogenesis

We compared the genotype of each mother with offspring from the same nest. We analyzed 188 offspring from 59 nests in total. For this analysis, we selected nests in which the mother and immature brood were present – nests in stages active brood nests or full brood nest. Nests in the active brood stage are nests where the mother currently perform provisioning of brood cells. These nests contained currently provisioned brood cells and in outermost brood cell was egg or this brood cell was only partially provisioned (Rehan and Richards 2010; Mikát et al. 2021). In contrast, full brood nests contained larvae or pupae in the innermost and outermost cells, as the females had already completed provisioning and were guarding their offspring until adulthood (Rehan and Richards 2010; Mikát et al. 2021). Sampled nests for this analysis were from the following locations: Albania (10 offspring, two nests); Crete (23 offspring, six nests); Cyprus (89 offspring, 32 nests), Italy (38 offspring; 10 nests) and Tajikistan (28 offspring, nine nests).

Results

Sex ratio of adults

We collected *C. dallatoreana* samples across its native range. In total, we found 476 adult females, one gynandromorph and no adult males. Of the adult females, we collected 253 in Cyprus, 137 in Crete, three in Albania, three in North Macedonia, 36 in Italy, 30 in Georgia, and 14 in Tajikistan. We found the gynandromorph in Tajikistan.

Ploidy

All analyzed adult females from the maternal generation (n = 132) were heterozygotes in at least one locus. One female was a heterozygote in only one locus, while all others (n = 131) were heterozygotes in at least two loci. Thus, we determined that *C. dallatoreana* females are diploid. The gynandromorph was homozygous in all loci, therefore we considered this individual to be haploid.

Heterozygosity

We generally detected high heterozygosity in our studied loci. Average heterozygosity across all locations and loci was 56.25%. However, heterozygosity differs between loci, with the highest proportion of heterozygotes at locus 36 (97.06%), and lowest proportion at locus 12 (4.41%). The proportion of heterozygotes in each locus across different geographical areas is shown in Table 1.

Allele frequency deviated from Hardy-Weinberg equilibrium for all loci in Georgioupoli (Crete) and in three of five variable loci in Lefkara (Cyprus). Heterozygosity was increased in some loci but decreased in others. Observed heterozygosity was significantly higher than expected for loci 36 and 9 in Georgioupoli (Crete) and 36 and 67 in Lefkara (Cyprus), but significantly lower for loci 17, 23 and 12 in Georgioupoli (Crete) and 17 in Lefkara (Cyprus) (Table 2). When we performed this analysis in reduced sample (n=26 for Lefkara, Cyprus, n=13 for Georgioupoli, Crete), which excludes possible close relatives sampled in the same shrub, we obtained the same pattern for Lefkara population and a very similar pattern for the Georgioupoli population (Suppl. material 2: table S4).

Table 1. Proportion of heterozygotes at each studied locus by geographical area. The category other includes samples from Albania (n = 3) and North Macedonia (n = 3).

		Proportion of heterozygotes in locus						
Country	**N**	17	36	23	9	12	67	mean
Crete	30	0.33	1.00	0.73	1.00	0.03	0.97	0.68
Cyprus	64	0.16	0.98	0.16	0.58	0.00	0.97	0.47
Georgia	11	0.73	0.91	0.64	0.72	0.18	0.82	0.67
Italy	12	0.58	0.83	0.25	0.75	0.00	0.50	0.49
Tajikistan	9	0.33	1.00	1.00	0.78	0.00	1.00	0.69
Other	6	0.50	1.00	0.33	1.00	0.00	0.67	0.58
Total	132	0.32	0.97	0.41	0.73	0.04	0.90	0.56

Table 2. Comparison of expected (HetEXP) and observed (HetOBS) proportions of heterozygotes. P-values of statistical tests from expected frequencies are shown: p(excess) = p-value of heterozygote excess test, p(deficit) = p-value of heterozygote deficit test, p(HW) = p-value test of difference from Hardy-Weinberg equilibrium in allele frequency. All calculation performed in software Genepop. Bold indicates significant values. Locus 12 in Cyprus population had only one allele, therefore excess or deficit of heterozygotes could not be calculated.

Lefkara (Cyprus), N=50						
Locus	p(deficit)	p(excess)	p(HW)	HetEXP	HetOBS	n alelles
17	**0.0000**	1.0000	**0.0000**	0.59	0.18	5
36	1.0000	**0.0000**	**0.0000**	0.54	0.98	4
23	1.0000	0.7339	1.0000	0.15	0.16	3
9	0.4326	0.5688	0.0900	0.63	0.58	3
12	NA	NA	NA	0.00	0.00	1
67	1.0000	**0.0000**	**0.0000**	0.64	0.96	3
Georgioupoli (Crete), N=26						
Locus	p(deficit)	p(excess)	p(HW)	HetEXP	HetOBS	n alelles
17	**0.0027**	0.9974	**0.0000**	0.61	0.27	6
36	1.0000	**0.0048**	**0.0000**	0.82	1.00	9
23	**0.0176**	0.9824	**0.0000**	0.70	0.69	4
9	1.0000	**0.0000**	**0.0000**	0.63	1.00	4
12	**0.0007**	1.0000	**0.0003**	0.15	0.00	3
67	0.3422	0.6578	**0.0000**	0.72	0.96	6

Diversity of genotypes

We found a high diversity of multilocus genotypes. In all localities, we collected at least two multilocus genotypes. In Cyprus, we collected the largest sample in Lefkara (n = 50). In this location, we collected females with 26 multilocus genotypes. Eighteen of these genotypes were collected only once. Two of the most common genotypes had a frequency of 14.0% (7/50). We sampled another three locations in Cyprus: Agios Theodoros (n = 4, three multilocus genotypes); Mathiatis (n = 3, three multilocus genotypes); and Pyrgos (n = 5, four genotypes).

In Crete, we collected the largest sample in Georgioupoli (n = 26). In this location, we found 15 multilocus genotypes. The most common genotype had frequency 36.4% (9/26). Another sampled location was Chania airport, where we found three multilocus genotypes in three sampled individuals.

In Italy, we sampled at Pescariello (n = 5, five genotypes), Cassino (n = 3, two genotypes) and Santa Marinella (n = 3, three genotypes). In Georgia, we analyzed females from Vashlovani (n = 4, three genotypes) and Kvareli (n = 3, three genotypes). In Tajikistan, we analyzed samples from Shariston (n = 4, two genotypes).

Comparison of maternal and offspring genotypes

Almost all offspring genotypes were identical to those of their mother (97.87%, 184/188 out of 59 nests). The same genotype in mother and offspring was found in all nests in Albania (two nests, 10 offspring), Crete (six nests, 23 offspring), and Italy (10 nests, 38 offspring). In Cyprus, we found one out of 89 offspring (32 nests) with a different genotype than its mother and in Tajikistan we found 3 such offspring out of 28 offspring (9 nests). However, all offspring for which we detected different genotypes from those of the mother had much lower detection peak for multiple microsatellite loci than most of the analyzed individuals. All four individuals contained at least one allele which was not shared with their mother. Two individuals from Tajikistan had both alleles different from the mother at least one locus. One individual from Cyprus and two from Tajikistan had a unique allele not found in any other individual. We can also exclude the effect of null alleles, because all four individuals were heterozygotes at least one locus with alleles that would disagree with the maternal genotype. Therefore, the apparent differences between offspring and maternal genotypes was the result of genotyping errors.

Discussion

Previously, *Ceratina dallatoreana* was believed to reproduce parthenogenetically (Daly 1966, 1983), but without direct genetic evidence. We observed parthenogenesis in several locations in the Mediterranean (Albania, Italy, Crete, Cyprus) and central Asia (Tajikistan), providing evidence for parthenogenesis from a large part of the native

range of the species. As males are extremely rare in North Africa (Daly 1983) and California, where the species was introduced (Daly 1966), parthenogenesis would appear to be the prevailing or only mode of reproduction across populations.

Thelytokous parthenogenesis is rare in bees. Outside of *Apis mellifera capensis* (Rabeling and Kronauer 2013; Goudie and Oldroyd 2014), it was previously in evidence only in the genus *Ceratina*, where populations of several species were found without known males. These evidence include *C. acantha* (Slobodchikoff and Daly 1971), *C. dentipes* (Snelling 2003; Shell and Rehan 2019; M. Mikát unpublished data), *C. parvula* (Terzo et al. 2007; M. Mikát, unpublished data) and *C. dallatoreana* (Daly 1966). These species are not closely related, belonging to different subgenera (Rehan and Schwarz 2015; Ascher and Pickering 2020). Parthenogenesis is probably not the prevailing mode of reproduction in *C. dentiventris* and *C. sakagamii* which are considered to be the most closely related to *C. dallatoreana*, because they do not show a skewed sex ratio (Daly 1983; Terzo 1998). However males have not been found in *C. rasmonti*, which is known from only a few individuals and is closely related to *C. dallatoreana* (Terzo 1998). Given the distribution of parthenogenesis across *Ceratina* lineages, there may be a trend for parthenogenesis to arise in the *Ceratina* genus. Future research that includes the sampling of more species with a high-resolution phylogeny is needed for understanding evolution of parthenogenesis in this genus.

Although we found several offspring with genotypes that were not identical to genotypes of mothers, we suspect that these cases were the result of genotyping errors such as allelic dropout or false alleles. Situations in which offspring showed different genotypes from the mother were usually not compatible with scenarios of sexual reproduction. These results were also incompatible with any mode of parthenogenesis, because we detected alleles in offspring that were not detected in the mother. In two of four cases, offspring bear in at least one locus both alleles different from the mother. In case of parthenogenesis we can suppose allele loss, but not the rise of novel alleles.

Offspring resulting from parthenogenesis should bear only alleles shared by their mother. However, the cytology of parthenogenesis determines the rate of loss of heterozygosity from mother to offspring (Pearcy et al. 2006). We did not observe any heterozygosity loss, as all offspring were genetically identical to their mothers (when four improperly genotyped individuals are excluded) and are therefore clones of their mothers. This is compatible with two types of parthenogenesis: apomixis and automixis with central fusion. Apomixis is the more likely of the two, because under automixis with central fusion there would be at least some loss of heterozygosity due to recombination (Goudie and Oldroyd 2014; Engelstädter 2017). Empirical studies on organisms with central fusion automixis using microsatellites showed at least some heterozygosity loss (Rey et al. 2011; Fougeyrollas et al. 2015). Studies of *Apis mellifera capensis* show that homozygotes arise due to recombination, but they often die during early development. Therefore, high heterozygosity would be preserved by selection (Goudie and Oldroyd 2014). As we did not find any case of a homozygous offspring with a heterozygous mother even at the developmental egg stage, apomixis is the more probable mechanism.

We have shown that thelytokous parthenogenesis is the prevailing mode of reproduction in *C. dallatoreana*. However, there remains the question of whether sexual reproduction is only extremely rare or not occur at all. The existence of males is rarely reported for this species, but most of the reports of males could have been confused with closely related species (Daly 1983; Terzo 1998). Males are undoubtedly reported from California, where *C. dallatoreana* is invasive and no similar species are present (Daly 1966). However, the existence of males alone does not prove their involvement in reproduction. Strictly apomictic species usually have only one or a few genotypes in one location or region (Lorenzo-Carballa and Cordero-Rivera 2009; Ryskov et al. 2017). Although we detected some genotypes repeatedly, there was usually a high genotype diversity in each location, suggesting that sexual reproduction sometimes occurs in *C. dallatoreana*, even if rare.

The best documented examples of thelytoky in aculeate Hymenoptera are found among advanced eusocial species, and features of thelytoky are influenced by their social organization (Goudie and Oldroyd 2018). On the other hand, *Ceratina* are often facultatively social (Groom and Rehan 2018; Rehan 2020). Although most studied species are able to establish social colonies, the larger proportion of the population is solitary, and social colonies contain only two or a few females (Sakagami and Maeta 1977; Rehan et al. 2009; Groom and Rehan 2018; Mikát et al. 2022). Reversion to strict solitary nesting is also evident in some species (Groom and Rehan 2018; Mikát et al. 2020). Social nests have not yet been documented in *C. dallatoreana* (Daly 1966; Mikát et al. 2022), although the number of nests so far analyzed does not preclude the possibility of occasional sociality. This is quite surprising, because parthenogenesis may be expected to facilitate group living due to high relatedness between mother and offspring (Hamilton 1964). Moreover, two other species of *Ceratina* where parthenogenesis probably occurs (*C. dentipes* and *C. parvula*) are facultatively social (Terzo et al. 2007; Rehan et al. 2009; Mikát et al. 2022). The social status of one parthenogenetic species, *C. acantha* (Slobodchikoff and Daly 1971), is not yet known. *Ceratina* bees are an excellent group for the study of social evolution, due to their within- and between-species variability in social behavior (Groom and Rehan 2018; Rehan 2020). The existence of parthenogenesis in *Ceratina* species that are not closely related provides us unique system for study how between-species variability in relatedness influences social evolution.

Acknowledgements

We are grateful to Daniel Benda, Karolína Dobešová, Klára Daňková, Slavomír Dobrotka, Zuzana Dobrotková, Karolína Fazekašová, Tereza Fraňková, Jiří Houska, Jiří Janoušek, Lukáš Janošík, Celie Korittová, Tereza Maxerová, Miroslav Mikát, Blanka Mikátová, Jindra Mrozek, Daniela Reiterová, Tadeáš Ryšan, Vít Procházka, Vojtěch Waldhauser and Jitka Waldhauserová for assistance in the field. We are also grateful to Vít Bureš and Celie Korritová for collecting additional bees. We are grateful to Jesse Huisken and Ben Pyenson for feedback on the manuscript. The Grant Agency of Charles University (Grant GAUK 764119/2019) and the Specific University Research Project Integrative Animal Biology (Grant SVV 260571/2021) supported this research.

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Supplementary material 1

Dataset

Authors: Michael Mikát, Jakub Straka

Data type: occurences, genetic

Explanation note: Primary data used for paper Genetic evidence for parthenogenesis in small carpenter bee, *Ceratina dallatoreana* in its native distribution area. Dataset contains information about lotaions and dates of collected samples, lenght of microsatellite loci and infromation to which analyses was sample included.

Link: https://doi.org/10.3897/jhr.95.87165.suppl1

Supplementary material 2

Faunistic notes and microsatellite primers

Authors: Michael Mikát, Jakub Straka

Data type: genetic, faunistic

Explanation note: 1) notes about distribution of species *C. dallatoreana* 2) Features of microsatellite loci of *C. dallatoreana* 3) Features of successfully amplified microsatellites for *C. dallatoreana* 4) frequencies of alleles of microsatellite loci of *C. dallatoreana* 5) Comparison between expected and observed proportion of heterozygotes on reduced dataset from Lefkara (Cyprus) and Georgiopoli (Crete).

Link: https://doi.org/10.3897/jhr.95.87165.suppl2

JHR 95: 215–230 (2023)
doi: 10.3897/jhr.95.98260
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Morphological specialisation for primary nectar robbing in a pollen specialist mining bee (Hymenoptera, Andrenidae)

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Academic editor: Jack Neff | Received 1 December 2022 | Accepted 30 January 2023 | Published 17 February 2023

https://zoobank.org/84CB0F4F-360C-4E78-8432-17196C6D620F

Citation: Müller A, Westrich P (2023) Morphological specialisation for primary nectar robbing in a pollen specialist mining bee (Hymenoptera, Andrenidae). Journal of Hymenoptera Research 95: 215–230. https://doi.org/10.3897/jhr.95.98260

Abstract

The European mining bee species *Andrena lathyri* (Andrenidae) is a narrow specialist of flowers of *Lathyrus* and *Vicia* (Fabaceae), from which both females and males gain nectar by primary nectar robbing. Both sexes are equipped with a unique proboscis, which is much longer and more strongly angled than in most other *Andrena* bees including the most closely related species. The comparison between the shape of the proboscis and the interior of the host flowers combined with field observations revealed that the specialised mouthparts of *A. lathyri* precisely correspond to the dimensions of the flower interior and the position of the nectary, representing one of the few known examples of a morphological adaptation to primary nectar robbing in bees. For nectar uptake, the bee's head is inserted laterally under the standard petal before it is moved towards the flower base, thereby slitting the calyx longitudinally to a depth necessary to reach the nectary from inside the flower with the specialised proboscis. Nectar-robbing individuals of *A. lathyri* are able to adapt their behaviour to the different calyx lengths of their host flower species by slitting the calyx over varying distances. Except for the slit in the calyx, primary nectar robbing by *A. lathyri* does not damage any flower parts allowing for normal fruit development.

Keywords

Anthophila, Apiformes, bee-flower relationships, Fabeae, *Lathyrus pratensis*, *Taeniandrena*, *Vicia sepium*

Introduction

Nectar that is deeply hidden inside a flower is usually only accessible to flower-visiting insects that possess long mouthparts. However, flower visitors with short mouthparts may also obtain deeply concealed nectar either by i) biting a hole into the plant tissue near the nectary through which the nectar is ingested ("primary nectar robbing"), ii) using an existing hole bitten by another flower visitor ("secondary nectar robbing"), iii) entering the flower through the normal entrance without touching the sexual flower organs for example due to small body size ("nectar thieving"), or iv) pushing the mouthparts between the petals of a polypetalous flower from the side or base, thereby gaining abbreviated access to the nectary ("base working") (Inouye 1980; Irwin et al. 2010). Compared to nectar-drinking insects that visit the flowers in a legitimate way, nectar robbers, nectar thieves and side workers usually do not pollinate the flowers during the process of nectar uptake.

Bees behaving as primary nectar robbers are usually not equipped with specialised morphological structures to get access to the nectar, but instead mostly use their unspecialised mandibles to bite through the plant tissue (Macior 1966; Inouye 1983). However, in the two bumblebee species *Bombus* (*Alpigenobombus*) *wurflenii* Radoszkowski and *B.* (*Bombus*) *occidentalis* Greene occurring in mountaineous areas of Europe and western North America, respectively, the apical edge of the female mandible is not regularly rounded as in most other bumblebees but equipped with several teeth, which are assumed to facilitate the perforation of the plant tissue (Inouye 1983; Reinig and Rasmont 1988; Rasmont et al. 2021). As both species are regular and frequent nectar robbers (Løken 1950; Maloof 2001; Goulson et al. 2013), the toothed mandibles likely represent a morphological specialisation for primary nectar robbing. The two species are only distantly related (Cameron et al. 2007), suggesting that this specialisation has independently evolved twice in the genus *Bombus*. In contrast to most other primary nectar robbers, carpenter bees of the genus *Xylocopa* do not use their mandibles to perforate the flower tissue, but instead slit the flowers with their maxillae (Schremmer 1972; Inouye 1983; Gerling et al. 1989). Interestingly, the maxillae appear to be highly adapted to nectar robbing since the galeae are heavily sclerotised, modified and tightly linked together forming a strong piercing organ, which is able to perforate even rather hard plant tissue (Schremmer 1972).

The European mining bee species *Andrena* (*Taeniandrena*) *lathyri* Alfken (Andrenidae) exclusively collects pollen on plants belonging to the Fabaceae (Westrich and Schmidt 1987). It is known to regularly act as a primary nectar robber on flowers of *Lathyrus* and *Vicia* (Westrich 1989; Teppner et al. 2016), a behaviour that may be obligatory in this species (Westrich 1989). *Andrena lathyri* differs from most other *Andrena* species including the closely related representatives of the subgenus *Taeniandrena* by a distinctly longer and strongly angled proboscis. This peculiar shape of the proboscis has to the best of our knowledge never been noticed by bee taxonomists nor has its function be explored by bee biologists. As both females and males of *A. lathyri* have identical mouthparts and regularly rob flowers, the peculiar proboscis is hypothesised here to be a morphological specialization for primary nectar robbing similar to the toothed mandibles of some *Bombus* species and the piercing maxillae of *Xylocopa*.

In the present study, we i) verify the narrow pollen host specialisation of *Andrena lathyri*, ii) confirm the species' habit as obligatory nectar robber, iii) analyse the morphology of the specialised proboscis by comparing it with that of closely related *A.* (*Taeniandrena*) species, iv) describe the bees' behaviour during nectar robbing and pollen collection, v) investigate the impact of primary nectar robbing on flower integrity and fruit formation, and vi) discuss the hypothesis that the peculiar proboscis of *A. lathyri* is a morphological adaptation to primary nectar robbing.

Methods

Bee species, flower species and study sites

Andrena lathyri is a 10–14 mm long ground-nesting solitary bee, which is distributed over large parts of Europe and Turkey (Gusenleitner and Schwarz 2022). In Central Europe, the species is widespread at lower elevations and regionally often abundant. It belongs to the subgenus *Taeniandrena*, which comprises about 35 Palearctic species with one species additionally introduced into the Nearctic (Gusenleitner and Schwarz 2002; Wood et al. 2021; Praz et al. 2022; Wood 2022; T. Wood, personal communication). All species of this subgenus, for which the pollen hosts are known, exhibit an exclusive or very strong preference for the pollen of Fabaceae (Westrich 1989; Praz et al. 2022). Together with *A. aberrans* Eversmann, which exclusively collects pollen on few genera of the tribe Genisteae, such as *Chamaecytisus* (Westrich 2018), *A. lathyri* is among the most strongly pollen-specialised representatives of *A.* (*Taeniandrena*) exploiting only two closely related genera of the tribe Fabeae, i.e., *Lathyrus* and *Vicia* (Westrich and Schmidt 1987; Schaefer et al. 2012). As the proboscis of *A. lathyri* is too short to reach the nectaries at the base of the *Lathyrus* and *Vicia* flowers, nectar cannot be ingested during legitimate flower visits. Instead, *A. lathyri* gains nectar by primary nectar robbing on its pollen hosts.

Lathyrus pratensis L. and *Vicia sepium* L. have typical legume flowers with a five-part calyx and corolla (Kugler 1970; Proctor et al. 2003; Fig. 6a–f). The calyx consists of five sepals, which are fused over most of their length ending in five apical lobes ("calyx teeth"). The corolla is composed of five petals, of which the uppermost petal is in dorsal position ("standard"), the median two petals are in lateral position ("wings") and the lowermost two petals are in ventral position largely concealed by the lateral wings. The lowermost two petals are ventrally and apically fused forming a boat-shaped structure ("keel") that encloses the single pistil and ten stamina, of which the dorsalmost stamen is free, whereas the filaments of the other nine stamina are fused to a staminal tube surrounding the pistil. The flowers have a secondary pollen presentation in that the pollen is shed at the late bud stage onto a dense brush of fine hairs near the apex of the style ("stylar brush"), from where it is removed by pollen-collecting bees. Nectar is produced at the base of the pistil in a nectary, which is longitudinally crossed by the filament of the uppermost stamen, either slightly above the nectary's upper rim in *L. pratensis* or slightly below the upper rim in *V. sepium* (Fig. 6e, f) The nectar is accessible only from above

the staminal tube, where there is a spacious empty flower interior due to the considerably arched base of calyx and standard. To drink nectar from the flowers of *L. pratensis*, flower visitors have to pass the proboscis through the rather narrow slit on either side of the stamen filament, while the nectar is slightly more easily accessible in *V. sepium* due to the lower position and the smaller width of the crossing stamen filament.

Field observations and experiments were performed in northern Switzerland near Rekingen (47°33'59"N, 8°18'41"E; site 1), Dagmersellen (47°13'28"N, 7°58'49"E; site 2) and Wädenswil (47°13'06"N, 8°40'45"E; site 3) from May to June 2021 and 2022.

Pollen host preferences

To verify the pollen host specialization of *Andrena lathyri* to flowers of *Lathyrus* and *Vicia*, we microscopically analysed the pollen contained in the hind leg scopa of 30 females collected at 30 different localities in Switzerland (n = 28) and Liechtenstein (n = 2) between 1914 and 2019 using the method described by Westrich and Schmidt (1986). Before removing pollen from the scopa, the amount of pollen was assigned to five classes, ranging from 5/5 (full load) to 1/5 (filled to one-fifth). The pollen grains were stripped from the scopa of one leg with a fine needle, embedded in glycerol gelatin on a slide and identified at a magnification of 400× to family, subfamily or genus level. While pollen of *Lathyrus* and *Vicia* can be easily distinguished from that of other Fabaceae taxa in Central Europe by light microscopy based on shape, ornamentation and size of the hydrated pollen grains, there are no reliable characters to separate *Lathyrus* from *Vicia* pollen in every case (Beug 2004). Therefore, pollen of these two closely related taxa was recorded as *Lathyrus*/*Vicia* type in the pollen samples examined.

Obligatory or facultative nectar robbing?

To clarify whether *Andrena lathyri* is an obligatory nectar robber on flowers of *Lathyrus* and *Vicia* or whether it exploits numerous other plant taxa for nectar, we analysed the flower-visiting data for *A. lathyri* contained in the database of the Wildbienen-Kataster Baden-Württemberg. At the time of data retrieval (August 2022), the database comprised 392 records of *A. lathyri*, which were distributed all over Baden-Württemberg, collected from 1988 to 2021 and provided by M. Haider, M. Klemm, V. Mauss, R. Prosi, A. Schanowski and H.-R. Schwenninger. For 189 out of these 392 records, the plant genus or plant species visited by *A. lathyri* was known.

Morphology of the proboscis

To analyse the morphology of the mouthparts of *Andrena lathyri*, the proboscis was examined under a stereomicroscope at a magnification of 40× and compared with that of nine other western Palearctic *Andrena* species of the subgenus *Taeniandrena*, i.e., *A. aberrans*, *A. afzeliella* (Kirby), *A. gelriae* Van der Vecht, *A. caesia* Warncke, *A. intermedia* Thomson, *A. leucopsis* Warncke, *A. poupillieri* Dours, *A. russula* Lepeletier, and *A. wilkella* (Kirby). For measurements, *A. wilkella* was selected as representative species for the

subgenus *Taeniandrena* as its proboscis was found to be morphologically identical to all *A*. (*Taeniandrena*) species other than *A. lathyri*. For five females and five males each of *A. lathyri* and *A. wilkella* originating from different localities in Switzerland, the length of the glossa from the lowermost point of the basiglossal sclerite to its apex and the intertegular width were measured with a micrometer scale to the nearest 0.025. For the same individuals, the angle between the dorsal surface of the labium's lateral sclerites and the anterior surface of the glossa was determined on close-up photographs taken with a Nikon D750 camera. The morphological terminology of the proboscis follows Michener (2000).

Nectar-robbing behaviour

To examine the behaviour of *Andrena lathyri* during primary nectar robbing, we observed females and males gaining nectar from flowers of *Lathyrus pratensis* and *Vicia sepium* with the aid of a threefold magnifying glass at site 1 on 16.6.2021 and at site 2 on 17.5.2022. In total, we observed about 90 nectar-robbing visits.

To understand the movements of the labium during nectar ingestion, two females of *Andrena lathyri* were observed in the laboratory drinking sugar water from a small bowl.

To investigate whether the long and angled proboscis of *Andrena lathyri* fits into the flower interior for nectar uptake, three consecutive sections of the labium of ten females and ten males originating from different localities in Switzerland were measured with a micrometer scale to the nearest 0.025 mm under a stereomicroscope at 40× magnification (Fig. 2a): i) length from the sclerotised base of the prementum to the sclerotised base of the labial palpi ("basal section"), ii) dorsal length of the intermediate section extending from above the sclerotised base of the labial palpi to and including the basiglossal sclerite ("intermediate section"), and iii) length of the anterior surface of the glossa from the dorsalmost point of the basiglossal sclerite to the tip of the glossa ("apical section"). The measured lengths were averaged over all 20 individuals, multiplied with ten and plotted onto a 10:1 drawing of an average-sized flower each of *Lathyrus pratensis* and *Vicia sepium* to optically evaluate the labium's fit to the flower interior and its ability to reach the nectary.

Pollen-collecting behaviour

To examine the behaviour of *Andrena lathyri* during pollen collection, we observed females harvesting pollen on flowers of *Vicia sepium* with the aid of a threefold magnifying glass at site 2 on 17.5.2022 and at site 3 on 24.5.2022. In total, we observed about 30 pollen-collecting visits.

Impact of nectar robbing on flower integrity and fruit development

To evaluate the damage caused to flowers by nectar-robbing individuals of *Andrena lathyri*, we collected 50 robbed flowers each of *Lathyrus pratensis* and *Vicia sepium* at site 1 on 4.5., 12.5. and 25.5.2022 and examined both corolla and calyx under a stereomicroscope at a magnification of 20–40×.

To investigate whether the fruits of flowers robbed by *Andrena lathyri* develop normally, 25 flowers of *Vicia sepium* that showed the typical sign of having been robbed, i.e., a longitudinal slit in the calyx (see below), were marked with coloured threads at site 3 on 24.5.2022. Two weeks later, the development of the fruits of the marked flowers was assessed.

Results

Pollen host preferences

All 30 female pollen loads of *Andrena lathyri* from Switzerland and Liechtenstein exclusively consisted of pollen of the *Lathyrus*/*Vicia* type (Fig. 1a). This finding supports the species' narrow pollen specialisation already postulated by Westrich and Schmidt (1987), who found 46 pollen loads from Germany, Austria, Poland and Greece to be composed only of *Lathyrus* and/or *Vicia* pollen.

Obligatory or facultative nectar robbing?

Based on the Wildbienen-Kataster dataset, 69 (87.3%) of 79 flower visits by females of *Andrena lathyri* and 102 (92.7%) of 110 flower visits by males were recorded on flowers of *Lathyrus*, such as *L. niger* (L.) Bernhardi, *L. pratensis* L. and *L. vernus* (L.) Bernhardi, and of *Vicia*, such as *V. angustifolia* L., *V. cracca* L., *V. sativa* L., *V. sepium* L. and *V. villosa* Roth (Fig. 1b). Considering that the females mostly collect both pollen and nectar during the same flower visit (see below) and the males exploit the flowers only for nectar, these figures suggest that *A. lathyri* only exceptionally gains nectar from flowers other than *Lathyrus* and *Vicia*, rendering the species an almost obligatory nectar robber.

Morphology of the proboscis

The comparison of the proboscis of *Andrena lathyri* with that of nine other *Andrena* species of the subgenus *Taeniandrena* revealed that the morphological differences between *A. lathyri* and its relatives are restricted to the labium, whereas the construction of the maxillae is identical.

The labium of *Andrena* (*Taeniandrena*) species consists of five main sclerotised parts (Fig. 2a, b): i) the prementum, which extends till the base of the labial palpi, ii) the two four-segmented labial palpi, which attach laterally to the end of the prementum, iii) a pair of lateral sclerites, which cover the base of the paraglossae from above and form together with the adjacent basiglossal sclerite an intermediate section between the end of the prementum and the anterior surface of the glossa, iv) the two paraglossae, which attach distal to the ventral end of the prementum, run along the lower margin of the two lateral sclerites and are distally bent outwards, and v) the glossa, which originates between the base of the paraglossae, tapers towards its apex and is densely beset with annulate hairs below the basiglossal sclerite.

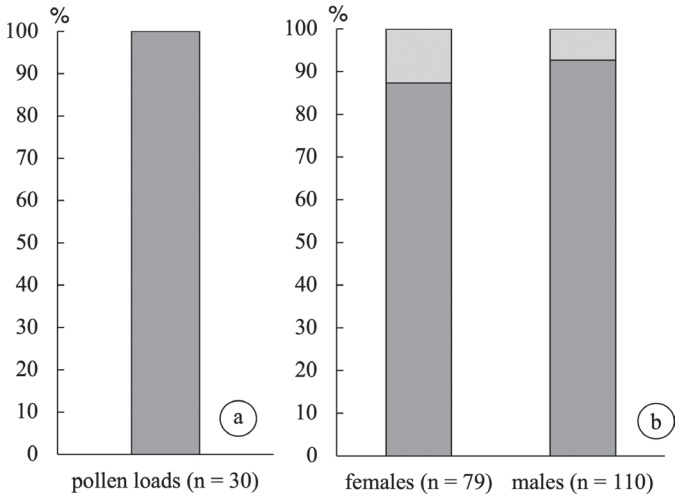

Figure 1. Pollen and nectar sources of *Andrena lathyri* **a** composition of female pollen loads **b** flower visits. Dark grey = *Lathyrus* and *Vicia* species, light grey = other plant species.

The labium of *Andrena lathyri* differs in two main respects from that of all other *A.* (*Taeniandrena*) species represented here by *A. wilkella* (Fig. 2). First, the glossa is on average 1.57 mm long in *A. lathyri* (range = 1.48–1.68 mm, n = 5 females and 5 males), whereas it is on average 0.50 mm long in *A. wilkella* (range = 0.45–0.55 mm, n = 5 females and 5 males). By correcting for the difference in body size, which is 10% smaller in *A. wilkella* based on intertegular width, the glossa of *A. lathyri* is about 2.9× longer than that of *A. wilkella*. Second, the angle between the dorsal surface of the lateral sclerites and the anterior surface of the glossa is on average 100.8° in *A. lathyri* (range = 91–109°, n = 5 females and 5 males), whereas it is on average 140.9° in *A. wilkella* (range = 130–152°, n = 5 females and 5 males). These differences result in a much longer and distinctly more strongly angled labium in *A. lathyri* compared to most other *Andrena* species including the closely related representatives of the subgenus *Taeniandrena*.

Nectar-robbing behaviour

The behaviour of nectar-robbing individuals of *Andrena lathyri*, which was very uniform, identical in both sexes and invariant between flowers of *Lathyrus pratensis* and *Vicia sepium*, can be divided into three phases. In the first phase, the bees crawled headfirst to the side of the flower and inserted the half-extended proboscis and the lower half of the head under the lower margin of the standard (Figs 3a, 6c, d). In the second phase, the bees moved the head between the inner side of the standard and the outer side of the wing towards the base of the flower (Fig. 3b); when the advancing head reached the calyx, the outer mandible was spread out (Fig. 3c), and by moving the head further towards the flower base, the calyx was torn open between the lowest and the second lowest calyx tooth (Fig. 3d); during advancing, the outer mandible was repeatedly moved up and down, which probably facilitated the process of tearing the rather hard calyx tissue by acting

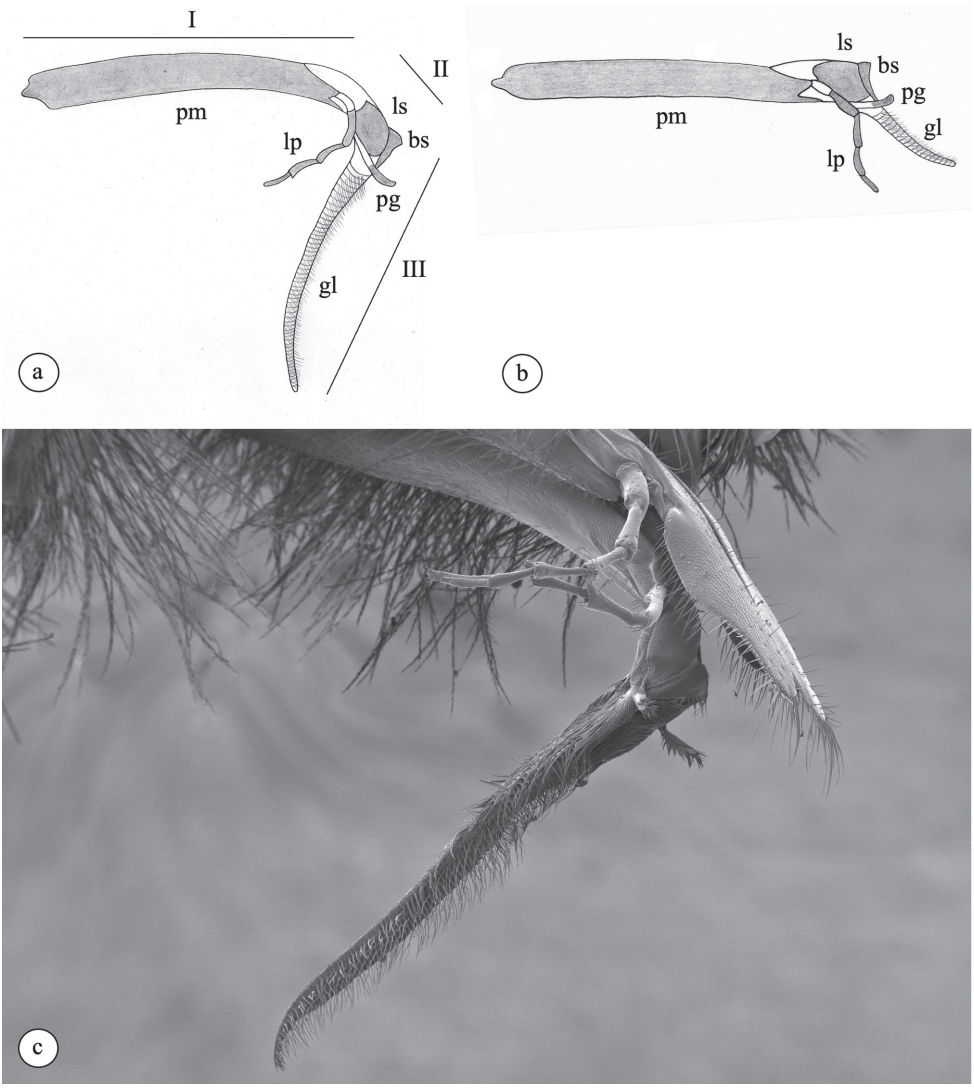

Figure 2. Mouthparts of *Andrena lathyri* and *Andrena wilkella* in lateral view **a** labium of *A. lathyri* **b** labium of *A. wilkella* **c** maxilla and labium of *A. lathyri*. pm = prementum, lp = labial palpus, ls = lateral sclerite, bs = basiglossal sclerite, pg = paraglossa, gl = glossa; I = basal section, II = intermediate section, III = apical section.

as an abutment or support, but not as cutting tool; instead, the calyx tissue tore due to the pressure of the advancing head; sometimes, a soft crackling sound was heard, which probably occurred when the tissue tore; at the end of this phase, the outer mandible rested on the calyx and the inner mandible, the proboscis and one compound eye were completely hidden inside the flower (Fig. 3d). In the third phase, the bees — holding

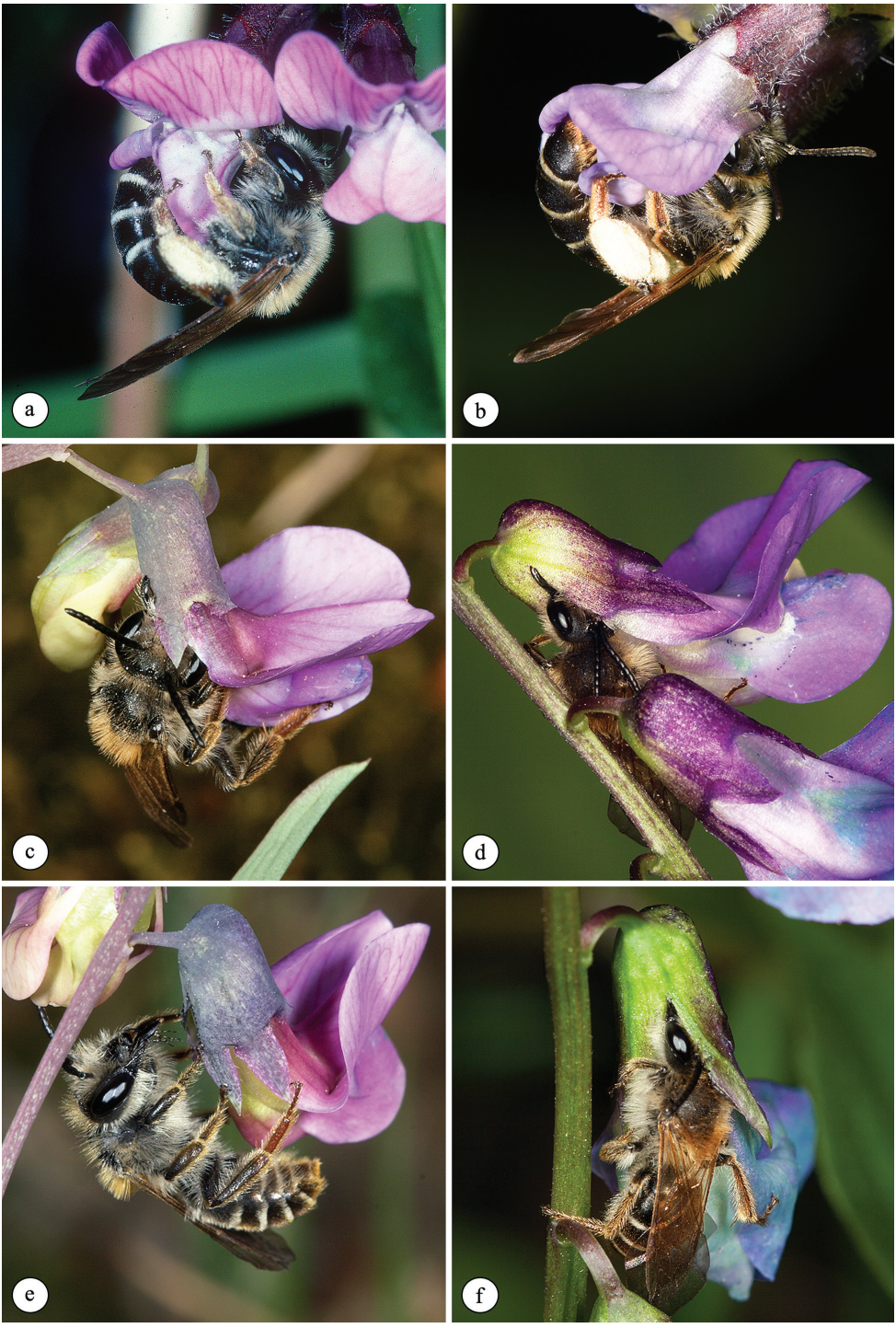

Figure 3. Nectar-robbing behaviour of *Andrena lathyri*. See text for details.

their head obliquely upwards from its ventral position relative to the flower —extended the proboscis inside the flower in longitudinal direction to the head upwards, so that the uppermost point of the angled proboscis was close to the upper roof of the flower base and the lowermost point, i.e., the apex of the glossa, reached the nectary from above to drink nectar (Fig. 4); after nectar uptake, the bees withdrew the proboscis from the calyx slit and left the flower (Fig. 3e).

In all observed nectar-robbing visits (n = 90), only one side of the calyx was slit, suggesting that the proboscis can empty the nectary from one side despite the longitudinally crossing stamen filament and that calyces slit on both sides originate from two different visits by *Andrena lathyri*.

How often individuals of *Andrena lathyri* gained nectar by secondary nectar robbing, i.e., by using an already existing calyx slit, could not be quantified exactly, because the observer's view of the flower base became obscured as soon as the bee crawled to the side of the flower to rob nectar. However, secondary nectar robbing occasionally occurred (Fig. 3f).

The observation of nectar-drinking females of *Andrena lathyri* in the laboratory revealed that the glossa can be moved far forwards and backwards in longitudinal direction to the body due to a ventral joint adjacent to the distal end of the prementum. In addition, the haired part of the glossa itself is movable to all sides, allowing for its precise guidance within the flower. In repose, the glossa is folded back over the dorsal surface of the prementum, which is embedded between the stipites of the maxillae within the proboscidial fossa on the underside of the head. For nectar ingestion, the prementum is moved downwards and forwards followed by the folding out of the glossa. At maximum extension of the proboscis, the sclerotised base of the prementum is situated roughly underneath the labrum resulting in a long maximal reach of the proboscis. By moving the prementum forward at different distances and/or bending the joint at the end of the prementum at varying angles, the mouthparts have a considerable flexibility to adjust to the specific interior of the host flowers, which is expected to slightly differ among the different species of *Lathyrus* and *Vicia* that are exploited by *A. lathyri* (see above).

The basal, intermediate and apical section of the labium of *Andrena lathyri* measured on average 1.91 mm, 0.30 mm and 1.74 mm, respectively (n = 10 females and males each). The true-to-scale plotting of these three sections, i.e. the labium at its maximum extension, onto flower drawings of *Lathyrus pratensis* and *Vicia sepium* revealed - in combination with the observed position of the bee's head during primary nectar robbing and the fact that the nectaries can be accessed only from above (see above) - that the bee's mouthparts precisely correspond to the dimensions of the flower interior and the position of the nectary (Fig. 4a–c).

Pollen-collecting behaviour

To collect pollen on flowers of *Vicia sepium*, the females of *Andrena lathyri* pushed the standard upwards with the front of the head, which created the necessary space for the movements of the fore legs (see below), and simultaneously pressed the wings sideways

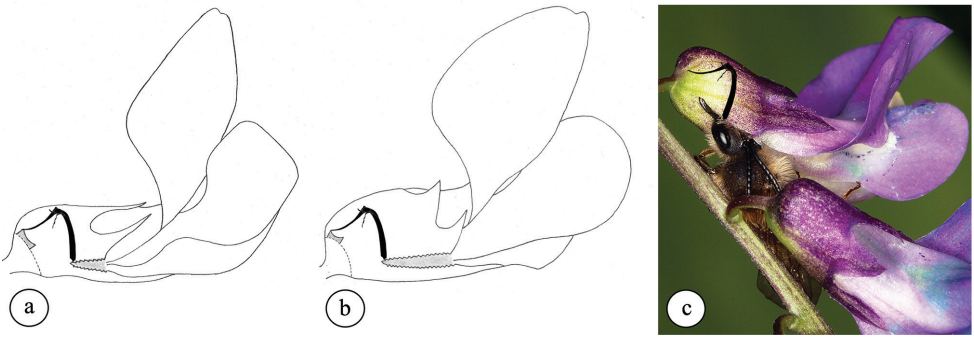

Figure 4. Maximally extended labium of *Andrena lathyri* plotted true to scale into the flower interior **a** *Lathyrus pratensis* **b, c** *Vicia sepium*. Black = labium, grey = nectary and calyx slit.

down with the middle and hind legs, which caused the keel to move downwards and the pollen-bearing stylar brush to emerge from the tip of the keel (Fig. 5a). The bees then harvested the pollen from the stylar brush by rapid strokes of the fore legs, which resulted in pollen masses adhering to both the pilosity of the foretarsi and the long hairs covering the underside of the maxillar stipites (Fig. 5b).

During most flower visits on *Lathyrus pratensis* and *Vicia sepium*, the females of *Andrena lathyri* first collected pollen, before crawling to one side of the flower to rob nectar. Occasionally, however, pollen-only and nectar-only visits also occurred.

Impact of nectar robbing on flower integrity and fruit development

Of the 50 corollae each of *Lathyrus pratensis* and *Vicia sepium* examined for damage by nectar-robbing individuals of *Andrena lathyri*, 81 (81%) were intact and 16 (16%) could not be properly assessed as the corolla was partly withered due to the late development stage of the flower; only three (3%) flowers of *V. sepium* were found to have a 1.8–5.8 mm long longitudinal crack near the lower margin of one of the two lateral wings, most probably caused by *A. lathyri* during nectar robbing.

Primary nectar robbing by *Andrena lathyri* on *Lathyrus pratensis* and *Vicia sepium* invariably resulted in a longitudinal slit in the calyx (Fig. 6a, b). This slit was located between the lowest and the second lowest calyx tooth in all 100 flowers examined with the exception of one flower of *V. sepium*, where the slit was located between the second lowest and the third lowest calyx tooth; interestingly, the corolla of the latter flower was one of the very few damaged by nectar robbing (see above), suggesting that this flower might possibly have been robbed by an unexperienced forager. The calyx was slit only on one side in 89 (89%) flowers examined, whereas it was slit on both sides in 11 (11%) flowers, probably resulting from two different flower visits (see above). The length of the calyx slit was significantly shorter in *L. pratensis* (range = 0.7–2.1 mm, median = 1.3 mm, n = 54) than in *V. sepium* (range = 2.0–3.9 mm, median = 2.8 mm, n = 57) (Mann-Whitney-U-test, $U = 2.5$, $p < 0.001$). In contrast, the distance between the end of the slit and the base of the flower, where the nectary is located in both

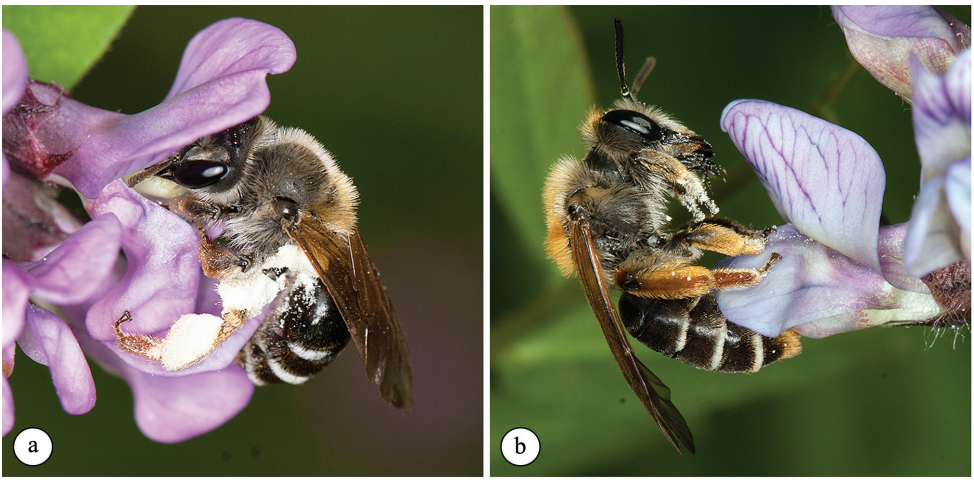

Figure 5. Pollen-collecting behaviour of *Andrena lathyri*. See text for details.

species, did not significantly differ between *L. pratensis* (range = 1.4–2.6 mm, median 2.1 mm, n = 54) and *V. sepium* (range = 1.4–2.9 mm, median = 2.2, n = 57) (Mann-Whitney-U-test, $U = 1325.5$, $p = 0.206$).

In 21 (84%) of the 25 marked flowers of *Vicia sepium*, the fruits developed normally in spite of having been robbed by *Andrena lathyri* (Fig. 6g, h), in one flower (4%) the ovary was withered and three flowers (12%) dropped off the plant for unknown reasons, overall suggesting a negligible negative impact of primary nectar robbing on fruit development.

Discussion

The present study verifies the narrow pollen specialization of *Andrena lathyri* to flowers of *Lathyrus* and *Vicia* (Fabaceae) and confirms the species' habit as a largely obligatory nectar robber on its pollen hosts.

Compared to the great majority of *Andrena* bees including the most closely related species (see Danforth et al. 2019), the proboscis of *A. lathyri* is exceptionally long due to a considerable elongation of the glossa and strongly bent due to an almost right angle between the short intermediate section of the labium and the glossa. This specialised proboscis precisely fits into the flower interior of *Lathyrus* and *Vicia* and its long and angled shape enables the bees to reach the nectary from above, which is the only way to gain nectar given the ventral position of the bee's head during nectar uptake. Because of this exact match between bee proboscis and host-flower interior, the specialised mouthparts of *A. lathyri* are interpreted here as a morphological specialisation for primary nectar robbing, which is in line with the findings that the proboscis is not actively involved in pollen harvesting and that females and males, which both rob flowers, possess identical mouthparts.

Figure 6. Flowers of *Vicia sepium* (left) and *Lathyrus pratensis* (right) **a, b** longitudinal slit in calyx due to primary nectar robbing by *Andrena lathyri* **c, d** flower with calyx removed showing lower side of standard (arrow), under which *A. lathyri* moves head and proboscis towards flower base **e, f** flower in top view with calyx, standard and wings removed showing nectary (ne), which is accessible only from above **g, h** normally developing fruits despite slit in the calyx (arrow) caused by *A. lathyri.*

Beside this morphological adaptation to primary nectar robbing, *Andrena lathyri* also exhibits behavioural specialisations, which include the insertion of proboscis and head under the lower margin of the standard of the *Lathyrus* and *Vicia* flowers and

the subsequent slitting of the calyx to a depth necessary to reach the nectary with the specialised proboscis. Interestingly, by slitting the calyx over a shorter distance in *L. pratensis* than in *V. sepium*, which has a longer calyx, nectar-robbing individuals of *A. lathyri* keep in both flower species the same distance between the end of the slit and the flower base, where the nectary is located. Slitting the calyx of *L. pratensis* over a similar length as in *V. sepium* would render nectar uptake by the specialised mouthparts impossible as in this case the proboscis would be too close to the flower base to reach the nectary from above. This finding reveals an amazing ability of nectar-robbing individuals of *A. lathyri* to adapt their behaviour to the different calyx lengths of their hosts.

The flowers of *Lathyrus* and *Vicia* species are characterised by a strongly arched base of calyx and standard leading to an almost right angle between the posterior side and the upper side of the flower base. This characteristic, which also occurs in other genera of the tribe Fabeae, such as *Pisum* or *Vavivolia*, results in a spacious flower interior above the nectary providing enough space for the movements of the specialised proboscis of *Andrena lathyri*. In contrast, the flower base of many other Fabaceae genera, such as *Genista*, *Hippocrepis*, *Lotus*, *Melilotus*, *Onobrychis* or *Trifolium*, is usually distinctly less strongly arched hardly leaving enough space for *A. lathyri* to gain nectar with its mouthparts. This difference in the shape of the flower base might contribute to the narrow flower specialisation of *A. lathyri* and probably renders host shifts to Fabaceae species other than those of the Fabeae difficult.

Primary nectar robbing by *Andrena lathyri* does not damage any flower parts except for a short slit on one side of the calyx, which is consistent with the findings that fruit development was not substantially impaired by nectar robbing and that at sites where *A. lathyri* was common many calyces at the base of well-developed fruits of both *Lathyrus pratensis* and *Vicia sepium* were slit. In contrast to the males of *A. lathyri*, which never come into contact with the sexual flower organs due to their illegitimate nectar visits, females are expected to pollinate the flowers during pollen collection, since both *Lathyrus* and *Vicia* have homogamous flowers with female and male reproductive organs maturing at the same time (Kugler 1970). Thus, primary nectar robbing by *A. lathyri* does not seem to have any significant negative effect on the reproduction of the bee's exclusive host plants, even more so as the flowers of *L. pratensis* and *V. sepium* are visited and pollinated by a multitude of different long-tongued bee species (Westrich 1989).

Conclusions

The peculiar proboscis of *Andrena lathyri* is one of the few known examples of a morphological adaptation to primary nectar robbing in bees and tightly binds the bee to its specific host plants, whose fruit development is not negatively affected by the illegitimate nectar gain.

Acknowledgements

The Wildbienenkataster Baden-Württemberg and Rainer Prosi generously provided an excerpt of all data of *Andrena lathyri* from Baden-Württemberg containing numerous flower-visiting records. Philipp Heller, Christophe Praz and André Rey gave hints to localities, where *A. lathyri* occurred in good numbers. Urs Weibel helped with fieldwork. Thomas Wood provided information on the morphology of the proboscis of *A.* (*Taeniandrena*) species. Michael Greef (ETH Zurich), Jessica Litman (Muséum d'Histoire Naturelle de Neuchâtel) and Christophe Praz loaned females of *A. lathyri* for pollen removal and analysis. Mark Winston helped with the morphological interpretation of the proboscis of *A. lathyri*. Anne Greet Bittermann from ScopeM (ETH Zurich) took the SEM image. Comments by J. Cane, J. Neff, E. Scheuchl and T. Wood improved the manuscript.

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JHR 95: 231–244 (2023)
doi: 10.3897/jhr.95.87752
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Niche modeling of bumble bee species (Hymenoptera, Apidae, *Bombus*) in Colombia reveals highly fragmented potential distribution for some species

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Academic editor: Christopher K. Starr | Received 14 June 2022 | Accepted 28 December 2022 | Published 17 February 2023

https://zoobank.org/7C72F539-B97F-4E66-BDD8-5AB0A37A12BF

Citation: Rojas-Arias L, Gómez-Morales D, Stiegel S, Ospina-Torres R (2023) Niche modeling of bumble bee species (Hymenoptera, Apidae, *Bombus*) in Colombia reveals highly fragmented potential distribution for some species. Journal of Hymenoptera Research 95: 231–244. https://doi.org/10.3897/jhr.95.87752

Abstract

Insect population decline has been reported worldwide, including those of pollinators important for ecosystem services. Therefore, conservation actions which rely on available rigorous species distribution data are necessary to protect biodiversity. Niche modeling is an appropriate approach to distribution maps, but when it comes to bumble bees, few studies have been performed in South America. We modeled ecological niches of nine Colombian *Bombus* species with MAXENT 3.4 software using bioclimatic variables available from WorldClim. This resulted in maps for each species that show the potential distribution area at the present time. Modeled species maps accurately represent potential niches according to the description of bioclimatic conditions in the species' habitat. We grouped the species into three clusters based on our results, as well as on distributional information from literature on the topic: High Mountain, Mid- Mountain and inter-Andean, and the Amazon and Eastern Plains Basin. Niche modeling depicted bumble bee species' distribution in Colombia, the results of which can serve as a useful tool for conservation policies in the country.

Keywords

biogeography, distribution, maxent, native bees, pollinators

Introduction

Bumble bee species (*Bombus* spp.) are one of the widest studied taxa of bees in the world and Colombia regarding their ecological traits, the genetic composition of populations, pathogens, and their role in pollination services (Cure and Rodríguez 2007; Gamboa et al. 2015; Jaramillo-Silva et al. 2018; Lotta-Arevalo et al. 2020). Buzz pollination carried out by bumble bees allows for the pollination of several crops of economic importance in Colombia, such as Solanaceae like potatoes, tomatoes, paprika, etc. (Cure and Rodríguez 2007; Riaño et al. 2015; Vinícius-Silva et al. 2017) or Passifloraceae like passion fruit, gulupa, curuba, etc. (Pinilla-Gallego et al. 2016). Also, bumble bees are important for the pollination of many plant species in natural ecosystems like paramos and cloud forests, which are key for water regulation in Colombia (Rubio 2012). For example, they serve in the pollination of several species of "frailejon" of the genus *Espeletia* (Fagua and Bonilla Gómez 2005).

However, a drastic decline in insects has been documented worldwide (Hallmann et al. 2017; Seibold et al. 2019; Klink et al. 2020). A massive decline in the species distribution of most bumble bees has been reported, especially in developed regions like Europe and North America (Goulson et al. 2008). In Europe, there have been reports of declines since the 50's. Just in the United Kingdom, three species out of twenty-five have gone extinct, and eight report major declines (Goulson 2003). Similar results have been reported in North America. In Illinois alone, four species have become locally extinct (Grixti et al. 2009). There is no population trend research of this kind performed in Colombia, and as one of the richest countries in terms of biodiversity (Rangel 2015), the country should increase research on its pollinators in order to develop preventive management of natural resources.

Biogeographic information about species is vital for conservation planning. The study of species distribution patterns has two complementary approaches: historical biogeography, which elucidates the causal mechanisms of current distribution, and ecological biogeography, which evaluates the ecogeographic factors that are currently shaping the distribution of species, looking for patterns in characteristics that are required for a species' long-term survival. It looks at abiotic characteristics like humidity, temperature, or salinity and ecological characteristics like interactions with other organisms or genetical features. Research in this last approach is usually performed at a local scale a short time frame (Pérez-Malváez and Gutiérrez 2003).

Niche modeling is one of the methods used in ecological biogeography. It represents the fundamental niche without considering the realized niche, which requires detailed field research and confirmed species samples. Niche modeling aims to find the potential distribution area of a species (Soberon and Miller 2009). It is important to have good quality and quantity of information for the models, as they can be biased if the data for a species is scarce.

Thus, niche modeling can provide potential distribution maps for bumble bees in Colombia, updating and complementing the previous available maps, made by Abramovich et al. (2004). This information could be an important tool to use in both future studies with an evolutionary and ecological aim, as well as in conservation efforts by

informing environmental conservation plans, political decisions, and public policies in Colombia, and by strengthening existing efforts like the Colombian Pollinators Initiative (CPI) (Nates-Parra 2016). As the importance of bee conservation continues to escalate, conservation actions are necessary to protect bees' biodiversity (Sárospataki et al. 2005).

Materials and methods

Study area

The geographical extent used for each species in the modeling process covers the entire continental Colombian territory, from the north (13°23.73'N) to the south (4°13.75'N) and from the west (81°44.13'W) to the east (66°50.63'W). No buffer was used.

There are nine species from the genus *Bombus* reported for Colombia: *B. pauloensis* Friese, 1913 (formerly *B. atratus* Franklin, 1913), *B. excellens* Smith, 1879, *B. funebris* Smith, 1854, *B. hortulanus* Friese, 1904, *B. melaleucus* Handlirschi, 1888, *B. pullatus* Franklin, 1913, *B. robustus* Smith, 1854, *B. rubicundus* Smith, 1854, and *B. transversalis* Oliver, 1789. Across the paramo ecosystem, four species can be found with different altitudinal distributions: *B. funebris* between 2500 and 4750 m, *B. hortulanus* between 2100 and 3600 m, *B. robustus* between 2100 and 3800 m, and *B. rubicundus* between 2500 and 3900 m (Pinilla-Gallego et al. 2016). Along the sub-Andean forest or the cloud forest, two species can be found: *B. excellens* between 1500 and 2600 m and *B. melaleucus* between 450 and 2100 m. The most abundant species in the low mountain strata is *B. pauloensis*. However, it has a wide altitudinal range between 150 and 3600 m (Liévano et al. 1991). Finally, two different species can be found across tropical forests with warm and wet climates: *B. pullatus* between 120 and 3500 m (especially in the foothills) and *B. transversalis* between 180 and 1100 m (restricted to the amazon forest and its foothills).

Occurrence data

We obtained occurrence points from the Wild Bee Research Lab of the Universidad Nacional de Colombia, Bogotá (**LABUN**), the Insect Collection of Universidad del Quindío (**CIUQ**), the Francisco Luis Gallego Entomological Museum (**MEFLG**), Universidad Nacional de Colombia, Medellín, the Bee Collection of the Universidad Militar Nueva Granada, the Entomological Museum of the School of Agronomy at Universidad Nacional de Colombia (**UNAB**), and the database from the Global Biodiversity Information Facility (**GBIF**). Occurrence points were adjusted using the altitude reference reported by Pinilla-Gallego et al. (2016). All occurrence point data were originally collected from 1938 until 2020, throughout different seasons and derived from various methods (See Suppl. material 1). The senior author checked all occurrence records to prevent recognizable errors in georeferencing and taxonomy. The number of points used for each specie was: *B. excellens* 46; *B. funebris* 150; *B. hortulanus* 1130; *B. melaleucus* 47; *B. pauloensis* 2128; *B. pullatus* 549; *B. robustus* 243; *B. rubicundus* 426; *B. transversalis* 43 (Fig. 1).

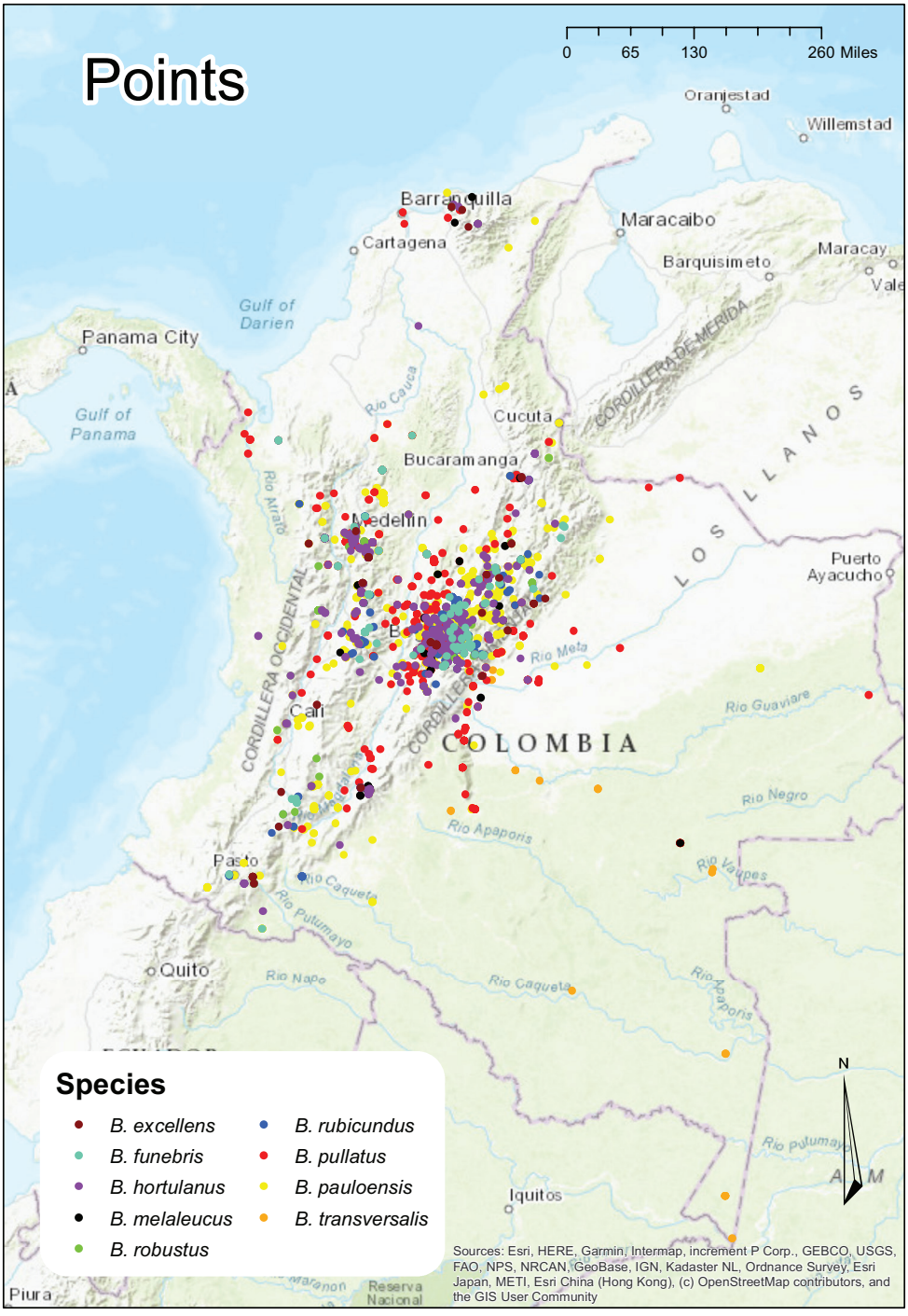

Figure 1. Distribution of the points used in the modeling of each specie.

Environmental variables

We used 19 environmental data layers for modeling (Table 1), which were downloaded from the WorldClim V. 2.1 website (Fick and Hijmans 2017). The layers represent the climate average from the year 1970 to the year 2000 at a spatial resolution of 30 Arc seconds (0.93 km^2).

Niche models

QGIS 2.8 (QGIS Development Team 2016) was used to clip and convert environmental layers, enabling their use in MAXENT. Niche models were developed using MAXENT 3.4 (Phillips et al. 2020). Specific settings were set at the following defaults: a maximum of 500 iterations, 10% test point (CrossValidate), without extrapolation and cumulative, keeping a limit convergence of 0.00001 and prevalence of 0.5, maximum number of background points 10000.

Two different statistical analyses were performed per species: the Jackknife test to evaluate the weight or importance of each variable (Timaná de la Flor and Romero 2015; Phillips and AT&T Research 2017), and the AUC (Area Under the Curve) test. Jackknife test is a method for validating the samples and model. It shows the representative variables for modeling each specie (Shcheglovitova and Anderson 2013). According to the results of the Jackknife test, we selected the three variables with the highest percentage of importance, whose values depend on each species. To obtain the AUC, the specific settings given above were chosen according to established knowledge about bumble bees' altitudinal distribution (Pinilla-Gallego et al. 2016). Subsequently, an ROC (Receiver Operating Characteristics) curve was created, and the AUC was calculated. The AUC value reflects the model's accuracy or its capacity for prediction. The AUC value moves between 0 and 1, with 1 representing a perfect prediction. Values over 0,9 are considered strong (Pliscoff and Fuentes-Castillo 2011). Finally, ArcMap 10.5 (ESRI 2015) was used for reclassifying and visually representing the ecological niche modeling results.

Table 1. Bioclimatic variables included in modeling were obtained from WorldClim.

Temperature	Precipitation
1 Annual Mean Temperature	12 Annual Precipitation
2 Mean Diurnal Range (Mean of monthly (max. temp.-min. temp.))	13 Precipitation of Wettest Month
3 Isothermality (BIO2/BIO7) (×100)	14 Precipitation of Driest Month
4 Temperature Seasonality (standard deviation ×100)	15 Precipitation Seasonality (Coefficient of Variation)
5 Max. temperature of Warmest Month	16 Precipitation of Wettest Quarter
6 Min. Temperature of Coldest Month	17 Precipitation of Driest Quarter
7 Temperature Annual Range (5–6)	18 Precipitation of Warmest Quarter
8 Mean Temperature of Wettest Quarter	19 Precipitation of Coldest Quarter
9 Mean Temperature of Driest Quarter	
10 Mean Temperature of Warmest Quarter	
11 Mean Temperature of Coldest Quarter	

Results

The models showed good results for all nine bumble bee species, with AUC values above 0.9, although the number of occurrences for *Bombus excellens*, *Bombus melaleucus*, and *Bombus transversalis* was low. *Bombus pauloensis* and *Bombus hortulanus* had the highest number of occurrences, being common in collections (Table 2). Based on our results and distributional information from scientific literature, we divided the bumble bee species into three distributional groups: high mountain bumble bees, mid-mountain/inter-andean bumble bees, and Amazon and eastern Plains basin bumble bees.

High Mountain bumble bees

The potential distribution areas for *B. funebris*, *B. hortulanus*, *B. robustus*, and *B. rubicundus* occurred only in high mountain departments, along a range of different small and fragmented areas, with a high probability of occurrence in the central part of the Eastern Andes Range (Fig. 2). *B. funebris* represented the most restricted and fragmented species. *B. hortulanus* occurrences had the lowest altitudinal points for this group. *B. robustus* data was similar to *B. hortulanus* but included some areas in the northern part of the Eastern and the Central Ranges. *B. rubicundus* showed similar potential distribution to *B. robustus* but with more restricted and fragmented areas.

Mid-Mountain and Inter-Andean bumble bees

This group of species showed a wider altitudinal and potential distribution than the high mountain species, along and between the three mountain ranges (Fig. 3). *B. excellens* showed a higher probability along most of the Central and Eastern mountain ranges, as well as in the mid-mountain area of the Sierra Nevada de Santa Marta.

For *B. melaleucus*, its potential distribution was indicated along the three mountain ranges. *B. pauloensis* exhibited a continuous distribution over the Andean region. *B. pullatus* was concentrated along low altitudinal areas of the inter-Andean valleys.

Table 2. Bumble bee species data for the area under the curve (AUC obtained by niche modeling), number of occurrences used for modeling, and the distributional group selected for each species according to our results and previous results by Pinilla-Gallego et al. (2016).

Species	AUC 1970-2000	Distributional group
Bombus excellens	0.951	Mid-mountain and inter-Andean
Bombus funebris	0.976	High Mountain
Bombus hortulanus	0.977	High Mountain
Bombus melaleucus	0.965	Mid-mountain and Inter-Andean
Bombus pauloensis	0.911	Mid-mountain and Inter-Andean
Bombus pullatus	0.917	Mid-mountain and Inter-Andean
Bombus robustus	0.983	High Mountain
Bombus rubicundus	0.973	High Mountain
Bombus transversalis	0.999	Amazon and Eastern Plains Basin

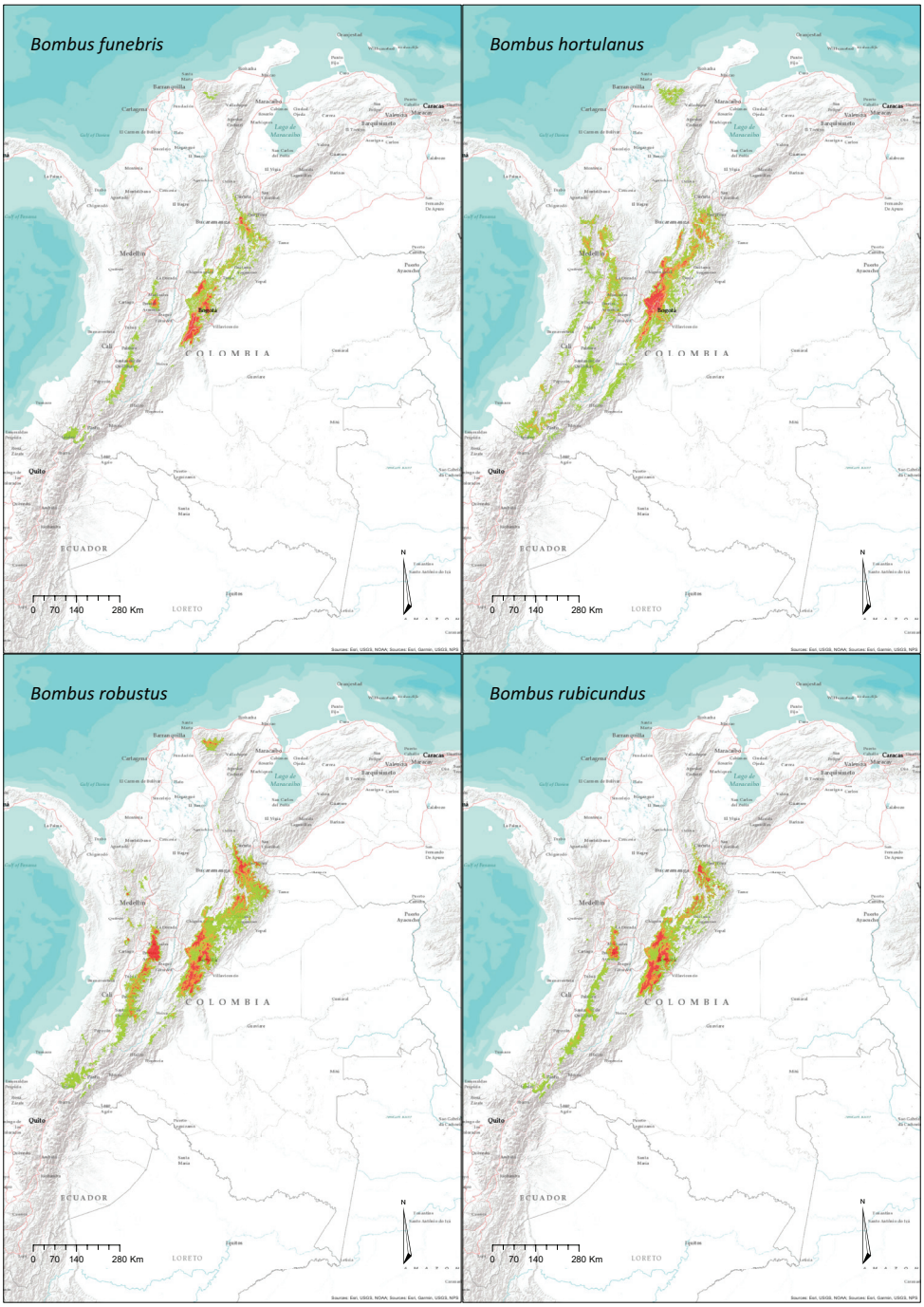

Figure 2. Potential distribution maps for High Mountain bumble bees **A** *B. funebris* **B** *B. hortulanus* **C** *B. robustus* **D** *B. rubicundus*.

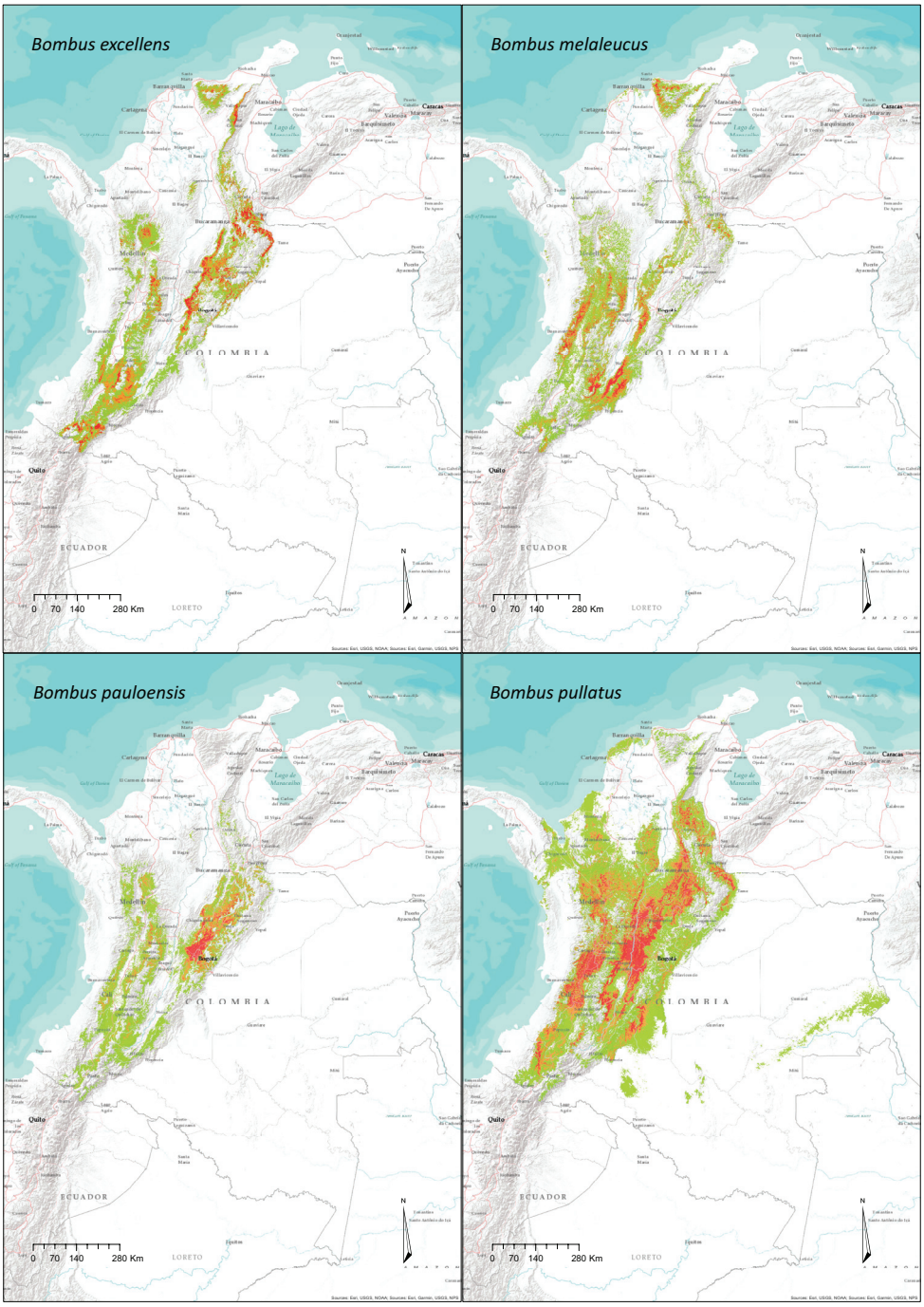

Figure 3. Potential distribution maps for Mid-Mountain and Inter-Andean bumble bees. **A** *B. excellens* **B** *B melaleucus* **C** *B. pauloensis* **D** *B. pullatus*.

Amazon and Eastern Plains Basin bumble bees

Bombus transversalis is the only species with a potential distribution along the Amazon Forest, the Andean foothills, and the Orinoquia region. The eastern part of Meta presented the highest altitudinal points for the species at 1150 m. The northern area of the biogeographic region of Chocó showed optimal bioclimatic conditions for *B. transversalis*, but no occurrence point was found or used there for this model (Fig. 4).

The variables with the highest weight for each species are presented in Table 3.

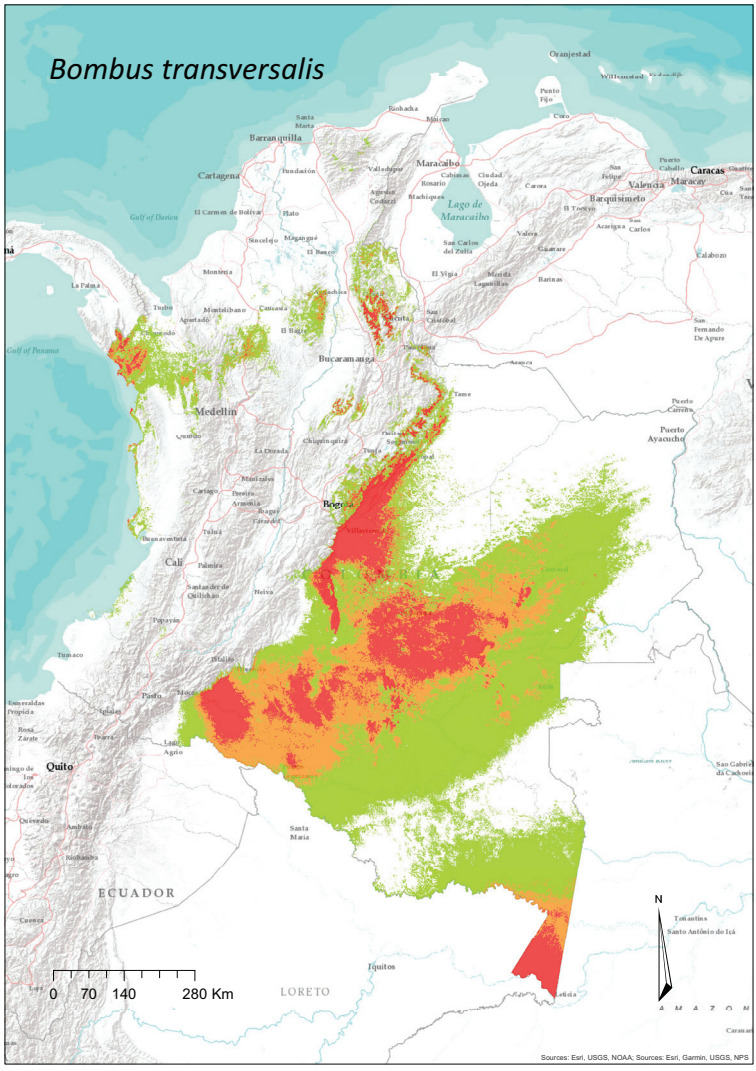

Figure 4. Potential distribution map for Amazon and Eastern Plains Basin bumble bees, *B. transversalis*.

Table 3. Bioclimatic variables had the highest weight in the model for each species, according to the results obtained by the Jackknife test.

Species (Distributional group)	Variables with the highest weight (Percent of contribution)		
	1st	2nd	3rd
Bombus excellens (Mid-Mountain and inter-Andean)	**8.** Mean Temperature of Wettest Quarter 94.35%	**1.** Annual Mean Temperature 94.27%	**6.** Min. Temperature of Coldest Month 94.04%
Bombus funebris (High Mountain)	**4.** Temperature Seasonality 95.50%	**6.** Min. Temperature of Coldest Month 94.54%	**1.** Annual Mean Temperature 94.03%
Bombus hortulanus (High Mountain)	**11.** Mean Temperature of Coldest Quarter 96.72%	**1.** Annual Mean Temperature 96.63%	**10.** Mean Temperature of Warmest Quarter 96.61%
Bombus melaleucus (Mid-Mountain and inter-Andean)	**5.** Max. Temperature of Warmest Month 95.20%	**11.** Mean Temperature of Coldest Quarter 95.16%	**1.** Annual Mean Temperature 95.14%
Bombus pauloensis (High Mountain)	**6.** Min. Temperature of Coldest Month 91.30%	**11.** Mean Temperature of Coldest Quarter 91.18%	**8.** Mean Temperature of Wettest Quarter 91.09%
Bombus pullatus (Mid-Mountain and inter-Andean)	**11.** Mean Temperature of Coldest Quarter 83.91%	**8.** Mean Temperature of Wettest Quarter 83.53%	**4.** Temperature Seasonality 76.67%
Bombus robustus (High Mountain)	**5.** Max. Temperature of Warmest Month 93.74%	**6.** Min. Temperature of Coldest Month 93.72%	**8.** Mean Temperature of Wettest Quarter 93.51%
Bombus rubicundus (High Mountain)	**6.** Min. Temperature of Coldest Month 96.70%	**1.** Annual Mean Temperature 96.70%	**8.** Mean Temperature of Wettest Quarter 96.48%
Bombus transversalis (Amazon and Eastern Plains Basin)	**13.** Precipitation of Wettest Month 98.88%	**11.** Mean Temperature of Coldest Quarter 97.87%	**6.** Min. Temperature of Coldest Month 96.94%

Discussion

These potential distribution maps for bumble bees in Colombia improve the previous maps available (Abrahamovich et al. 2004) for the species, with more precise areas of potential distribution and a visual schema for patterns already described in the literature (Pinilla-Gallego et al. 2016).

The four high-mountain species are associated with the paramo and high Andean ecosystems (Pinilla-Gallego et al. 2016). Paramo ecosystems are characterized as biogeographical islands, highly isolated areas with a great deal of endemism and low genetic flow between populations (Lotta-Arevalo et al. 2020). As a result, the potential distribution of these species is highly fragmented. *B. excellens*, *B. melaleucus*, and *B. pullatus* are associated with mountain forests, and it is remarkable that, even though they showed a wide suitable area (Fig. 3A, B, D, respectively), there is a low number of occurrences. This indicates a small population size and high vulnerability. For example, *B. excellens* can only be found in the cloud forest, an ecosystem with an accelerated rate of deforestation (Nates-Parra 2006). *B. pauloensis* (Fig. 3C) shows a wide altitudinal and potential distribution consistent with its high plasticity in the habitat chosen for nesting (Liévano et al. 1991; Nates-Parra et al. 2006), and this is reflected by its wide distribution along the mountain areas. Its potential distribution will help conservation planning, as *B. pauloensis* positively impacts national agricultural productivity by pollinating staple foods such as fruits and vegetables (Cure and Rodríguez 2007; Riaño et al. 2015; Poveda et al. 2018) due to its adaptability to disturbed habitats. According to its biogeographical history, for *B. transversalis* the Andes Mountain Range represents a physical barrier for emigration. Thus, the potential distribution area in the biogeographical region of Chocó is not likely (Abrahamovich et al. 2004). The eastern

area of Meta stands out as an expansion of previously recorded areas to include the low areas of the Andean foothills.

The most important bioclimatic variables for bumble bees in Colombia are related to temperature (Table 3). Thus, drastic temperature changes put bumble bees in a condition vulnerable to threat under a climate change scenario (Gonzalez et al. 2021). Also, the potential distribution for most species overlaps with densely populated areas. Therefore, these maps are a tool for the detailed location of bumblebee biodiversity for conservation planning as bumble bee' populations and their associated services will probably soon be reduced (Williams and Osborne 2009; Pinilla-Gallego et al. 2016).

Conclusion

The potential distribution for Colombian bumble bee species is reported here. Seven of them show a restricted distribution, shaped mainly by temperature restrictions. These results are a direct contribution to knowledge about Colombian bumble bee species, constituting useful knowledge for conservation, territorial planning, protection plans, and environmental management. Thus, by obtaining the most suitable areas for a species, this study can provide the ideal location to breed and reproduce the native species. These species contribute to improving the agricultural productivity of several regions by pollination. Likewise, this information can help avoid the introduction of foreign species for this purpose (Nates-Parra 2016). It is necessary to increase research efforts on bees for them to be included in conservation planning.

Acknowledgements

We would like to thank M. Sc. Andrea Lorena García and M. Sc. Daniela Hoyos-Benjumea from the Insect collection at Universidad del Quindío, Ph.D. Diego Riaño from the bee collection in Universidad Militar Nueva Granada, Ph.D. Francisco Serna from the entomological museum Universidad Nacional, School of Agronomy, Bogotá, and Ph.D. Sergio Orduz and biol. Carlos Londoño from the entomological museum Francisco Luis Gallego for their contributions to occurrence data. We are indebted to Ph.D. Sydney A. Cameron for comments and suggestions that improved this manuscript.

We acknowledge financial support by Stiftung Universität Hildesheim.

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Supplementary material 1

Points used for the Niche modeling of Bumblebee species (Hymenoptera, Apidae, *Bombus*) in Colombia

Authors: Laura Rojas-Arias, Daniel Gómez-Morales, Stephanie Stiegel, Rodulfo Ospina-Torres

Data type: COL (excel document).

Explanation note: Ocurrence data used for the modeling.

Link: https://doi.org/10.3897/jhr.95.87752.suppl1

JHR 95: 245–260 (2023)
doi: 10.3897/jhr.95.98222
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Taxonomy of the genus *Peucobius* Townes (Hymenoptera, Ichneumonidae, Sisyrostolinae)

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Academic editor: Gavin Broad | Received 1 December 2022 | Accepted 8 February 2023 | Published 24 February 2023

https://zoobank.org/99C6279A-FA04-4E86-8AC6-ED33C9FD87B3

Citation: Khalaim AI, Ruíz-Cancino E, Coronado-Blanco JM (2023) Taxonomy of the genus *Peucobius* Townes (Hymenoptera, Ichneumonidae, Sisyrostolinae). Journal of Hymenoptera Research 95: 245–260. https://doi.org/10.3897/jhr.95.98222

Abstract

The genus *Peucobius* Townes previously comprised two species occurring in the Nearctic region: *P. fulvus* Townes and *P. piceus* Townes. In the current study we revise this genus, transfer it to the subfamily Sisyrostolinae (**comb. nov.**), and describe two new species – *P. bennetti* Khalaim & Ruíz-Cancino, **sp. nov.** from Central Mexico and *P. shimizui* Khalaim, **sp. nov.** from Japan. The genus *Lygurus* Kasparyan occurring in Russian Far East and Taiwan is morphologically similar to *Peucobius*; characters for distinguishing these two genera are provided for the first time with the use of colour photographs. Identification keys to four world species of *Peucobius*, and to species of *Lygurus* and *Peucobius* occurring in the East Palaearctic region, are provided. We suggest that species of *Peucobius* are associated with xyelid sawflies (Xyelidae) whose larvae feed in staminate pine cones.

Resumen

El género *Peucobius* Townes previamente incluía dos especies en la región Neártica: *P. fulvus* Townes y *P. piceus* Townes. En el presente artículo se revisa el género, se transfiere a la subfamilia Sisyrostolinae (**comb. nov.**) y se describen dos especies nuevas – *P. bennetti* Khalaim & Ruíz-Cancino, **sp. nov.** de la zona central de México y *P. shimizui* Khalaim, **sp. nov.** de Japón. El género *Lygurus* Kasparyan que ocurre en el Lejano Oriente Ruso y en Taiwán es similar morfológicamente a *Peucobius*; se señalan por primera vez las características que los distinguen, incluyendo fotografías a color. Se elaboraron las claves para las cuatro especies de *Peucobius* del mundo, y las de las especies de *Lygurus* y *Peucobius* que ocurren en la región Paleártica Oriental. Se sugiere que las especies de *Peucobius* están asociadas con moscas sierra (Xyelidae), cuyas larvas se alimentan en conos estaminados de pinos.

Keywords
Japan, key, *Lygurus*, Mexico, Nearctic region, new combination, new species, North America, parasitoids

Introduction

The genus *Peucobius* Townes was described in the subfamily Phrudinae for two Nearctic species (Townes 1971: 29), and no further works on this genus were published for almost the following forty years (Yu et al. 2016), until a phylogenetic revision of the family Ichneumonidae by Quicke with co-authors (Quicke et al. 2009: 1354) in which the genus *Peucobius* and six other "microphrudine" genera (*Astrenis* Förster, *Earobia* Townes & Townes, *Notophrudus* Porter, *Phaestacoenitus* Smits van Burgst, *Phrudus* Förster and *Pygmaeolus* Hellén) were transferred to Tersilochinae s.l. on the basis of a combined molecular and morphological analysis. Five other genera previously treated in Phrudinae (*Brachyscleroma* Cushman, *Erythrodolius* Seyrig, *Icariomimus* Seyrig, *Lygurus* Kasparyan and *Melanodolius* Saussure) were placed within the resurrected subfamily Brachyscleromatinae Townes (Quicke et al. 2009: 1354), and one more genus, *Laxiareola* Sheng & Sun, was described afterwards from Oriental China (Sheng and Sun 2011). Subsequently, Bennett with co-authors (Bennett et al. 2013) revised the genus *Erythrodolius* and established the subfamily name Sisyrostolinae Seyrig instead of Brachyscleromatinae Townes due to the priority of Seyrig's name. They also pointed out that not all former phrudine genera were included in the analysis of Quicke et al. (2009), and therefore some of the omitted genera (e.g., *Notophrudus* and *Peucobius*) could end up being more closely related to Sisyrostolinae than the *Phrudus* group of genera (Bennett et al. 2013: 425–426).

Recently, Bennett with co-authors (Bennett et al. 2019) performed a combined morphological and molecular phylogenetic analysis of Ichneumonidae, and in both combined analyses *Peucobius fulvus* Townes was sister to *Erythrodolius calamitosus* Seyrig (Sisyrostolinae), not *Phrudus* sp. (Tersilochinae s.l.) which would support the move of *Peucobius* to Sisyrostolinae (Bennett et al. 2019: 116–118). However, the monophyly of the subfamily Sisyrostolinae was not confirmed as *Brachyscleroma* did not cluster with *Peucobius*+*Erythrodolius*.

The aims of this study are to revise new material of *Peucobius*, describe two new species from Mexico and Japan, and discuss the relationships of *Peucobius* to Sisyrostolinae and the *Phrudus* group of Tersilochinae. Identification keys to world species of *Peucobius*, and to East Palaearctic species of *Peucobius* and the morphologically similar genus *Lygurus* will be provided.

Materials and methods

The specimens examined in this study were borrowed from or deposited in the following collections: Instituto de Biología, Universidad Nacional Autónoma de México,

D.F., México (**UNAM**), the National Institute for Agro-Environmental Sciences, Tsukuba, Japan (**NIAES**), Canadian National Collection of Insects, Ottawa, Ontario, Canada (**CNC**), and the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia (**ZISP**). Photographs of *Lygurus* spp. (Figs 21–26) were taken from non-type females from the Russian Far East deposited in ZISP.

Morphological terms follow Broad et al. (2018). Wings of *P. shimizui* sp. nov. (Fig. 18) were slide-mounted using Solakryl BMX. Layer photographs were taken in ZISP, with a Canon EOS 70D digital camera attached to an Olympus SZX10 stereomicroscope. Partly focused images were assembled with Helicon Focus Pro 6 software. Contour map of North America (Fig. 27) was taken from D-maps (2018).

Results and taxonomy

The genus *Peucobius* is found to belong to the subfamily Sisyrostolinae (comb. nov.) as it shares the following important morphological features (see Quicke et al. 2009; Bennett et al. 2013): antenna with cylindrical scape, proboscidial fossa strongly narrowed, hind wing with vein M+Cu long relative to vein 1-M, metasoma with large laterotergites and mostly sclerotized sternites, and thin and long ovipositor which is slightly swollen before the apex and has neither notch nor nodus (Figs 11, 20).

Unlike Tersilochinae s.l., *Peucobius* possesses unspecialized flagellomeres (in Tersilochinae almost all species possess finger-shaped structures at least on several flagellomeres, see Vikberg, Koponen 2000; Khalaim pers. obs.), maxillary and labial palp formula 5+4 (in Tersilochinae this formula is usually 4+3, though in microphrudines genera it is 5+4) and fore tibia with a tooth on the distal outer side (in Tersilochinae distal end of fore tibia is unspecialized, i.e. without tooth).

Within Sisyrostolinae, *Peucobius* is very similar to the small East Palaearctic and Oriental genus *Lygurus* Kasparyan from which it can be distinguished by the following characters: laterotergite 2 not separated by a crease; clypeus weakly convex and without a transverse ridge (Fig. 15); and short second metasomal tergite which is about 0.7 times as long as anteriorly broad (Fig. 19). Further taxonomic study of these two genera is required to clarify their generic limits and phylogenetic relationships.

Thus, the subfamily Sisyrostolinae currently comprises seven genera: *Brachyscleroma* Cushman (Afrotropical and Oriental regions), *Erythrodolius* Seyrig (Afrotropical and Neotropical regions), *Icariomimus* Seyrig (Afrotropical region), *Laxiareola* Sheng & Sun (Oriental region), *Lygurus* Kasparyan (Eastern Palaearctic and Oriental regions), *Melanodolius* Saussure (Afrotropical) and *Peucobius* Townes (Holarctic region) (Yu et al. 2016).

Genus *Peucobius* Townes, 1971

Type species. *Peucobius fulvus* Townes, 1971, by original designation.

Comparative diagnosis. *Peucobius* can be distinguished from all other genera of Sisyrostolinae by the combination of the following characters: 1) fore wing without areolet, i.e. vein 3rs-m absent (Fig. 18) (areolet present in *Brachyscleroma*); 2) tarsal claws simple (pectinate in *Laxiareola*); 3) anterior transverse carina of propodeum present (Fig. 3) (carina absent in *Melanodolius* and some species of *Erythodolius*); 4) clypeus unspecialized, evenly rounded in profile, without a transverse ridge, medial point or multiple or medial crenulations (Figs 5, 15) (transverse ridge present in *Lygurus*; medial point present in some species of *Erythrodolius*; medial crenulations present in some species of *Erythrodolius* and *Icariomimus*).

Within Sisyrostolinae, *Peucobius* has the smallest species with fore wing length 2.5–3.0 mm, while the genus with the next smallest species, *Lygurus*, has fore wing length 4.0–5.0 mm, and some other genera are much larger (e.g., 7.0–19.0 mm in *Erythrodolius* and 17.0–30.0 mm in *Melanodolius*).

Description. Small insects with fore wing length 2.5–3.0 mm. Head and mesosoma mostly finely granulate to subpolished, impunctate or partly with fine punctures. Head, in dorsal view, with gena strongly rounded posterior to eyes. Clypeus wide, 2.5–3.5 times as broad as long, lenticular, weakly convex, more or less flat medially, with a row of long fine setae on lower margin. Mouthparts short, unspecialized; maxillary and labial palp formula 5+4. Mandible bidentate, with teeth subequal by length or either upper or lower tooth somewhat longer than other. Malar space 0.8–1.0 times as long as basal mandibular width, with scabrous area between eye and mandibular base. Flagellum filiform, with 14–17 flagellomeres. Epomia absent. Notaulus short or absent. Scutellum with lateral longitudinal carinae very short. Sternaulus absent or as weak impression ventrolaterally in anterior part of mesopleuron. Epicnemial carina present. Posterior transverse carina of mesopleuron absent. Propodeum more or less fully carinate (as in Fig. 3), sometimes carinae partly obliterated. Fore wing as in Fig. 18; areolet absent, pterostigma relatively large, vein 2m-cu with one large bulla. Hind wing with nervellus (cu1&cu-a) intercepted below centre. Legs slender; fore tibia with a tooth-like projection on distal outer side; tarsal claws not pectinate. First metasomal tergite 1.8–2.5 times as long as posteriorly broad, with large glymma at base. Second tergite transverse; thyridum (if discernible) very small, oval, at base of tergite 2. Laterotergites 2 and 3 not separated by crease. Ovipositor long and slender, weakly upcurved, slightly swollen before apex (Fig. 11).

Remarks. In addition to two previously known Nearctic species of *Peucobius*, one species from Central Mexico and one from Japan are described. A distribution map (Fig. 27) of three North American species and identification key to the four species of *Peucobius* occurring in the world are given below. We also provide a key to a new species of *Peucobius* discovered from the East Palaearctic region and two species of the morphologically similar genus *Lygurus*.

The new species from Mexico differs well from two other North American species, *P. fulvus* and *P. piceus* (see the key below). *Peucobius fulvus* and *P. piceus* are known to us from their original descriptions (Townes 1971) and several specimens from USA and Canada. The length of mandibular teeth is found to work well for distinguishing these

morphologically very similar species from each other, while the two features provided by Townes (1971), i.e. sculpture of mesoscutum and colouration of hind coxa, are less reliable and therefore must be used with caution.

Key to world species of *Peucobius*

1 Frons with short but distinct ridge between antennal sockets. Propodeum with area basalis substituted by a single, longitudinal, median carina; areola pointed anteriorly (Fig. 10). Mesopleuron entirely black (Fig. 8). Mexico..... ..... ***P. bennetti* sp. nov.**

– Frons without longitudinal ridge between antennal sockets. Propodeum with area basalis distinct and more or less contiguous with areola (Fig. 3). Mesopleuron with subtegular ridge and area below sternaulus yellow or reddish orange (Figs 1, 2) **2**

2 Metacarpus (R1) of fore wing ending significantly behind radial cell (Fig. 18). Metasoma predominantly brownish black (Fig. 12). East Palaearctic region (Japan)..... ***P. shimizui* sp. nov.**

– Metacarpus (R1) of fore wing ending just beyond radial cell (Figs 1, 2). Metasoma predominantly reddish orange (Fig. 1) or brown (Fig. 2). Nearctic region **3**

3 Mandible with lower tooth subequal to, or slightly longer than the upper tooth. Mesoscutum centrally dull. Hind coxa yellow or pale brown (Fig. 1).... ..... ***P. fulvus* Townes**

– Mandible with lower tooth distinctly longer than the upper tooth. Mesoscutum centrally polished. Hind coxa darkened with brown or black (Fig. 2) .... ***P. piceus* Townes**

Key to *Lygurus* and *Peucobius* species occurring in East Palaearctic region

1 Second tergite very short, 0.65 times as long as anteriorly broad (Fig. 19); laterotergite 2 not separated by crease (as in Fig. 8). Flagellum of female with 15 flagellomeres (Fig. 13). Clypeus weakly convex, without transverse ridge (Fig. 15). Ovipositor sheath 0.7 times as long as body (Fig. 12). Body yellowish orange and black (Fig. 12). Fore wing length about 2.6 mm..... ***P. shimizui* sp. nov.**

– Second tergite distinctly elongated, about 1.5 times as long as anteriorly broad; laterotergite 2 separated by sharp crease (Fig. 24). Flagellum of female with 19–24 flagellomeres. Clypeus with transverse ridge (Fig. 22). Ovipositor sheath longer than body (Fig. 21). Body almost entirely black (Fig. 21). Fore wing length 4.0–5.0 mm **2**

2 Propodeum with areola distinctly transverse. Ovipositor sheath 1.4 times as long as body. Russian Far East (Kasparyan 1983) ..... ***L. townesi* Kasparyan, 1983**

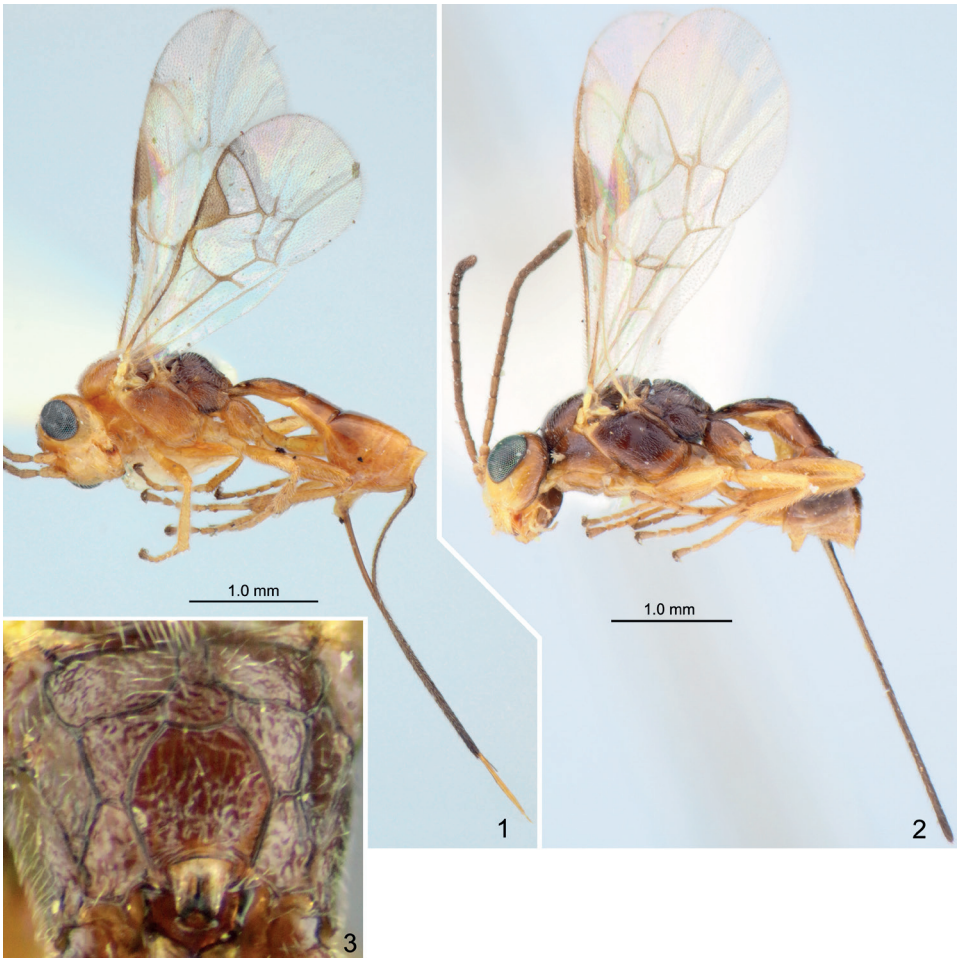
– Propodeum with areola somewhat elongated. Ovipositor sheath 1.1 times as long as body (Fig. 21). Russian Far East and Taiwan (Chiu and Wong 1987: 4; Kasparyan and Khalaim 2007: 564)..... ***L. marjoriae* Chiu, 1987**

***Peucobius fulvus* Townes, 1971**
Fig. 1

Remarks. The specimens from Maryland were swept from *Pinus virginiensis* [*P. virginiana* Mill.] with staminate cones (Townes 1971: 31).

Material examined. 1 female (CNC), Canada, Ontario, Spencerville, Limerick Forest, 29.V.1956, coll. W.R.M. Mason (det. V. Vikberg, 2004). 1 female (paratype, ZISP), USA, Maryland, Takoma Park, 25.IV.1942, coll. H. & M. Townes.

Distribution. Canada (Ontario) (new country and provincial record for genus), USA (Maryland, Michigan, North Carolina) (Townes 1971).



Figures 1–3. *Peucobius fulvus*, female (Canada, Ontario) (**1**) and *P. piceus*, female (USA, Colorado) (**2, 3**). **1, 2** habitus, lateral view **3** propodeum, dorsal view.

***Peucobius bennetti* Khalaim & Ruíz-Cancino, sp. nov.**
https://zoobank.org/8AC4CA3C-0DE3-4CF3-899C-D9CF76BD0730
Figs 4–11

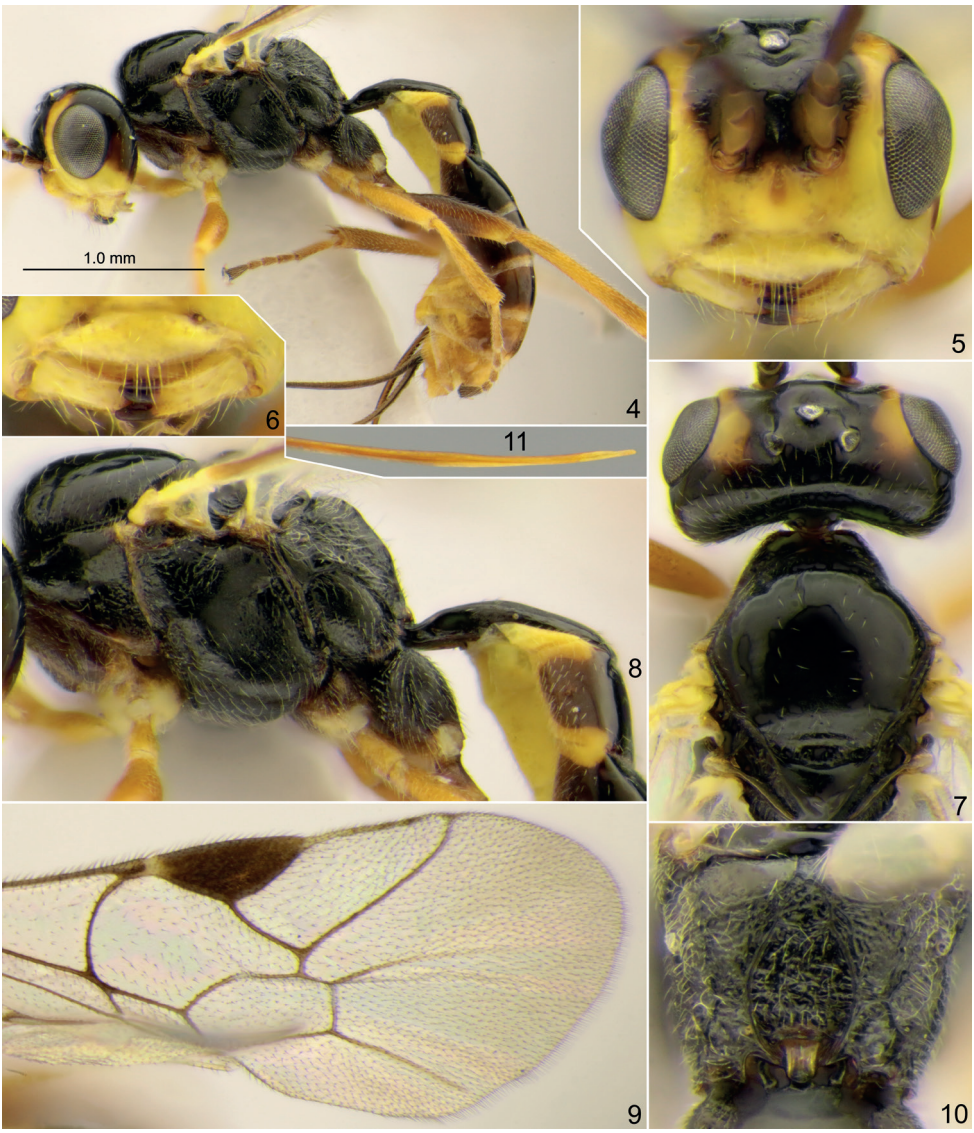
Comparison. *Peucobius bennetti* sp. nov. differs from the two other North American species, *P. fulvus* and *P. piceus*, by having the frons with a sharp longitudinal carina between the antennal sockets (weakly convex or with rounded longitudinal swelling in *P. fulvus* and *P. piceus*), the occipital carina completely absent in the lower half of the head (complete, or sometimes indistinct near the hypostomal carina in *P. fulvus* and *P. piceus*), deep notaulus (absent or very shallow in *P. fulvus* and *P. piceus*), area basalis of propodeum substituted by a longitudinal keel (Fig. 10) (distinct in *P. fulvus* and *P. piceus*, see Fig. 3), and black and yellow body (Fig. 4), while in *P. fulvus* and *P. piceus* the body is paler with yellowish markings less contrasting (Figs 1, 2).

Peucobius bennetti sp. nov. also differs from the Eastern Palaearctic *P. shimizui* sp. nov. by having a shorter metacarpus (R1) of the fore wing, ending just behind the radial cell (Fig. 9), while in *P. shimizui* sp. nov. the metacarpus (R1) extends well beyond the radial cell (Fig. 18).

Description. Female. Body length 4.1 mm. Fore wing length 3.0 mm.

Head with gena strongly rounded posterior to eyes (Fig. 7); gena in dorsal view 0.75 times as long as eye width. Clypeus 3.5 times as broad as long (Fig. 6), more or less flat medially and with rounded transverse ridge laterally, smooth, with fine punctures in upper half, with a row of long fine setae on lower margin. Mouthparts short; maxillary palp with 5 palpomeres, palpomeres 4 and 5 short, distinctly shorter than palpomeres 2 and 3; labial palp with 4 short palpomeres. Mandible with lower tooth slightly longer than the upper (Fig. 6). Malar space about 0.8 times as long as basal mandibular width, with broad scabrous area between eye and mandibular base. Flagellum with 17 flagellomeres, filiform; three basal flagellomeres 2.5–2.8 times as long as broad; subapical flagellomeres subsquare. Face with conspicuous median swelling, with short and narrow longitudinal furrow on this swelling, with fine and sharp punctures on smooth background laterally, and smooth and almost impunctate centrally on median swelling. Frons with short and sharp longitudinal carina between antennal sockets (just above groove on median swelling); polished and impunctate medially, with fine and sharp punctures laterally. Vertex and gena polished, with very fine setiferous punctures. Occipital carina thin and sharp in upper half of head, evenly convex mediodorsally, and obliterated in lower part (completely absent below level of eye midline).

Mesoscutum polished, centrally almost impunctate, with scattered very fine punctures anteriorly and laterally (Fig. 7). Notaulus present on anterolateral side of mesoscutum, short and deep. Scutellum polished, almost impunctate. Mesopleuron subpolished, dull, with very fine inconspicuous punctures in lower part. Sternaulus as weak impression ventrolaterally in anterior part of mesopleuron. Epicnemial carina extending somewhat above level of lower corner of pronotum, with upper end not



Figures 4–11. *Peucobius bennetti* sp. nov., holotype female **4** body, lateral view **5** head, front view **6** clypeus and mandibles, antero-ventral view **7** head and mesoscutum, dorsal view **8** mesosoma and base of metasoma, lateral view **9** fore wing **10** propodeum, dorsal view **11** apex of ovipositor, lateral view.

reaching anterior margin of mesopleuron and slighty curved backwards. Propodeal spiracle separated from pleural carina by almost one times its own maximum diameter. Propodeum almost entirely covered with weak irregular wrinkles, its carinae partly indistinct; area basalis substituted by a single, longitudinal carina; areola about as long as broad, pointed anteriorly, contiguous with area petiolaris posteriorly (Fig. 10).

Fore wing as in Fig. 9. Vein 2rs-m long, somewhat longer than abscissa of M between 2rs-m and 2m-cu. Metacarpus (R1) very short, ending just behind the distal end

of radial cell. Hind wing with nervellus (cu1&cu-a) intercepted in lower 0.45. Legs moderately slender. Fore tibia with long tooth on distal outer side. Hind tibia with two short spurs which are slightly curved at apex.

First metasomal tergite 1.8 times as long as posteriorly broad, with large glymma in basal half (Fig. 8); dorsally tergite 1 polished, finely striate in basal half. Second tergite distinctly transverse, 0.65 times as long as anteriorly broad. Tergites 2 to 6 dorsally highly polished and almost glabrous (with row of fine setae at hind margins), laterally tergites 2 and 3 with rather dense setae and following tergites laterally with sparse setae. Ovipositor slightly swollen before apex and tapered to a point posterior to swelling (Fig. 11); sheath as long as fore wing, or 3.0 times as long as hind tibia.

Head predominantly black; clypeus, face, frontal orbits (extending above to level of hind end of lateral ocelli) and lower third of gena yellow (Figs 4, 5, 7); median third of gena brownish. Mouthparts and mandible (except brownish black teeth) yellow. Antenna black, scape and pedicel ventrally brownish yellow. Mesosoma black; propleuron and front margin of pronotum dark brown (Fig. 8). Tegula yellow. Pterostigma brown with yellowish spot at base (Fig. 9). Fore leg with coxa and trochanters yellow (coxa slightly darkened at extreme base); femur, tibia and tarsus brownish yellow (femur apically yellowish). Mid leg predominantly brownish yellow with coxa extensively darkened with brown and tarsus infuscate. Hind leg with coxa almost entirely brownish black (except pale brown extreme apex), trochanters and femur brownish yellow with extensive dark brown markings on dorsal side, and tibia and tarsus more or less entirely brownish yellow. Tergite 1 black. Metasoma posterior to first tergite dorsally and laterally dark brown (anteriorly) to brown and yellow (posteriorly), ventrally and on hind margins of tergites yellow (Fig. 4). Ovipositor sheath dark brown basally to black apically.

Male. Unknown.

Etymology. The species is named in honour of the Canadian expert in Ichneumonidae, Andrew M.R. Bennett (CNC).

Material examined. ***Holotype*** female (UNAM), Mexico, Tlaxcala, Nanacamilpa, Ejido Los Búfalos, 19°28'N, 98°35'W, bosque pino-encino (*Pinus* + *Quercus* forest), 2830–2900 m, Malaise trap, 4.IV–3.V.2016, coll. Y. Marquez & A. Contreras.

Distribution. Mexico (Tlaxcala).

***Peucobius piceus* Townes, 1971**

Figs 2, 3

Remarks. The specimens from Utah were swept from *Pinus contorta* Douglas bearing staminate cones, and the specimens from California were probably swept from *Pinus monophylla* Torr. & Frém. (Townes 1971: 30).

Material examined. 1 female (CNC), USA, Colorado, Nederland, 8500 ft (= 2590 m), 18.VI.1961, coll. W.R.M. Mason (det. V. Vikberg, 2004).

Distribution. USA (California, Colorado, New Mexico, Utah).

***Peucobius shimizui* Khalaim, sp. nov.**
https://zoobank.org/98ACA320-4515-4FAA-9419-B542B04C0154
Figs 12–20

Remarks. The species was collected on *Pinus densiflora* in spring period together with a large quantity of individuals of *Gelanes* spp. (Tersilochinae).

Comparison. This species differs from the three Nearctic species in having a longer metacarpus (R1) of the fore wing (Fig. 18) (ending just behind the radial cell in all other species, see Figs 1, 2, 9).

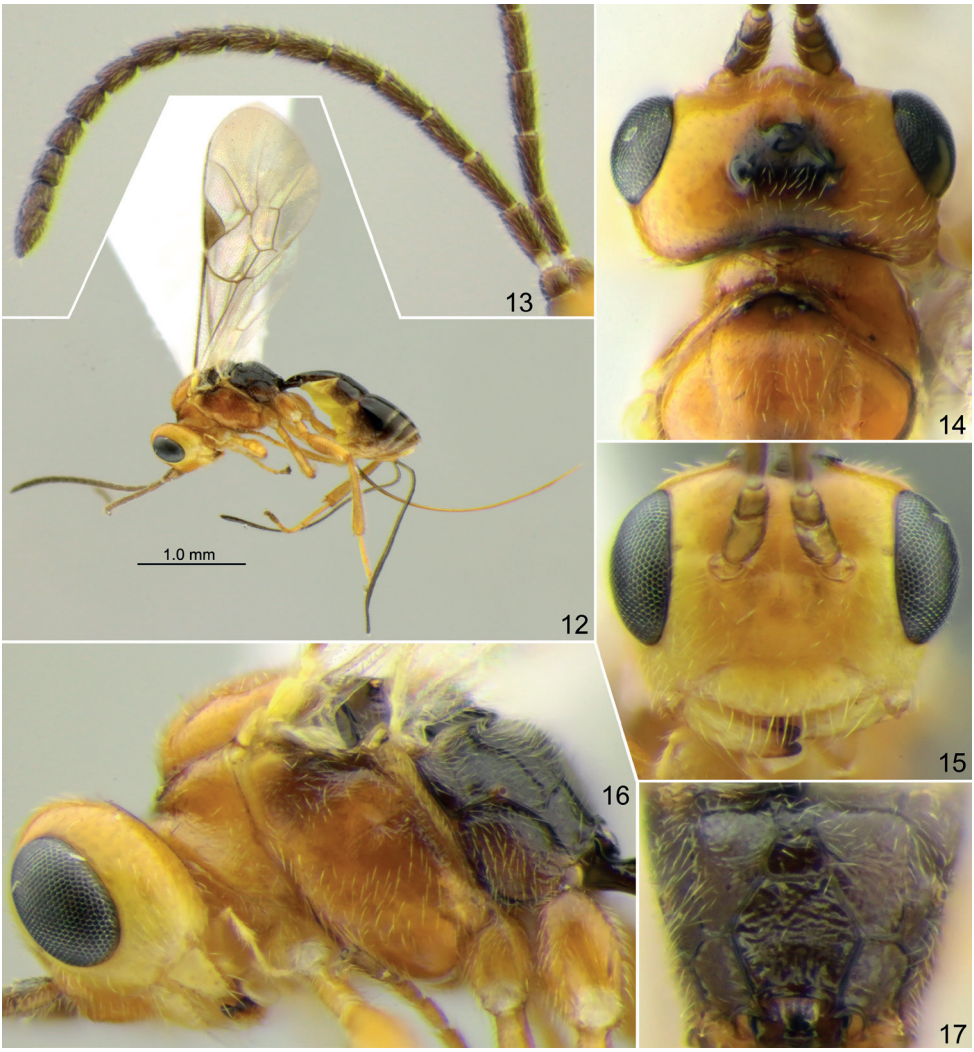
Description. Female. Body length 2.8 mm. Fore wing length 2.6 mm.

Head with gena strongly rounded posterior to eyes (Fig. 14); gena in dorsal view 0.65 times as long as eye width. Clypeus 3.3 times as broad as long (Fig. 15), very weakly convex, smooth, with fine punctures in upper 0.4, with a row of long fine setae on lower margin. Mouthparts short; maxillary palp with 5 palpomeres, palpomeres 4 and 5 short, distinctly shorter than palpomeres 2 and 3; labial palp with 4 short palpomeres. Mandible with lower tooth distinctly broader and somewhat longer than upper (Fig. 15); both teeth in holotype and paratype very obtuse (rounded apically), possibly abraded. Malar space as long as basal mandibular width, with broad scabrous area between eye and mandibular base. Flagellum with 15 flagellomeres, filiform; flagellomeres 2 to 4 about 2.6 times and subapical flagellomeres 1.1–1.3 times as long as broad. Face with weak median swelling and small median tubercle in upper part of this swelling, without longitudinal furrow; face with very fine punctures on smooth background, smooth and impunctate centrally on median swelling. Frons without ridge between antennal sockets, with very fine (partly indistinct) punctures on smooth background, laterally slightly scabrous. Vertex and gena polished, with very fine setiferous punctures. Occipital carina thin, complete, evenly convex mediodorsally.

Mesoscutum and scutellum subpolished, with very fine punctures. Notaulus present on anterolateral side of mesoscutum, weak, with more or less distinct longitudinal wrinkle. Mesopleuron scabrous, impunctate and dull in upper part; with fine punctures on more or less smooth background in lower part. Sternaulus absent. Epicnemial carina extending to about level of lower corner of pronotum, with upper end not reaching anterior margin of mesopleuron. Propodeal spiracle adjacent to pleural carina in holotype and separated from this carina by half diameter of spiracle in paratype. Propodeum scabrous, weakly shining; area basalis small, confluent with areola; areola separated posteriorly from area petiolaris by very weak wrinkle (Fig. 17).

Wings as in Fig. 18. Vein 2rs-m long, much longer than abscissa of M between 2rs-m and 2m-cu. Metacarpus (R1) short, projecting somewhat behind distal end of radial cell. Hind wing with nervellus (cu1&cu-a) intercepted in lower 0.35. Legs moderately slender. Fore tibia with distal outer end pointed (this tooth-like projection shorter than in *P. bennetti*). Hind tibia with two short straight spurs.

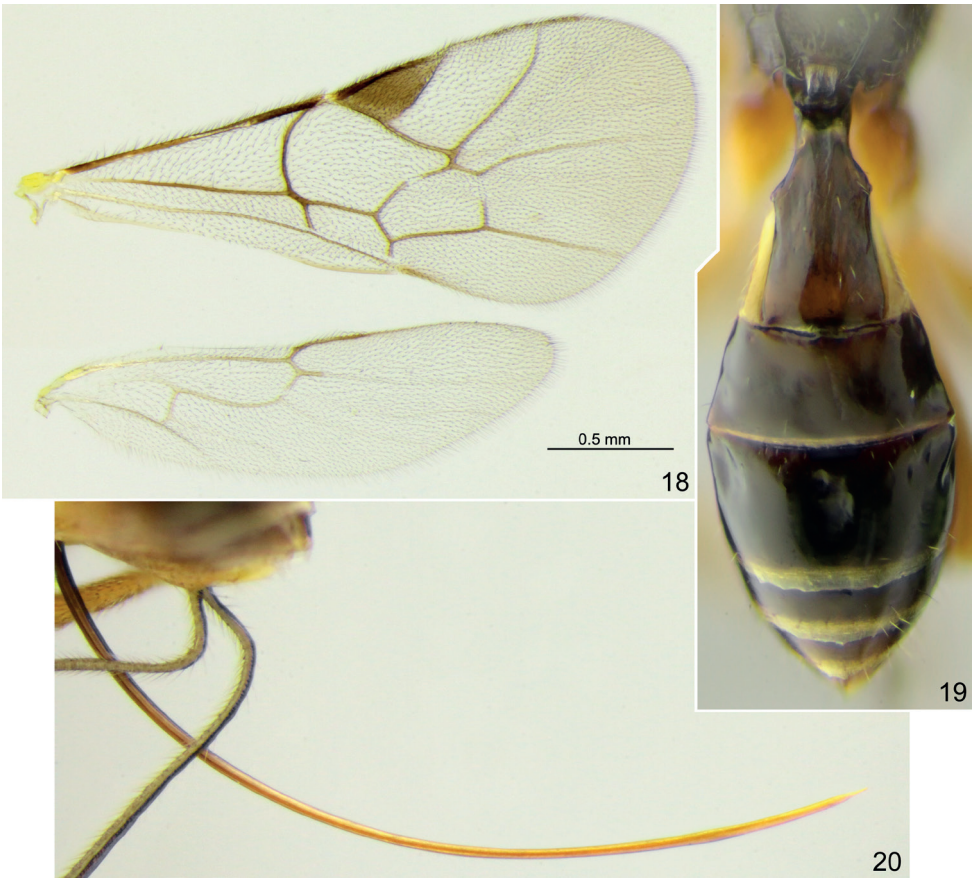
First metasomal tergite twice as long as posteriorly broad, with large glymma in basal half and spiracle in basal 0.4 (Fig. 19). Second tergite distinctly transverse, 0.7 times as long as anteriorly broad. All metasomal tergites dorsally polished and almost



Figures 12–17. *Peucobius shimizui* sp. nov., holotype (**11, 13–15**) and paratype (**12, 16**) females **12** habitus, lateral view **13** antenna **14** head, dorsal view **15** head, front view **16** head and mesosoma, lateral view **17** propodeum, dorsal view.

glabrous (with a row of fine setae at hind margins), tergite 2 and following tergites with setae laterally. Ovipositor slightly swollen before apex and tapered to a point posterior to swelling (Fig. 20); sheath 0.65–0.7 times as long as fore wing, or about 3.5 times as long as hind tibia.

Head predominantly yellow; face and frons medially, vertex and upper part of gena yellowish orange; interocellar area, vertex medially and upper part of occiput blackish (Figs 14, 15). Mouthparts and mandibles (except dark reddish-brown teeth) yellow. Antenna dark brown, scape and pedicel ventrally pale brown. Mesosoma



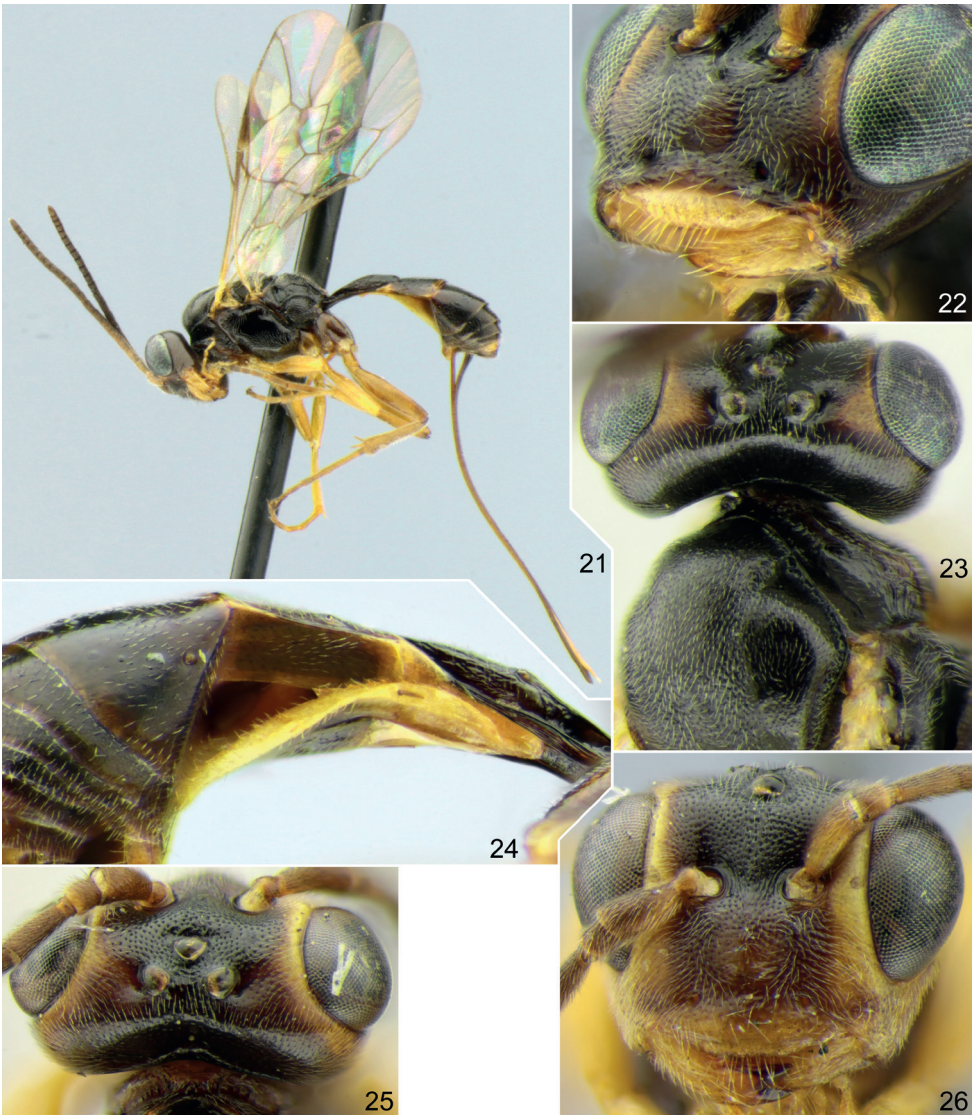
Figures 18–20. *Peucobius shimizui* sp. nov., holotype female **18** wings **19** metasoma, dorsal view **20** ovipositor, lateral view.

predominantly yellowish orange (Fig. 16); metanotum and propodeum dark reddish brown to black; scutellum laterally and metapleuron darkened with reddish brown; mesoscutum with more or less distinct reddish brown or dark brown marks anteriorly, centrally and posteriorly. Tegula yellow. Pterostigma pale brown (Fig. 18). Legs rather uniformly yellowish orange to brownish orange, hind coxa somewhat darker (with more intense brownish orange colouration). Tergite 1 dark brown. Metasoma posterior to first tergite dark brown dorsally, brown laterally and yellow ventrally; tergites with pale yellow bands on posterior margins dorsally, pale band very narrow on tergite 2 and broader on following tergites (Fig. 19). Ovipositor sheath blackish.

Male. Unknown.

Variation. The two specimens are almost identical in size, structure and colouration.

Etymology. The species is named in honour of the collector of the type material, Japanese expert in Ichneumonidae, So Shimizu (Kobe University, Japan).



Figures 21–26. *Lygurus marjoriae* (**21–24**) and *L. townesi* (**25, 26**), females **21** habitus, lateral view **22** lower part of head, antero-ventro-lateral view **23, 25** head, dorsal view **24** metasoma, lateral view **25** ovipositor, lateral view **26** head, front view.

Material examined. ***Holotype*** female (NIAES), Japan, Honshu I., Kantō Reg., Ibaraki Pref., Kasama City, Hizawa, 36°24'39.3"N, 140°15'6.8"E, ca. 90 m, sweeping on *Pinus densiflora*, 28.IV.2017, coll. So Shimizu.

***Paratype*.** 1 female (NIAES), same data as holotype.

Distribution. Japan (Honshu I.).

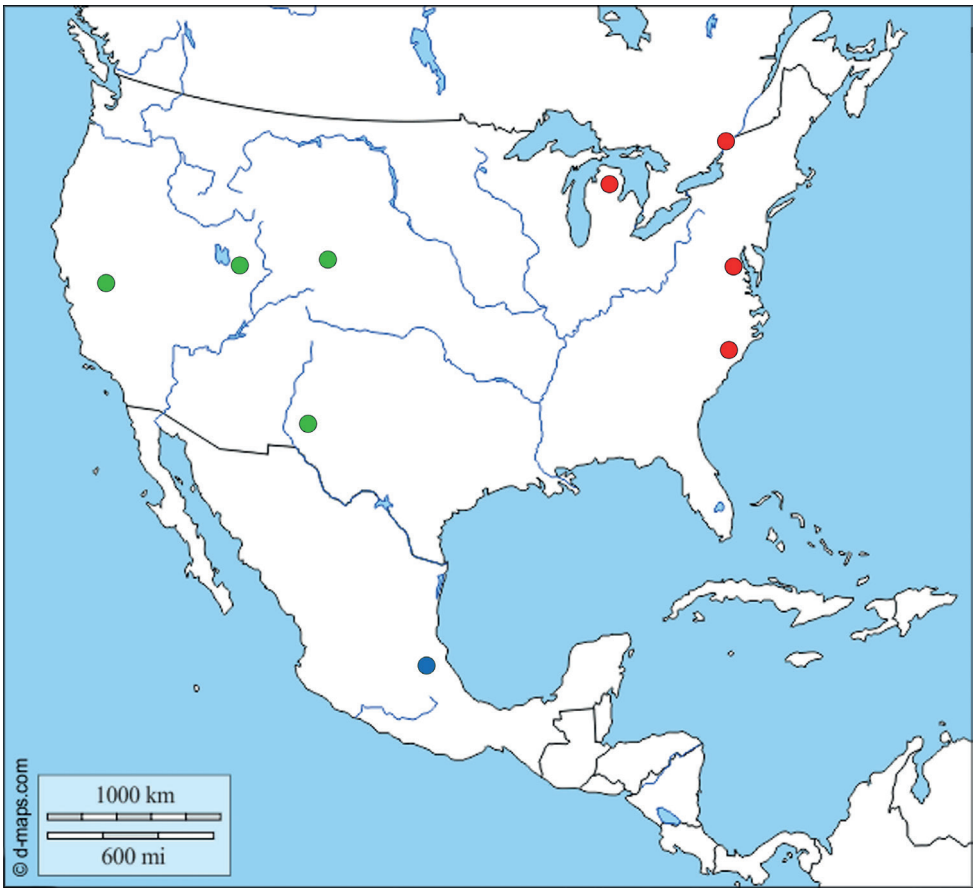


Figure 27. Distribution map of *P. fulvus* (**red**), *P. bennetti* sp. nov. (**blue**) and *P. piceus* (**green**) in North America.

Discussion

The two Nearctic species described by Townes were collected by sweeping branches of pines bearing staminate cones (Townes 1971: 29), and *P. bennetti* from Mexico was collected in pine-oak forest on the same dates and at the same locality as two other parasitoids, *Gelanes horstmanni* Khalaim (Tersilochinae) and *Idiogramma elbakyanae* Khalaim (Tryphoninae), were collected (Khalaim and Ruíz-Cancino 2017). The fourth species of the genus, *P. shimizui* from Japan, was also collected on *Pinus* in spring together with a large quantity of individuals of *Gelanes* spp. In the body habitus, yellow/orange and black colour pattern of the body, and possession of a long and thin ovipositor, *P. bennetti* and *P. shimizui* resemble species of *Gelanes* and *Idiogramma* very much.

There is no direct host record for any *Peucobius* species, but genera *Gelanes* Horstmann and *Idiogramma* Förster are typical parasitoids of xyelid sawfly larvae (Xyelidae) developing in staminate pine cones, and therefore we suggest that the genus *Peucobius*,

being frequently collected together with *Gelanes* and *Idiogramma* species on their host plants, and being morphologically very similar to these taxa, quite possibly parasitizes the same host.

Acknowledgements

We are thankful to Alejandro Zaldívar-Riveron (UNAM), So Shimizu (Kobe University, Japan) and Andrew M.R. Bennett (CNC) for loaning specimens, to Dmitri R. Kasparyan (ZISP) for his comments on the taxonomy of *Lygurus*, Yulia V. Astafurova (ZISP) for her assistance in taking colour photographs of *Lygurus* spp., and to Andrew M.R. Bennett (CNC), Rikio Matsumoto (Osaka Museum of Natural History, Osaka, Japan) and Alexandra Viertler (Institute of Ecology and Evolution, University of Bern, Bern, Switzerland) for reviewing the paper. Sincere thanks to A.M.R. Bennett for his hard work on the review of the manuscript, valuable remarks and suggestions on the taxonomy and structure of the paper. The work of the senior author was partly supported by the Russian State Research Project no. 122031100272-3.

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JHR 95: 261–274 (2023)
doi: 10.3897/jhr.95.100689
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

A new species of *Mesoneura* (Hymenoptera, Tenthredinidae) associated with a xerothermic oak forest in the Western Carpathians, Slovakia

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Academic editor: Marko Prous | Received 17 January 2023 | Accepted 10 February 2023 | Published 24 February 2023

https://zoobank.org/96BB415C-5DE0-410E-8802-5CD577E83DEE

Citation: Roller L, Kočišek J (2023) A new species of *Mesoneura* (Hymenoptera, Tenthredinidae) associated with a xerothermic oak forest in the Western Carpathians, Slovakia. Journal of Hymenoptera Research 95: 261–274. https://doi.org/10.3897/jhr.95.100689

Abstract

A new species of tenthredinid sawfly, *Mesoneura tematinensis* Roller, **sp. nov.**, was discovered in the Tematinske kopce Mountains in the Western Carpathians in Slovakia. Adults of both sexes and larvae of different stages are described and illustrated. Based on morphology and DNA barcoding, the new species is closely related to *Mesoneura opaca* (Fabricius), a widespread oak sawfly in Europe, with which it occurs in the same locality and shares a common host plant. Larvae of the new species are part of a rich assemblage of a total of 13 Symphyta species that feed on leaves of the pubescent oak *Quercus pubescens* in a thermophilic supra-Mediterranean forest. A key to the European species of *Mesoneura* Hartig is provided.

Keywords

assemblage, biology, key, larva, *Quercus pubescens*, Symphyta, taxonomy

Introduction

Thermophilic forests dominated by the pubescent oak (*Quercus pubescens*), especially in southern Europe, provide a suitable habitat for a unique assemblage of sawflies (Lacourt 2020). In Central Europe, this habitat is rare, threatened and often protected. As part of the ongoing research on the Symphyta fauna of Slovakia, we surveyed

several sites with pubescent oaks, which provided new data on the occurrence of rare species, including some first records for the territory of Slovakia (Roller 2004; Macek et al. 2020; Smetana et al. 2020). The surveys in the Tematínske kopce (Tematín Mountains) with Malaise traps also brought the discovery of the rare sawfly *Periclista lenta* Konow, 1903 (= *vernalis* Lacourt, 1985), whose biology is unknown (Roller 2004; Macek et al. 2020). While searching for larvae of *P. lenta*, we found for the first time the larvae of an unknown sawfly species belonging to the genus *Mesoneura* Hartig, 1837.

Mesoneura is a small genus of the family Tenthredinidae and the subfamily Nematinae with nine extant species distributed in the Palaearctic region (Wei et al. 2013; Taeger et al. 2018). Only two species, *Mesoneura opaca* (Fabricius, 1775) and *M. lanigera* Benson, 1954, occur in Europe (Liston 2012). The larvae of both species feed on fresh leaves of various oaks (*Quercus* spp). *Mesoneura opaca* is associated with most oak species throughout Europe, while *M. lanigera* is known only from the Transcarpathian region, Crimea, the North Caucasus (Stavropol region) and Cyprus, where its hosts are *Q. pubescens* and *Q. infectoria* (Ermolenko 1967; Liston and Späth 2008). In this article we describe the third European species of *Mesoneura* and provide information on its biology and the assemblage of sawflies it is involved in.

Materials and methods

Sampling of Symphyta was carried out in several stands of thermophilic supra-Mediterranean forests in two neighbouring localities in western Slovakia. The pubescent oak stands were sampled on several days in April and May 2018, 2019, 2020 and 2021. Larvae were collected by tree beating and adults were caught with an entomological net (RNDr. Ondrej Šauša-Entomologické pomôcky). In addition, samples from the Malaise trap, which had been set up in 1999 at the site where the newly described species was found (Roller 2004), were re-examined. The species were identified using the keys of Macek et al. (2020) and Lacourt (2020).

Representatives of each captured larval instar were preserved in 96% ethanol with isopropanol and other larvae were reared on the oak leaves. After feeding was completed, larvae were transferred to glass jars with soil substrate and stored outdoors until the following spring. Adults were mounted dry or stored in ethanol at -20 °C until DNA analysis and then mounted.

Morphological terminology and measurement conventions were adopted from Viitasaari (2002). Genitalia were separated from fresh insects without maceration or digestion of soft tissues and temporarily mounted on slides in ethanol for examination and photography. The detached parts were then glued onto a card and pinned together with the specimen. Adults and larvae were examined and photographed using a Leica M205C stereomicroscope and a Leica Flexacam C1 camera, including operating software with a Z-stack projection tool. Images of the penis valve and ovipositor lancet were taken with a Nikon Coolpix P7700 camera connected to a Nikon Eclipse 600 microscope with Nomarski DIC optics.

Total DNA extracted from an adult insect leg or the abdominal wall of a larva was used for barcoding. DNA sequences of approximately 1,078 bp of the cytochrome c oxidase subunit I (COI) gene region were obtained using primers SymF1 and A2590-R (Normark et al. 1999; Prous et al. 2016) and kits and reagents were described previously (Semelbauer et al. 2021). PCR amplification conditions were as follows: initial denaturation step at 95 °C for 5 min, 36 cycles of 30 sec denaturation at 95 °C, 30 sec annealing at 53 °C, 1 min 20 sec extension at 72 °C, followed by a final extension of 10 min at 72 °C. The data of the analysed sawflies are stored in the GenBank database.

Abbreviations for collections

IZ SAS Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia;
SNM Slovak National Museum, Nature Science Museum, Bratislava, Slovakia.

Links to genetic data

ON226738 https://www.ncbi.nlm.nih.gov/nuccore/ON226738.1/;
ON227048 https://www.ncbi.nlm.nih.gov/nuccore/ON227048;
ON228194 https://www.ncbi.nlm.nih.gov/nuccore/ON228194;
ON231583 https://www.ncbi.nlm.nih.gov/nuccore/ON231583;
ON231584 https://www.ncbi.nlm.nih.gov/nuccore/ON231584.

Results and discussion

Taxonomy

***Mesoneura tematinensis* Roller, sp. nov.**
https://zoobank.org/C8A61A3D-87AC-48DB-ABD8-4B6B72C27A06

Type material. ***Holotype*** ♀. **Type locality.** Slovakia SW, Považský Inovec Mts, Tematínske kopce, Lúka env, Kňaží vrch nature reserve., 450 m a.s.l. Labelled [white and printed]: "Slovakia SW, Tematín. kopce, Lúka env., Kňaží vrch, 48°39'49.29"N, 17°55'37.04"E, L. Roller leg." "Larva on *Quercus pubescens*, Larva: 21.V.–7.VI.2021, Adult: 27.–30.III.2022, L. Roller leg." "LR73" (tissue sample ID for genetic data) "Holotypus, *Mesoneura tematinensis* Roller, 2023, des. L. Roller [red and printed]. Good condition: right mid and hind legs removed as tissue samples. SNM. ***Paratypes*.** 1 ♀, the same data as the holotype, IZ SAS; 1♀, the same locality data, but "ex larva on *Quercus pubescens* coll. 11.V.2019, adult 7.IV.2020, L. Roller leg." [white label, printed], IZ SAS; 1 ♂ 2 ♀♀ "Slovakia SW, Tematín. kopce, Lúka env., Hradlová nivka, 48°40'3.37"N, 17°54'53.09"E, (330 m a.s.l.)", "Larva on *Quercus pubescens*, Larva: 21.V. –7.VI.2021, Adult: 27.–30.III.2022, L. Roller leg.", ♂ bears white label "LR72" and one ♀ "LR74" (tissue sample ID for genetic data) [white labels, printed], "Paratypus, *Mesoneura tematinensis* Roller, 2023, des. L. Roller [red and printed]. SNM.

Larvae from *Quercus pubecens*: 1 mature feeding larva, Kňaží vrch, 8.V.2020, "LR2l" (tissue sample ID for genetic data); 6 small feeding larvae, Kňaží vrch, 8.V.2021 and 21.V.2021; 4 small feeding larvae, Hradlová nivka, 21.V.2021; 2 prepupae, one from each type locality, 4.VI.2021. IZ SAS and SNM.

Description. Female (Figs 1, 4; Holotype Fig. 1C) (variability in specimens other than the holotype in brackets). Body length 8.7 (7.7–8.7) mm, wing length 8.0 (7.2–8.1) mm.

Colour (Fig. 1A–C). Body black; following parts ochre to reddish-brown: apical half of clypeus, labrum, bases of mandibles; apical 2/3 of forewing costa and basal 2/3 of pterostigma; apical 1/3 and dorsal margin of profemur, apical 1/4 of mesofemur and apex of metafemur; apices of tibiae and tarsomeres; posterior margin of pronotum; tegula; flecks on mesonotum: anterolaterally on medial lobe (nearly whole lobe), medially anterior to scutellum, medially on scutellum (absent); postscutellum and metascutellum (absent to extensively); central mesepisternum (absent); ventral margins of lateral terga; dorsal parts of terga 1–3 and 8–9 extensively, terga 4–7 slightly (absent); sterna 2–7 extensively; and cerci; following parts pale to whitish-yellow: palps; trochanters and trochantelli; basal 2/3 to almost entire tibiae, tarsomeres extensively; cenchri; and posterior margins of terga and sterna slightly. Wings translucent; basal 1/3 of costa and anterior face of subcosta on forewing, hindwing costa, longitudinal veins in basal 1/3 of wings whitish-yellow; remaining veins and apical 1/3 of pterostigma dark to black.

Head (Figs 1B, C, 4C). Shiny and punctate, with most dense punctures on face, frons and lower gena; pale dense and long pubescence, hairs on postocellar area distinctly longer than diameter of ocellus; narrowed behind eyes; frontal area poorly-defined, shallowly depressed; frontal pit deep, narrowly elliptical; malar space almost half as long as diameter of lateral ocellus; postocellar area about 2.5 times as long as diameter of lateral ocellus, with shallow median furrow; OOL : POL : OOCL = 1 : 1.1 : 1; clypeus emarginated; antenna reaching postscutellum; antennomeres 3 and 4 about same length, following segments gradually becoming slightly shorter; and antennomere 9 about 3.5 times longer than its width.

Thorax (Fig. 1B–D). Shiny and punctate, interspaces weekly alutaceous, sunken lateral meso- and metanotum densely punctate; pale dense and long pubescence, longest hairs on mesepisternum twice as long as ocellar diameter; distance between cenchri as long as or slightly longer than cenchrus; metafemur slightly longer than 1/2 of metatibia, metatarsus slightly longer than metafemur, metatarsomere 1 slightly shorter than three following tarsomeres together; and claws with subapical tooth slightly shorter than apical tooth.

Abdomen (Fig. 1B, C, E–G). Cylindrical, slightly tapering from segment 6 to blunt apex; terga and sterna with microsculpture, alutaceous on most surfaces and finely strigulate on posterior margins of terga; hypopygium sinuous posteriorly with broadly incised median lobe (Fig. 1G); sawsheath broadly emarginated posteriorly, trifid and 3.5 times as wide as cercus in dorsal view; cerci longer than sawsheath in dorsal view; lancet of ovopositor with 14 annuli, annular sutures extending from serrulae to 1/2–2/3 of height of lamnium, serrulae flat and lacking denticles, and basal 5–6 annuli with strongly sclerotised and finely serrated ctenidial ridges (Figs 1F, 4C).

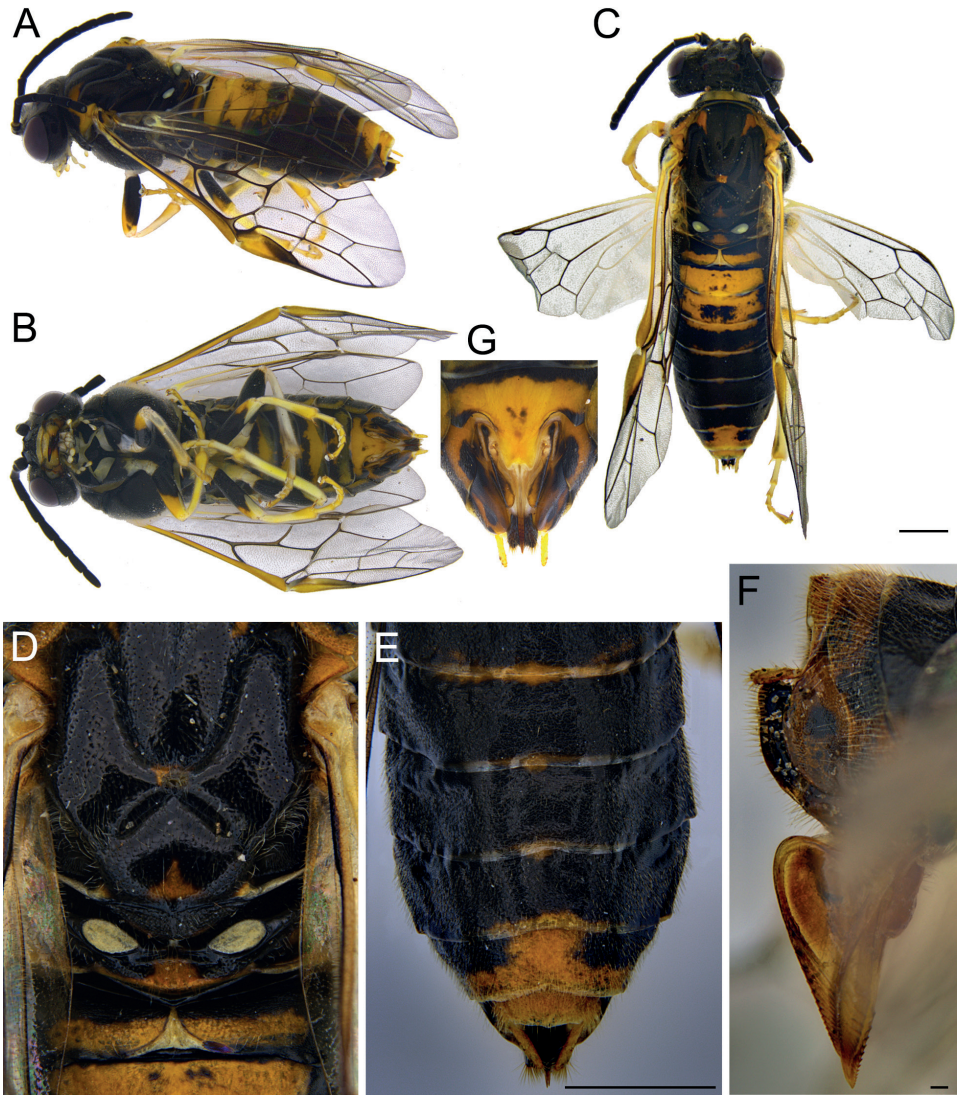

Figure 1. *Mesoneura tematinensis* sp. nov., female **A** dorsolateral habitus **B** ventral habitus **C** dorsal habitus, holotype **D** meso and metathorax and 1st abdominal segment dorsal **E** abdominal segments 5–10 dorsal **F** apex of abdomen with retracted saw lateral **G** hypopygium and sawsheath ventral. Scale bars: 1 mm (**C, E**); 0.1 mm (**F**).

Male (Fig. 2). Body length 7.8 mm, wing length 6.7 mm.

Colour similar to female with the following differences: anterior half of clypeus, labrum and palps whitish-yellow; thorax largely black, only outer margins of tegulae and posterior margins of pronotum reddish-brown; metatibia with tips and metatarsomeres largely blackish-brown; pro- and mesotrochanters and trochantelli largely black; terga and sterna black with pale outer margins; penis valves and harpes largely reddish-brown; and posterior margin of tergum 8 and sternum 9 slightly reddish-brown.

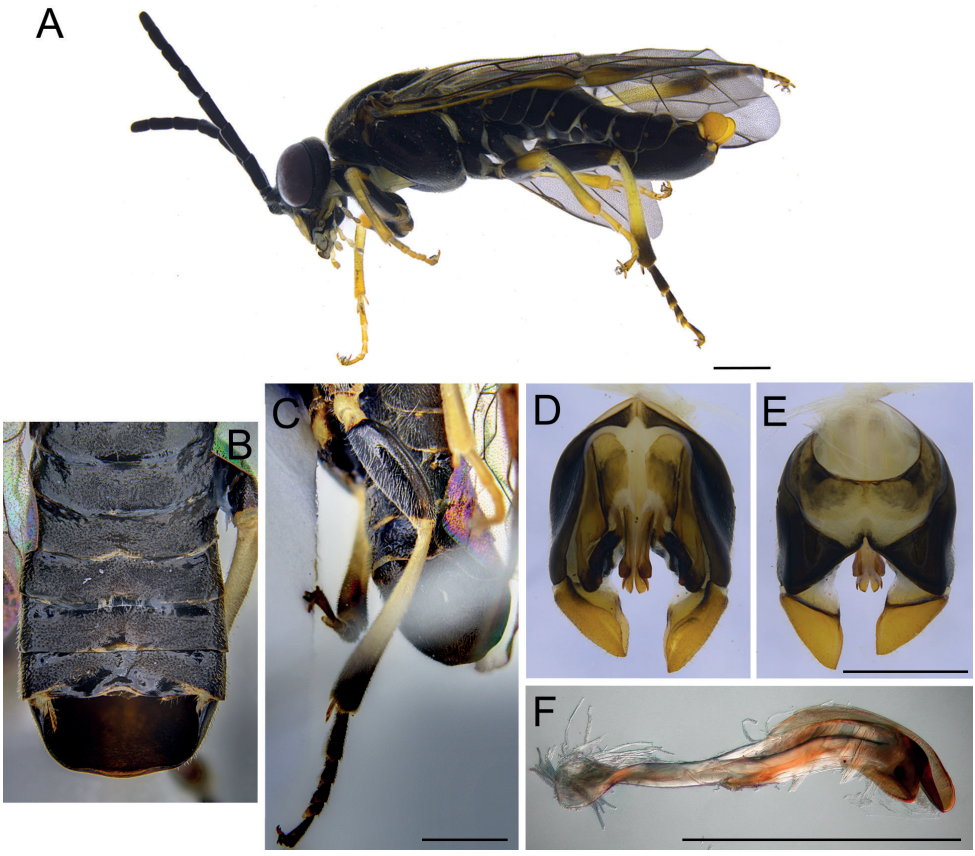

Figure 2. *Mesoneura tematinensis* sp. nov., male **A** lateral habitus **B** abdomen dorsal **C** abdomen and hind leg lateral **D** genital capsule dorsal **E** genital capsule ventral **F** penis valve. Scale bars: 1 mm.

Morphology similar to female with the following differences: antenna as long as head and thorax; malar space very short, almost indistinct; metafemur swollen and short, about 0.7 length of metatibia (Fig. 2C); posterior half of tergum 8 with glabrous, shallow median depression (Fig. 2B); sternum 9 obtusely produced medially; genital capsule and penis valve (Fig. 2D–F).

Larva (Figs 3, 4).

Mature feeding larva (Figs 3A, C–F, 4E). Body length (maximum of larva preserved in etanol) 18.5 mm. Ground colour green, head grey-green; body dusted with whitish wax (Fig. 3A); ocularia, mandibles apically and claws of thoracic legs black; basal margin of coxa and adjacent anterior venter narrow black (Fig. 3D); cuticle of trunk with dense dark granules (Fig. 3F, in wax deprived larva); thoracic segments with sparse, fine, short setae with distinct pinacula arranged as in Fig. 3C, D; thoracic legs with longer setae; abdominal segments with six annulets, annulets 1, 2, 3, 4 and 5, subspiracular and surpedal lobes, and hypopleurite with fine setae distributed as in Fig. 3E.

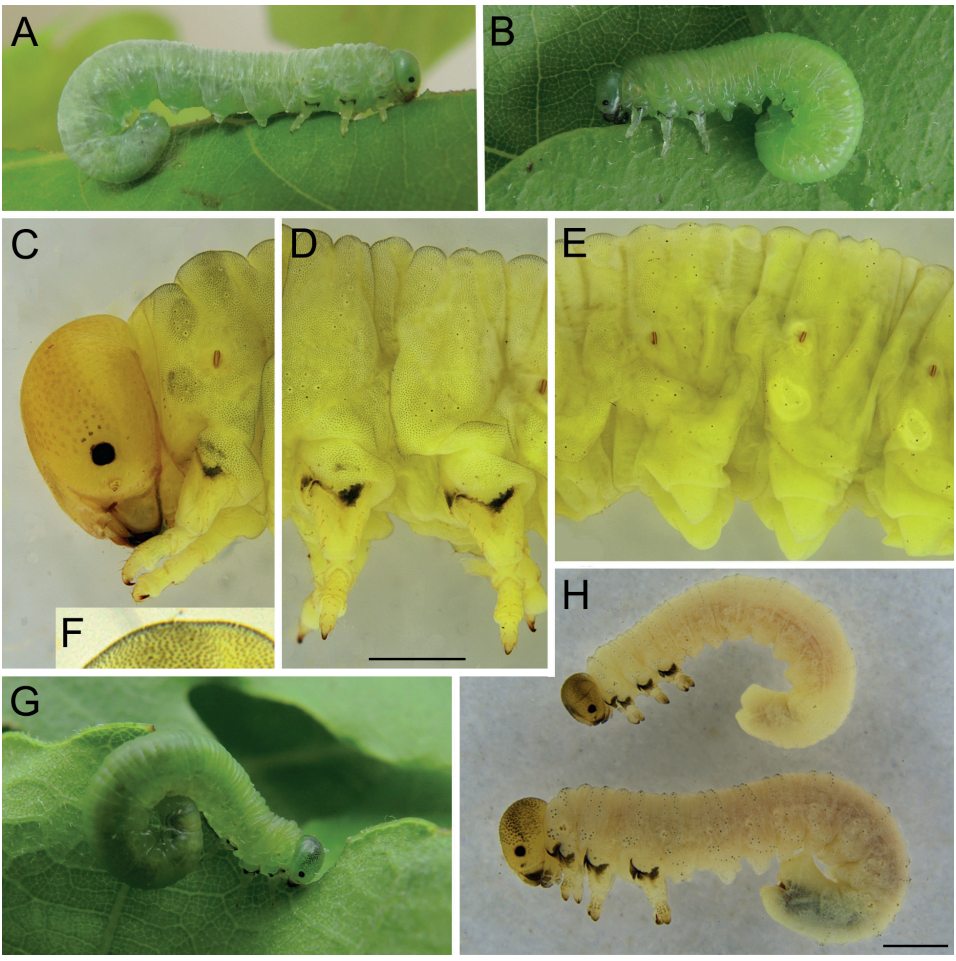

Figure 3. Larva of *Mesoneura tematinensis* sp. nov. **A** live mature feeding larva **B** live prepupa **C–F** mature larva in ethanol, freed from wax, head and prothorax (**C**), meso and metathorax (**D**), abdominal segments 2 and 3 (**E**), granular surface on prothorax tergum (**F**) **G** live smaller feeding larva **H** smaller larvae in ethanol. Note the dark markings on the head and thoracic legs and the dark pinacula on the body of larvae preserved in ethanol. Scale bars: 1 mm.

Prepupa (Fig. 3B). Length 12–13 mm. Trunk green to yellowish green, glossy, without whitish dust; head turquoise.

Smaller larva, probably second to fourth instar (Fig. 3G–H). Trunk yellowish green without whitish dust, head turquoise; black markings on head and thoracic segments more distinct than in mature feeding larva, additionally with black parietal and weak facial flecks; fine setae with distinct pinacula distributed as in Fig. 3H.

Genetic data. One female, one male and one larva were DNA barcoded and their approximately 1,050 bp sequences are available (Accessions ON231583, ON231584

and ON226738). All three sequences are identical and differ significantly from the available barcodes of the related taxa *Mesoneura opaca*, *M. lanigera* and *M. truncatatheca*, including *M. opaca* from the locality of the new species (accession for larva ON228194 and female ON227048). The closest relative of *M. tematinensis* appears to be *M. opaca*, which has a sequence dissimilarity of at least 5% (N = 13).

Etymology. Tematinensis is a latinised adjective referring to the Tematín Mountains, the area of origin of the new species.

Differential diagnosis

The body colouration and morphology of the sawsheath, lancet and cerci in females and the penis valve in males distinguish *M. tematinensis* from the other nine extant species of this genus. The cerci protruding behind the tip of sawsheath and the broadly incised middle lobe of the hypopygium may place *M. tematinensis* in the *M. opaca* group, which consists of the other two species *M. lanigera* and *M. opaca* from Europe (Wei et al. 2013). The European species of *Mesoneura* can be distinguished using the following key.

Key to the species of *Mesoneura* Hartig in Europe

1	Female	**2**
–	Male	**4**
2	Abdomen with terga 2–8 predominantly black	***M. opaca***
–	Abdomen with at least part of terga 2–8 largely reddish yellow	**3**
3	Terga 5–7 largely reddish yellow, pterostigma largely pale with darker base	***M. lanigera***
–	Terga 5–7 largely black, pterostigma bicoloured with darker apical third	***M. tematinensis***
4	Abdomen with reddish band	***M. lanigera***
–	Abdomen without reddish band	**5**
5	Abdominal terga 5–8 with sharply defined medial depression, pterostigma completely dark	***M. opaca***
–	Abdominal terga 5–8 without distinct medial depression, pterostigma bicoloured, partly pale	***M. tematinensis***

In addition, *M. lanigera* differs from *M. tematinensis* in that the female has a lancet with a higher number of annuli (about 20) and the male has a thicker penis valve with a longer valvispina (Benson 1954; Liston 2012).

The newly described species is most similar to *M. opaca*, with which it occurred in the same locality and shared a common host plant. In addition to the features used in the key, females can be distinguished by the pterostigma, which has a distinct dark apical third in *M. tematinensis*, while in *M. opaca* it is monochrome yellowish-brown (material from Slovakia) or has a darker base (some individuals imaged in ECatSym, Taeger et al. 2018). The body hair is also significantly longer in *M. tematinensis*. This is

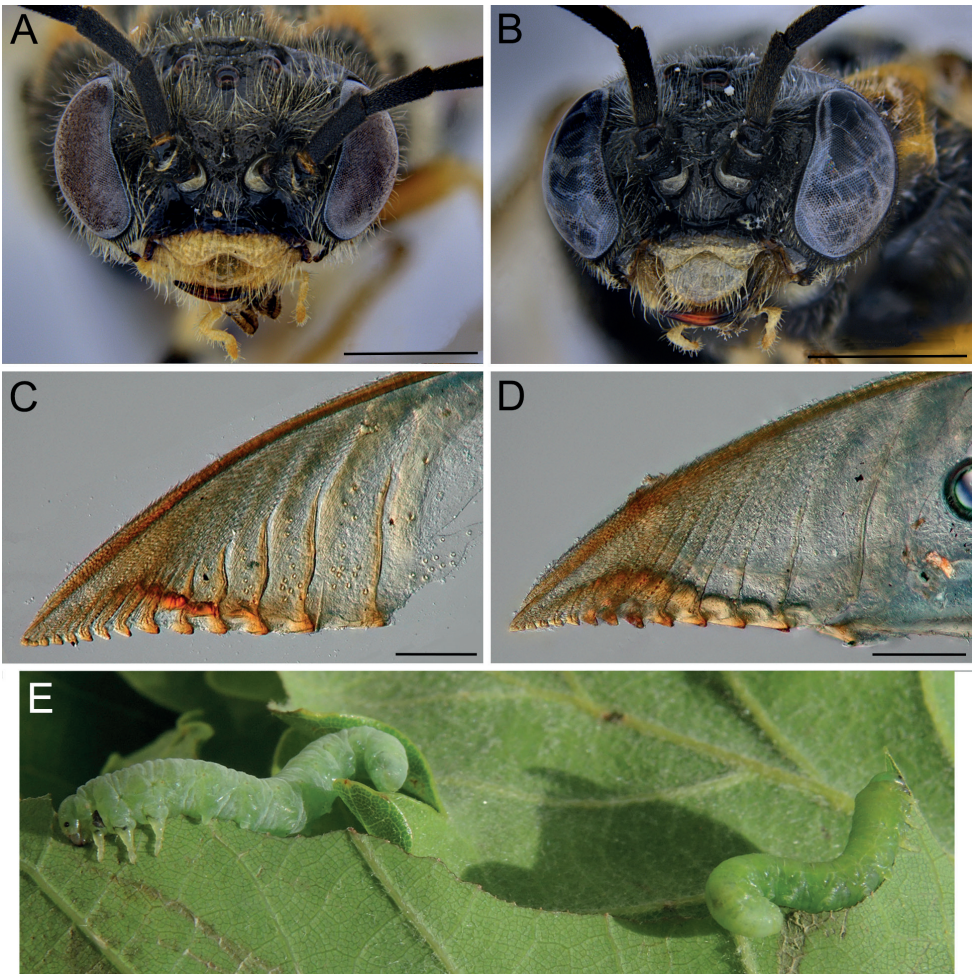

Figure 4. Differences between *Mesoneura tematinensis* sp. nov. (**A, C, E** left) and *M. opaca* (**B, D, E** right). Note the different length of pubescence on the head (**A** versus **B**), the ctenidia of ovipositor lancet (**C** versus **D**) and the habitus of full grown feeding larva (**E** left versus right). Scale bars: 0.1 mm (**A, B**); 1 mm (**C, D**).

well observed on the head, where the hairs in the postocellar region are almost 2 times as long as the ocellar diameter in *M. tematinensis* (Fig. 4A) and only as long as the ocellar diameter in *M. opaca* (Fig. 4B; N = 15). The lancet of *M. tematinensis* is characterised by strongly sclerotised ctenidial ridges (Fig. 4C) instead of ctenidial teeth, which are largely present in *M. opaca* (Fig. 4D). Males of both species have a distinct penis valve, which is curved and slender in *M. tematinensis* (Fig. 2F), while it is straighter and more robust in *M. opaca* (fig. 4 in Liston 2012). The feeding larvae can also be distinguished. *M. tematinensis* has black horizontal stripes on the bases of the thoracic legs, which are absent in *M. opaca* (Fig. 4E). Furthermore, the mature feeding larva of *M. tematinensis* has a distinct whitish dusting. Finally, the body size of adults and larvae

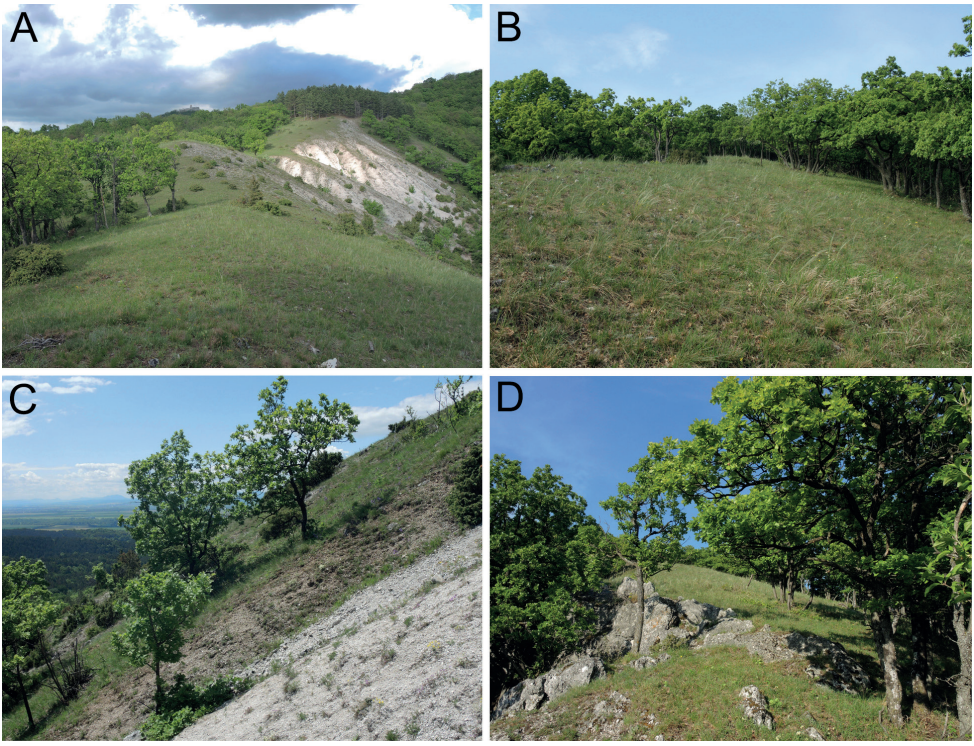

Figure 5. Localities of *Mesoneura tematinensis* sp. nov. photographed when the larvae appeared **A** Hradlová nivka **B** Kňaží vrch, holotype locality **C** solitary oaks in Hradlová nivka **D** edge of the oak stand in Kňaží vrch.

of *M. tematinensis* is larger than that of *M. opaca*. In females it is 7.66–8.8 mm (N = 5, mean 8.3 mm, median 8.4 mm) for *M. tematinensis* and 6.2–7.45 mm (N = 18, mean and median 6.9 mm) for *M. opaca*.

Material of *Mesoneura opaca* examined: Total 18 ♀♀. S and SW Slovakia [leg. L. Roller and in IZ SAS]: 5 ♀♀, Tematínske kopce, Lúka env., Kňaží vrch, 23.IV.2000, 30.IV.2021, 10.V.2021; 4 ♀♀, Malé Karpaty Mts., Devínska Kobyla – Sandberg, 24.IV. and 26.IV.1994, 14.IV.2017; 1 ♀, Podunajská nížina, Rusovce – park, 19.IV.2020; 1 ♀, Burda, Kamenica nad Hronom, 21.IV.2019; 3 ♀♀, Borská nížina, Šaštínsky les, 24.IV.2006; 2 ♀♀, Borská nížina, Rohožník, 10.V.2010, 20.IV.2011; 1 ♀, Borská nížina, Jakubov, 1.V.1994; 1 ♀ Borská nížina, Sološnica, 25.IV.1994.

Biology

The species inhabits pubescent oaks in a thermophilic supra-Mediterranean forest (Fig. 5A, B). Despite continuous sampling with the Malaise trap and with an entomological net at approximately one-week intervals during oak leaf sprouting in spring, no adults were collected. All adults examined were reared from the larvae of the previous year and appeared from late March to early April. The flight period can only be

predicted to the time of early sprouting of the oak leaves. The finding of the male specimen is particularly noteworthy. The related *M. opaca* is considered to be predominantly parthenogenetic, with few males reported (Liston 2012). Parthenogenesis also seems to be the preferred reproductive strategy in *M. tematinensis*, as only one male was reared out of a total of six adults.

The larvae feed externally on fresh leaves of the pubescent oak. Depending on the weather conditions in the respective season, they were found on fresh oak leaves for about 15 days from the end of April to the beginning of June. They occurred singly on branches of solitary trees and trees at the edge of open areas (Fig. 5C, D). Larvae about 1 cm in size (probably second to third instars) were found on about half-grown leaves, and mature larvae (probably fourth or fifth instar) were feeding on fully grown but still light green leaves. The mature larvae remained on the leaves for a few days longer than most other phyllophagous oak sawflies (*M. opaca*, *Periclista* and *Apethymus* species), of which only *Pristiphora fausta* was still present. Shortly after ecdysis to prepupa, the larvae burrowed into the soil where they overwintered. It is noteworthy that after several failed attempts with different soil substrates we reared the adults only with the soil we had taken from near the oaks where the larvae were found. Our rearing shows that *M. tematinensis* has only one brood per year.

Accompanying sawfly species

When sampling stands where *M. tematinensis* occurs, 13 other Symphyta species were found associated with the pubescent oak (Table 1). With the exception of *Harpiphorus lepidus*, *Periclista lenta* and *Janus cynosbati*, this association was confirmed by collecting feeding larvae and their growth into adults. *Harpiphorus lepidus* is known to feed on oak leaves (Enslin 1914) and its larvae probably hatch after our sampling period

Table 1. Numbers and stages of Symphyta species collected from pubescent oaks at *Mesoneura tematinensis* sp. nov. type localities.

Species	Malasie trap	Individual sampling
Apethymus cereus (Klug, 1818)		6 L; 3 A ex L
Apethymus serotinus (Müller, 1776)	15 A	4 L
Harpiphorus lepidus (Klug, 1818)	1 A	1 A
Periclista albida (Klug, 1816)	1 A	4 L; 1 A; 1 A ex L
Periclista albipennis (Zaddach, 1859)		10 L; 2 A ex L
Periclista lenta Konow, 1903	3 A	
Periclista lineolata (Klug, 1816)	1 A	7 L; 1 A; 2 A ex L
Periclista pubescens (Zaddach, 1859)	1 A	8 L; 3 A ex L
Periclista pilosa Chevin, 1971		12 L; 2 A; 4 A ex L
Mesoneura opaca (Fabricius, 1775)	7 A	18 L; 3 A; 2 A ex L
Pristiphora fausta (Hartig, 1837)		6 L
Pamphilius sylvarum (Stephens, 1835)		2 L
Janus cynosbati (Linné, 1758)	1 A	

Sampling with a Malaise trap in 1999; individual sampling in 2018–2021; L = larva, A = adult, A ex L = adult reared from larva.

(April–May). *Periclista lenta* is a very rare species with unknown larvae (Macek et al. 2020). However, it is very likely that it develops on oak like all other members of the genus *Periclista*, and the pubescent oak is the only oak species found in the sampled stand. The larvae of *Janus cynosbati* feed inside oak shoots and the larvae of the other ten species were found on fresh oak leaves at the same time as *M. tematinensis*. This indicates that the stands studied harbour a very rich assemblage of phyllophagous Symphyta. Particularly noteworthy is the occurrence of all *Periclista* species found in Central Europe, with the exception of *P. albiventris* (Klug, 1816). However, the species generally occurred in very low numbers, with *Mesoneura opaca* being the most common. Most of the oaks examined were free of Symphyta larvae, and a maximum of five larvae of two to three species were found on a single tree.

Remarks

The discovery of a new species of the genus *Mesoneura* on pubescent oak in Slovakia can be considered surprising, as the sawfly fauna of oaks (*Quercus* spp.) is relatively well known in Central Europe and its study has a long tradition in Slovakia (Patočka et al. 1962; Roller 2004; Smetana et al. 2020). Pubescent oak has a large distribution range in Southern and Central Europe and reaches the northern limit of its range in Slovakia. It was therefore unlikely that a previously unknown taxon associated with this tree would appear for the first time in the Tematín Mountains. However, the supra-Mediterranean oak stands in the Tematín Mountains are very well preserved, as evidenced by the occurrence of thermophilic insect species rare in Central Europe, such as the large predatory bush cricket *Saga pedo* and the true cicadas *Tibicen plebejus* and *Cicada orni* (Vidlička et al. 2002; Májsky and Janský 2006). *Mesoneura tematinensis* is thought to have a wider range and is likely to be overlooked due to its secretive lifestyle in relatively extreme habitats. In order to monitor its occurrence at suitable sites, we recommend collecting the larvae at the time of fresh, almost fully developed oak leaves.

Acknowledgments

We thank Jan Macek (National Museum, Prague), Andrew Liston (Senckenberg German Entomological Institute, Muenchenberg), Marko Prous (University of Oulu) and Hideho Hara (Hokkaido Research Organisation, Bibai) for fruitful discussions on the identity of the newly described *Mesoneura*. We are grateful to managers of the Biele Karpaty Protected Landscape Area, Nové Mesto nad Váhom, especially Jozef Májsky and Soňa Michalčíková, for advice on the study sites and facilitating sampling. We also thank Ľubomír Vidlička and Oto Majzlan (Institute of Zoology of SAS, Bratislava) for providing sawfly material from the Malaise trap collection.

The study was partly funded by the Slovak Funding Agency VEGA (No. 2/0070/23) and the European Regional Development Fund (ERDF) through the Operational

Programme for Research and Innovation, No. ITMS2014 + 313021 W683: "DNA Barcoding of Slovakia (SK-BOL), as part of the international initiative International Barcode of Life (iBOL)".

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JHR 95: 275–293 (2023)
doi: 10.3897/jhr.95.96025
https://jhr.pensoft.net

RESEARCH ARTICLE

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

The North American bees of the genus *Ptilothrix* Cresson, 1878 (Hymenoptera, Apidae, Emphorini), with the description of two new species

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Academic editor: Jack Neff | Received 6 October 2022 | Accepted 15 February 2023 | Published 24 February 2023

https://zoobank.org/55CB05E0-743C-4CDB-BD29-3D114676F090

Citation: Flórez-Gómez N, Danforth B (2023) The North American bees of the genus *Ptilothrix* Cresson, 1878 (Hymenoptera, Apidae, Emphorini), with the description of two new species. Journal of Hymenoptera Research 95: 275–293. https://doi.org/10.3897/jhr.95.96025

Abstract

Ptilothrix Cresson is a genus of New World bees with an amphitropical distribution. Like other genera in the tribe Emphorini, *Ptilothrix* have narrow pollen preferences. These solitary ground-nesting bees exhibit a remarkable nesting behavior in which females carry water from ponds to facilitate the excavation of the hard soil where they nest. With 16 described species, there are few taxonomic studies and, before this work, a lack of taxonomic treatments for the species in North America. Thus, in this study we revised and recognized four species for the region: *Ptilothrix bombiformis* Cresson, *Ptilothrix sumichrasti* Cresson, *Ptilothrix chiricahua* Florez-Gomez & Danforth, **sp. nov.** and *Ptilothrix zacateca* Florez-Gomez & Danforth, **sp. nov.** We describe and illustrate males and females of the two new species. We also present diagnoses for the four species, a key to identify them, and a map of their geographic distributions.

Keywords

Anthophila, Apoidea, Chiricahua, Mexico, taxonomy

Introduction

The genus *Ptilothrix* Cresson, 1878 is a group of bees restricted to the Western hemisphere with an amphitropical distribution. In North America the genus is found from Ontario, Canada to Oaxaca, Mexico and in South America it occurs from northern

Brazil to northern Patagonia, Argentina (Michener 2007; Roig-Alsina 2007; Sharkey et al. 2020). Like many members in Emphorini, species in this genus are pollen specialists that are associated with plants in the families Malvaceae, Cactaceae, Convolvulaceae, Zygophyllaceae and Onagraceae (Cazier and Linsley 1974; Rust 1980; Hazeldine 1997; Tellería 2003; Sipes and Tepedino 2005). *Ptilothrix* species are solitary bees that nest in aggregations in flat or slightly sloping areas of hardpacked sandy soil that is softened with water they collect by alighting on nearby bodies of water (Linsley et al. 1956; Martins et al. 1996; Michener 2007).

Within Emphorini, *Ptilothrix* is phylogenetically related to *Diadasina* and *Melitomella* (Roig-Alsina and Michener 1993; Freitas et al. 2020), both South American genera. Morphologically, the genus can be distinguished from the other genera in the tribe by the lack of arolia, elongated first flagellomere, tongue not surpassing the forecoxae, upper margin of the clypeus surpassing the lower margin of the antennal sockets, and widely spaced tibial scopal hairs with straight branches (Roig-Alsina 1998; Michener 2007; Roig-Alsina 2007).

The genus currently includes 16 described species (Ascher and Pickering 2020), 14 of which are in South America. Taxonomic studies are scarce, the only previous revision is by Roig–Alsina (2007) for the *albidohirta* group, which comprises three species that are known for Bolivia and Argentina. In North America there are three reported species: *P. bombiformis* Cresson, *P. sumichrasti* Cresson and one undescribed species that has been identified in bee surveys as *P.* nr. *sumichrasti* (Michener 2007; Minckley and Radke 2021). Herein we describe this species and one new species from Mexico, we also present diagnosis of the North American species, an identification key to recognize males and females, and a map of their geographic distribution.

Methods

We examined specimens deposited in the following collections: Cornell University Insect Collection (**CUIC**), American Museum of Natural History (**AMNH**), Snow Entomological Museum, University of Kansas (**SEM**), USDA–ARS Pollinating Insects Research Unit, Utah State University (**BBSL**), Central Texas Melittological Institute (**CTMI**), Texas A&M University (**TAMU**), Minckley collection at the University of Rochester (**MCUR**), Illinois Natural History Survey Insect Collection (**INHS**), Entomology Research Museum at UC Riverside (**UCRC**) and Colección Nacional de Insectos, Departamento de Zoología, Instituto de Biología UNAM, Mexico (**CNIN**). Additionally, we examined the lectotypes of *P. sumichrasti* and *P. bombiformis* obtained on loan from the Academy of Natural Sciences of Drexel University (**ANSP**). Designated holotypes and paratypes were deposited at CUIC, AMNH, SEM, CTMI and BBSL.

The morphological terminology used in this study follows that proposed by Michener (2007). Abbreviations in the descriptions and key are T, S, and MOD for metasomal terga, metasomal sterna and median ocellar diameter, respectively. We made the descriptions based on holotype and paratype specimens, measurements were made using a Zeiss Stemi SV11 microscope and are expressed in millimeters (mm).

Additionally, we dissected male genitalia, seventh and eighth sterna (S7 and S8), these structures were cleared using a 10% NaOH solution and stored in glycerin. We took habitus images with a Cannon EOS 6D and a 65 mm macro lens. To stack single photographs we used a Stackshot 3× Cognisys device and Zerene stack software. Photographs of other structures were taken using a Zeiss Stemi SV6 microscope with an Axiocam 105 color camera.

Finally, the known distribution obtained from the specimen labels of the four species were mapped using QGIS software and topographic layers downloaded from EarthEnv. For *Ptilothrix bombiformis*, we additionally included data from the Global Biodiversity Information Facility (**GBIF**).

Results

Genus *Ptilothrix* Smith, 1853

***Ptilothrix chiricahua* sp. nov.**
https://zoobank.org/96871ED3-1B4A-48BD-8714-00BC4B91D5B9
Figs 1, 2A, B, 3A, B, 4B, 5

Diagnosis. Body size of females from 8.6–12 mm, males 9–10.8 mm. This species is distinguished by the polished and shiny ocellocular area, with few scattered punctures close to the eye margin in both sexes. Morphologically similar to *P. sumichrasti*, but males differ by the yellowish pubescence on T7, and the shape of S7, S8 and the genital capsule (Figs 2A, B, 3A, B). As in *P. sumichrasti*, this species is characterized by the relatively short first flagellomere (when compared with other *Ptilothrix*), length no more than twice its apical width; fulvous pubescence on pronotum, scutum, scutellum, pale yellowish to white in some specimens, and whitish on the mesepisternum and propodeum. Metasoma with apical bands of yellowish appressed hairs on T1–T4. Anterior area of T3–T5 with black, short appressed hairs.

Description. Male. Total length 9.16 mm (paratypes 9–10.8 mm, n=10). ***Head.*** Integument black, except mandible brown with middle area lighter brown; apex yellowish in some paratypes. Mandible with rounded apical margin. Pubescence whitish on labrum, clypeus, around antennal sockets, frons and gena, becoming pale yellowish on vertex, whitish in some paratypes. Labrum rectangular, disc densely punctate, margin raised and impunctate. Clypeus protuberant in lateral view, with punctures densely distributed, separated by a distance less than a puncture diameter. Punctation of lower paraocular area, supraclypeal area and frons punctation as in clypeus. Inner ocular margins subparallel. Ocellocular area polished, with few scattered punctures and short pilosity closer to the eye margin, distance 0.52 mm. Antennae dark brown, scape 0.68 mm long, first flagellomere longer than broad, 1.7 times longer than its apical width. Head length 2.6 mm. Head width 3.2 mm. Gena width 0.62 mm. Lower interocular distance 1.8 mm. Upper interocular distance 2 mm. Lower interocular distance 0.8 mm. Antennocular distance 0.28 mm. MOD 0.228 mm.

***Mesosoma*.** Integument black. Pubescence fulvous on the pronotum, scutum, scutellum and metanotum; becoming whitish towards the propodeum, mesepisternum and metepisternum; some paratypes with pubescence entirely whitish. Propodeal triangle with short appressed, whitish hairs. Scutum with homogeneously distributed punctures separated by a distance up to a puncture diameter. Mesepisternal disc with punctures densely distributed, separated by a distance up to a puncture diameter, interspaces shiny. Hypoepimeral area with close punctures on the upper area, impunctate and polished towards the scrobal groove. Tegula translucent brownish. Tuft of yellow pubescence at base of wings, membrane of wings subhyaline, venation brown. Length of forewing 8 mm. Intertegular distance 2.6 mm. Scutum length 2.35 mm. ***Legs*.** Integument dark brown, except tarsi reddish brown, with whitish pubescence. Hind coxa and femur enlarged. Hind basitarsus slightly curved, 5 times the length of the second tarsomere. Posterior tibial spurs slightly hooked at the apex.

***Metasoma*.** Tergal integument black, sterna brown. T1 and T2 covered with whitish erect pilosity and posterior margin with a band of yellowish appressed hairs. T3–T6 with appressed short, dark hairs, margin with bands of yellowish pilosity. T7 covered with yellowish pubescence, apical margin with two pointed projections forming a medial U–shaped notch. Sternal pubescence erect, yellowish. S6 with a median tuft of hairs. Metasomal pubescence whitish in some paratypes. S7 and S8 as in Fig. 2A, B. Gonobase rounded, middle area of gonostylus slightly broadened (Fig. 3A, B).

Female. Total length 10.62 mm (8.6–12 mm n=10). ***Head*.** Integument color as in males. Maxillary palpus 6–segmented, segments 1–4 with short setae on the outer margin. Mandible with rounded apical margin. Pubescence color as in males, but hairs shorter. Labrum densely punctate, apex subtriangular with margin raised, impunctate. Inner ocular margins nearly parallel. Clypeus as in male, interspaces between punctures up to a puncture diameter. Punctation in paraocular area as in males. Ocellocular area polished and with few scattered punctures and short pilosity closer to the eye margin, distance 0.56 mm. Antennae dark brown, scape 0.71 mm long, first flagellomere 1.8 times longer than its apical width (Fig. 4B). Head length 2.7 mm. Head width 3.45 mm. Gena width 0.44 mm. Lower interocular distance 2.02 mm. Upper interocular distance 2.2 mm. Interocular distance 0.98 mm. Antennocular distance 0.3 mm. MOD 0.25 mm. ***Mesosoma*.** Color, punctures and pubescence color as in males, but hairs shorter. Wings and tegula as in males. Length of forewing 8.5 mm. Intertegular distance 2.66 mm. Scutum length 2.56 mm. ***Legs*.** Integument and pubescence color as in male. Hind tibia with whitish scopa, apical margin with a tuft of white pubescence, inner surface rugose and with short and appressed hairs. Scopa on hind basitarsus whitish on the outer surface and brownish on the inner surface. ***Metasoma*.** Integument as in male. T1–T4 pubescence as in male. T5 with black, short and appressed hairs on the anterior area, prepygidial fimbria yellowish with brown hairs intercalated, entirely brown in some paratypes. Pygidial fimbria dark brown. Pygidial plate with rounded apex.

Material examined. ***Holotype*.** United States of America • ♂; New Mexico; Hidalgo Co.1 mi. S Rodeo; 23 Aug. 2002; B.N. Danforth leg.; on *Kallstroemia*; CUIC.

***Paratypes*.** Mexico – **Sonora** • 1 ♀; Rancho San Bernardino. 28 km E Agua Prieta, Ciénaga; 27 Jul. 2000; K. Toal leg.; MCUR 1519 • 1 ♀; same data as for preceding;

Figure 1. *Ptilothrix chiricahua* sp. nov. **A** male holotype habitus, lateral view **B** male holotype habitus; frontal view **C** male holotype dorsal view **D** male holotype metasoma **E** female paratype habitus, lateral view **F** female paratype habitus, frontal view **G** female paratype dorsal view **H** female paratype metasoma.

22 Jun. 2000; MCUR 1874 • 1 ♂; 30 km E Agua Prieta; 31°19'08"N, 109°15'04"W; 12 Aug 2001; A. Romero leg., MCUR SBV043745.

United States of America – **Arizona** • 1 ♂; Cochise Co. 10 mi SW Apache; 31.571°N, 109.26°W; 27 Aug. 2014; B.N. Danforth leg.; CUIC • 5 ♀, 4♂; Cochise

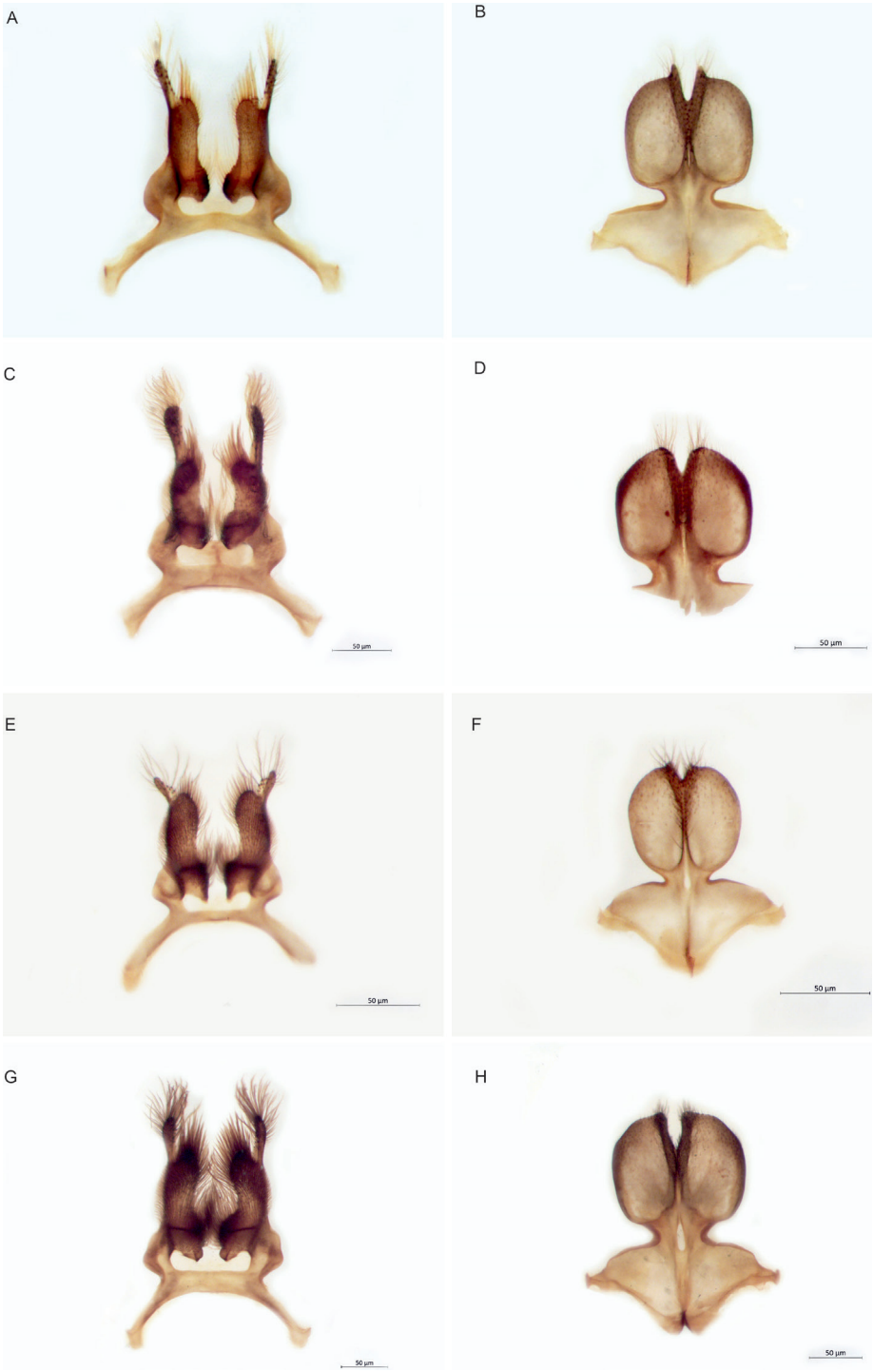

Figure 2. A *Ptilothrix chiricahua* sp. nov. male S7 **B** *Ptilothrix chiricahua* sp. nov. male S8 **C** *Ptilothrix zacateca* sp. nov. male S7 **D** *Ptilothrix zacateca* sp. nov. male S8 **E** *Ptilothrix sumichrasti* male S7 **F** *Ptilothrix sumichrasti* male S8 **G** *Ptilothrix bombiformis* male S7 **H** *Ptilothrix bombiformis* male S8.

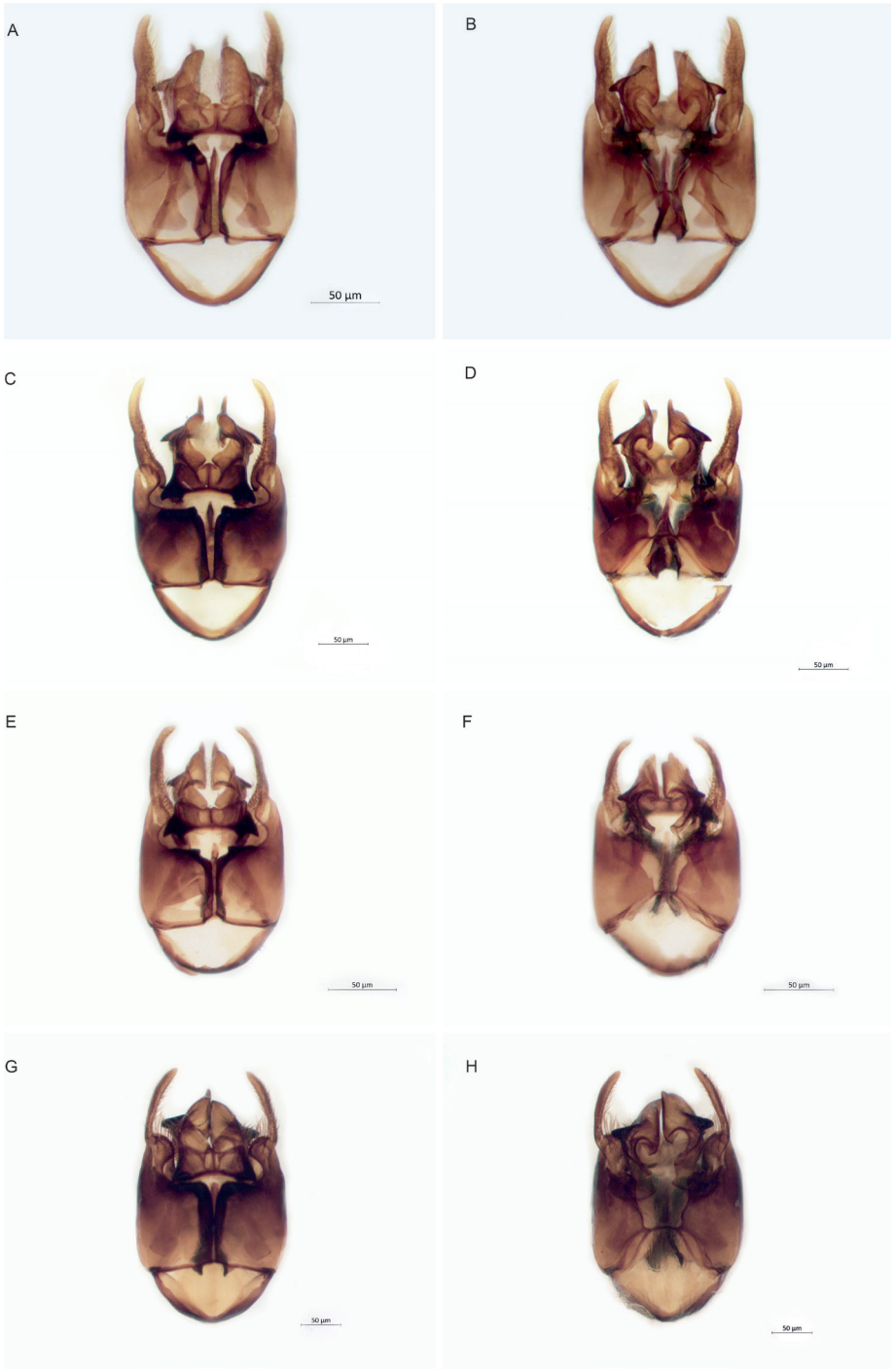

Figure 3. A, B *Ptilothrix chiricahua* sp. nov. male genital capsule **A** dorsal view **B** ventral view **C, D** *Ptilothrix zacateca* sp. nov. male genital capsule **C** dorsal view **D** ventral view **E, F** *Ptilothrix sumichrasti* male genital capsule **E** dorsal view **F** ventral view **G, H** *Ptilothrix bombiformis* male genital capsule **G** dorsa view **F** ventral view.

Figure 4. Ocellocular area and antenna of **A** female lectotype of *Ptilothrix sumichrasti* **B** female paratype of *Ptilothrix chiricahua* sp. nov. **C** female paratype of *Ptilothrix zacateca* sp. nov.

Co. Portal 6 mi E; 31.876°N, 109.058°W; 31 Jul. 2010; B.N. Danforth leg.; CUIC • 2 ♀, 1 ♂; Cochise Co. Portal vicinity; 31°52.55'N, 109°03.46'W; 26 Jul. 2008; B.N. Danforth leg.; CUIC • 2 ♀; Cochise Co. Portal 6 mi E.; 31.876°N, 109.058°W; 29 Aug. 2010; J.L. Neff leg.; on *Kallstroemia grandiflora*; CTMI 34537 to 34538 • 2 ♂; same data as for preceding; CTMI 34539 to 34540 • 1 ♂; Cochise Co. Portal Rd. 1.4 mi. N. rd 80; 31.880°N, 109.036°W; 25 Jul. 2006; J. L. Neff leg.; on *Kallstroemia grandiflora*; CTMI 31553 • 1 ♀; Cochise Co. Douglas 16 mi N; 31°28.10'N, 109°15.12'W; alt. 1250 m; 22 Jul. 2000. J. L. Neff leg.; on *Kallstroemia grandiflora*; CTMI 11971 • 1 ♀; Pima Co. Silver Bell, 5 mi E; 32.383°N, 111.417°W; 12 Aug. 1974; J. L. Neff leg.; on *Kallstroemia grandiflora*; CTMI 97071 • 2 ♂; same data as for preceding; 7–12 Aug. 1973; CTMI 102430 to 102431 • 1 ♂; same data as for preceding; 28 Sep. 1974; CTMI 65420 • 1 ♂; Pima Co. Rillito; 34.208°N, 111.150°W; 17 Jul 1974; J. L. Neff leg.; on *Kallstroemia grandiflora*; CTMI 65570 • 1 ♀; Pima Co. Silver Bell Bajada IBP Desert Scrub Site; J. L. Neff leg.; on *Kallstroemia grandiflora*; CTMI 97073. – **New Mexico** • 8 ♀, 3 ♂; same data as for holotype • 1 ♀; Hidalgo Co. 0.5 mi N Rodeo; 19 Sep. 2001; Danforth & Magnacca leg.; CUIC • 6 ♀, 3 ♂; Hidalgo Co. 7 mi N Rodeo; 31.933487°N, 109.024734°W; 27 Jul.2018; B.N. Danforth leg.; on *Kallstroemia*; CUIC • 7 ♀, 1 ♂; Hidalgo Co. Rodeo; 22 Sep. 1999; Danforth & Magnacca leg.; on *Kallstroemia*; CUIC • 2 ♀; Hidalgo Co. Rodeo 20 mi. N. San Simón Ciénaga; 12 Sep. 1999; Danforth & Magnacca leg.; CUIC • 9 ♀, 1♂; Hidalgo

Co. Rodeo 4 mi N.; 18 Sep. 1999; Danforth & Magnacca leg.; on *Kallstroemia*; CUIC • 1 ♀; Hidalgo Co. Rodeo 2 mi N; 15 Jul. 1974; JM Linsley leg.; on *Kallstroemia grandiflora*; INHS 375568 • 1 ♂; same data as for preceding; INHS 375569.

Other material. Mexico – **Sonora** • 1 ♀; 30 km E Agua Prieta; 31°30'00"N, 109°28'00"W; 1 Aug 2001; E. Serrano leg., MCUR SBV069744 • 1 ♀; same data as for preceding; MCUR SBV069979 • 1 ♀; same data as for preceding; MCUR SBV070114 • 1 ♀; same data as for preceding; MCUR SBV070141 • 1 ♀; same data as for preceding; 1–15 Aug. 2001; R.L. Minckley leg.; MCUR SBV079342 • 1 ♀; same data as for preceding; MCUR SBV079339 • 1 ♀; same data as for preceding; MCUR SBV079331 • 1 ♀; Rancho San Bernardino. 28 km E Agua Prieta, Ciénaga; 27 Jul. 2000; K Toal leg.; MCUR 1329 • 1 ♀; same data as for preceding; 10 Jul. 2000; MCUR 232 • 1 ♀; same data as for preceding; MCUR 2555 • 1 ♀; same data as for preceding; MCUR 226 • 1 ♀; same data as for preceding; MCUR 182.

United States of America – **Arizona** • 1 ♂; Portal. Cochise Co.; 7 May 1977; R Brooks leg.; on *Kallstroemia*; SEM 1304613 • 1 ♂; Pima, San Luis Wash; 31.63227°N, 111.429°W; alt. 1038 m; 19 Aug. 2016; T.L. Griswold leg.; BBSL 1035487 • 1 ♂; same data as for preceding; BBSL 1035497 • 3 ♂; same data as for preceding; BBSL 1035575 to 1035577 • 1 ♀; same data as for preceding; BBSL 1035579 • 1 ♀; same data as for preceding; BBSL 1035496 • 1 ♂; same data as for preceding; on *Kallstroemia grandiflora*; BBSL 1035010 • 1 ♀; Pima, Brown Canyon; 31.75558°N, 111.525°W; alt. 1131 m; 21 Aug. 2016; T.L. Griswold leg.; BBSL 1035607 • 1 ♀; same data as for preceding; BBSL 1035609 • 1 ♂; Pima; Tequila Tank, 0.67 air km ESE; 31.632°N, 111.444°W; alt. 1062 m; 19 Aug. 2016; T.L. Griswold leg.; BBSL 1035465 • 1 ♀; Cochise; Portal, 3.7 mi N; 31.959°N, 109.151°W; alt. 1412 m; 21 Aug. 2002; T.L. Griswold leg.; BBSL 689511 • 1 ♀; same data as for preceding; BBSL 689526 • 1 ♀; same data as for preceding; BBSL 689530 – **New Mexico** • 1 ♂; Hidalgo Co. 3 mi N Rodeo; 12 Aug.1978; R. Brooks leg.; on *Kallstroemia*; SEM 1304615 • 1 ♂; Hidalgo, Jct. Stateline & Hwy 80; 31.87985°N, 109.004°W; alt. 1250 m; 20 Aug. 2002; T.L. Griswold leg.; on *Kallstroemia* sp.; BBSL 689267 • 1 ♂; same data as for preceding; BBSL 689295 • 1 ♂; same data as for preceding; BBSL 689301 • 1 ♀; same data as for preceding; BBSL 689297 • 1 ♂; Hidalgo, Animas; 31.9292°N, 108.806°W; alt. 1352 m; 18 Aug. 2002; T.L. Griswold leg.; BBSL 690364 • 1 ♀; same data as for preceding; 31 Aug. 2002; BBSL 689365 • 1 ♀; same data as for preceding; BBSL 689368 – **Texas** • 2 ♀; Jeff Davis Co. TNC Davis Mountains Preserve McIvor Center vicinity; 8 Jul.- 27 Aug. 2022; J.A. Hanson, D. Heffern leg.; TAMU.

Comments. *P. chiricahua* is associated to mixed Chihuahuan desert grassland habitats of Southern Arizona, New Mexico, Western Texas and Northern Mexico (Fig. 5). This species has been mainly collected on flowers of *Kallstroemia grandiflora* in the late summer/fall and, based on examination of scopal loads, appears to be a pollen specialist on *K. grandiflora*. Butler (1967) and Michener (2007) also reported that this species has been collected in cotton flowers (Malvaceae).

Etymology. This species is named in honor to the Chiricahua Apache people whose historical homeland encompasses the area where this bee now occurs.

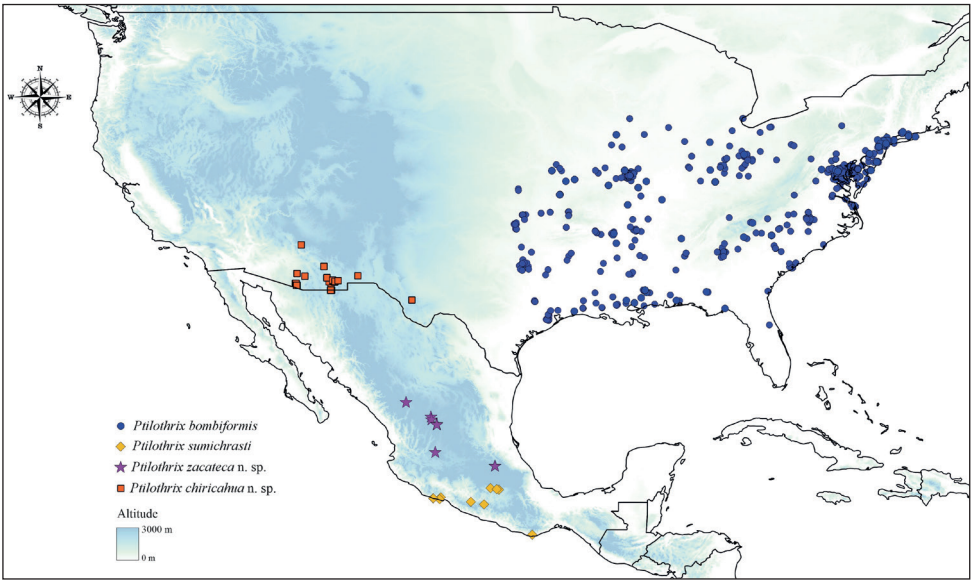

Figure 5. Distribution map of the North American species of the genus *Ptilothrix*: *Ptilothrix chiricahua* sp. nov., *Ptilothrix zacateca* sp. nov., *Ptilothrix sumichrasti* and *Ptilothrix bombiformis*.

***Ptilothrix zacateca* sp. nov.**
https://zoobank.org/9A25784C-75A2-4BD3-978B-C9B17B25F1CD
Figs 2C, D, 3C, D, 4C, 5, 6

Diagnosis. Female body size from 11.3–12.7 mm, male 10.5–13 mm. This species can be recognized by the overall whitish to pale yellowish pubescence, except for that on the female basitarsi, which is dark yellowish to pale brown. Differing from *P. sumichrasti* and *P. chiricahua* by the longer first flagellomere (length at least twice its apical width), presence of erect, overall longer pubescence, especially on the anterior areas of T2 and T3, more robust body and slightly larger size. Both sexes with tergal hair bands on T1–T4 in females, T1–T6 in males.

Description. Male. Total length 12.34 mm (paratypes 10.5–13 mm, n=7). ***Head.*** Integument black, except brown middle area of the mandible, mandibular apex yellowish in some paratypes. Uniformly whitish pubescence covering all of the head except ocellocular area where hairs are sparser. Mandible with rounded apical margin. Maxillary palpus 6–segmented. Labrum rectangular, disc densely punctate, margin raised and impunctate. Clypeus slightly protuberant in lateral view, with coarse punctation. Inner ocular margins subparallel. Frons, paraocular area and vertex with close punctures, separated by a distance up to a puncture diameter. Ocellocular integument micro–sculptured and with punctures towards the eye margin, small area around the lateral ocellus polished and shiny, distance 0.63 mm. Antennae black, scape 0.8 mm,

first flagellomere 2.5 times longer than its apical width. Head length 3.25 mm. Head width 3.85 mm. Gena width 0.8 mm. Lower interocular distance 2.45 mm. Upper interocular distance 2.5 mm. Interocular distance 1.03 mm. Antennocular distance 0.41 mm. MOD 0.25 mm

Mesosoma. Integument black. Overall pubescence whitish on pronotum, scutum, scutellum, mesepisternum, metepisternum and propodeum; pale yellowish on the scutum of some specimens. Scutum with close punctures regularly distributed, separated by less than a puncture diameter with shiny interspaces. Scutellar punctures even more closely spaced than those of the scutum. Disc of mesepisternum punctured as in scutum, deep punctures and very close one to each other, imbricate interspaces between punctures. Hypoepimeral area deeply and densely punctate, micro–sculptured towards the scrobal groove. Propodeal triangle with whitish short pubescence, middle line glabrous. Tegulae translucent brown. White tuft of hairs at the base of wings, membrane of wings slightly infuscate, venation brownish. Length of forewing 10.83 mm. Intertegular distance 3.25 mm. Scutum length 3.0 mm. ***Legs.*** Integument black to dark brown, except tarsi reddish brown. Pubescence whitish overall. Hind coxa and femur enlarged. Hind basitarsus slightly curved, 6 times the length of the second tarsomere. Hind tibial spurs dark brown, slightly hooked at the apex.

Metasoma. Overall integument black, margin of sterna subhyaline. T1–T4 with dense, whitish erect pubescence, posterior margin with band of white hairs. T5–T6 with dense and erect black pubescence on the anterior area, margin with a band of whitish dense pubescence. T7 covered with black pubescence, margin with two pointed projections forming a middle notch. Sterna with dense, whitish, erect pubescence. S6 with a median tuft of hairs. S7 and S8 as in Fig. 2C, D. Gonobase rounded, gonostylus filiform (Fig. 3C, D).

Female. Total length 12.67 mm (11.3–12.7 mm n=8). ***Head.*** Integument color as in male, pubescence as in male, but less abundant. Mandible color and shape as in males. Labrum densely punctate, with subtriangular, raised, impunctate apex. Clypeus with coarse punctures separated by a distance up to twice a puncture diameter. Punctation of paraocular area and frons as in males, puncture size smaller than those on clypeus. Inner ocular margins nearly parallel. Integument of ocellocular area as in males, distance 0.76 mm. Antennae black, scape 0.93 mm long, first flagellomere 2.3 times longer than its apical width (Fig. 4C). Head length 3.48 mm. Head width 4.49 mm. Gena width 0.83 mm. Lower interocular distance 2.81 mm. Upper interocular distance 2.85 mm. Interocular distance 1.22 mm. Antennocular distance 0.47 mm. MOD 0.30 mm. ***Mesosoma.*** Color, punctures and pubescence as in males, but pubescence shorter. Wing color and tegula as in male. Wings and tegula as in males. Length of forewing 10.62 mm. Intertegular distance 3.75 mm. Scutum length 3.37 mm. ***Legs.*** Overall black integument except brown tarsi. Hind tibia with whitish scopa, apical margin with a tuft of white pubescence, inner surface rugose and with short, whitish and appressed hairs. Scopa on hind basitarsus entirely dark yellowish to pale brown. ***Metasoma.*** Integument as in male. T1– T2 with erect whitish hairs, posterior margin with bands of whitish pubescence. T3 and T4 with whitish erect pubescence on the anterior area

Figure 6. *Ptilothrix zacateca* sp. nov. **A** male holotype habitus, lateral view **B** male holotype habitus; frontal view **C** male holotype dorsal view **D** male holotype metasoma **E** female paratype habitus, lateral view **F** female paratype habitus, frontal view **G** female paratype dorsal view **H** female paratype metasoma.

shorter than those in T1–T2, and with bands of pubescence. Prepygidial fimbria brownish, with whitish hairs intercalated laterally. Pygidial plate with rounded apex. Sterna with erect long, whitish–yellowish pubescence on the disc, denser at the margins.

Material examined. ***Holotype.*** Mexico • ♂; Zacatecas; Guadalupe; 28 Jun. 1953; C. & P. Vaurie leg.; D. Rockefeller Mex exped.; AMNH

Paratypes. Mexico – **Durango** • 2 ♂**;** 14 mi N.E of Durango; alt. 1889 m; 17 Jun. 1956; H.A. Scullen leg.; BBSL – **Jalisco** • 1 ♀**;** Villa Guadalupe; 26 Jul. 1951; H. E. Evans leg.; SEM SM0803860 • 1 ♂; same data as for preceding; SEM SM0803859 – **Zacatecas** • 3 ♂**;** same data as for holotype • 6 ♀**;** Fresnillo; alt. 2133 m; 15 Aug. 1947; C.D. Michener leg.; D. Rockefeller Mex exped.; AMNH. – **Hidalgo** • 1 ♀**;** 3 mi Pachuca; Kansas University Mexico exped.; 24 Jun. 1953; on *Argemone*; UCRC 546145 • 1 ♂; same data as for preceding; UCRC 546145.

Comments. This species is endemic to Mexico. Records are from the mountain regions in the Sierra Madre Occidental from 1800–2000 m (Fig. 5). Its preferred host plants are unknown, although two specimens were collected on *Argemone* flowers, suggesting a possible association. But labels of most of observed material did not include information about related plants and specimens did not have pollen loads to facilitate these associations. It has been collected from June–August.

Etymology. This species is named in honor to the Zacatecos, the indigenous group that inhabited the state of Zacatecas, the area where the holotype was collected.

Ptilothrix sumichrasti Cresson, 1878

Figs 2E, F, 3E, F, 4A, 5, 7

Ptilothrix sumichrasti Cresson, E. T. (1878). (Lectotype: ANSP #f: Mexico).

Diagnosis. Body size of females from 10–10.6 mm, males 8.2–10 mm. This species is morphologically similar to *P. chiricahua* but is recognizable by the punctation pattern in the ocellocular area, which is micro–sculptured, with punctures towards the eye margin and a small area around the lateral ocellus polished and shiny in both sexes. Males are clearly distinguishable by the brown pubescence covering T7, in addition to the shape of S7, S8 and genital capsule (Figs 2E, F, 3E, F). This species shares with *P. chiricahua* the following characters: fulvous pubescence on pronotum, scutum, scutellum, whitish towards the mesepisternum and propodeum. Metasoma with apical bands of yellowish appressed hairs from T1–T4. Anterior area of T3–T5 with black, short appressed hairs. First flagellomere length less than twice its apical width, being shorter than the rest of species in the genus *Ptilothrix* (Fig. 4A).

Material examined. Mexico – **Guerrero** • 1 ♂; Iguala; CNIN 2887 – **Michoacán** • 1 ♀; La Mira, 4 km N Playa Azul; 2 Nov. 1987; T. Griswold leg.; BBSL 725135 • 1 ♂; Los Amates, 26 km N Playa Azul; 2 Nov. 1987; T. Griswold leg.; on *Ipomoea*; BBSL 725132 • 2 ♂; Caleta de Campos, 4 kmN Playa Azul; 3 Nov. 1987; T. Griswold leg.; BBSL 725133 to 725134 –**Oaxaca** • 1 ♂; 4 mi. N Pochutla. 11 Oct. 1975; J.L. Neff leg.; CTMI – **Puebla** • 6 ♀; Tilapa, Carr. Fed. Azúcar de Matamoros–Cuautla; 18°37.96'N, 98°34.96'W; 4 Sep.1998; T. Griswold leg.; BBSL 335125 to 335130.

Guatemala • 3 ♀; C.A; alt. 300 m; 10 Jan.1923; AMNH 262013 to 262015.

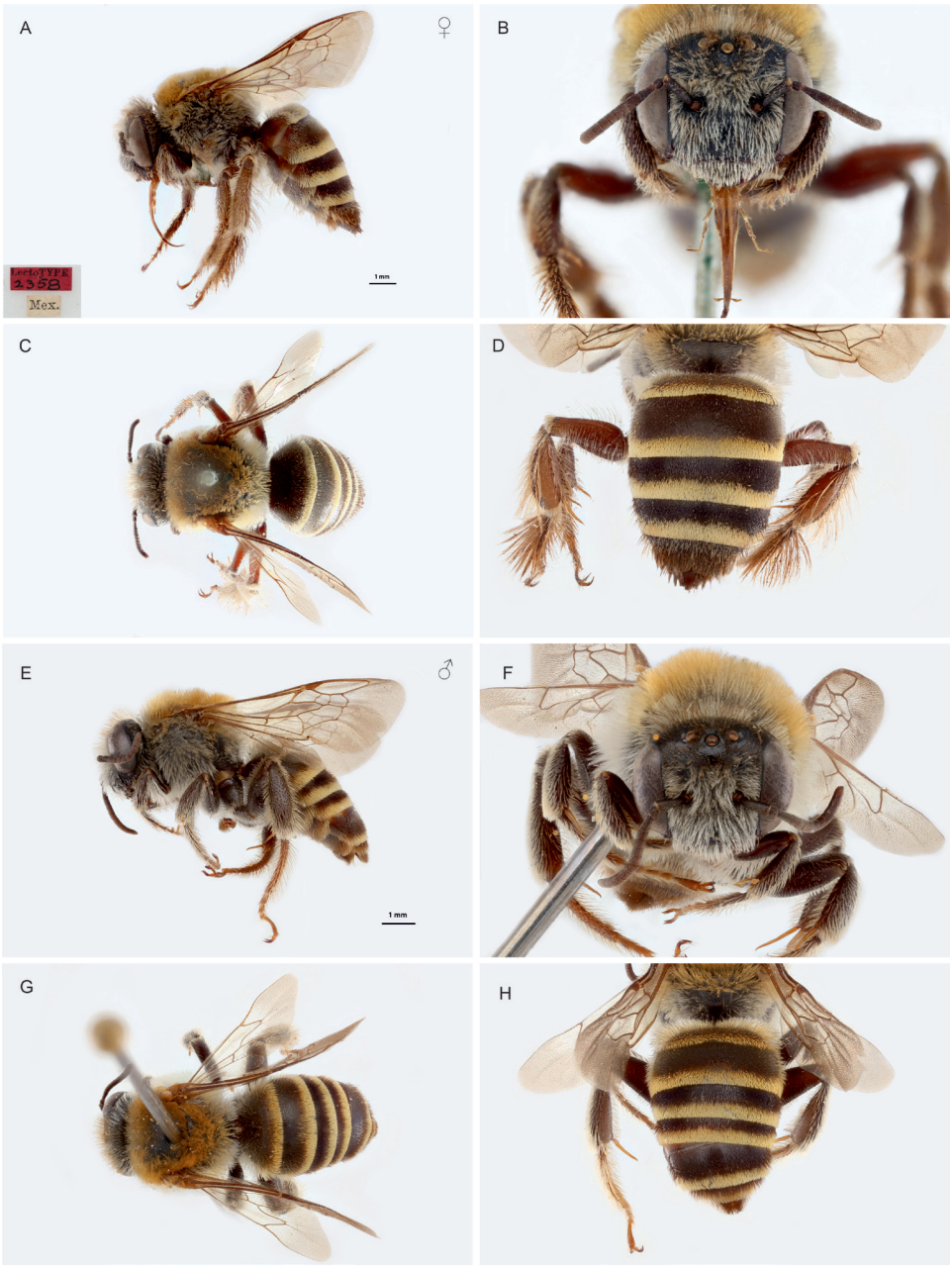

Figure 7. *Ptilothrix sumichrasti* **A** female lectotype habitus, lateral view **B** female lectotype habitus; frontal view **C** female lectotype, dorsal view **D** female lectotype habitus metasoma **E** male habitus, lateral view **F** male habitus, frontal view **G** male dorsal view **H** male metasoma.

Comments. Records of this species are from lowlands in Guatemala and Mexico in the Transmexican Volcanic Belt and the Balsas River Basin (Fig. 5), it is seemingly associated with flowers of *Ipomoea* and has been collected from October to January.

***Ptilothrix bombiformis* Cresson, 1878**
Figs 5, 6G, H, 7G, H, 8

Emphor bombiformis Cresson, 1878.
Emphor fuscojubatus Cockerell, 1913.
Ptilothrix bombiformis Cresson, (1878). (Lectotype: ANSP #f: Kansas, Snow).

Diagnosis. Large bees, female body size from 13.5–19 mm, males from 12.5–18.6 mm. This species is clearly recognizable by the white to pale yellow pubescence on the head and mesosoma, and entirely black metasoma, except T1 which has whitish pubescence on the lateral sides in some specimens. Differs from *P. sumichrasti*, *P. chiricahua* and *P. zacateca* by the absence of yellowish metasomal bands, darkened wings, entirely black scopa, and overall larger size. First flagellomere length twice its apical width, as in other *Ptilothrix* species except *P. sumichrasti* and *P. chiricahua*. Shape of male S7, S8 and genital capsule as shown in Figs 2G, H, 3G, H.

Material examined. United States of America – **Alabama** • 1 ♂; Morgan; 34.6059°N, 86.9833°W; alt 178 m; Aug.1944; GE Bohart leg.; BBSL 511531 • 2 ♀; Houston, Cowarts; 31.2°N, 85.3047°W, 1 Aug. 1916; AMNH 00260371 to 00260372. – **Arkansas** • 1 ♂; Monroe, Cotton Plant, 5.4 mi SWbS; 34.9327°N, 91.2726°W; alt. 54 m; 21 Jul. 2015; P.L. Stephenson leg.; BBSL 1027862 • 1 ♂; same data as for preceding; BBSL 1027868 • 1 ♀; Prairie Des Arc, 6.8 mi SE, Cache River NWR; 34.9231°N, 91.3959°W; alt. 50 m; 24 Jun. 2015; P.L. Stephenson leg.; BBSL 1027864 • 1 ♂; same data as for preceding; BBSL 1027863 • 1 ♂; Monroe, Brinkley, 7.6 mi SW; 34.8521°N, 91.3242°W; alt. 49 m.; 16 Jul. 2015; PL Stephenson leg.; BBSL 1027865 • 1 ♂; same data as for preceding; BBSL 1027867 • 1 ♂; St. Charles, 4.4 mi SWbS; White River NWR. 55 m. 34.3117, –91.1213. 24–VII–2015. P.L. Stephenson; BBSL 102786. – **Illinois** • 13 ♂; Calhoun, Two Rivers NWR, HQ TI3S-RIW-S. 16; 38.94888°N, 90.5889°W; 2 Jul. 2012; B Loges leg.; AMNH 00260559 to 00260571 – **Indiana** • 1 ♀; Ripley, Friendship; 38.9703°N, 85.1477°W, alt. 194 m; on *Hibiscus syriacus*; 29 Jul. 1950; LW Chandler leg.; BBSL 511562 – **Iowa** • 2 ♂; Louisa, Port Louisa NWR (HSB6) Rush Lake Rd.; 41.22°N, 91.12°W; 3 Jul. 2012; J Young; AMNH 00260557 to 00260558 – **Kansas** • 1 ♂; Crawford Pittsburg, 404 West Jefferson Street; 37.40049°N, 94.7119°W; alt. 286 m; on *Hibiscus syriacus*; 14 Jun. 2010; BBSL 1046881 • 1 ♀; same data as for preceding; 15 Jul. 2003; BBSL 1046887 • 2 ♂; same data as for preceding; 15 Jul. 2003; BBSL 1046885 to 1046886 • 2 ♂; Crawford, Pittsburg; 37.41092°N, 94.6993°W; alt. 283 m; on *Hibiscus* sp.; 20 Jul. 2003; BBSL 1046882 to 1046883 • 2 ♀; Douglas, Lawrence, Mary's Lake; 38.9284°N, 95.2171°W; alt. 253 m; 8 Aug. 1996; B Alexander leg.; BBSL 207987 to 207988 – **Kentucky** • 1 ♂; Franklin, Frankfort, Lakeview Park; 38.21638°N, 84.8303°W; alt. 231 m; 25 Jul. 1999; DM Gordon leg.; on *Helianthus* sp.; BBSL 1047096 • 2 ♀ ; same data as for preceding; on *Ipomoea* sp.; 16–19 Jul. 2000; BBSL 1047109 to 1047110 • 7 ♂; same data as for preceding; BBSL 1047097 to 1047106 • 2 ♀ ; Franklin. Frankfort, 504 Piaute Trail; 38.2149°N, 84.8362°W; alt. 227 m; 1 Jun 1999; DM Gordon leg.; BBSL 1047111 to 1047112 – **Louisiana** • 2 ♀; Saint Tammany, Abita Creek Preserve; 30.517°N, 89.967°W; 07 Aug. 2002; D Prowell leg.; on *Hibiscus aculeatus*; BBSL 664704

Figure 8. *Ptilothrix bombiformis* **A** female lectotype habitus, lateral view **B** female lectotype habitus; frontal view **C** female lectotype, dorsal view **D** female lectotype habitus metasoma **E** male habitus, lateral view **F** male habitus, frontal view **G** male dorsal view **H** male metasoma.

to 664705 • 2 ♂; same data as for preceding; BBSL 664706 to 664707 – **Mississippi** • 1 ♀; Pearl River, Poplarville; 30.84°N, 89.5342°W; alt. 102 m; 18 Aug.1998; B Sampson leg.; on *Hibiscus cannabinus*; BBSL 511533 – **Virginia** • 1 ♀; Warren; 39.006°N, 78.071°W; alt. 159 m; 03 Aug. 2014; E Bodnar; BBSL 1096111.

Comments. This species is the most widely distributed in North America, its geographical range from Ontario, Canada (Sharkey et al. 2020) to South Texas and Florida (Fig. 5). It is mainly associated to flowers of *Hibiscus*, although occasionally visits species of *Ipomoea*. It is active mid– to late summer.

Key to North American species of *Ptilothrix*

1 Metasoma with bands of yellowish or whitish pubescence from T1–T4; wings infuscate or subhyaline; female hind tibia with white or yellow scopa, yellow or brownish on basitarsus..... **2**

– Metasoma pubescence entirely black, without bands, T1 sometimes with yellow pubescence on lateral areas; wings darkened, female hind tibia and basitarsus with entirely black scopa ..... ***Ptilothrix bombiformis***

2 First flagellomere up to two times longer than its apical width (Fig. 4A, B); T3 with appressed, short black pubescence on the anterior area, posterior margin with bands of yellow or white pubescence; body size of females from 8.6–12 mm, males 8.2–10.8 mm..... **3**

– First flagellomere more than two times longer than its apical width (Fig. 4C); T3 with erect pubescence on the anterior area, posterior margin with bands of white pubescence; body size of females from 11.3–12.7 mm, males 10.5–13 mm..... ***Ptilothrix zacateca* sp. nov.**

3 Ocellocular area predominantly impunctate, polished and shiny (Fig. 4B); male T7 covered with yellowish pubescence; male S7 and S8 as in Fig. 2A, B .... ***Ptilothrix chiricahua* sp. nov.**

– Ocellocular area micro–sculptured, with scattered punctures and a small polished area around the lateral ocellus (Fig. 4A); male T7 covered with brown pubescence; male S7 and S8 as in Fig. 2E, F ..... ***Ptilothrix sumichrasti***

Discussion

The genus *Ptilothrix* is a species–rich group, especially in South America (Roig–Alsina 1998), however the precise number of species is hard to assess because no taxonomic revisions exist and there are a considerable number of new species and synonymies. In this study we added two new, previously undescribed species to the four total known from North America. We found that characters in the male genitalia, S7 and S8, integument punctation and metasomal pubescence are useful to distinguish North American species.

Ptilothrix bombiformis is the most widely distributed species in North America and one of the most commonly collected, it is associated to the genus *Hibiscus* (Malvaceae), although occasionally can visit flowers of *Ipomoea* (Convolvulaceae) (Rust 1980; Sharkey et al. 2020). *Ptilothrix chiricahua*, is also abundant in collections and is associated with flowers of *Kallstroemia grandiflora* (Zygophyllaceae) and cotton (*Gossypium* spp., Malvaceae) (Butler 1967; Cazier and Linsley 1974). Collection efforts focusing

on these species have benefited from the fact that their preferred pollen sources are known, facilitating their observation and study. In contrast, *Ptilothrix sumichrasti* and *Ptilothrix zacateca* are rare species that have been associated with the genus *Ipomoea*. Only one study, done by Linsley et al. (1956), has described biological aspects of *P. sumichrasti*. This work was conducted in Fresnillo, Zacatecas, Mexico. Based on our analysis of the geographic distributions of Mexican *Ptilothrix*, *P. sumichrasti* does not occur in this area. We suspect that the observations by Linsley et al. were of *P. zacateca* sp. nov, but we were not able to locate voucher specimens from this study to confirm their identity. If this study actually was on the biology of *P. zacateca*, then this species has an association with *Ipomoea longifolia* Benth. and *Ipomoea pringlei* A. Gray. From the examined specimens, we could not get information about floral hosts for this species, and the few labels of *P. sumichrasti* indicated a relationship with the genus *Ipomoea*. This finding highlights the importance of including floral associations in specimen labels, in order to facilitate future collections and further study of host–plant use in this lineage of pollen specialist bees, especially in Mexico and Guatemala, where they are uncommon.

Acknowledgements

We thank the Cornell University Insect Collection (CUIC) for letting us use the imaging system to take photographs. We are grateful to the following curators who facilitated visits to their collections and lent us specimens: Jason Weintraub and Jon Gelhaus (ANSP), Jerome Rozen (AMNH), Terry Griswold (BBSL), Michael Engel (SEM), Robert Minckley (University of Rochester), John Neff (CTMI), Douglas Yanega (University of California, Riverside), Michelle Kohler (INHS), Ismael Hinojosa (CNIN), and Donald Harvey, Timothy McMahon, Dennis Johnson, who shared their personal collections with us. We thank the reviewer and editor for their valuable comments on the manuscript. This project was supported by NSF–DEB grant number 2127745 (Collaborative Research: Bees of the World – Phylogenomics, Biogeography, and Evolution of Host–Plant Associations) to Elizabeth Murray (Washington State), Michael Branstetter (USDA ARS), Paul Maxfield (Natural History Museum of Utah), and Bryan Danforth.

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