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Eretmocerus iulii Laudonia et Melone sp. n.: parasitoid associated with *Aleurocanthus spiniferus*

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Abstract

A meticulous study has led to description and identification of a novel member of the aphelinid genus *Eretmocerus* as a species associated with nymphs of the orange spiny whitefly, *Aleurocanthus spiniferus* (Quaintance) (Hemiptera Aleyrodidae). The detailed morphological analysis of the parasitoid has revealed distinctive characteristics that differentiate it from known species within the genus. Molecular analysis has been conducted to provide additional evidence to the morphological analyses. Molecular data obtained from DNA sequencing of the mitochondrial COI and 28S ribosomal genes has been compared with sequences from existing databases, revealing a genetic divergence from other available *Eretmocerus* species and genetically defining the studied entity. The integrated methodology employed in this study enabled the delineation of a new species within the *Eretmocerus serius* Silvestri group, *Eretmocerus iulii* Laudonia et Melone sp. n. (Hymenoptera Aphelinidae).

Key words: Chalcidoidea, *Eretmocerus serius* group, taxonomy, natural enemy, sustainable pest management, orange spiny whitefly.

Introduction

The aphelinid genus *Eretmocerus* Haldeman 1850 (Hymenoptera Chalcidoidea Aphelinidae) currently contains 90 species (Noyes, 2019), all well-known as primary parasitoids of whiteflies (Hemiptera Aleyrodidae) (Zolnerowich and Rose, 2008). Adult females, equipped with a curved tip of the ovipositor, deposit their eggs outside the body of whitefly nymphs between the ventral part of the hosts and the upper blade of the leaves of the host plants (Gerling *et al*., 1998). The larvae develop as solitary ecto-endoparasitoids and pupate in the mummified fourth instar nymphs. The adults emerge through a typical exit hole in the host integument, at the dorsal level of the parasitized nymph. The entrance hole of the 1st instar parasitoid larva remains visible on the ventral side of the abandoned puparium of the host, thus calling, for brevity, what remains of the fourth instar nymph completely emptied by the parasitic attack (Rose and Zolnerowich, 1997; Gerling *et al*., 1998; Zolnerowich and Rose, 2008).

Aleurocanthus spiniferus (Quaintance 1903) (Hemiptera Aleyrodidae), the orange spiny whitefly (OSW), originating from China and South and Southeast Asia, is one of the important pests infesting mainly *Citrus* spp. and more than 100 plant species belonging to different botanical families (Porcelli, 2008; Bubici *et al*., 2020; Nugnes *et al*., 2020). This species is widely present in the tropical and subtropical regions of the Old World, often overlapping in some areas with the distribution of *Aleurocanthus woglumi* Ashby 1915. In Europe, *A. spiniferus* and *Aleurocanthus camelliae* Kanmiya et Kasai 2011 are the only species attributed to the genus *Aleurocanthus* Quaintance et Baker 1914 (Nugnes *et al*., 2020; CABI, 2021; Rizzo *et al*., 2021). However, only OSW is considered a quarantine pest for the European area (EU, 2019) and, currently, it is spreading across all countries of the Mediterranean Basin (Cioffi *et al*., 2013; Šimala and Masten Milek, 2013; Radonjić *et al*., 2014; Bariselli *et al*., 2019; Kapantaidaki *et al*., 2019; Rapisarda and Longo, 2021; EPPO, 2024). Due to the difficulties of phytosanitary control methods of *A. spiniferus*, it is essential to investigate and develop new procedures for biological control of this harmful organism following a more eco-sustainable pest management. Among the natural parasitoids of OSW cited in the literature, several species of the genus *Encarsia* Forster 1878 were reported (Clausen *et al*., 1978; Nakao and Funasaki, 1979; Noyes, 2019; CABI, 2021), along with *Eretmocerus*. Within the latter genus, *Eretmocerus serius* Silvestri 1927 and *Eretmocerus serius* var. *orientalis* Silvestri 1927 (Silvestri, 1927), later redescribed as *Eretmocerus orientalis*, were the only known species associated with *A. spiniferus* (Gerling, 1969).

A new association of an *Eretmocerus* species with *A. spiniferus* was recently reported for Europe (Melone *et al*., 2024). The preliminary taxonomic study, conducted on numerous specimens, demonstrated that they represent an undescribed species, clearly differentiated from all the other described species of *Eretmocerus*, and it is attributable to the *serius* group (*sensu* Gerling, 1969). This study provides a morphological description, a key for species discrimination, and molecular characterization of this new species, representing the first report of an OSW parasitoid in Europe.

Materials and methods

Sampling of hosts and collection of parasitoids

Samplings were conducted in five localities in Italy (table 1) on *Citrus limon* (L.) Burm. F. and *Citrus* × *aurantium* L. No phytosanitary treatment had been carried out on the plants in previous years. Thirty fully developed

Table 1. Information about the specimens involved in this study, the respective mt-haplotyping results and the sequence accession numbers. CE: Caserta Province; NA: Naples Province; AP: Ascoli Piceno Province.

Specimen code	Sex	Location	Coordinates	Date of record	Accession Number COI	Accession Number 28S-D2	Mt-Haplotype
AA_580	♀	Bari	41°06'40.0"N 16°52'53.1"E	14.VII.23	PP844640	PP849179	hA
AA_581	♀	Casagiove (CE)	41°04'42.5"N 14°19'00.4"E	31.V.23	PP844641	PP849180	hA
AA_582	♀				PP844642	PP849181	hA
AA_684	♂				PP844648	PP849187	hA
AA_685	♀				PP844649	PP849188	hA
AA_584	♂	Palermo	38°07'48.7"N 13°19'40.3"E	30.IX.23	PP844643	PP849182	hA
AA_585	♂				PP844644	PP849183	hB
AA_586	♂				PP844645	PP849184	hC
AA_682	♀	Portici (NA)	40°48'55.4"N 14°21'03.8"E	05.VI.23	PP844646	PP849185	hA
AA_683	♀				PP844647	PP849186	hD
AA_686	♂	Grottammare (AP)	42°59'19.6"N 13°52'09.4"E	09.VIII.23	PP844650	PP849189	hA
AA_687	♀				PP844651	PP849190	hA

OSW-infested leaves were collected from newly developed shoots during each sampling. The collected leaves were stored in a thermal bag in sealed, labeled plastic bags and transported or sent to the laboratories of the Institute for Sustainable Plants Protection (Portici, Italy) of the National Research Council (CNR-IPSP) and of the Department of Agricultural Science of the University of Naples Federico II (UNINA), to perform more in-depth analyses.

Each leaf was observed under a stereomicroscope searching for parasitized stages of OSW. The black integument of the host makes identifying active parasitization difficult; therefore, apparently healthy 4th-instar nymphs were collected by detaching them from the leaves and placing them in gelatin capsules (13.59 mm × 5.57 mm) as cohorts of 5-10 specimens. They were stored in Petri dishes at 25 ± 2 °C, 65 ± 10% RH and 16:8 L:D.

The isolated samples were checked once a day and any emerged parasitoids were collected in individual labeled Eppendorf tubes and killed by freezing at −20 °C.

Among the 120 emerged parasitoids, 75 were females and 45 males, all attributable to the *serius* group of *Eretmocerus* due to the peculiar characteristics of the female antenna (Silvestri, 1927; Compere, 1936; Gerling, 1969; Hayat, 1998).

For subsequent taxonomic studies under the optical microscope, specimens were preserved air dried and in 70% ethanol. The newly emerged specimens designated for molecular analysis were preserved in absolute ethanol.

Morphological analysis

Specimens used for taxonomic studies were mounted on glass slides using Canada balsam phenol as a permanent medium (Noyes, 1982; Ravikumar *et al*., 2014). Each was numbered and reported on the label. Morphological terminology and format for species descriptions follow Rose and Zolnerowich (1997) (see supplemental material).

Observations and measurements were acquired using a Leica DMLS microscope. The measurements were carried out on 20 females and 3 males, all taken in Casagiove (Caserta, Italy) between 31 May and 1 June 2023. Means (µm) and related standard deviations were then calculated and the ratios were used for specific discrimination. Photos were taken with an Axiocam HRC digital camera attached to a Zeiss Axiophot 2 microscope (Carl Zeiss, Oberkochen, Germany). CombineZP® software was employed to combine multiple planes of focus in order to obtain fully-focused images.

Molecular analysis

Genomic DNA extraction from individual samples listed in table 1 was performed following a non-destructive Chelex-proteinase K protocol (Gebiola *et al*., 2009) with the following modifications: DNA extraction buffer consisted of 7 µl of proteinase K (20 mg/ml) and 50 µl of 5% Chelex 100 resin suspension, incubation at 55 °C lasted 3 hours, and 40 µl of supernatant was recovered. Extracted DNA was stored at −20 °C until use. After DNA extraction, each sample was rinsed with distilled water and mounted on slides for morphological examination using the methods previously described for preparation (see additional material in results).

Based on genetic databases GenBank and BOLD (www.ncbi.nlm.nih.gov/genbank/) (www.boldsystems.org), (last accessed on 31 December 2023), the mitochondrial cytochrome c oxidase subunit I (COI) (for an amplicon size of ~ 600 bp) was sequenced. Primer pair LepF1-LepR1 (Hajibabaei *et al*., 2006) was used to obtain COI fragments with the thermocycling conditions as in Nugnes *et al*. (2017). Furthermore, the ribosomal gene 28S was sequenced with primers ITS2f-D2Rev with PCR conditions as in de Benedetta *et al*. (2022). All PCRs were performed on an Eppendorf Mastercycler Nexus X2 thermocycler (Eppendorf, Milan, Italy) and amplicons were directly sequenced (BMR Genomics, Padova, Italy). Obtained sequences were manually edited by comparing two-sense chromatograms using BioEdit 7.0 software (Hall, 199) and COI sequences were virtually translated using EMBOSS Transeq (www.ebi.ac.uk/Tools/st/emboss_transeq/) to amino acids to detect frameshift mutations and nonsense codons.

The edited sequences were compared with each other and within the genetic databases GenBank and BOLD (last

Figure 1. *Eretmocerus iulii* Laudonia et Melone holotype slide.

accessed on 15 May 2024). MEGA software - version 10.2.4 (Stecher *et al*., 2020) was employed to calculate sequence distances and standard errors based on uncorrected *p*-distance of mt COI. Groups were based on the sampling site.

Results

Eretmocerus iulii Laudonia et Melone sp. n.

Morphological analysis

Type material:

Holotype ♀ on slide n. 9 on the label (figure 1), (ITALY: Campania, Caserta Province, Casagiove, 31.V.2023, from *Aleurocanthus spiniferus* Quintance, coll. G. Melone). Paratypes: 19 ♀♀ and 3 ♂♂ on slides, same references as holotype, some were collected on 01.VI.2023. Additional material: 7 ♀♀ and 5 ♂♂ on slides, same references as reported in table 1.

The holotype and 18 paratypes (15 ♀♀ and 3 ♂♂) are deposited in the entomological collection of the Department of Agricultural Sciences - Division of Biology and Protection of Agricultural and Forest Systems (BIPAF), University of Naples "Federico II", Portici, Italy. Furthermore, 2 ♀♀ paratypes at the University of California, Riverside, California, USA (UCR); 2 ♀♀ paratypes at the National Museum of Natural History, Washington, D.C., USA (USNM). All measures are available in the supplementary file deposited at https://github.com/Frank-Nug/Eretmocerus-iulii.git.

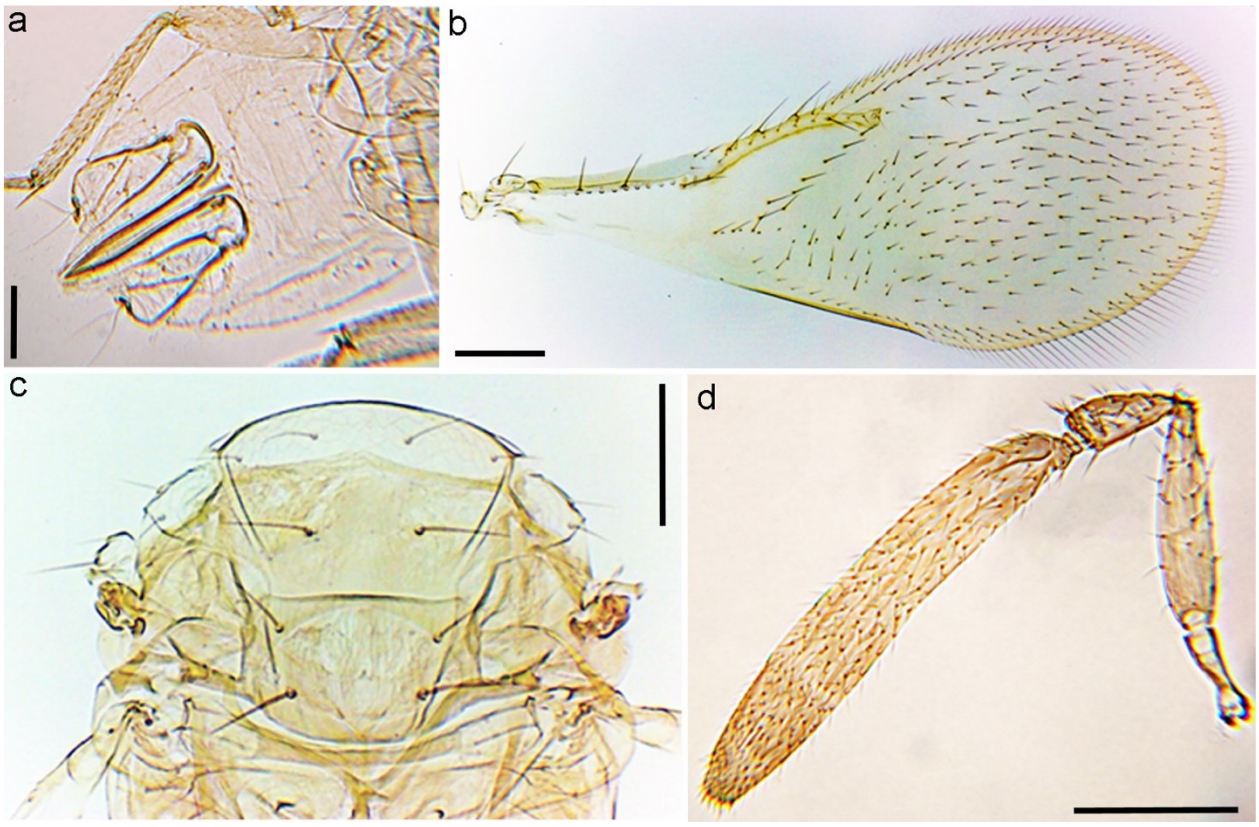

Figure 2. *Eretmocerus iulii* female. **a)** ovipositor; **b)** forewing; **c)** thorax; **d)** antenna. Bars: 100 µm.

Description:

Female. Body yellow, legs pale yellow with tarsal segments slightly darker; wings hyaline. Head yellow with grey-green eyes and red ocelli. Antenna slightly darker than head. Ovipositor dark yellow.

Body length: 1.06 mm (± 0.09) (n = 20); holotype: 1.09 mm. Antenna (figure 2d) 0.60 mm (± 0.05) in length (n = 5); radicle 3.32× (± 0.39) longer than wide; scape 4.31× (± 0.30) as long as wide, 2.51× (± 0.18) the radicle length, 2.06× (± 0.11) the pedicel length, and 0.45× (± 0.01) club length; pedicel 2.28× as long as wide (± 0.19), 1.22× (± 0.10) length of radicle, 0.49× (± 0.02) length of scape, 0.22× (± 0.01) club length. First funicular segment extremely short and ring-like; second funicular segment short and sub-trapezoidal, wider at apex. Club subcylindrical, narrowed at apex, 6.56× (± 0.16) as long as wide, 2.22× (± 0.07) length of scape, and 4.58× (± 0.24) length of pedicel.

Mesoscutum (figure 2c) 0.72× (± 0.03) as long as wide, anteriorly with reticulate sculpture, laterally and posteriorly with substrigulate sculpture; usually 3 pairs of setae are present on mid lobe, only one specimen shows an extra seta on the rear left side (n. 3 on the label). Sidelobe of mesoscutum each with 2 setae and substrigulate sculpture; axilla with substrigulate sculpture and 1 seta. Scutellum with elongate reticulate sculpture medially and substrigulate sculpture laterally, 2 pairs of setae and 2 placoid sensilla. Propodeum with substrigulate sculpture. Endophragma usually reaching the posterior half of second gastral tergite.

Forewing (figure 2b) is 2.10× (± 0.07) longer than the maximum width of disc and 8.23× (± 0.71) the longest posterior fringe. Wing basal area asetose, while the distal portion of the costal cell with 2-6 setae, usually 3 on the left wing and 4 on the right one. Marginal vein 1.56× (± 0.05) longer than the stigmal vein, usually with 2 prominent setae, although in one specimen, 3 setae on the right wing and 2 on the left one were found (n. 15 on the label); 9-14 cilia arranged in a row are present between the apex of the stigmatic vein and the posterior part of the wing, delimiting the linea calva above. Linea calva with 6-13 tubercles posteriorly and arranged on the ventral surface of the wing. The remaining part of the wing disc, excluding the setae present along the entire margin of the wing, covered by approximately 170-250 short setae. The mid-tibial spur (figure 2a) is 0.37× (± 0.02) shorter than the respective basitarsus. The ovipositor is slightly exerted (figure 2a), 0.74× (± 0.02) the length of the club, 1.63× (± 0.07) the scape length, and 0.87× (± 0.03) the length of mid-tibia (figure 2a).

Male. Similar in colour to female. Body length: 0.91 mm (± 0.05) (n = 3). Antenna typical for the genus, 3-segmented, with club 4.62× (± 0.16) the length of the scape.

Forewing 2.09× (± 0.02) as long as the maximum width disc and the maximum width of the disc is 6.50× (± 0.08) the longest posterior fringe. Wing basal area asetose and the distal portion of the costal cell usually with 2-4 setae. Marginal vein 1.65× (± 0.01) as long as the stigmal vein, with 3-4 prominent setae, but the character appears to be unstable. Of the three specimens examined, the first has 3 prominent setae on both wings, the second has 4 prominent setae on the left wing and 3 on the right one, and the last has 4 prominent setae on both wings.

Etymology

The name is in reference to Stefania Laudonia's beloved grandson, Giulio.

Taxonomic notes

This taxonomic study, conducted on several specimens, demonstrated that *E. iulii* is attributable to the *E. serius* group characterized by the antenna of the female with the club entire and highly developed, about 7 or 8 times as long as wide, with subparallel margins and rounded apex, and above all, by the first funicular segment ring-like and the second one trapezoidal (Silvestri, 1927; Compere, 1936). These antennal characters are also associated with a short marginal fringe of the forewing (Gerling, 1969).

Four species are currently attributed to the *serius* group: *E. serius*, *E. orientalis* Gerling 1969, *E. silvestrii* Gerling 1969 and *E. gunturiensis* Hayat 1972 (Silvestri, 1927; Gerling, 1969; Hayat, 1972; 1998). Silvestri described *E. serius* from specimens of both sexes that emerged from *A. woglumi* collected in Singapore. Furthermore, Silvestri described *E. serius* var. *orientalis* from two specimens (1 ♀ and 1 ♂) from *Aleurocanthus inceratus* Silvestri 1927 in Van Phu, Vietnam and from *A. spiniferus* in Foochow (Fuzhou), China (Silvestri, 1927). Subsequently, Gerling (1969) examined type material of Silvestri and elevated *E. orientalis* to the species rank and described one new species, *E. silvestrii*. The holotypes and paratypes of these species were lent in 1984 to M. Rose of the Department of Entomology of Montana State University, USA, and are no longer present in the Silvestri Museum of the University of Naples. Attempts to recover this type material have been unsuccessful. Females of *E. iulii* differ from those of the other species of the *serius* group by the characteristics of the mesonotum and several dimensional values of the antennae, forewing, ovipositor, and middle leg, as given in the key below.

Key to females of the *Eretmocerus serius* group of species

1. Mid lobe of mesoscutum with 4 pairs of setae . . . . . *E. silvestrii*
- Mid lobe of mesoscutum with 3 pairs of setae 2
2. Antennal club 8-9× as long as wide; marginal vein always with 4 setae . . . . . *E. gunturiensis*
- Antennal club less than 8× as long as wide, marginal vein with 2 or 3 setae 3
3. Antennal club about 1.4× length of ovipositor; ovipositor shorter than midtibia . . . . . *E. iulii* sp. n.
- Antennal club about 1.1× length of the ovipositor, ovipositor as long as or longer than midtibia 4
4. Stigmal vein subequal to marginal one . . *E. orientalis*
- Stigmal vein almost half marginal vein . . . . . *E. serius*

Other morphological characteristics of *E. iulii* appear to vary between different specimens and even in the same specimen, for example, the number of cilia on the forewing. In literature, however, the characteristics mentioned above are described and sometimes used as discriminants for the identification of the species of the group (Silvestri, 1927; Gerling, 1969; Hayat, 1972; 1998). In particular, the number of cilia on the forewing can vary from 9 to 14 between different specimens of *E.iulii*. Still, in most cases, there is a difference between the right and left wing of the same individual. Regarding the setae on the forewing disc, their number appears to be related to the size of specimens, varying from about 170 in the smaller ones to 250 in the larger ones.

Molecular analyses

After editing and trimming, COI barcoding region sequences with a maximum length of 602 bases have been obtained. Sequences blasting against genetic databases did not give any high similarity percentage with *Eretmocerus* species available. The highest resulted similarity was 90.47% with *Eretmocerus orchamoplati* Schmidt 2011 (accession number JF750712).

Sequencing analyses of *E. iulii* specimens (table 1) revealed the presence of 4 haplotypes, with a mean genetic distance of 0.08% (± 0.043 SE). The maximum intragroup distance was found among the specimens collected in Palermo, followed by Portici ones with mean genetic distances of 0.19% (± 0.147 SE) and 0.17% (± 0.163 SE), respectively. Three out of four mt-haplotypes were found in Palermo, while in Portici, two out of four. However, haplotype A (hA) was found in both the sites, and between Palermo and Portici samples, the maximum intergroup distance was found, 0.16% (± 0.111 SE). The ribosomal gene 28S sequences of samples from each site were identical, and there was no polymorphism in any site. Blasting searches showed no similarity higher than 95% with any *Eretmocerus* spp. sequences available in the databases.

Discussion

The integrative approach used in this study allowed to delimit a new species belonging to the *Eretmocerus* genus and attributable to its *serius* species group. Morphological evidence converges towards assessing species status to *E. iulii*. The specimens of this genus can be morphologically distinguished from those of the *serius* group by various characteristics, including the length of the club, features of the forewing, middle leg, ovipositor, and mesonotum.

Based on the COI portion evaluated in this study, molecular analyses showed a low haplotype diversity, although they highlighted four haplotypes found in different sites. This variability is even lower than that found in the populations of another *Eretmocerus* species, where genetic variability between 1% and 1.7% was observed (Schmidt *et al*., 2011). Furthermore, the variability here found resulted perfectly congruent with the concept of a single species; as such, the distances found are so negligible and well below the minimum genetic divergence thresholds (2-3%) that it could aid in distinguishing between species (Zhang *et al*., 2022). The affiliation to a single species is fully supported by the ribosomal genes investigated, which, showing no differences, classify all the analysed samples from every site under the name *E. iulii*. It is known that even a single polymorphism in the ribosomal genes could indicate affiliation with another taxonomic entity (Gebiola *et al*., 2015; Nugnes *et al*., 2017).

Among the four found haplotypes, haplotype A (hA) was present at all investigated sites and was the only one found in both Grottammare and Casagiove. Additionally, haplotype A was also found in Portici and Palermo, where it coexists with haplotype D (hD) and haplotypes B and C (hB and hC), respectively (table 1).

It is to note the specific case of Sicily. Until now, *E. iulii* has not been found on any other host, making its direct link with *A. spiniferus* plausible. Since *A. spiniferus* is a not native invasive species in Europe and Italy, it logically follows that its parasitoid might also be exotic. The highest variability of haplotypes was found in Sicily, suggesting that *E. iulii* may have been introduced there and then spread to the mainland. However, it should be noted that *A. spiniferus* was only reported in Sicily in 2021 (Rapisarda and Longo, 2021), and the presence of *E. iulii* is much lower in Sicily compared to other investigated sites, particularly Casagiove and Portici (Melone *et al*., 2024), thereby undermining the previously stated hypothesis.

Another important factor to underline is the finding of *E. iulii* only on populations of *A. spiniferus* belonging to Haplogroup 2, which is the only haplogroup found in Italy (Nugnes *et al*., 2020). The same study also emphasized that the genetic distances revealed in the populations could suggest that individuals belonging to the two haplogroups might even pertain to different species, supporting what was previously noted by Uesugi *et al*. (2016). Therefore, it is not excluded that if haplogroup 1 actually represents an independent specific entity, *E. iulii* may not be able to parasitize it due to its specialization on haplogroup 2. Consequently, a potential entry in Italy or Europe of *A. spiniferus* belonging to haplogroup 1 could initiate an invasive process without facing an effective natural enemy already present in the new territories.

However, further studies will be crucial for understanding the distribution of *E. iulii* in *A. spiniferus* infested zones, its own biological behaviours, and its ability to limit the spread and the populations of its host.

The morphological analyses conducted have led to the description of a new species, finding support in molecular analyses. After all, morphological variability has been observed, and considering the small size of entities belonging to the genus *Eretmocerus*, it is essential not to base identification on one or a few specimens. Therefore, it is necessary to proceed with a high number of females to obtain a more objective evaluation in the identification process and to employ, where possible, molecular analysis as well.

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(Supplemental material available at https://github.com/FrankNug/Eretmocerus-iulii.git)