

# An integrative taxonomic study of north temperate *Cotesia* Cameron (Hymenoptera, Braconidae, Microgastrinae) that form silken cocoon balls, with the description of a new species

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## Abstract

Using CO1 sequence analysis, we investigated the relationships of Western Palearctic and Nearctic *Cotesia* that spin aggregated cocoons in the shape of a ball, and as adults are morphologically very similar. The analysis included the conceptual taxa *C. tibialis*, *C. ofella*, *C. vanessae*, *C. ruficrus*, *C. xyлина* and *C. yakutatensis*, as well as the newly described species *C. trivaliae* **sp. nov.** The examined specimens of *C. tibialis*, *C. ofella*, *C. vanessae*, *C. ruficrus* and *C. trivaliae* **sp. nov.** were collected in several European countries, and *C. xyлина* and *C. yakutatensis* in Canada and the USA. Molecular analyses showed that *C. ruficrus* is not closely related to the other studied taxa. Based on the genetic distances as well as biology and morphology, *C. vanessae* and *C. ofella* are confirmed as solid taxa. The species *C. yakutatensis* comprises two entities. Having 8 haplotypes, *C. tibialis* also emerges as a species complex, divided into two clusters. With 26 detected haplotypes, *C. xyлина* shows the highest diversity, being composed of three segregates. The

conceptual species *C. tibialis*, *C. xylyna* and *C. yakutatensis* seem to be species complexes containing several candidates for recognition as distinct species. One from the European *C. tibialis* complex is here described as new, and the impediments to be overcome before the description of further species are outlined.

### Keywords

DNA barcoding, genetic distance, hosts, species aggregates

## Introduction

*Cotesia* Cameron, 1891 is a large genus of parasitoid wasps with about 340 species described worldwide (Fernández-Triana et al. 2020). Like other members of the subfamily Microgastrinae (Hymenoptera: Braconidae), *Cotesia* species are koinobiont parasitoids of lepidopteran larvae, mostly those known as “macrolepidoptera”. The great majority oviposit into an early larval instar, though a few species oviposit into fully-developed eggs (e.g., *C. hyphantriae* Riley, 1887). Fully grown parasitoid larvae usually erupt from a later, often final, larval instar, but never from the host pupa (Shaw and Huddleston 1991). Many species of *Cotesia* (including those discussed in the present paper) are gregarious parasitoids, but a substantial proportion are solitary (e.g., *C. vestalis* (Haliday, 1834)) (Shaw 2007; Gupta and Fernández-Triana 2014). Since *Cotesia* species attack caterpillars, some of which are serious pests in agroecosystems, several species have been used as biological control agents, e.g., *C. flavipes* Cameron, 1891 against *Busseola fusca* (Fuller, 1901) and *Sesamia calamistis* (Hampson, 1910) (Overholt et al. 1997) and several other cases (e.g., Jiang et al. 2004; van Driesche 2008; Avila et al. 2013).

Cocoon-spinning by *Cotesia* species usually takes place externally on, under, around, or near the dying caterpillar, which can live several days after the emergence of the parasitoid larvae (Shaw and Huddleston 1991). The larvae of some gregarious *Cotesia* species such as those in the *C. tibialis* (Curtis, 1830) group, and *C. vanessae* (Reinhard, 1880), make typical ball-shaped silken cocoon masses (Nixon 1974). The erupting parasitoid larvae cooperate in spinning a communal web that encloses their individual, separately spun but connected cocoons. Sometimes caterpillars are induced to additionally cover the parasitoid cocoon mass with their own silk; for example, the hosts of the unrelated *C. glomerata* (Linnaeus, 1758) (Brodeur 1992).

There is a major problem in properly understanding the host repertoires of parasitoids based on the published literature that has been uncritically compiled in sources such as Taxapad (Yu et al. 2016), because the many sources of error and accumulated misinformation (discussed by many authors, including Shaw 1994, 2023; Noyes 1994 and, in relation to Microgastrinae in particular, Fernández-Triana et al. 2020) go unrecognised and severely obscure reality. Taxapad is an extremely valuable resource, but it was not designed to give reliable host information. Here we largely ignore data from Taxapad (which, for the commonest taxon of this study, *C. tibialis*, is so bloated with unreliable data as to suggest a host repertoire of more than a hundred Lepidoptera species over as many as 17 families), but instead give only host information that we believe

to be reliable. One of the commonly collected apparent species that make cocoon masses that, to a greater or lesser extent, look like fluffy balls is *C. tibialis*. The cocoon mass is usually about 10–20 mm in diameter, depending especially on the number of parasitoid larvae that spin the cocoon (VŽ personal observation). *Cotesia tibialis* is widespread throughout the Palaearctic region and has already been considered to possibly be a complex of species, including two seasonal forms identified by Nixon (1974). This issue is discussed in detail by Lazarević et al. (2022). The colour of the cocoons is variable; they can be almost white to yellowish. Morphological variation in the adults is also considerable and may have contributed to the existence of many supposed synonyms, but it also suggests the possibility of as yet unrecognised additional species. Confirmed records refer to hosts belonging to owlet moths (Noctuidae), especially those from the major subfamily Noctuinae (*sensu lato*). The hosts (Noctuinae unless indicated) recorded several times come from caterpillars feeding in low vegetation in the following genera: *Agrotis* Ochseneimer, 1816, *Autographa* Hübner, 1821 (Plusiinae), *Lacanobia* Billberg, 1820, *Mamestra* Ochseneimer, 1816, *Noctua* Linnaeus, 1758, *Orthosia* Ochseneimer, 1816, *Xestia* Hübner, 1818 and *Xylena* Ochseneimer, 1816 (Yu et al. 2016).

Morphologically and by cocoon architecture, *Cotesia ofella* (Nixon, 1974) is very similar to *C. tibialis*. There are not many published data on the hosts for this parasitoid, but certainly its host repertoire includes noctuid species; in this case, *Acronicta aceris* (Linnaeus, 1758), *A. rumicis* (Linnaeus, 1758) (Nixon 1974; Razowski and Wiackowski 1999), *A. auricoma* (Denis & Schiffermüller, 1775) (MRS, unpublished) as well as *Simyra dentinosa* Freyer, 1838 (Karimpour et al. 2001), both genera from the subfamily Acronictinae. Beside these noctuids, Nixon (1974) noted *Spilosoma lubricipeda* (Linnaeus, 1758) (Erebidae) as a host of *C. ofella*, but this may be an error resulting from morphological similarity of that caterpillar to certain low-feeding Acronictinae larvae. The cocoon masses of *C. ofella* are mostly yellowish, sometimes intensely yellow, which may be useful in some cases for preliminary discrimination from other species, for example between *C. tibialis* and *C. ofella* (VŽ personal observation).

Another species morphologically similar to *C. tibialis* is *Cotesia berberis* (Nixon, 1974). Nothing is known about the biology of this rarely found species, recorded from just three countries (Nixon 1974; Papp 1986, 1987); although the ball-shaped cocoon mass of the type series was collected on *Berberis* sp. (Berberidaceae) (Nixon 1974) it is by no means certain that the host had fed on that plant. The outer layer of the cocoon mass is spun from yellowish silk and resembles the late summer forms of *C. tibialis*. The cocoon texture is somewhat looser than in *C. tibialis*. Due to the lack of fresh material, we are unable to consider this species further.

Unlike the previous three species, *C. vanessae* is predominantly recorded from some Nymphalidae (Nymphalini) as well as certain Noctuidae. Definite summer hosts are caterpillars of the nymphalids *Aglais urticae* (Linnaeus, 1758), *Vanessa atalanta* (Linnaeus, 1758) and *V. cardui* (Linnaeus, 1758) (Nixon 1974; Shaw et al. 2009), while winter hosts are noctuids (Nixon 1974; Hervet et al. 2014). The cocoon mass is a white ball of silk that is usually dense enough to fully conceal the individual cocoons

within. Literature data indicate that *C. vanessae* is widespread in the Palaearctic region (including North Africa), but it has also been recently recorded in the Nearctic region as a parasitoid of pest species of plusiine noctuids in greenhouses and fields in southern Ontario and Alberta, Canada (Hervet et al. 2014; Fernández-Triana et al. 2020).

*Cotesia ruficrus* (Haliday, 1834), a taxon with cosmopolitan distribution recorded in all regions, spins a cocoon mass that more weakly conceals the individual cocoons but overlaps with the above species in the host repertoire. In the north temperate area, this parasitoid is frequently recorded from the pest noctuids *Helicoverpa armigera* (Hübner, 1808), *Leucania loreyi* (Duponchel, 1827), *Mythimna separata* Walker, 1865 and *Spodoptera exigua* (Hübner, 1808), but it undoubtedly has a much wider host repertoire (MRS, unpublished).

In the Nearctic region, there are at least two more conceptual species that make ball-like cocoon masses, *C. xyliana* (Say, 1836) and *C. yakutatensis* (Ashmead, 1902). Hosts parasitized by *C. xyliana* include the following species of Noctuidae: *Mamestra configurata* Walker, 1856, (Wylie and Bucher 1977) *Peridroma saucia* (Hübner, 1808) (Roberts et al. 1977; Marsh 1979), *Xestia c-nigrum* (Linnaeus, 1758) (Muesebeck 1921; Marsh 1979), with records from *Epiglaea apiata* (Grote, 1874) and *Xylena nuptera* (Lintner, 1874) also probably reliable (Franklin 1950). The other Nearctic species, *C. yakutatensis* has a narrower recorded host range, including the plusiine noctuids *Autographa californica* (Speyer, 1875) (Muesebeck 1921; Clancy 1969; Marsh 1979; Miller and West 1987), *Autoplusia egea* (Guenée, 1852) (Clancy 1969; Marsh 1979), *Trichoplusia ni* (Hübner, 1803) (Miller and West 1987) and the noctuid *Xestia c-nigrum* (Linnaeus, 1758) (Marsh 1979).

Considering the morphological resemblance of these seven nominal *Cotesia* species, similar architecture of the cocoons, as well as sometimes overlapping host repertoires, it is of interest, and also importance in view of the biocontrol potential of some taxa in this group, to examine their relationships and especially their integrity.

## Material and methods

### Collecting and processing specimens

Samples were mostly collected from cocoon masses (Fig. 1) spun and attached to the top of stems of various low plants, often grasses. Part of the parasitoid material was obtained by rearing parasitized caterpillars that were identified, while most of the parasitoids emerged from cocoons that were collected in the field without caterpillars present. Consequently, host information is unknown in these cases. The vast majority of *Cotesia* specimens were stored in 2 ml plastic microtubes filled with 96% ethyl alcohol for later molecular analyses. Individuals included in the molecular analysis are listed in Suppl. material 1: table S1. Selected specimens of both sexes were dried and glued to cardboard, and some were dissected in a Berlese mounting medium to study details of morphological structures. Identification of the European specimens using



**Figure 1.** Aggregated ball-like cocoon **A** cooperative work of all parasitoid larvae in spinning the cocoon mass (*C. vanessae* ex *Aglais urticae*) **B** spun cocoon mass (*C. tibialis* ex *Mythimna conigera*).

morphological characters is mostly based on Nixon's (1974) work, whereas identification of the North American material was mostly based on Muesebeck (1921).

Photographs were taken using a Leica Flexacam C3 on a Leica M165C stereomicroscope with a magnification of 7.3 $\times$ . For micrographs, we used a Leica DFC490 camera (Leica Microsystems, Wetzlar, Germany), adapted to a microscope Leica 2500 (Leica Microsystems, Wetzlar, Germany), at a total magnification of 5.0–20.0 $\times$ . The equipment is in the Laboratory of Zoology at the Faculty of Sciences and Mathematics, Department of Biology and Ecology, University of Niš, Serbia.

Our material is deposited in the collections of the Faculty of Sciences and Mathematics, University of Niš, Serbia; the Naturalis Biodiversity Center, Leiden, The Netherlands; the National Museums of Scotland, Edinburgh, UK; and the Canadian National Collection of Insects, Arachnids and Nematodes, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada. Many of the specimens whose sequences were obtained from public databases are housed in depositories less accessible to us and, partly for that reason but also because many lack host data, a detailed morphological analysis of all segregates has been postponed until such a time that it can be done in conjunction with both DNA barcoding and host data.

### DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from whole specimens of parasitoids using Dneasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. For material reared from hosts, caterpillar identity was confirmed by DNA barcoding following the emergence of parasitoids. The genomic DNA used for analysis was extracted from the caterpillar head.

Taxonomic relationships among the relevant species within the genus *Cotesia* were investigated using sequence data of the barcoding region of the mitochondrial cytochrome oxidase subunit I (CO1). Standard primer pairs LCO1490/HCO2198 in combination with other primers (Table 1) were used to amplify barcoding CO1 fragments. Each PCR reaction was carried out in a volume of 20  $\mu$ l, including 1  $\mu$ l of

**Table 1.** The list of primers used to retrieve barcoding fragments of CO1 in *Cotesia* samples.

Primer name	5' primer sequence 3'	Primer direction	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	Forward	Folmer et al. (1994)
HCO2198	TAAACTTCAGGCTGACCAAAAAATCA	Reverse	
LCO1490puc	TTTCAACWAATCATAAAGATATTGG	Forward	Cruaud et al. (2010)
HCO2198puc	TAAACTTCWGGRTGWCCAAARAATCA	Reverse	
LCO1490hem	TTTCAACTAAYCATAARGATATYGG	Forward	Germain et al. (2013)
HCO2198hem	TAAACYTCDGGATGBCCAAARAATCA	Reverse	
Aph2Fd	ATAATTGGWGGATTTGGWAATTG	Forward	Mitrović and Tomanović (2018)
Aph2Rd	GTWCTAATAAAAATTAATWGCWCC	Reverse	
Lys2Rd	GTWCTAATAAAAATTAATTGCHCC	Reverse	
Pr2Fd	ATAATTGGAGGRTTTGGWAATTG	Forward	

extracted DNA, 11.8  $\mu\text{l}$   $\text{H}_2\text{O}$ , 1  $\mu\text{l}$  of each primer 0.5  $\mu\text{M}$ , 2  $\mu\text{l}$  High Yield Reaction Buffer A with  $1\times\text{Mg}$ , 1.8  $\mu\text{l}$  of  $\text{MgCl}_2$  2.25 mM, 1.2  $\mu\text{l}$  of dNTP 0.6 mM, 0.2  $\mu\text{l}$  DNA polymerase 0.05 U/ $\mu\text{l}$ . The amplification protocol included: i) initial denaturation for 5 min at 95 °C; ii) 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 30 sec at 72 °C; and iii) final extension at 72 °C for 7 min. Amplified products were run on 1% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator. Barcoding CO1 fragments were sequenced using automated equipment (Macrogen Europe, Amsterdam, the Netherlands).

In addition, 63 sequences of CO1 barcoding fragments of *Cotesia* parasitoids were obtained from the public databases GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and the Barcoding of Life Data Systems (BOLD, <http://www.boldsystems.org/>) and included in the molecular analysis (Suppl. material 1: table S1). Many of the specimens concerned (indicated by codes starting with the letters MRSJFT) are in NMS, whereas specimens with codes starting with the CNC, CNCHYM, HYM, MIC and WMIC are in the CNC. Specimens with other codes are mostly deposited in the Canadian Center for DNA Barcoding and in those cases, not all determinations could be checked.

## Molecular analyses and tree constructions

Sequenced CO1 fragments were manually edited in FinchTV ver. 1.4.0 (<https://digitalworldbiology.com/FinchTV>) and aligned using the ClustalW program integrated into MEGA5 (Tamura et al. 2011). According to the obtained Akaike Information Criterion scores, the best fit model for the estimation of evolutionary divergence was the Tamura-Nei model (Tamura and Nei 1993). The Bayesian inference analysis was done by running two Markov Chain Monte Carlo searches each with one cold and three heated chains, for 3 million generations (Fig. 2 and Suppl. material 1: fig. S1) and 1,2 million generations (Fig. 3), sampling every 100 generations. The first 7,500 trees (Fig. 2 and Suppl. material 1: fig. S1) and 3,000 trees (Fig. 3) were discarded as a burn-in. The average standard deviation of split frequencies was below 0.01. The Bayesian tree was constructed using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck

2003). The program FigTree 1.3.1. was used to view the consensus tree with posterior probabilities (Rambaut 2006–2009). To root the tree, we used the reference barcode sequence of *Glyptapanteles pallipes* (Reinhard, 1880) (GenBank Acc. No. [KJ459198](#)) as the outgroup within the same subfamily. For the far outgroup, which belongs to a different braconid subfamily, we used the sequence of *Aphidius sussi* Pennachio & Tremblay, 1989 (GenBank Acc. No. [MT432023](#)).

## Haplotype analysis

CO1 sequences were aligned, trimmed to the same size of 501 bp (a compromise to achieve uniformity for a large number of sequences), and analysed for haplotype diversity and evolutionary distances. The haplotype data file was generated using DnaSP ver. 5.10.01 (Librado and Rozas 2009). A median-joining haplotype network (Bandelt et al. 1999) was constructed using the PopART program (<http://popart.otago.ac.nz>).

## Terminology for description of the new species

Terminology of body characters follows van Achterberg (1993). For wing venation terminology follows Nixon (1965, 1974). The length of the first metasomal tergite (T1) and the length of the discoidal segments in the fore wing (1CU1 + 2CU2) are measured linearly as the shortest distances, not as total curvature length; for T1 it is the distance between the base and the apex.

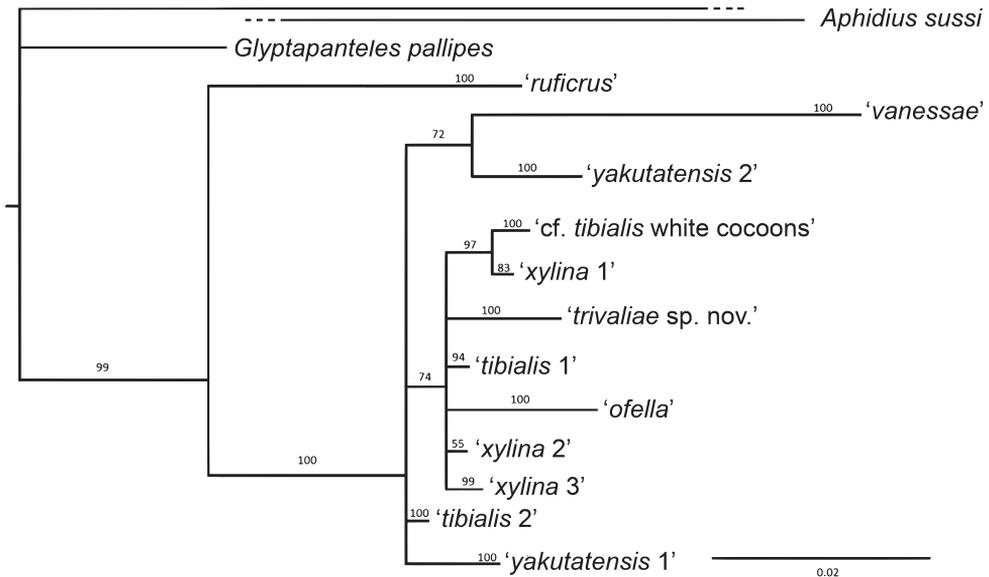
## Results

Preliminary morphological sorting into possible taxa is reflected in Suppl. material 1: table S1. Molecular analysis including 105 CO1 barcoding sequences of different *Cotesia* specimens segregated them into 12 distinct groups (Fig. 2, Suppl. material 1: table S1, fig. S1), which were assigned the following provisional names: ‘*tibialis* 1’; ‘*tibialis* 2’; ‘cf. *tibialis* white cocoons’; ‘*xylina* 1’; ‘*xylina* 2’; ‘*xylina* 3’; ‘*Cotesia* sp.’ (= *trivaliae* sp. nov.); ‘*ofella*’; ‘*yakutatensis* 1’; ‘*yakutatensis* 2’; ‘*vanessae*’ and ‘*ruficrus*’.

From the trimmed CO1 sequences, 56 haplotypes were determined with 108 variable sites detected (Suppl. material 1: table S1).

All three Bayesian inference trees (Figs 2, 3, Suppl. material 1: fig. S1) and the Median-joining network (Fig. 4), showed a general delineation of 12 groups of CO1 barcode haplotypes. Three sequences, determined as *C. ruficrus* originating from Serbia, were separated from the remaining *Cotesia* as a distinctive group we named ‘*ruficrus*’, supported by 100-posterior probability (Fig. 2 and Suppl. material 1: fig. S1). The two haplotypes identified within the ‘*ruficrus*’ group (H1, H2) are distant 1.5%, while they are clearly distinguished from all other groups (Table 2).

Four sequences determined as *C. vanessae* originating from Spain (3) and Canada (1) clustered together and formed the ‘*vanessae*’ group. Two haplotypes, H3 and



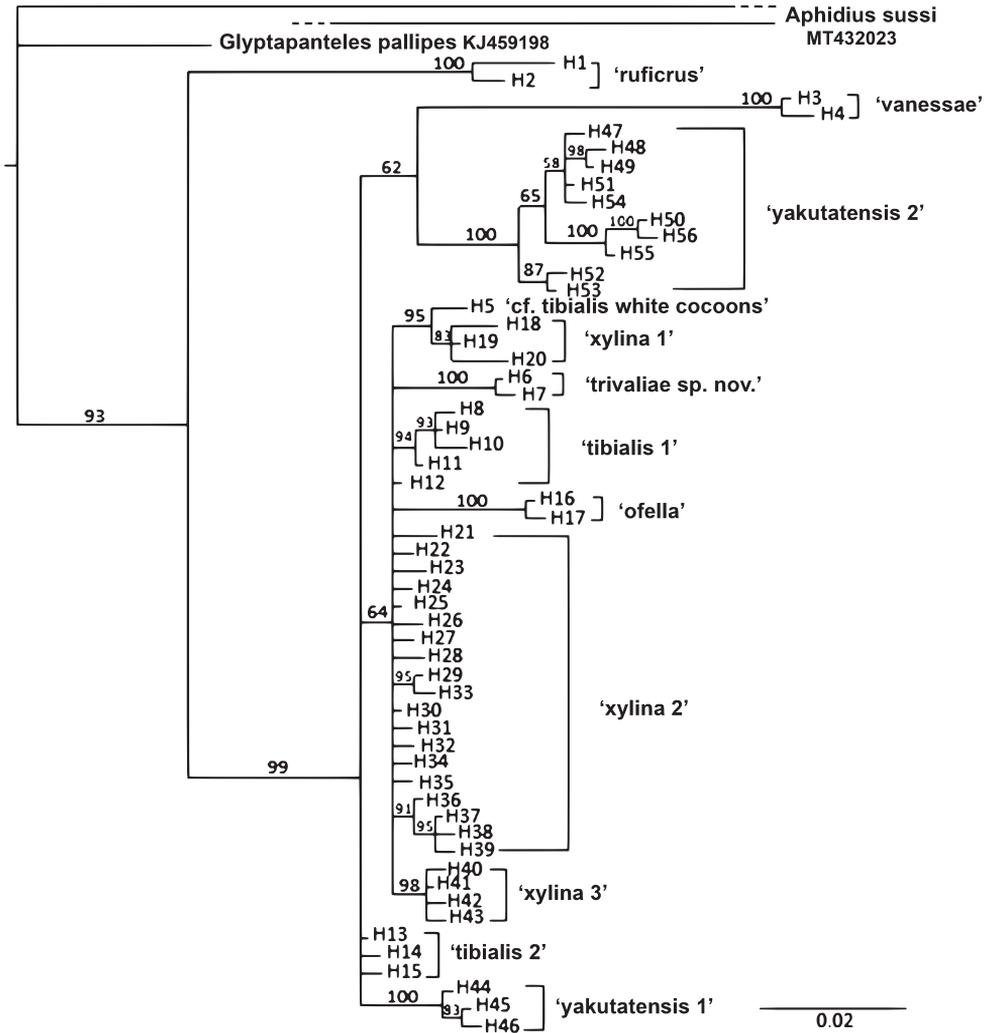
**Figure 2.** A condensed Bayesian tree inferred from the CO1 barcoding fragments of *Cotesia* specimens. Bayesian posterior probabilities are shown above branches; scale bar indicates substitutions per site (0.02). Potential scale reduction factors (PSRF) were all approximately equal to one. Description of *Cotesia* CO1 barcode sequences included in the analysis is given in Suppl. material 1: table S1. Outgroups: *Aphidius sussi* – Acc. No. MT432023; *Glyptapanteles pallipes* Acc. No. KJ459198.

**Table 2.** Average between-groups genetic distances (%) calculated using the Tamura-Nei method.

Group	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]
[1] ' <i>ruficrus</i> '											
[2] ' <i>vanessae</i> '	11.5										
[3] 'cf. <i>tibialis</i> white cocoons'	9.3	6.8									
[4] <i>trivaliae</i> sp. nov.	8.7	7.1	3.0								
[5] ' <i>tibialis</i> 1'	8.6	6.5	1.4	2.3							
[6] ' <i>tibialis</i> 2'	7.8	6.4	1.8	2.3	1.1						
[7] ' <i>ofella</i> '	8.4	7.8	2.8	3.3	3.0	2.9					
[8] ' <i>xylina</i> 1'	9.3	6.9	1.1	3.1	1.6	1.9	2.9				
[9] ' <i>xylina</i> 2'	8.6	6.5	1.8	2.3	1.2	1.1	2.9	1.9			
[10] <i>xylina</i> 3'	8.6	6.5	1.8	2.0	1.2	1.1	2.7	1.9	1.2		
[11] ' <i>yakutatensis</i> 1'	7.7	6.8	3.3	3.1	3.1	2.2	4.0	3.4	3.0	3.0	
[12] ' <i>yakutatensis</i> 2'	8.5	6.9	4.3	4.4	4.1	3.2	5.1	4.4	4.0	4.0	3.4

H4 were identified, with a genetic distance of 0.4% (Fig. 3). This group undoubtedly separates from other groups, with an average distance ranging from 6.4 to 11.5% (Table 2).

*Cotesia* 'cf. *tibialis* white cocoons' consisted of sequences extracted from three independent samples originating from Poland. Since all three sequences were identical, they appear on the tree under the same haplotype (H5). The average genetic distance of



**Figure 3.** A Bayesian tree inferred from the *Cotesia* CO1 barcoding haplotypes. Bayesian posterior probabilities are shown above branches; scale bar indicates substitutions per site (0.02). Potential scale reduction factors (PSRF) were all approximately equal to one. Description of *Cotesia* haplotypes is given in Suppl. material 1: table S1. Outgroups: *Aphidius sussi* – Acc. No. MT432023; *Glyptapanteles pallipes* Acc. No. KJ459198.

this group from 'vanessae', 'ruficrus', '*Cotesia* sp.' (= *trivaliae* sp. nov.), 'ofella', 'yakutatensis 1' and 'yakutatensis 2' is relatively large (Table 2). However, lower distances were detected in comparison to 'tibialis 1', 'tibialis 2', 'xylina 1', 'xylina 2' and 'xylina 3'.

Sixteen CO1 sequences of specimens from Serbia (3), Austria (3), Slovenia (2), Finland (1), Germany (1) and Poland (6), initially determined as *Cotesia* cf. *tibialis* (3) or *C. tibialis* (12), were grouped within the same clade 'tibialis 1'. Five haplotypes (H8, H9, H10, H11, H12) were identified with in-group average distance of 0.4%. This

group separates from '*ruficrus*', '*vanessae*', '*ofella*', '*yakutatensis* 1' and '*yakutatensis* 2' in a range from 3.0 to 8.6% (Table 2). Lower genetic distances were detected in comparison with 'cf. *tibialis* white cocoons' and '*Cotesia* sp.', as described above, including '*tibialis* 2', '*xylina* 1', '*xylina* 2' and '*xylina* 3'.

Eight barcode CO1 sequences originating from Serbia, two from the Netherlands and one from Austria clustered within the '*tibialis* 2' group (Suppl. material 1: table S1). Three haplotypes (H13, H14, H15) were detected, with an average distance of 0.3%. The '*tibialis* 2' group can undoubtedly be discriminated from '*ruficrus*', '*vanessae*', '*ofella*' and '*yakutatensis* 2' (Table 2). On the other hand, low genetic distances were determined in comparison with '*Cotesia* sp.', 'cf. *tibialis* white cocoons', '*tibialis* 1', '*xylina* 1', '*xylina* 2', '*xylina* 3' and '*yakutatensis* 1' (Table 2).

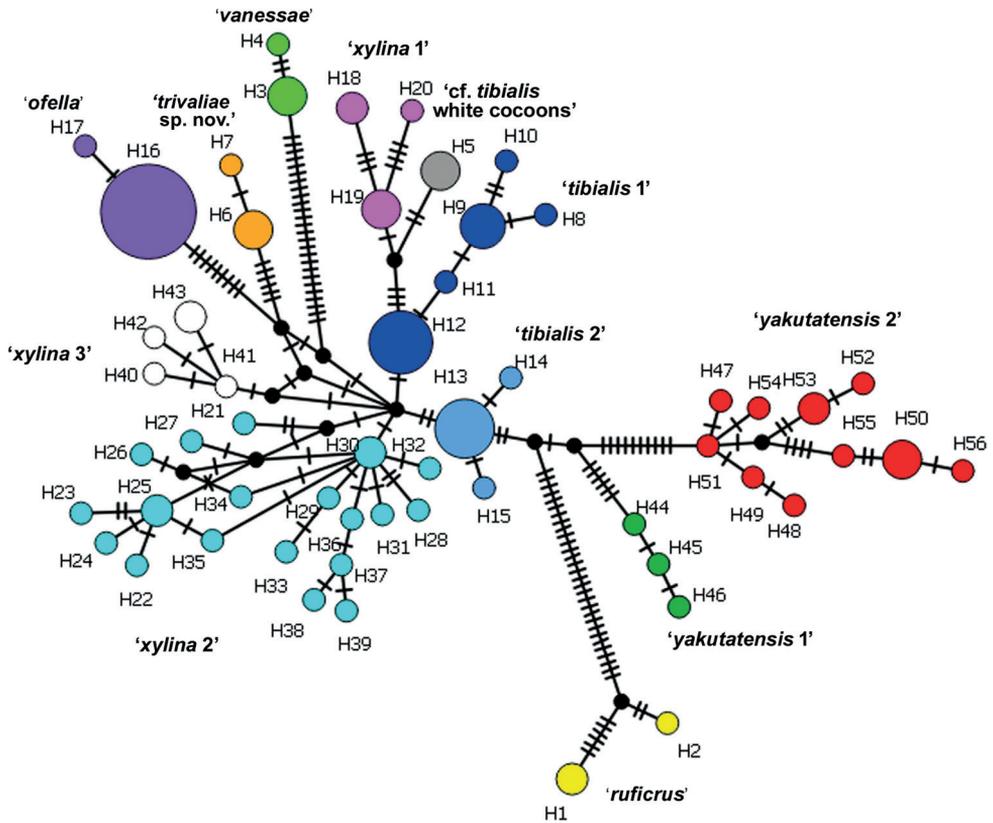
With only one haplotype (H5), 'cf. *tibialis* white cocoons' is inserted into the '*tibialis*'/'*xylina*'/'*ofella*' part of the tree. Comparing the average genetic distances between this group and all '*tibialis*' and '*xylina*' segregates, 'cf. *tibialis* white cocoons' is closest to '*xylina* 1' with an average genetic distance of 1.1%, then to '*tibialis* 1' with an average genetic distance of 1.4%, and equally distant from the groups '*tibialis* 2', '*xylina* 2' and '*xylina* 3' with an average genetic distance of 1.8%.

Within the '*ofella*' group there are two haplotypes (H16, H17) that differ among themselves with an average distance of 0.2%. This group of haplotypes is separated from other groups with a range of 2.9 to 8.4% (Suppl. material 1: fig. S1, Table 2).

Analysis of 32 specimens initially determined as *C. xyлина*, originating from Canada (31) and the USA (1) revealed the separation of barcode sequences into three groups to which the following names were assigned: '*xylina* 1', '*xylina* 2' and '*xylina* 3' (Fig. 2 and Suppl. material 1: fig. S1). Within the group '*xylina* 1' three haplotypes were determined (H18, H19, H20) with an average distance of 0.9%. The '*xylina* 2' clade encompasses 19 haplotypes (from H21 to H39) which differ among them on average 0.7%. Four haplotypes (H40, H41, H42, H43) were recognized in the '*xylina* 3' group with an average genetic distance of 0.3%. Average distances between the three groups were as follows: '*xylina* 1' vs '*xylina* 2' 1.9%; '*xylina* 1' vs '*xylina* 3' 1.9%; '*xylina* 2' vs '*xylina* 3' 1.2%.

Barcode sequences of 16 specimens determined as *C. yakutatensis* originating from Canada (15) and the USA (1) were analysed. In total, 13 haplotypes were determined (Suppl. material 1: table S1), separated into two clades on the Bayesian tree (Fig. 3) and MJ network (Fig. 4). One branch was assigned the name '*yakutatensis* 1' which is comprised of three haplotypes (H44, H45, H46) (Suppl. material 1: table S1). The rest of the haplotypes (H47 to H56) clustered as a separate group '*yakutatensis* 2' (Suppl. material 1: table S1; Figs 3, 4). The average genetic distance between haplotypes in the '*yakutatensis* 1' group is 0.3% and in the '*yakutatensis* 2' group 1%. The average distance between the two *yakutatensis* groups was 3.4% (Table 2).

Seven sequences clustered together revealing a new taxon *Cotesia trivaliae* sp. nov., described below. This includes three sequences from Poland (host unknown) and one from Slovenia, ex *Orthosia* sp. (Suppl. material 1: table S1) from specimens that could



**Figure 4.** Median-joining network designed for 56 CO1 haplotypes of different *Cotesia* segregates. Black dots are median vectors representing the missing unsampled intermediary haplotype(s). Mutational steps are marked with short black lines. Haplotypes H1, H2 (yellow circles) = '*ruficrus*'; H3, H4 (light green circles) = '*vanessae*'; H5 (grey circle) = '*cf. tibialis* white cocoons'; H6, H7 (orange circles) = '*trivaliae* sp. nov.'; H8–H12 (blue circles) = '*tibialis* 1'; H13–H15 (light blue circles) = '*tibialis* 2'; H16, H17 (purple circles) = '*ofella*'; H18–H20 (pink circles) = '*xylina* 1'; H21–H39 (turquoise circles) = '*xylina* 2'; H40–H43 (white circles) = '*xylina* 3'; H44–H46 (green circles) = '*yakutatensis* 1'; H47–H56 (red circles) = '*yakutatensis* 2'.

not initially be determined morphologically with sufficient certainty and were designated as '*Cotesia* sp.' Additionally, out of three reared series (ex *Orthosia gracilis* (Denis & Schiffermüller, 1775)) of this species from Scotland, and a non-reared series from England (Suppl. material 1: table S1), two were initially determined as *C. tibialis*, and two as *Cotesia cf. tibialis*. Molecular analysis clustered these specimens within the '*C. trivaliae* sp. nov.' group. Two haplotypes (H6, H7) were identified with a genetic distance of 0.2%. This particular group is distinguished from other groups with a genetic distance ranging from 2.0 to 8.7%. Lower average distances were detected in comparison with '*tibialis* 1', '*tibialis* 2', '*xylina* 2' and '*xylina* 3' (Table 2). Also, this group differs from *C. ofella* by 3.3%.

## Description of the new species

### *Cotesia trivaliae* Žikić & Shaw, sp. nov.

<https://zoobank.org/734CD2C6-46EF-4443-8427-2E52A48B5D5D>

Figs 5, 6

**Diagnosis.** The new species shares some morphological similarities with *C. tibialis*, including the shape of the first metasomal tergite (T1) laterally, with a medial keel near the proximal part, and the apical truncation of the hypopygium (features used by Nixon (1974) to characterise *C. tibialis*). Additionally, the coloration of the legs, particularly the presence of a dark spot on the apical part of the otherwise reddish yellow hind femora, is similar to that described by Nixon (1974) for the early summer brood of his concept of *C. tibialis*, and there is a spine (albeit extremely weak) on the fifth segment of the fore tarsus. The ovipositor sheath is as short as in *C. tibialis*, and the aedeagus is similarly shaped in the two species. However, *C. trivaliae* sp. nov. can be clearly distinguished from *C. tibialis* based on the following characteristics: male antenna is completely brown in *C. trivaliae* sp. nov., while in *C. tibialis* 4–5 apical segments are yellowish. The length index of the 1<sup>st</sup> and the 2<sup>nd</sup> part of discoides (1-CU1) / (2-CU1) of fore wings is about 0.7 in *C. trivaliae* sp. nov., relative to *C. tibialis* where this ratio is 0.8–0.9. Dorsally, (T1) length/width index ranges from 0.9 to 1.1 in *C. trivaliae* sp. nov., while it is 1.2–1.3 in *C. tibialis*. Communal cocoons in *C. trivaliae* sp. nov. are spun differently from those of *C. tibialis*, also *C. ofella*. In *C. tibialis* and *C. ofella* (Fig. 6D–F), they are fluffy to varying degrees, and individual cocoons are not visible because they are covered with very densely spun communal silk. Consequently, the structure appears as a ball mass 15–25 mm in length and about 10 mm in width. Cocoon masses of *C. trivaliae* sp. nov. are more oblong, usually 10–15 mm long and 5–6 mm wide, and much less fluffy, with at least some individual cocoons visible through sparsely spun silk. The average genetic distance revealed above between *C. trivaliae* sp. nov. and other morphologically similar taxa indicates that it is a different species. The only known host of *C. trivaliae* sp. nov. is *Orthosia gracilis*, substantially different from the known hosts of the close taxa, although it must have other as yet undiscovered hosts to enable it to complete its annual cycle.

**Type material.** *Holotype*: POLAND ♀; Kampinos National Park, Granica; 05.VI.2018; ex cocoon mass in grassland; V. Žikić leg.; dry mounted. Paratypes: POLAND 41 ♀ 29 ♂; Kampinos National Park, Granica; 05.VI.2018; ex same brood (3 ♀ 3 ♂ dry mounted, 3 ♀ 3 ♂ microscopic slide mounted, 1 ♀ barcoded, the rest kept 34 ♀ 23 ♂ in 96% alcohol); POLAND 40 ♂; Kampinos National Park, Granica, 05.VI.2018, (1 ♂ barcoded, 3 ♂ dry mounted, 1 ♂ microscopic slide mounted, 35 ♂ in 96% alcohol); “same data as for preceding” 15 ♀ 12 ♂ (1 ♀ barcoded, 3 ♀ 3 ♂ dry mounted, 3 ♀ 1 ♂ microscopic slide mounted, 8 ♀ 8 ♂ in 96% alcohol); “same data as for preceding” 54 ♀ 6 ♂ (1 ♀ barcoded, 3 ♀ 3 ♂ dry mounted, 3 ♀ 1 ♂ microscopic

slide mounted, the rest kept in 96% alcohol); V. Žikić leg.; SLOVENIA 1 ♀; Ljubljana; ex *Orthosia* sp.; 01.VI.2018; Š. Modic leg.; (1 ♀ barcoded, slide mounted); GREAT BRITAIN 28 ♀ 16 ♂ Scotland, Berwickshire, Foulden ex *Orthosia gracilis*, collected 10.VI.2017, (17 ♀ 5 ♂ barcoded; 8 ♀ 11 ♂ barcoded; 3 ♀ from a further brood of low emergence; VII.2017; M. R. Shaw leg.; GREAT BRITAIN 1 ♀ 1 ♂; Scotland, Fife, Fleecefauld, ex *O. gracilis* collected 14.VII.2012, emerged 04.VIII.2012 (1 ♀ 1 ♂ barcoded); M. R. Shaw leg.; GREAT BRITAIN 3 ♀; England, Kent, Swanscombe, ex cocoon mass collected on *Phragmites australis* 10.IV.2017, emerged 02.V.2017 (3 ♀ barcoded) M. Jennings leg.

**Depositories.** The holotype ♀ of *C. trivaliae* sp. nov., and paratypes have been deposited in the collection of the Faculty of Sciences and Mathematics, the University of Niš, Serbia. A single female from Slovenia has been deposited in the collection of the Faculty of Sciences and Mathematics, the University of Niš, Serbia. Additionally, 3 ♀ 3 ♂ from Poland, as well as all specimens from Great Britain have been deposited at the Department of Natural Sciences, National Museums of Scotland.

**Etymology.** The new species is named in honour of the gothic rock band Trivalia.

**Distribution.** The currently known distribution of the new species is Poland, Slovenia and the UK (England and Scotland).

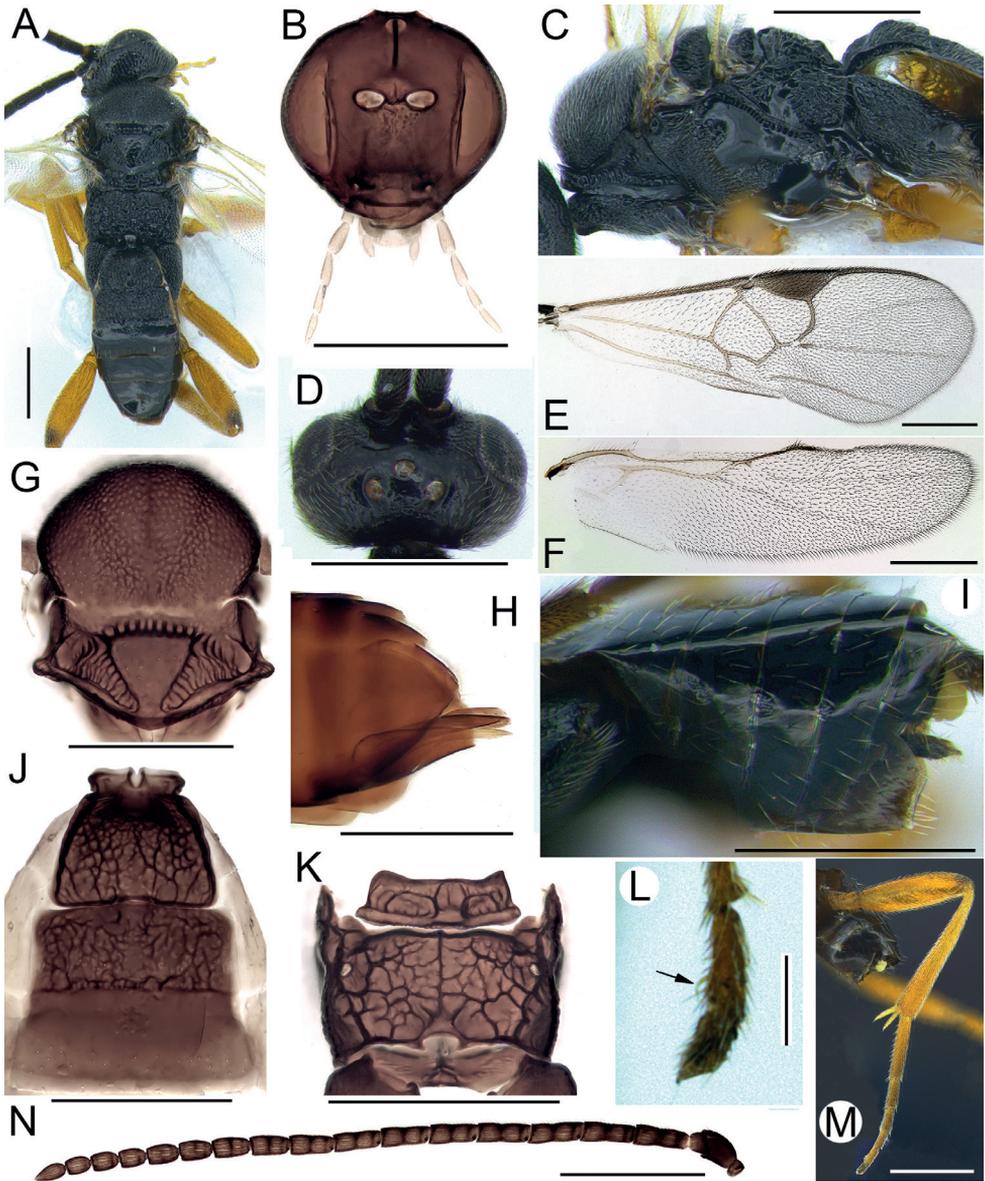
**Description. Female:** (Fig. 5). Body length 2.6 mm (range 2.5–2.7 mm) (Fig. 5A).

**Head:** In frontal view (Fig. 5B), about 1.6 times as wide as long (from widest eye to eye), temple about 0.9 times as long as eye at first narrowing behind roundly, ocelli in moderately low triangle (Fig. 5D), diameter between anterior/posterior ocelli and between posterior ocelli 0.5. Index of intertentorial distance/tentori-ocular distance = 2.7. Face above clypeus smooth, while below antennal sockets sculptured. Stemmaticum and vertex smooth. Antennae (Fig. 5N) as long as body, 1<sup>st</sup> flagellar segment as long as 2<sup>nd</sup>, penultimate segment 2.2 times shorter than 1<sup>st</sup>, length/width of penultimate segment = 1.1, last segment 1.4 times longer than penultimate, pointed at apex.

**Mesosoma:** Mesoscutum in dorsal view (Fig. 5G) punctate, middle part slightly rugose, prescutellar sulcus deep, with 9 foveae. Scutellum smooth and shiny, with about thirty punctures. Propodeum (Fig. 5K) strongly rugose, median keel completely developed. Mesopleuron (Fig. 5C) in larger part shiny, marginally sculptured bearing dense setae, mid-mesoscutal line large with 24–25 deep punctures. Metapleuron rugose, small lower area smooth and shiny.

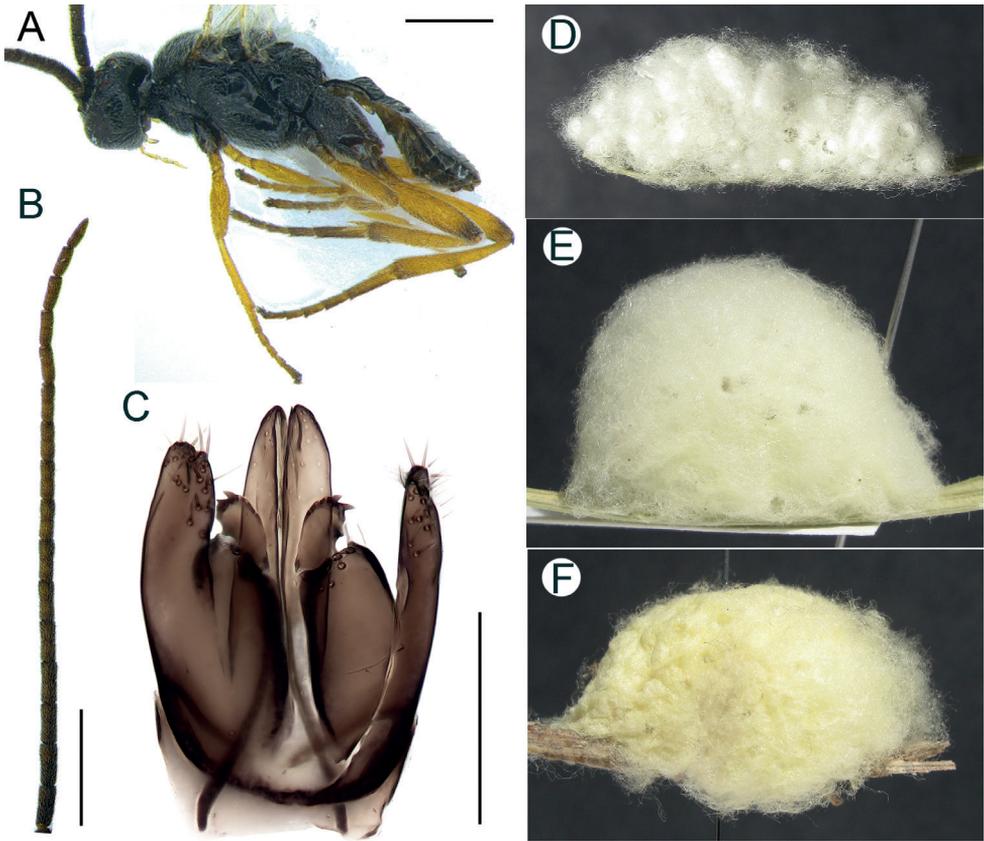
**Legs:** Fore leg spine on 5<sup>th</sup> tarsomere present, extremely weak; hard to see (Fig. 5L). Hind leg (Fig. 5M) with femur 3.9 times as long as wide, tibial spurs subequal, inner spur not reaching middle of hind basitarsus.

**Wings:** Fore wing length 2.5 mm (range 2.4–2.5 mm), and 2.5 times as long as wide at maximal distances (Fig. 5E), pterostigma 2.2 times longer than wide, metacarp (1-R1) about as long as pterostigma, 1<sup>st</sup> part of discoideus (1-CU1) about 0.7 times as long as 2<sup>nd</sup> (2-CU1), vein (cu-a) 0.4 times as short as (CU1), with posterior third bent forward. Hind wing 4.3 times as long as wide at maximal distances, vannal lobe straight without setae in mid part (Fig. 5F).



**Figure 5.** *Cotesia trivaliae* sp. nov. female **A** habitus **B** head frontal view **C** mesosoma and T1 lateral view **D** head dorsal view **E** fore wing **F** hind wing **G** mesoscutum dorsal view **H** ovipositor lateral view **I** metasoma lateral view **J** metasoma dorsal view (T1-T3) **K** propodeum dorsal view **L** 5<sup>th</sup> tarsomere of front leg, arrow points spine **M** hind leg outer face **N** antenna. Scale bars: 500 µm (**A–K, M, N**); 100 µm (**L**).

**Metasoma:** In dorsal aspect T1 heavily rugose all over, widened behind (Fig. 5J), almost as long as wide, in profile T1 bearing conspicuous short medial keel (Fig. 5C). Second tergite (T2) rectangular, 0.4–0.5 times as long as wide, sculptured, posterior margin crenulate (Fig. 5J). Third tergite (T3) almost equal to second, smooth, shiny,



**Figure 6.** *Cotesia trivaliae* sp. nov., male (**A–C**) **A** habitus **B** antenna **C** aedeagus ventral view; cocoon masses (**D–F**) **D** *C. trivaliae* sp. nov. **E** *C. tibialis* **F** *C. ofella*. Scale bars: 500 µm (**A**, **B**); 100 µm (**C**).

slightly sculptured at mid base, bearing sparsely distributed hairs practically in a single row (Fig. 5J). Hypopygium in lateral view short, truncate at apex (Fig. 5I). Last tergite in line with apex of hypopygium (Fig. 5I). Ovipositor sheath short (Fig. 5H).

**Colour:** Head, mesosoma including tegula, all coxae and metasoma black. Trochanter brownish, rest of leg parts yellowish; hind femora apically with small dark spot. Antennae brown. Palpi yellow. Wing venation distinctly yellowish to brown, metacarp, pterostigma and radialis brown.

**Male:** (Fig. 6 A–C). Morphology and colouration (Fig. 6A) similar to female except for sexual characters. All antennal segments (Fig. 6B) brown, antenna about 1.2 times as long as body. Aedeagus (Fig. 6C) with two-toothed digiti at apex, teeth well developed, relatively large and sharp, digitus length/width ratio = 2.1, digitus 2.8 times as short as volsella, cuspis inconspicuous.

**Cocoon mass:** (Fig. 6D). Relatively small, usually elongated, 10–15 mm long, and about 5–6 mm wide, weakly fluffy, communal silk sparsely spun over individual cocoons leaving them partly visible, light-yellow coloured.

## Discussion

Analysis of the barcode sequences of selected parasitoid species from the genus *Cotesia* shows clear separation of some taxa, as well as close relatedness among a significant number of others. What is common to the presently analysed species of *Cotesia* that make ball-like cocoon masses is that all of them parasitize members of the family Noctuidae, probably exclusively except for *C. vanessae* (*C. ruficrus* is considered to be outside this strict grouping, see below). The Bayesian tree (Figs 2, 3, Suppl. material 1: fig. S1) and the Median-joining network (Fig. 4) clarified somewhat a separation of 12 provisional clades to which names were assigned: ‘*ruficrus*’; ‘*vanessae*’; ‘*ofella*’; ‘cf. *tibialis* white cocoons’; ‘*Cotesia* sp (= now *trivaliae* sp. nov.)’; ‘*yakutatensis* 1’; ‘*yakutatensis* 2’; ‘*tibialis* 1’; ‘*tibialis* 2’; ‘*xylina* 1’; ‘*xylina* 2’; ‘*xylina* 3’.

Molecular analyses confirmed evident delineation as separate entities for the haplotypes of the specimens determined as *C. ruficrus*, *C. vanessae*, *C. ofella*, *C. trivaliae* sp. nov. (see below) and, for *C. yakutatensis*, two entities ‘*yakutatensis* 1’ and ‘*yakutatensis* 2’. The large genetic difference between *C. ruficrus* and the other *Cotesia* is reflected in as many as 30 mutations from the bifurcation spot, as shown in the haplotype network (Fig. 4). Too little sampling of the probably much more complex *C. ruficrus* has been done to comment on its integrity in a wider context.

All the *Cotesia* species studied here exclusively use noctuid larvae to complete their annual life cycle except *C. vanessae* which (at least in Europe) parasitises a restricted group of hosts from the Nymphalidae (Nymphalinae) during summer. These, however, do not overwinter in the larval stage, and *C. vanessae* critically depends, like the others, on overwintering as larvae inside overwintering noctuid larvae that either diapause or feed only sporadically through the winter, especially low-feeding or sub-surface resting species in the subfamilies Noctuinae and Plusiinae. It seems possible that the summer hosts of *C. vanessae* reflect an extension from its ancestral host repertoire. *Cotesia vanessae* differs from the other 11 defined groups with a genetic distance in the range of 6.4–7.8%.

In the case of *C. yakutatensis* specimens of North American origin included in this analysis, there is a clear discrimination between ‘*yakutatensis* 1’ and ‘*yakutatensis* 2’ as two separate lineages. Their within-group genetic distances are significantly lower than the between-group distance (Table 2; Figs 2–4). Considering that this parasitoid may have a narrow host repertoire (but see below), with only four species recorded (see details above in the Introduction section), this finding is potentially of interest for morphological re-examination and further investigation of possible host-associated genetic divergence patterns. This is important if *C. yakutatensis* is ever to be tested as a potential biological control agent since one of its reported hosts is *Autographa californica*, a pest of great economic importance in Canada and the USA (Vail et al. 1989). It should, however, be borne very much in mind that the rearing of parasitoids from their hosts in N. America is in its infancy in comparison with European efforts, and the apparently narrow host repertoire of *C. yakutatensis* (*sensu lato*) is quite likely an artifact due to low sampling; furthermore, none of the sequenced specimens was reared from known hosts.

DNA barcoding showed no clear discrimination between the specimens from groups 'tibialis 1', 'tibialis 2', 'cf. tibialis white cocoons', 'xylina 1', 'xylina 2' and 'xylina 3'. *Cotesia tibialis* is already considered to be a complex of species with variable morphology of the body, wings and cocoon (e.g., Nixon 1974). Considering the many synonyms related to *C. tibialis*, as well as Nixon's attempt to decipher this species complex, the results we obtained indicate that in addition to the morphological variability observed, it is indeed a genetically very variable taxon, which forms three groups in our analyses (Fig. 2 and Suppl. material 1: fig. S1). In addition, this is also confirmed by the many haplotypes detected in this group. Furthermore, these three *C. tibialis* groups were mixed with three more groups that were formed from the taxon previously identified as *C. xylina*. In this analysis, *C. xylina* was found to have even more haplotypes than *C. tibialis*, as many as 26; even though some specimens may have been misidentified, it is also likely that *xylina* comprises a complex of species. Unfortunately, none of the barcoded specimens had a known host. The DNA barcodes for *C. xylina* were taken from public databases which had been obtained from specimens sampled in Canada and the USA, from different localities, sometimes separated by significant geographical distances. In short, *C. xylina* appears to be a genetically diverse species, in a way similar to *C. tibialis*. Although these two species inhabit different geographical regions, the genetic distances between them are generally small (Table 2). The clade 'cf. tibialis white cocoons' resulting from white cocoons collected in Poland differs from 'xylina 1' (Canada) which also spins white cocoons, by only 1.1%. Such a low percentage of genetic distance often indicates merely intraspecific variability. Unfortunately, the available material was collected from already-spun cocoon masses found on grass and conclusions on the closeness of these two groups/species are troublesome without knowing their hosts.

There is a definite separation between the groups 'tibialis 1' and 'tibialis 2'. Thus 'tibialis 1' is connected with 'cf. tibialis white cocoons' and 'xylina 1', whereas 'tibialis 2' is genetically close to the segregates 'xylina 2' and 'xylina 3'. Based on the admittedly small number of identified caterpillars for the samples included in the analysis, it seems that this separation is reflected by the hosts. While 'tibialis 1' was reared from several hosts (the overwintering hosts *Noctua interposita* (Hübner, 1790) and *Xestia xanthographa* (Denis & Schiffermüller, 1775) both collected in Austria, and the summer hosts *Cucullia chamomillae* (Denis & Schiffermüller, 1775) from Finland and *Anarta myrtilli* (Linnaeus, 1761) from Germany), 'tibialis 2' was reared only from *Mythimna conigera* (Denis & Schiffermüller, 1775) collected in Serbia.

Finally, within *Cotesia tibialis* (*sensu lato*), we found a third group 'cf. tibialis white cocoons'. It stands in a common lineage with 'xylina 1' but is distant from the other 'xylina' groups and also 'tibialis 2', though still not enough to be identified as a potentially new taxon (Table 2) and we are further hampered by lacking precise data on the hosts.

*Cotesia ofella* could be discriminated as a separate lineage, being closest to *C. trivaliae* sp. nov. (Fig. 2 and Suppl. material 1: fig. S1). Unlike the great haplotype diversity found in *C. tibialis*, *C. xylina*, and *C. yakutatensis*, the species *C. ofella* molecular data is less variable. This narrowly oligophagous species was obtained from

several low-feeding hosts that belong to the subfamily Noctuinae (*Noctua comes* (Hübner, 1813) and *N. fimbriata* (Schreber, 1759)), and the subfamily Acronictinae (*Acronicta rumicis* and *Simyra albovenosa* (Goeze, 1781)). In addition, there are several series in the National Museums of Scotland from *Acronicta auricoma*, another low-feeding species, but these specimens have not been sequenced and so are not included in this study. However, it is clear that Acronictinae are the summer hosts and Noctuinae the overwintering ones. The analysed 20 samples were collected in several distant territories such as France, Great Britain, Hungary, Poland, Slovenia, and several spots in Serbia (Suppl. material 1: table S1). Regardless of its wide distribution and several host species from which it was reared, only two barcode haplotypes were detected differing by a single mutation (Fig. 4).

Seven specimens clustered in the separate group initially marked as ‘*Cotesia* sp.’ but described above as *C. trivaliae* sp. nov. are the only ones collected from caterpillars of the genus *Orthosia* (the single species *O. gracilis*, which is one of the few in the genus that is low-feeding rather than arboreal). Based on the general morphology of the adult body and the architecture and colour of the cocoon, they were originally identified as, or close to, *C. tibialis*. They are closely related to the *tibialis*–*xylina* groups, but genetically, morphologically, and biologically distinct enough to be described as a new species. It is crucial that we are able to describe this species as new because it differs morphologically from the concept of *C. tibialis* and synonyms as defined by their type specimens, and also because our reared material presents a consistent host repertoire that (although possibly incompletely known) is distinct. But the situation regarding the other probable new species involved in the *C. tibialis*, *C. xyлина* and *C. yakutatensis* complexes is far more problematic. In the first place, we have no molecular data for the relevant type specimens that would be needed to fix the use of available names, and neither permissions nor resources to try to obtain that. Second, biological data for the specimens analysed genetically is almost entirely lacking, so the important consideration of host repertoire differences cannot be addressed at this time. We recognise that rearing fresh material is a necessity for further progress, with next-generation techniques to obtain DNA from the old specimens in collections deemed to have reliable host data a further possibility – and we hope that this preliminary study will prompt and help to enable that. Third, a thorough morphological study of the sequenced specimens (and the relevant types, and reared specimens beyond our own) is necessary; but it is premature to conduct that until we can incorporate more reared material that still needs to be obtained. Thus, our study is held at this preliminary phase, but it is hoped that our results will promote further research that is clearly warranted.

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## Supplementary material I

### Supplementary information

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Data type: docx

Explanation note: **table S1.** The list of *Cotesia* specimens submitted to the molecular analyses. **fig. S1.** A Bayesian tree inferred from the CO1 barcoding fragments of *Cotesia* specimens.

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