Revisiting the host use and phylogeny of Colastomion Baker (Hymenoptera, Braconidae, Rogadinae), with a new host record from Japan

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Abstract
We report the solitary parasitism by Colastomion formosanum (Watanabe) (Hymenoptera, Braconidae, Rogadinae) on the larva of Nevrina procopia (Stoll) (Lepidoptera, Crambidae) feeding on Turpinia ternata Nakai (Staphyleaceae) in Amami Ōshima Is., Japan. This is the first host record for the genus Colastomion Baker outside of Papua New Guinea. We have also inferred the phylogenetic relationships of Colastomion species using Bayesian and maximum likelihood approaches, based on the mitochondrial cytochrome oxidase 1 gene. The results indicate two major clades—solitary and gregarious parasitoids—within Colastomion. Colastomion formosanum belongs to the clade of solitary parasitoids that specifically parasitize the crambid subfamily Spilomelinae. Plant-host-parasitoid associations and the evolutionary scenario of the host use of Colastomion are discussed.

Keywords
Colastomion formosanum, Crambidae, endoparasitoid, mtCO1, mummy, Nevrina procopia

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Introduction

The family Braconidae is one of the largest lineages in Hymenoptera, containing 21,221 valid species worldwide (Yu et al. 2016). Braconid wasps are parasitoids of various insects (e.g. Lepidoptera, Coleoptera, and Hymenoptera), including important agricultural pests (Wharton 1993; Quicke 2015; Maeto 2018). Their amazing diversity has resulted from the complicated biological interactions with host insects as well as contrasting lifestyles, e.g. solitary vs. gregarious, ecto- vs. endoparasitic, or idio- vs. koinobiont (Shaw 1988). Understanding their biology and phylogeny lends us a greater appreciation of the evolutionary pattern of host-parasitoid systems. Further, from a practical standpoint, the study of Braconidae can also be useful to leverage their parasitic abilities for biological pest control (Quicke 2015).

The subfamily Rogadinae Förster sensu stricto contains diverse koinobiont endoparasitoids of Lepidoptera and comprises 63 genera and 1,243 species that are distributed across all zoogeographical regions, except for polar regions (Quicke 2015; Yu et al. 2016). One of the marked biological features of the rogadine parasitoids is the mummification of their host larvae (Chen and He 1997; Zaldívar-Riverón et al. 2008).

Colastomion Baker is an uncommon genus of Rogadinae and comprises 15 species that occur throughout Papua New Guinea, southern East Asia, and Africa (Quicke et al. 2012; Yu et al. 2016). Crambid larvae have been reported to be hosts of Colastomion only in Papua New Guinea (Quicke et al. 2012). While the host larvae of Colastomion are typically mummified like other rogadine parasitoids, the mummy has been illustrated for only one species of Colastomion to date (Zaldívar-Riverón et al. 2008). Also, although the phylogenetic placement of Colastomion within Rogadinae has been largely established (Zaldívar-Riverón et al. 2008), the phylogenetic relationships within the genus has not been firmly estimated (Quicke et al. 2012).

Recently, the first and second authors (KS and SS) conducted field studies in Amami Ôshima Is., Kagoshima Pref., Japan, and obtained Colastomion specimens from host caterpillars and in a light trap. Here, we identified the Colastomion specimens as a species and conducted phylogenetic analyses based on the mitochondrial cytochrome oxidase 1 (CO1). These provide the first host record of Colastomion outside of Papua New Guinea, detailed mummy morphology, and the evolutionary scenario of host use within Colastomion.

Materials and methods

Field collection and rearing

A field study was conducted at Naze-Ôaza-Chinase (28°21’N, 129°26’E, 16 m alt.), Amami Ôshima Is., Kagoshima Pref., southwest Japan on 11 April 2019. The study site was the evergreen broad-leaved forest dominated by Ficus spp., Ligustrum japonicum Thunb. and Pittosporum tobira (Thunb.) W.T.Aiton. KS collected larvae of Nevrina procopia (Stoll)
Revisiting the host use and phylogeny of *Colastomion* as they were feeding on *Turpinia ternata* Nakai (Staphyleaceae). The larvae rolled young leaves roughly and hid themselves with the rolled leaves (Fig. 1). Collected larvae were reared in plastic cases under laboratory conditions (25 °C, 16:8 h light:dark). Emerged insects were killed in a freezer.

Further specimen was collected in a field study at Nishinakama (28°15’47.3”N, 129°24’56.1”E, 120 m alt.), Sumiyō Town, Amami Ōshima Is. on 12 July 2019, using High Intensity Discharge light traps by SS.

**Specimens examined, repositories and identification**

The examined specimens of *Colastomion* from Japan are deposited in the Institute for Agro-Environmental Sciences, NARO, Tsukuba, Japan (NIAES) and Osaka Museum of Natural History, Osaka, Japan (OMNH): 1 ♀, Naze-Ōaza-Chinase, Amami City, Amami Ōshima Is., Kagoshima Pref. (28°21’N, 129°26’E, 16 m alt.), 11.IV.2019, K. Sakagami leg. (by rearing host) (NIAES) [DDBJ–LC485659]; 1 ♀, Nishinakama, Sumiyō Town, Amami Ōshima Is., Kagoshima Pref. (28°15’47.3”N, 129°24’56.1”E, 120 m alt.), 12.VII.2019, S. Shimizu leg. (light trap) (NIAES) [DDBJ–LC499982]; 1 ♀, Mt. Yui-dake, Setouchi Town, Amami Ōshima Is., Kagoshima Pref., 24.VIII.2004, H. Makihara leg. (sweeping net) (OMNH); 1 ♂, Yona, Kunigami Village, Okinawa-hontō Is., Okinawa Pref., 29.VI.2013, S. Fujie leg. (sweeping net) (OMNH).

We identified *Colastomion* specimens based on Watanabe (1932, 1934), Tenma (2002), and Quicke et al. (2012): the specimens were morphologically similar to *C. formosanum*. To make sure that *Colastomion* specimens are *C. formosanum*, all the specimens were compared to photos of the Taiwanese specimens of *C. formosanum* deposited in the Senckenberg Deutsches Entomologisches Institut, Müncheberg, Germany (SDEI), which include the holotype of the species as follows: holotype ♂, Kankau, Changkou (Koshun), 22.IV.1912, H. Stauter leg.; 1 ♂, Kankau, Changkou (Koshun), VIII.1912, H. Stauter leg.; 1 ♀, Kankau, Changkou (Koshun), IV.1912, H. Stauter leg. The photos were provided by Taeger (2020), published online (https://doi.org/10.6084/m9.figshare.11984355.v1).

**Morphological observation, photo technique and terms**

Morphological observation was conducted using a stereoscopic microscope (SZ61, OLYMPUS, Tōkyō, Japan). Multi-focus photographs were taken using a single lens reflex camera (α7II, Sony, Tōkyō, Japan) with micro-lens (LAOWA 25 mm F2.8 2.5–5X ULTRA MACRO, Anhui Changgeng Optics Technology Co., Ltd, Hefei, China and A FE 50mm F2.8 Macro SEL50M28, Sony, Tōkyō, Japan). The photos were captured in RAW format and developed using Adobe Lightroom Creative Cloud. Then, they were stacked using Zerene Stacker and edited in Adobe Illustrator 2019. Morphological terms follow those of Quicke et al. (2012).
Figure 1. Leaves of *Turpinia ternata* Nakai rolled by a caterpillar of *Nevrina procopia* (Stoll).
Molecular technique

Part of the mitochondrial protein-coding cytochrome c oxidase 1 (CO1) gene, often referred to as “barcoding gene”, of two individuals of C. formosanum was sequenced for phylogenetic analysis. DNA was extracted from the right middle leg using the DNeasy Blood and Tissue Kit (Qiagen, Düsseldorf, Germany). For amplification, the following primers were used: LCO1490 (5’-GGTCAACAAATCATATAAGATATTGG-3’) and HCO2198 (5’-TAAACTTCAGGGTGACCAAAAAATCAA-3’) (Folmer et al. 1994). Polymerase chain reactions (PCR) were conducted using KOD FX NEO kit (Toyobo, Osaka, Japan), and PCR conditions were 94 °C for 2 min as initial denaturation, followed by 35 cycles of denaturation (10 sec at 98 °C), annealing (30 sec at 48 °C), and extension (30 sec at 68 °C), and then a final extension at 72 °C for 10 min. PCR product was purified using Illustra GFX kit (GE Healthcare Life Sciences, Marlborough, USA). The purified product was amplified with the same primers using the BigDyeTM Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Waltham, USA). Cycle sequencing products were purified using the 3.0 M sodium acetate, 95% ethanol, 70% ethanol, and Hi-Di formamide. Cycle sequencing reactions were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Waltham, USA), and the forward and reverse sequences were assembled using the DNA Dynamo Sequence Analyze Software (Blue Tractor Software, North Wales, UK). Finally, we deposited the obtained sequence to DNA Data Bank of Japan (DDBJ).

Phylogenetic analyses

In order to exclude the taxon sampling bias, a single sequence for each species was selected from 42 sequences of CO1 of the nine Colastomion species from Papua New Guinea and Benin deposited in the DNA databases (Quicke et al. 2012). Megarhogas maculipennis Chen & He and Myocron sp., closely related genera to Colastomion (Zaldivar-Riveron et al. 2008), were selected as outgroups. A total of 10 sequences from ingroup and two from outgroups were used for analyses (see Table 1).

Multiple sequence alignment was conducted in MAFFT v7.409 (Katoh and Toh 2008) using the L-INS-I algorithm. The aligned sequences were checked visually. Subsequently, they were manually optimized for phylogenetic analysis.

Using Bayesian Inference (BI) and maximum likelihood (ML) approaches, phylogenetic analyses were performed. Evolutionary models were determined using Kaksan4 v4.0 (Tanabe 2011). The best-fit models were selected based on the lowest corrected Akaike information criterion (AICc) for ML and the lowest Bayesian Information Criterion (BIC) for BI.

For the ML analysis, we used RAxML v8.2.10 (Stamatakis 2014) with 1,000 bootstrap replications, the codon separate model, and GTRGAMMA as a substitution model.
For the BI analysis, we used MrBayes v3.2.2 (Ronquist et al. 2012) with two independent runs of a Bayesian Markov chain Monte Carlo (MCMC) analyses of eight chains each, heating at 0.1, as well as random starting trees with trees sampled every 1,000th generations for 10,000,000 generations. If the average standard deviation of split frequencies was below 0.01, chain stationarity was checked with Tracer v1.6 (Rambaut and Drummond 2007) and two converged MCMC runs were considered adequate (Ronquist and Huelsenbeck 2003). The anterior half of the generations were discarded as a conservative burn-in and estimates were obtained for the harmonic means of the likelihood scores from the remaining half generations using the sump command. A final check of the convergence of the runs by the value of the potential scale reduction factor was conducted and a majority-rule consensus tree was obtained using the sumt command. The phylogenetic tree was edited using FigTree v1.4.3 (Rambaut 2006–2016) and Adobe Illustrator 2019.

We consider the node to be supported by either the Bayesian posterior probabilities (PP) > 0.95 or the bootstrap (BT) > 80%.

**Results**

**Rearing and mummy morphology**

One adult female of *Colastomion* emerged from a mummified final instar larva of *N. procopia* on 30 April 2019 (Figs 2, 3). The mummy remained unfixed within rolled leaves. It was mildly hardened, having a posterolateral and irregular, noncircular emergence hole (Fig. 4). Five adults of *N. procopia* emerged from unparasitized pupae, of which two emerged on 30 April, two on 8 May, and one on 9 May 2019.
Identification and adult morphology

All Japanese Colastomion specimens were identified as *C. formosanum* because of yellow face, antennae (although apical segments were brown) and legs (although middle and hind coxae and telotarsi were brown), sharply contrasting with brown mesosoma.
and metasoma (Fig. 2A, B); epicnemial area finely strigose, precoxal sulcus shallowly impressed and with some rugae, and pleural sulcus crenulate (Fig. 2C); notauli deep and weakly crenulate (Fig. 2D); propodeum rugose and with complete midlongitudinal carina (Fig. 2E); 1st metasomal tergite 1.7–1.8× longer than posteriorly wide; 2nd tergite as long as maximally wide (Fig. 2F); pterostigma entirely dark brown; fore wing cu-a postfurcal to 1-M (Fig. 3); and subquadrate vein 2-SC+R of hind wing (Fig. 3).

A female specimen from Taiwan shows a distinct protuberance on the base of the first metasomal tergite (Taeger 2020), whereas it is absent in males (including holotype) from Taiwan (Taeger 2020) and females and a male from Japan. The protuberance will probably be due to ontogenetic deformation in the individual. On the other hand, the sculpture of epicnemial area and precoxal sulcus tends to be stronger in females than in males, which is most likely a sexual variation.

**Phylogeny**

The Bayesian majority-rule consensus tree of *Colastomion* obtained from the CO1 sequences is shown in Figure 5. Both the BI and ML topologies were consistent with each other. *Colastomion formosanum* was identified as a sister group of the well-supported clade (*C. crambidiphagus* and *C. parotiphagus*) by both the BT and PP. Monophyly
of the clade of solitary parasitoids (C. crambidiphagus, C. formosanum, C. maclayi, C. parotiphagus, and C. wanang) plus C. pukpuk was good supported only by the PP. On the other hand, the clade of gregarious parasitoids (C. masalaii, C. gregarius, and C. madangensis) was not fully supported.

**Discussion**

We have found that *N. procopia* feeding on rolled leaves of *T. ternata* are the host species of *C. formosanum* in Japan. This supports the hypothesis of the host specificity of *Colastomion* to the crambids subfamily Spilomelinae as mentioned by Quicke et al. (2012), whereas the genus *Nevrina* of the tribe Udeini is a new host genus and the Staphyleaceae is a new host plant family for *Colastomion* (Table 2). Interestingly, Quicke et al. (2012) has reported that two solitary species of *Colastomion* use some species of crambid moths on a certain plant: *C. crambidiphagus* parasitizes several species of different tribes of Spilomelinae feeding only on the Convolvulaceae, and *C. parotiphagus* uses various host tribes of moth while mostly feeding on the Rubiaceae. Moreover, all gregarious species (*C. gregarius, C. madangensis, and C. masalaii*) consistently use Margaroniini on the Moraceae (Table 2). These may indicate that adult wasps search the host larvae by plant cues, such as herbivory-induced plant volatiles (Arimura et al. 2009) or oviposition-induced plant volatiles (Hilker and Fatoaros 2015). It is therefore important to understand the specificity of parasitoids not only in host insects but also in host plants.
Table 2. Host tribes (Crambidae: Spilomelinae), host plant families, and lifestyles of Colastomion. Sources: Quicke et al. (2012), except for C. formosanum. Systematics of host tribes follows Mally et al. (2019).

<table>
<thead>
<tr>
<th>Species</th>
<th>Host tribe</th>
<th>Host plant family</th>
<th>Lifestyle</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. crambidiphagus</td>
<td>Hydririni, Udeini</td>
<td>Convolvulaceae</td>
<td>Solitary</td>
</tr>
<tr>
<td>C. formosanum</td>
<td>Udeini</td>
<td>Staphyleaceae</td>
<td>Solitary</td>
</tr>
<tr>
<td>C. gregarius</td>
<td>Margaroniini</td>
<td>Moraceae</td>
<td>Gregarious</td>
</tr>
<tr>
<td>C. maclayi</td>
<td>Udeini</td>
<td>Rubiaceae</td>
<td>Solitary</td>
</tr>
<tr>
<td>C. madangensis</td>
<td>Margaroniini</td>
<td>Moraceae</td>
<td>Gregarious</td>
</tr>
<tr>
<td>C. masalaii</td>
<td>Margaroniini</td>
<td>Moraceae</td>
<td>Solitary/gregarious</td>
</tr>
<tr>
<td>C. parotiphagus</td>
<td>Agroterini</td>
<td>Malvaceae</td>
<td>Solitary</td>
</tr>
<tr>
<td>ditto</td>
<td>Margaroniini</td>
<td>Rubiaceae</td>
<td>Solitary</td>
</tr>
<tr>
<td>ditto</td>
<td>Unidentified</td>
<td>Lauraceae, Ulmaceae</td>
<td>Solitary</td>
</tr>
<tr>
<td>C. pukpuk</td>
<td>Unidentified</td>
<td>Rubiaceae</td>
<td>Unknown</td>
</tr>
<tr>
<td>C. wanang</td>
<td>Udeini</td>
<td>Myrtaceae, Vitaceae</td>
<td>Solitary</td>
</tr>
</tbody>
</table>

Colastomion formosanum (Figs 2, 3) is a solitary endoparasitoid that forms a hard mummy (Fig. 4). Solitary parasitism in Colastomion is considered the ancestral relative to gregarious parasitism because Megarhogas, which is closely related to Colastomion (and is included as an outgroup in our phylogenetic analyses), and most species in Rogadini are solitary (Quicke and Shaw 2005; Zaldívar-Riverón et al. 2008), while the phylogenetic placement of gregarious species is still unresolved (Fig. 5). The formation of a hard mummy as displayed by C. formosanum remains unusual within Rogadinae, although various types of host mummies appear to act as a protective roll during parasitoid metamorphosing (Zaldívar-Riverón et al. 2008; Maeto 2018). The hosts of most rogadine genera are killed as prepupae within host cocoons, which can protect the parasitoid larvae and pupae, and thus relatively frail mummies are the norm. In contrast, the hosts of genera that form hard mummies are killed in the larval stage, and the hardness of the mummies plays a vitally important protective role. In the case of C. formosanum, the host is killed in the larval stage probably because the immature host caterpillar is large enough for the parasitoid. Host stage of mummifying, which may vary according to relative size of host caterpillars to parasitoids, would therefore be relevant to the hardness of mummies.

Colastomion formosanum was originally described from Taiwan (as Formosa) (Watanabe 1932), later recorded from Hainan Is., China (Chen and He 1997), and was most recently recorded from Okinawa-hontô Is. and Iriomotejima Is., Japan (Tenma 2002). Our collection of C. formosanum from Amami Ôshima Is., Japan, has therefore expanded the northernmost border of the genus Colastomion as well as the species. It is most likely that C. formosanum has advanced northward from the tropics where species of Colastomion have highly diversified (Table 1; Fig. 5), although further investigations are needed to reveal the biogeographical history of Colastomion. C. formosanum is found only in the subtropical islands of China and Japan, whereas its host moth N. procopia is distributed much more broadly, from southern Japan to China, India, Papua New Guinea and western Africa (Swinhoe 1916; Chandra 1994; Nasu et al. 2013; Poltavsky et al. 2018). It would be interesting to know whether C. formosanum is truly an insular species of East Asia or is also further distributed in continental parts.
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