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TAXONOMY, PHYLOGEOGRAPHY AND MYRMECOPHILY OF THE SPIDER GENUS MASTIGUSA (ARANEAE, CYBAEIDAE)

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Abstract

The genus *Mastigusa* Menge, 1854 includes small entelegyne spiders represented by extant and fossil species presenting characteristic features in male and female genitalia. The genus has a palearctic distribution, being present in Europe, North Africa, and the Near East, and shows ecological plasticity, with free-living, cave-dwelling and myrmecophile populations. The taxonomic history of the genus has been problematic, both regarding its phylogenetic placement and the delimitation of the species it includes. Three extant species are currently recognized, but the characters used to discriminate them have been inconsistent, leading to confusion about their identification and distribution. In the present thesis we addressed the taxonomic issues regarding *Mastigusa* by combining molecular and morphological data in an integrative taxonomy approach. For the first time, we included the genus in a molecular phylogenetic matrix solving a long going debate regarding its familiar placement, obtaining a well-supported placement in the family Cybaeidae. We used multi-locus molecular phylogenetic and DNA barcoding techniques as a starting point for identifying divergent lineages within the genus and revise the taxonomic status of the three known *Mastigusa* species, identifying a new species from the Iberian Peninsula, Algeria and the United Kingdom: *M. raimondi* sp. n. This taxonomic revision allowed a phylogeographic and ecological study of *Mastigusa* across its distribution range, carried out using phylogenetics and ecological niche modelling techniques, aiming at a comparison of the lifestyles and ecological requirements of the different species on a geographic scale. The Italian Alps were finally used as a testing ground for investigating the ecology and host preference of myrmecophile *Mastigusa arietina* populations living in association with ant species belonging to the *Formica rufa* species group. Spiders were found in association with five different *Formica* species, demonstrating little specificity and the tendency of associating with the locally present host species.

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Introduction

Araneae (spiders) is the major order belonging to the class Arachnida, with over 50,000 currently recognized species and several hundred new species being described every year (World Spider Catalog 2023). Spiders are considered a megadiverse order, with over 4,000 genera in 132 different families (World Spider Catalog 2023) and an ancient evolutionary history starting around 400 million years ago, in the Devonian period (Kallal et al. 2020; Magalhaes et al. 2020). Since then, they have colonized almost every terrestrial ecosystem and some freshwater ones, thanks to their great adaptability and dispersal efficiency (Turnbull 1973). In terrestrial ecosystems, spiders are among the most abundant and common predators and are estimated to consume between 400 and 800 million tons of prey each year (Nyffeler and Birkhofer 2017), mostly represented by other arthropods. For the abovementioned reasons, they significantly impact on ecosystem functioning (Turnbull 1973; Yang and Gratton 2014). Spiders not only offer countless research opportunities for taxonomists, ecologists, and evolutionary biologists, but are also particularly interesting for applied science and biomimetics, due to the considerable number of different chemical compounds constituting their venom, which find medical and agricultural applications (Saez and Herzig 2019; Wu et al. 2019). However, the main technological interest in spiders lies in the spider silk they produce, considered one of the toughest natural fibers on Earth (Greco and Pugno 2020). Reconstructing the phylogeny of such a big, ancient, and diverse group is challenging. Morphological phylogenies based on quantitative analyses started the process of disentangling the relationships among the major spider lineages (e.g., Griswold et al. 1998; Agnarsson 2004; Ramirez 2014), and in recent times the application of molecular phylogenetics and phylogenomic techniques are boosting the process allowing the generation of larger datasets, confirming and in some instances challenging the morphology-based hypotheses (Garrison et al. 2016; Wheeler et al. 2017; Fernandez et al. 2018; Kallal et al. 2020).

The spider genus *Mastigusa* Menge, 1854 includes small spiders (2 - 4 mm) characterized by extreme modifications of male and female genitalia (Roberts 1995), distributed in the Palearctic (World Spider Catalog 2022). It was first described from a specimen enclosed in Eocenic Baltic amber, named *Mastigusa acuminata* Menge, 1854. Seven other fossil species, all from Baltic amber, were later described: *M. arcuata* Wunderlich, 2004, *M. bitterfeldensis* Wunderlich, 2004, *M. laticymbium* Wunderlich, 2004, *M. magnibulbus* Wunderlich, 2004, *M. media* Wunderlich, 1986, *M. modesta* Wunderlich, 1986 and *M. scutata* Wunderlich, 2004 (Fig. 1). The first extant

species was described in 1871 by Thorell as *Cryphoeca arietina* Thorell, 1871. In 1897, Chyzer & Kulczynski moved *C. arietina* to the genus *Tuberta* Simon, 1884 and described *Tuberta arietina macrophthalma* Kulczyński, in Chyzer & Kulczyński, 1897. A year later, Simon (1898a) transferred the two taxa to the genus *Tetrilus* Simon, 1886, describing a new species, *T. lucifuga* (Simon, 1898). Only in 1986 Wunderlich, by comparing male genital morphology, realized that the three extant species were closely related with the fossil *M. acuminata*, moving them to the genus *Mastigusa* as *M. arietina* (Thorell, 1871), *M. macrophthalma* (Kulczynski, 1897), and *M. lucifuga* (Simon, 1898). The morphological identification and delimitation of these three extant species has always been troubled (for details, see **Chapter 2**), and the phylogenetic placement of *Mastigusa* also suffered from uncertainty. Indeed, the genus was placed in different families, all belonging to the "marronoid clade", characterized by the lack of strong synapomorphies (**Chapter 1**), but it was never included in a phylogenetic dataset before the present work.

The ecology of *Mastigusa* is also peculiar, as free-living, cave-dwelling and myrmecophile populations are known to exist (Castellucci et al. 2022). Given the uncertainties regarding the identity and distribution of extant species, it was difficult to discern the roles of taxonomy, biogeography, or climate in determining the adaptation of distinct populations to different lifestyles. While free-living populations, even if not so common for some of the species, can be found all over the known distribution range, cave-dwelling populations are only reported in the Southern Iberian Peninsula and North Africa (Simon 1898b, 1913; Fage 1931; Bristowe 1939).

Myrmecophily is defined as the ability of some organisms to live in close relationship with ants, from foraging in the periphery of the colony up to spending the whole life inside ant nests (Wasmann 1894; Donisthorpe 1927; Hölldobler & Wilson 1990). These taxa manage to avoid the ants' defensive strategies by using anatomical modification, behavioral responses, or chemical adaptations (Lenoir et al. 2001; Parker 2016). Myrmecophily in spiders has been observed in only 13 out of the 132 known spider families (Cushing 1997, 2011), and is still a little studied topic if compared to other taxonomic groups (Castellucci et al. 2022). Myrmecophile *Mastigusa* populations are common in Central and Northern Europe, with individuals living in close relationship with ants belonging mostly to the genera *Formica* Linneaus, 1758 and *Lasius* Fabricius, 1804 (Castellucci et al. 2022).

In this thesis we carried out a detailed study of the genus *Mastigusa* covering different aspects of its biology and evolution. For the first time, we included the genus in a phylogenetic analysis by sequencing both nuclear and mitochondrial markers for individuals sampled in *Mastigusa* populations spanning its whole distribution range and combining the produced data in a molecular matrix covering all the spider families where *Mastigusa* was previously placed (**Chapter 1**). This resulted in a revised familiar placement for the genus, in the first insights into the genetic differentiation within *Mastigusa* populations, and in an estimate of the divergence time of this genus from its sister group and between the different lineages within *Mastigusa.*

The molecular phylogeny obtained was used as a starting point for an integrated taxonomic revision of the genus carried out by combining molecular and morphological evidence (**Chapter 2**), and a molecular characterization of populations from the whole distribution range of *Mastigusa*. The revision resulted in the description of a species new to science, *M. raimondi* sp. n., and to the re-circumscription and redescription of the other *Mastigusa* species.

A taxonomic revision of the genus was a fundamental step required for an accurate analysis of the drivers shaping the ecological plasticity that can be observed within this genus. A phylogeographical and ecological study of the redefined *Mastigusa* species was carried out through molecular analyses and ecological niche modelling techniques (**Chapter 3**), which highlighted significant differences in the ecological requirements and lifestyles of each species.

Finally, for acquiring information on the relationships that myrmecophile *Mastigusa* populations have with their host ant species, a large-scale survey was carried out in the Italian Alps (**Chapter 4**). This resulted in novel insights on the host preference of myrmecophile *Mastigusa* populations and to co-occurrence records with new host ant species.

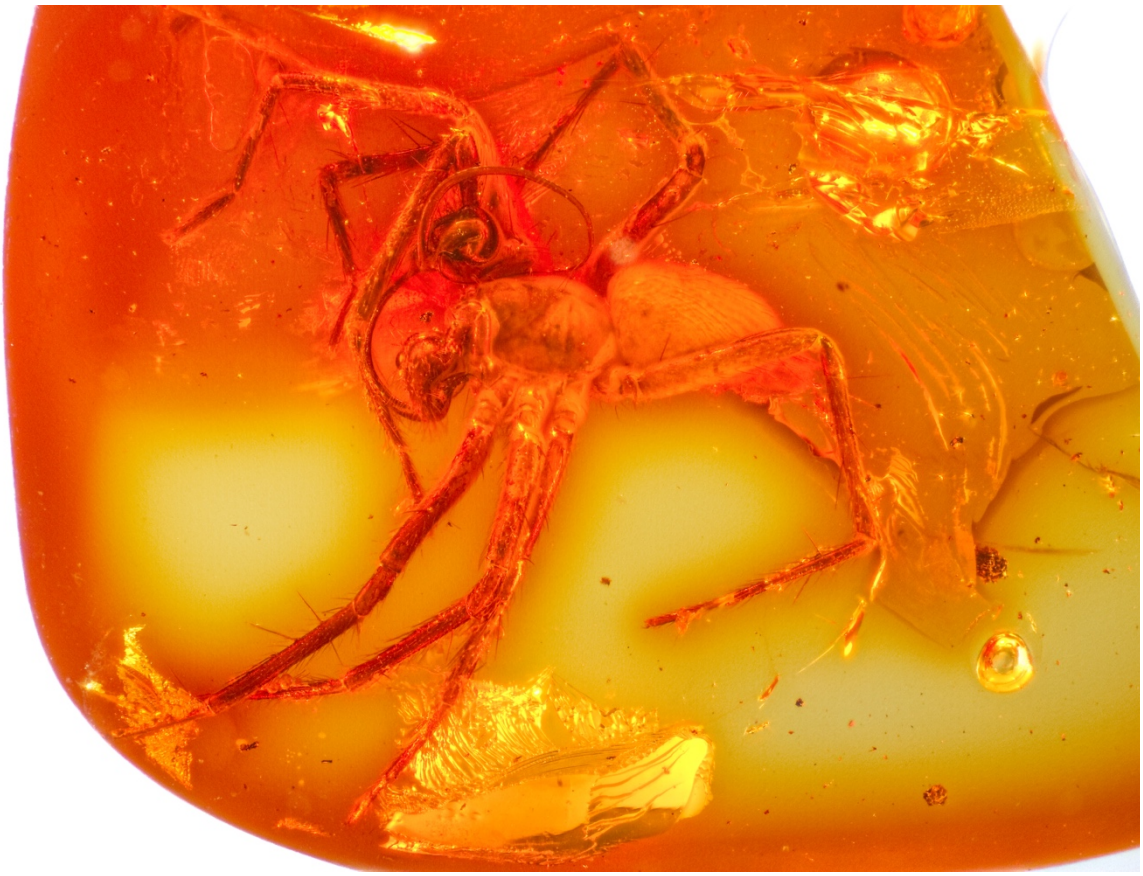

Figure 1. *Mastigusa bitterfeldensis* holotype (SMF Be1394) in Eocenic Baltic Amber.

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1. Molecular systematics and phylogenetics of the spider genus *Mastigusa* Menge, 1854 (Araneae, Cybaeidae)

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1.1. ABSTRACT

The palearctic spider genus Mastigusa Menge, 1854 is characterized by a remarkable morphology and wide ecological variability, with free-living, cave dwelling and myrmecophile populations known. This genus has a long and tangled taxonomic history and was placed in different families in the past, all belonging to the “marronoid clade”, an informal grouping of families characterized by the lack of strong synapomorphies. Three species are currently recognized, but their identity and circumscription has been long debated. A molecular approach was never applied for trying to solve these uncertainties, and doubts still remain both about its phylogenetic placement and about the taxonomic status of the described species. For the first time the genus Mastigusa is included in a molecular phylogenetic analysis and strong support is found for its placement within the family Cybaeidae, in sister relationship with the genus Cryphoeca Thorell, 1870. An analysis of Mastigusa populations spanning across the distribution range of the genus identifies a high and previously overlooked genetic diversity, with six distinct genetic lineages showing a strong geographic pattern. Divergence times between Mastigusa and its sister genus and between the distinct Mastigusa lineages are estimated, and the groundwork is laid for a taxonomic revision of the species belonging to the genus.

Keywords: marronoid clade; RTA clade; phylogeny; Europe; dating

1.2. INTRODUCTION

Spiders (Araneae) represent a megadiverse order of arthropods, with over 50,000 described species in 132 families (World Spider Catalog 2023). Reconstructing the phylogeny of such a big and diverse group is not an easy task, since it would require an extensive taxon sampling with representatives from each known family. The first attempt to reconstruct the “tree of life” for all spider families was carried out by Wheeler et al. (2017) and based on the usual low number of molecular markers available with Sanger sequencing datasets. Since then, phylogenomic dataset have been developed, thereby providing more data to disentangle the relationships between the major groups (Bond et al. 2014; Garrison 2016; Fernandez et al. 2018; Kallal et al. 2020; Kulkarni et al. 2020). Though, the taxon sampling remains limited if compared to the study of Wheeler et al. (2017). Moreover, most phylogenomic studies were focused on the phylogenetic placement of orb weavers and their close relatives.

The genus Mastigusa Menge, 1854 includes small spiders (3 - 4.5 mm) characterized by a remarkable morphology of the genitalia: the male palp exhibits an extremely long and bent conductor forming a ram-like structure that can exceed the length of the prosoma (Fig. 1a). The embolus is equally long and, in the unexpanded palp, is embedded in a groove on the conductor. The female inner genitalia are also peculiar, showing extremely long and tangled non-symmetrical copulatory ducts matching the long male embolus (Fig. 1b). These spiders are currently known from Europe, Algeria, Russia, and Iran (World Spider Catalog 2023), showing a wide ecological variability, with free-living, cave-dwelling and myrmecophile populations observed, and a still little is known biology (Castellucci et al. 2022).

The phylogenetic placement of this genus among spider families has always been problematic. Wunderlich (1986) placed it in the family Agelenidae, sub-family Cicurininae, later moving it to Dictynidae, sub-family Cryphoecinae, due to the morphology of the spinnerets and the size and shape of the bulbus in the male pedipalp (Wunderlich 2004). The latter paper represents the most recent discussion of its phylogenetic placement, but Wunderlich’s suggested placement of Mastigusa was not based on any phylogenetic analyses and this may be the reason why the World Spider Catalog continued to list Mastigusa as a member of Cicurininae (that in the meantime also became a sub-family of Dictynidae), instead of Cryphoecinae (World Spider Catalog 2023). In 2017, Wheeler et al. published a multi-locus molecular phylogeny including all known spider families, where they moved the genus Cicurina

Menge, 1871 (type genus of Cicurininae) from Dictynidae to Hahniidae. Although not included in the analysis, Mastigusa was also moved to Hahniidae, where it is now placed (World Spider Catalog 2023). In the same paper, the dictynid sub-family Cryphoecinae was recognized as a synonym of the family Cybaeidae. To date, the genus Mastigusa has never been included in a phylogenetic study.

Currently, the genus Mastigusa includes eight fossil species, retrieved in Baltic amber from the Eocene (Wunderlich, 2004), and three extant species are currently recognized: Mastigusa arietina (Thorell, 1871), known form Europe, Algeria, Russia, and Iran (World Spider Catalog 2023), Mastigusa lucifuga (Simon, 1898), only known from the type specimen, a female collected in the French Pyrenees, and Mastigusa macrophthalma (Kulczyński, 1897), known from Hungary, the Balkans, Caucasus, and Russia (World Spider Catalog 2023). The delimitation of the three species has always been problematic, with different authors considering them either as species (Simon 1898b, 1937; Locket & Millidge, 1953; Loksa 1969; Tyschchenko, 1971; Wunderlich 1986, 2004; Azarkina and Trilikauskas 2012) or sub-species (Chyzer & Kulczynsk 1887, Bristowe 1939, Roberts 1985). In his revision of the genus, Wunderlich (1986) distinguishes M. arietina and M. macrophthalma by eye characters (relative dimension of posterior and anterior median eyes), but mostly relying on characters in the chelicerae (number of teeth in the retrolateral margin of the cheliceral furrow) and male genitalia (shape and diameter of the conductor). On the other hand, he does not rule out the synonymy between M. lucifuga and M. arietina, given that the male of M. lucifuga is not known and that the only differences observed in the M. lucifuga type are in the dimension of the posterior median eyes, a character showing a certain degree of variation within M. arietina. Other authors only relied on the relative dimension of the posterior and anterior median eyes to discriminate the three species, not considering the morphology of the genitalia and chelicerae (Heimer and Nentwig 1991; Roberts 1995; Aakra et al. 2016). Given the weakness of eye characters due to interspecific variability, identifications solely based on them had always been problematic, leading to confusion about the actual identity and distribution of the three species.

The so-called “marronoid” clade, as named by Wheeler et al. (2017), is an informal sub-group of the RTA clade (the most diverse group of spiders (World Spider Catalog 2023)) including both cribellate and ecribellate representatives from nine spider families (Agelenidae, Amaurobiidae, Cybaeidae, Cycloctenidae, Dictynidae, Desidae,

Hahniidae, Stiphidiidae, Toxopidae) and more than 3300 species (World Spider Catalog 2023). Most of the marronoid families are characterized by a lack of distinctive morphological features; for this reason, they were in the past placed in a few big families, such as Agelenidae, Amaurobiidae, Desidae and Dictynidae, from which they were gradually moved to a larger selection of families (Wheeler et al. 2017). Few molecular phylogenetic studies have focused on these families (Miller et al. 2010; Spagna and Gillespie 2008; Spagna et al. 2010; Crews at al. 2020) and recent phylogenomic datasets still present a limited taxon sampling for these groups (Garrison et al. 2016; Fernandez et al. 2018; Kallal et al. 2020; Kulkarni et al. 2020). Thus, the relationship between marronoid families remains mostly unresolved. All families in which Mastigusa has been proposed to be placed belong to the marronoid clade, thus a dataset with a broad taxon sampling covering all of them is necessary for trying to solve its phylogenetic placement.

Uncertainties regarding the phylogenetic placement of Mastigusa and the taxonomic status of the three described species, also caused by the confusion in the morphological characters used to discriminate them, call for a re-examination of this genus with the aid of molecular data. This could help to clarify both the position of the genus within the spider tree of life and the actual diversity that it holds, further allowing comparative studies concerning the ecology and evolution of these spiders.

1.3. MATERIALS AND METHODS

1.3.1. Material acquisition and morphological species identification

Fresh *Mastigusa* specimens were collected during different fieldwork sessions in Italy, Denmark, Spain, and Croatia between 2018 and 2021. Specimens were hand collected under stones or logs and inside anthills of *Formica rufa* species group ants, one of the main *Mastigusa* hosts. Details about the methods used for collecting spiders in anthills are described elsewhere (Castellucci et al. 2022). Additional fresh material was acquired from colleagues, including specimens from Spain, United Kingdom, Belgium, and Georgia. Specimens were stored in 95% ethanol and at -20ºC prior to DNA extraction. For a full list of the *Mastigusa* specimens included in the molecular analyses see Table 1.

Collected specimens were examined and measured using a Leica M205A stereomicroscope equipped with a Leica DFC450 C camera and Leica Application

Suite v3.6 software and photographed with a BK+ Imaging System from Visionary Digital equipped with a Canon EOS 7D reflex camera. Identification of *Mastigusa* species was carried out following the original species descriptions (Thorell 1871; Chyzer and Kulczynski 1897; Simon 1898a) and the revision by Wunderlich (1986), and by comparison with type material for *M. arietina*, *M. macrophthalma* and *M. lucifuga*.

1.3.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from leg tissue using the NucleoSpin® DNA Insect kit (Macherey-Nagel) following the manufacturer's protocol. Polymerase chain reaction (PCR) was used for the amplification of partial fragments of the mitochondrial markers cytochrome c oxidase subunit 1 (COI), 12S ribosomal RNA and 16S ribosomal RNA and the nuclear markers histone H3 (H3), large subunit of ribosomal RNA (28S) and small subunit of ribosomal RNA (18S). PCR was carried out following the protocols of Wheeler et al. (2017). A list of the primers and annealing temperatures used is reported in Suppl. Table S1. PCR products were screened via gel electrophoresis on a 1% agarose gel and purified using ExoSAP-IT Product Cleanup Reagent (Thermo Fisher Scientific). Forward and reverse strands for amplified products were sent to Macrogen Europe (Amsterdam, The Netherlands) for Sanger sequencing. Chromatograms were visualized and inspected using SeqTrace v.0.9.0 (Stucky 2012). The search for potential contaminants was carried out using BLASTn (Zhang and Madden 1997) on NCBI. Sequences produced in this work were submitted to NCBI GenBank (accession numbers are given in Suppl. Table S2).

Additional sequences were obtained from the NCBI GenBank database, deriving mostly from the works of Spagna and Gillespie (2008), Miller et al. (2010), Wheeler et al. (2017) and Crews et al. (2020), to provide maximum coverage of marronoid taxa, including representatives of all the nine families identified by Wheeler et al. 2017 as belonging to the group. A broader taxon sampling was chosen for the candidate families for the placement of Mastigusa (Cybaeidae, Dictynidae, Hahhniidae). Other non-marronoid RTA families were included, mostly for calibration purposes, given the lack of reliable fossils within the marronoid families (Magalhaes et al. 2020). Non-RTA outgroups were included. For a list of the taxa included with accession numbers see Suppl. Table S3.

1.3.3. Alignment and phylogenetic analyses

Sequences were aligned using MAFFT v7.503 (Katoh and Standley 2013). Protein coding genes (PCGs) were aligned using the L-INS-i algorithm, while the X-INS-i algorithm was used for the ribosomal RNAs (rRNAs). The aligned protein coding genes were screened for the presence of stop codons by translating the nucleotide sequences into amino acids using AliView v1.28. Gblocks v0.91.1 (Castresana 2000) was used to exclude misaligned positions, with differential settings for PCGs and rRNAs. For PCGs the codon flag was selected, while the nucleotide flag was selected for rRNAs. The minimum number of sequences for a conserved position was set to 50% of the sequences included in the alignment (PCGs and rRNAs), the minimum number of sequences for a flanking position was set to 70% (PCGs) and 60% (rRNAs), the maximum number of contiguous nonconserved positions was set to 8 (PCGs) and 10 (rRNAs), the minimum length of a block was set to 10 (PCGs) and 5 (rRNAs), the allowed gap position was set to all (PCGs) and to with half (rRNAs). The alignments were then concatenated using FASconCAT v1.1 (Kück and Meusemann 2010). The concatenated dataset was partitioned into 10 subsets, one for each of the four rRNAs (12S, 16S, 18S, 28S) and one for each of the three codon positions for the two PCGs (COI, H3). Selection for best partitioning scheme and evolutionary models was performed using ModelFinder (Kalyaanamoorthy et al. 2017) as implemented in IQ-TREE v1.6.12 (Nguyen et al. 2015). Best partitioning scheme and evolutionary models selected are reported in Suppl. Table S4. Maximum likelihood (ML) phylogenetic inference was performed using IQ-TREE, nodal support was estimated using 1000 replicates of UltraFast bootstrap (Minh et al. 2013). A second ML analysis was performed using the same settings but adding some topological constraints based on nodes that resulted highly supported in the phylotranscriptomic work by Kallal et al. (2020). This was done to constrain some of the relationships between families given the known limited resolution power of classic Sanger markers at higher phylogenetic level in spiders (Garrison et al. 2016; Wheeler et al. 2017). The backbone tree used to set constraints is reported in Suppl. Fig. S1. Constrained and unconstrained ML trees were, then, compared with topology tests implemented on IQ-TREE: the RELL approximation (Kishino et al. 1990), as bootstrap proportion (BP), Kishino-Hasegawa test (KH) (Kishino and Hasegawa 1989), Shimodaira-Hasegawa test (SH) (Shimodaira and Hasegawa 1999), expected likelihood weights (ELW) (Strimmer and Rambaut

2002) (10,000 RELL replicates) and approximated unbiased test (AU) (Shimodaira 2002).

1.3.4. Time-Tree inference

Bayesian inference for divergence times estimation was carried out using Beast v2.6.7 (Bouckaert et al. 2014) using four fossil calibration points derived from Magalhaes et al. 2020. All fossils considered reliable by Magalhaes et al. (2020) from the RTA clade and the closely related UDOHs (Uloboridae, Deinopidae, Oecobiidae and Hersiliidae) were included, modeling the calibration with a gamma distribution as prior distribution, setting the minimum age for the fossil as offset and the alpha parameter as in Magalhaes et al. (2020). For details about the fossils and parameters used see Suppl. Table S5. Monophyly constraints were applied at higher phylogenetic level for matching our best working maximum likelihood hypothesis. The concatenated dataset was again partitioned by gene and by codon position for the two PCGs, with linked tree models, unlinked site models and linked clock models only for the mitochondrial partitions. A relaxed lognormal clock was used with a birth-death model as tree prior. Two independent runs were performed with 200 million generations each and sampling every 1000 states. Convergence between the two runs was checked with Tracer v1.7.2 (Rambaut et al. 2018) and adequate ESS were assessed (>200). Log files and tree files from the two runs were combined using LogCombiner v2.6.7. A maximum clade credibility tree was generated with TreeAnnotator v2.6.7 with a 25% burn-in.

1.4. RESULTS

1.4.1. Marronoid phylogeny

Beside the constrained nodes, the constrained and unconstrained ML tree topologies mostly overlap (Fig. 2; Suppl. Figure S2 and S3). In both analyses, families are all recovered as monophyletic, with the same internal relationship between taxa. The only differences observed are: i) the position of the clade composed by Dirksia cinctipes (Banks, 1896), Ethobuella tuonops Chamberlin & Ivie, 1937 and Brommella monticola (Gertsch & Mulaik, 1936), ii) the internal relationships within the genus Cicurina and iii) the position of the genus Cybaeota within Cybaeidae (Suppl. Figures S2 and S3). The phylogenetic position of Mastigusa and the relationships within the genus were

completely identical in the unconstrained and constrained ML trees. Moreover, when the two analyses were compared with topological tests, they did not significantly differ from each other (Suppl. Table S6). The constrained tree was therefore chosen as the best one (Fig. 2). In our analysis, Titanoecidae and Phyxelididae, two families considered non-RTA clade (Griswold et al. 1999, 2005; Wheeler et al. 2017) and not included in the phylogenomic datasets, result nested within the RTA clade with a good nodal support (bootstrap=90). The superfamily Zodarioidea (Zodariidae + Penestomidae) is recovered as monophyletic, although with moderate bootstrap value (79), and clusters within the RTA clade along with Titanoecidae and the dictynid genus Lathys Simon, 1884, that are in sister relationship. The marronoid clade sensu Wheeler et al. 2017 is not recovered as monophyletic because of the exclusion of Lathys and the inclusion of Phyxelididae. Though, this redefined marronoid clade is strongly supported (bootstrap=100). Within this group, we find a strongly supported (bootstrap=98) clade composed by Phyxelididae and Amaurobiinae amaurobids (Amaurobius C. L. Koch, 1837, Callobius Chamberlin, 1947 and Pimus Chamberlin, 1947), which is in sister relationship with the other marronoids. Amaurobiidae is not recovered as monophyletic, as Macrobuninae amaurobids (Anisacate Mello-Leitão, 1941, Rubrius Simon, 1887 and Zanomys Chamberlin, 1948) cluster elsewhere on the tree. The remaining marronoids form a monophyletic clade with maximum support. Among these, the families Cycloctenidae, Stiphidiidae, Desidae and Agelenidae are recovered as monophyletic (bootstrap=100, 99, 91 and 100, respectively). Dictynidae is not recovered as monophyletic due to the exclusion of Brommella Tullgren, 1948 and Lathys Simon, 1884. The remaining dictynids are recovered as monophyletic with maximum support (bootstrap=100). Hahniidae is not recovered as monophyletic due to the exclusion of Cucirina and Mastigusa. Other hahniids form a well-supported monophyletic clade (bootstrap=99). Cybaeidae is not recovered as monophyletic due to the exclusion of Ethobuella tuonops and Dirksia cinctipes and includes Mastigusa, in sister relationship with Cryphoeca Thorell, 1870 (bootstrap=100). Cybaeids (excluding E. tuonops and D. cinctipes and including Mastigusa) are in sister relationship with Toxopidae (bootstrap=98), that is recovered as monophyletic with maximum support. The genus Cicurina is recovered as monophyletic but its position remains unresolved, as it clusters with the Cybaeidae + Toxopidae clade with low support (bootstrap=55). One of the Brommella specimens (Brommella sp. ZZ-2016) clusters with maximum support with hahniids, while the other specimen (Brommella

monticola) forms a strongly supported clade (bootstrap=100) with the cybaeids Dirksia cinctipes (Banks, 1896) and Ethobuella tuonops Chamberlin & Ivie, 1937. This clade is sister to dictynids (bootstrap=74).

1.4.2. Mastigusa *spp. phylogenetic relationships*

The genus Mastigusa appears monophyletic (bootstrap=100) and sister to the Holarctic genus Cryphoeca (bootstrap=100; Fig. 2). The clade Mastigusa is split in two strongly supported sister clades, one composed of specimens from Italy, Denmark, Belgium and Georgia (bootstrap=100), and the other composed of specimens from Croatia, Spain and the United Kingdom (bootstrap=99; Fig. 3). Within the first clade, Georgian specimens are sister to a strongly supported Central European group (bootstrap=99). Within the second clade, specimens from the United Kingdom cluster with the Spanish specimens from Sierra Nevada with high support (bootstrap=95). This group is sister to a strongly supported clade (bootstrap=100) including Croatian specimens and the Spanish specimen from the Pyrenees (Fig. 3)

1.4.3. Divergence times

The estimated age for the diversification of the RTA clade is dated at 105.6 million years ago, in Lower Cretaceous (95% HPD=98.2–113.7 Mya), while that of marronoid clade is dated at 81.9 Mya, in Upper Cretaceous (95% HPD=74.6–94.9 Mya). The split between Mastigusa and its sister genus Cryphoeca is estimated at 49.1 Mya, in the Eocene (95% HPD=38.1–62.4 Mya). Within Mastigusa, the clade composed of Central European and Georgian populations diverged 25.2 Mya, in the Oligocene (95% HPD=17.4–35.9 Mya) from the one comprising populations from Spain, the United Kingdom and Croatia. The divergence between the sub-groups is estimated to have happened in the Miocene, as follows: Central Europe – Georgia=12.4 Mya (95% HPD=6.4–20.5 Mya); Sierra Nevada+United Kingdom – Croatia+Pyrenees=16 Mya (95% HPD=10.2–23.5 Mya); Spain – United Kingdom=8.3 Mya (95% HPD=3.6–15 Mya); Croatia – Pyrenees=11.7 Mya (95% HPD=6.6–18.7 Mya) (Fig. 3).

1.5. DISCUSSION

1.5.1. Marronoid phylogeny and phylogenetic placement of Mastigusa

Based on morphological data, Griswold (1999, 2005) recognized Titanoecidae and Phyxelididae as belonging to the superfamily Titanoecoidea and placed them outside the RTA clade, considering the lack of an RTA in this groups as ancestral and not the result of secondary loss. In our analysis, the two families do not form a monophyletic clade and they are nested withing the RTA clade with good support (bootstrap>70). Titanoecoidea was also recovered as non-monophyletic by Wheeler et al. (2017) but, in their analyses, both families are placed outside the RTA clade, although with low support. Concerning marronoid taxa, the paraphyly of Amaurobiidae, with Amaurobiinae and Macrobuninae not clustering together, is confirmed in our analysis, in agreement with previous works (Miller et al. 2010; Wheeler et al. 2017; Crews et al. 2020). The non-monophyly of Dictynidae, Cybaeidae and Hahniidae is likely due to the position of problematic taxa whose familiar placement has been debated, as Lathys, Dirksia, Brommella, Ethobuella and Cicurina. The genera Mastigusa and Cicurina, both currently included in Hahniidae, do not cluster within this family in our analyses. The placement of Mastigusa within Cybaeidae, and in sister relationship with Cryphoeca, agrees with Wunderlich suggestions (Wunderlich 2004). He included the genus in Cryphoecinae, at that time considered a sub-family of Dictynidae and this is strongly supported by our analysis. Despite its actual placement in Cicurininae, we do not observe phylogenetic proximity between Mastigusa and Cicurina, so consider its actual placement in Hahniidae as not justified. Present data, on the other hand, suggests the inclusion of Mastigusa in Cybaeidae. The position of Cicurina remains unresolved in our analysis since its relationship with Cybaeidae+Toxopidae is weakly supported (bootstrap=55). It's association with Hahniidae in Wheeler et al. (2017) is scarcely supported (bootstrap=67), while in Crews et al. (2020) the genus never clusters with Hahniidae. The genus is currently the only hahniid that is included in a phylogenomic analysis but given the uncertainties regarding its placement we do not find it as an adequate candidate for investigating the relationships between Hahniidae and the other marronoid lineages.

A Mesozoic origin and diversification of the major RTA groups is confirmed by our divergence time estimates that date the clade to the Cretaceous, in accordance with Garrison et al. (2016). However, RTA fossils are absent from all the Cretaceous ambers, like Burmese amber, and are so far only known since the Eocene (Magalhaes

et al. 2020). More recent studies, on the other hand, date it to the Jurassic (Fernandez et al. 2018; Magalhaes et al. 2020; Kallal et al. 2020).

1.5.2. Mastigusa *spp. phylogenetic relationships*

Distribution-wise, Central Europe fits with the known distribution of M. arietina, even though, as stated before, distributional information regarding Mastigusa species should be treated carefully due to their problematic identification. The phylogenetic proximity of specimens from the Italian Alps, Belgium, and Denmark, which do not form two separate clusters, suggests continuity of gene flow between the areas, which does not raise concerns regarding a possible undersampling in Central Europe. The Georgian specimens show a certain degree of genetic divergence regarding to the Central European ones, but no clear morphological differences were identified between them. Both M. arietina and M. macrophthalma are currently reported from the Caucasus region but again, the reliability of such reports is dubious. In the second clade we observe a clear separation between specimens from southern Spain (Sierra Nevada) and United Kingdom, on one side, and specimens from Croatia, clustering with the single specimen from the Pyrenees, on the other side. This clustering pattern is rather interesting, particularly considering that the only specimen from Pyrenees, a male, is morphologically consistent with the other specimens from Spain and the specimens from the United Kingdom. The Croatian specimens analyzed were collected in one of the type localities of M. macrophthalma and are morphologically consistent with other samples from Croatia and Slovenia, including type material for this species. They are, moreover, morphologically distinct from the specimens from Southern Spain, the United Kingdom and the Pyrenees. Morphology-wise, the clade composed by specimens from Southern Spain and the United Kingdom (and the single specimen from the Pyrenees), do not fit with any of the currently described Mastigusa species, showing marked differences with the observed type-material, mostly regarding the morphology of the male palp. Iberian populations have always been considered as M. arietina, while in the United Kingdom both M. arietina and M. macrophthalma have been historically reported, but again only based on eyes characters. The overall morphology the dimensions for these specimens, and their distribution fit with M. arietina, but the male palp consistently shows marked differences with all the other specimens observed. Our molecular data strongly suggests that these specimens could belong to a new, previously overlooked,

Mastigusa species. Future studies including an accurate morphological examination of a higher number of specimens from the Iberian Peninsula, United Kingdom and neighboring countries are necessary to deliberate on the taxonomic status of these populations. Moreover, none of the specimens included in our molecular analyses showed morphological traits that could be reconciled with the M. lucifuga type, only differing from M. arietina by having considerably smaller posterior median eyes, although variation has been observed in the dimension of posterior median eyes both in the Central European clade and in the populations from Spain and the United Kingdom. Doubts remain on the single male specimens from the Pyrenees: it could be close to M. lucifuga, having been collected near the type locality of this species (Eastern Pyrenees), but since the M. lucifuga male is not known it is hard to make clear statements in this sense. The appearance of the genus Mastigusa around 50 Mya, in the early Paleogene, is compatible with the known existence of eight fossil species from Baltic amber, dated at 23-48 Mya (Sadowski et al. 2017; Wunderlich 1986, 2004). The Miocene sees a great diversification in the genus with the appearance of the six extant lineages.

All specimens included in the Central European clade were collected inside ant nests belonging to the genera Formica L. 1758 and Lasius F. 1804. Myrmecophile Mastigusa populations have been observed in several countries in Central and Northern Europe (Westring 1861; Palmgren 1976; Roberts 1985; Heimer & Nentwig 1991; Scharff&Gudik-Sørensen 2006; Aakra et al. 2016; Parmentier et al. 2016; Castellucci et al. 2022). The Georgian population, closely related to the Central European clade, was observed outside of ant nests, with different life stages found below rocks with no ants in the immediate proximity. Few records of Mastigusa specimens collected outside of ant nests exist for Central and Northern Europe (e. g. Palmgren 1976; Kielhorn and Blick 2007; Isaia et al. 2015). Specimens from Croatia were collected again under rocks, with presence of different life stages and egg sacks. Moreover, no records of myrmecophile populations are known from Croatia or neighboring countries like Slovenia and Bosnia-Herzegovina. Specimens from Spain and the United Kingdom were all collected outside of ant nests, in pitfall traps or under stones and logs. No bibliographic records exist regarding myrmecophile Mastigusa populations in the Iberian Peninsula, while both myrmecophile and free-living populations are known from the United Kingdom (Donisthorpe 1908, 1927; Jackson 1913; Bristowe 1939; Locket and Millidge 1953). Interestingly, cave dwelling Mastigusa populations are

known only from the Iberian Peninsula and Algeria (Simon 1898b, 1913; Fage 1931; Bristowe 1939). No cave-dwelling populations are known from Central, Northern or Eastern Europe, even if the presence of free-living Mastigusa populations is documented in highly carsic areas like the Western Italian Alps (Isaia et al. 2015; Castellucci et al. 2022) or the classic Karst of Slovenia, Croatia, and North-Eastern Italy (Chyzer & Kulczynski 1897; Kostanjšek & Kuntner 2015; Castellucci et al. 2022). These areas have been strongly investigated both on a speleological and bio-speleological level, also with a focus on spiders (Deltshev 2008; Isaia et al. 2011; Mammola et al. 2019), so the lack of observation of cave dwelling populations in these areas is unlikely to be the result of a sampling bias.

The monophily of Mastigusa, its placement within the family Cybaeidae, and its sister-group relationship to Cryphoeca are well supported in our phylogenetic analysis. The genus Mastigusa shows a great, and previously overlooked, genetic diversity with several lineages showing a strong geographic pattern. The separate lineages appear to show marked ecological differences, that could be based on taxonomy, geography, climate, or a combination of the three. Given the molecular evidence obtained, a detailed morphological examination of a great number of specimens from the included localities and neighboring countries will be necessary for a taxonomic revision of the genus and for understanding the drivers leading to the observed ecological variability.

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1.8. TABLES

Code	Nation	Locality	Habitat	Collecting date	Lat	Lon	Elevation (m a.s.l.)	Legit
MABE01	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.
MABE02	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.
MMHR4	HR	6km west of Sljeme, Funzine	Under stones, *Fagus sylvatica* forest with some *Picea abies*	29/06/21	45°20.79500'	014°41.59333'	915	Castellucci F.
MMHR5	HR	6km west of Sljeme, Funzine	Under stones, *Fagus sylvatica* forest with some *Picea abies*	29/06/21	45°20.79500'	014°41.59333'	915	Castellucci F.
MAS_DK_01	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.
MAS_DK_03	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.
MAS_DK_09	DK	Tokkekøb Hegn, Lillerød	In *Formica* sp. nest, broad leaf forest with some conifers	12/04/18	55°53.24886'	012°23.16618'	60	Castellucci F.
MAGE04	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.
MAGE05	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.
MAGE06	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.
MAVSC1	IT	Chabod trail, Valsavarenche	In *Formica* sp. nest, *Larix decidua* forest with scarce *Pinus cimbra* and *Picea abies*	08/07/20	45°32.59620'	07°13.33200'	2024	Castellucci F.
MAVSC2	IT	Chabod trail, Valsavarenche	In *Formica* sp. nest, *Larix decidua* forest with scarce *Pinus cimbra* and *Picea abies*	08/07/20	45°32.59620'	07°13.33200'	2024	Castellucci F.
EDBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.
MADBC2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.46940'	012°09.25550'	1477	Castellucci F.
MAS_IT_01	IT	Casera Casavento, Claut	In *Formica* sp. nest, *Picea abies* forest	08/09/18	46°16.08600'	012°35.74800'	934	Castellucci F.
MD2844	ES	Sola de Boi, Lleida	In pitfall trap, white oak forest	15-29/6/13	42°32.97480'	000°52.35240'	1760	Crespo L. et al.
MD372	ES	Soportujar, Granada	In pitfall trap, white oak forest	31/5/13-14/6/13	36°57.69060'	-003°25.12860'	1787	Crespo L. et al.
MASN01	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.
MASN02	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.
MAUK01	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	-003°42.63333'	130	Gallon R.
MAUK05	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	-003°42.63333'	130	Gallon R.
MAUK06	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	-003°42.63333'	130	Gallon R.

Table 1. *Mastigusa* specimens included in the molecular analyses with collecting information. Country codes: BE=Belgium; DK=Denmark; ES=Spain; GE=Georgia; HR=Croatia; IT=Italy; UK=United Kingdom.

1.9. FIGURES

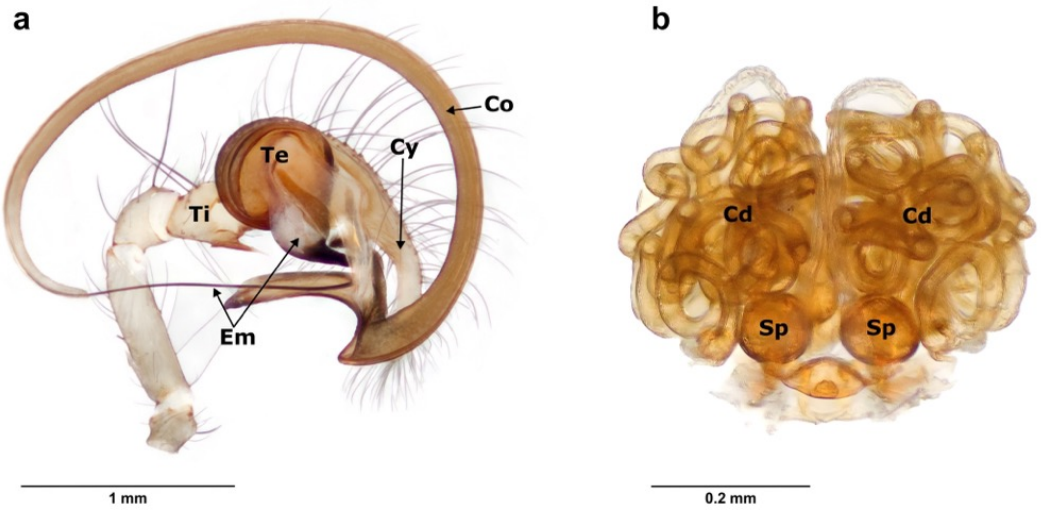


Figure 1. Male and female genitalia of *Mastigusa arietina*. a: left male pedipalp, prolateral view. b: female genitalia, excised and cleared, ventral view. Abbreviations: Cd=copulatory ducts; Co=conductor; Cy=cymbium; Em=embolus; Sp=spermatecha; Te=tegulum; Ti=tibia. Image credits: F. Castellucci and R.J. Jensen.

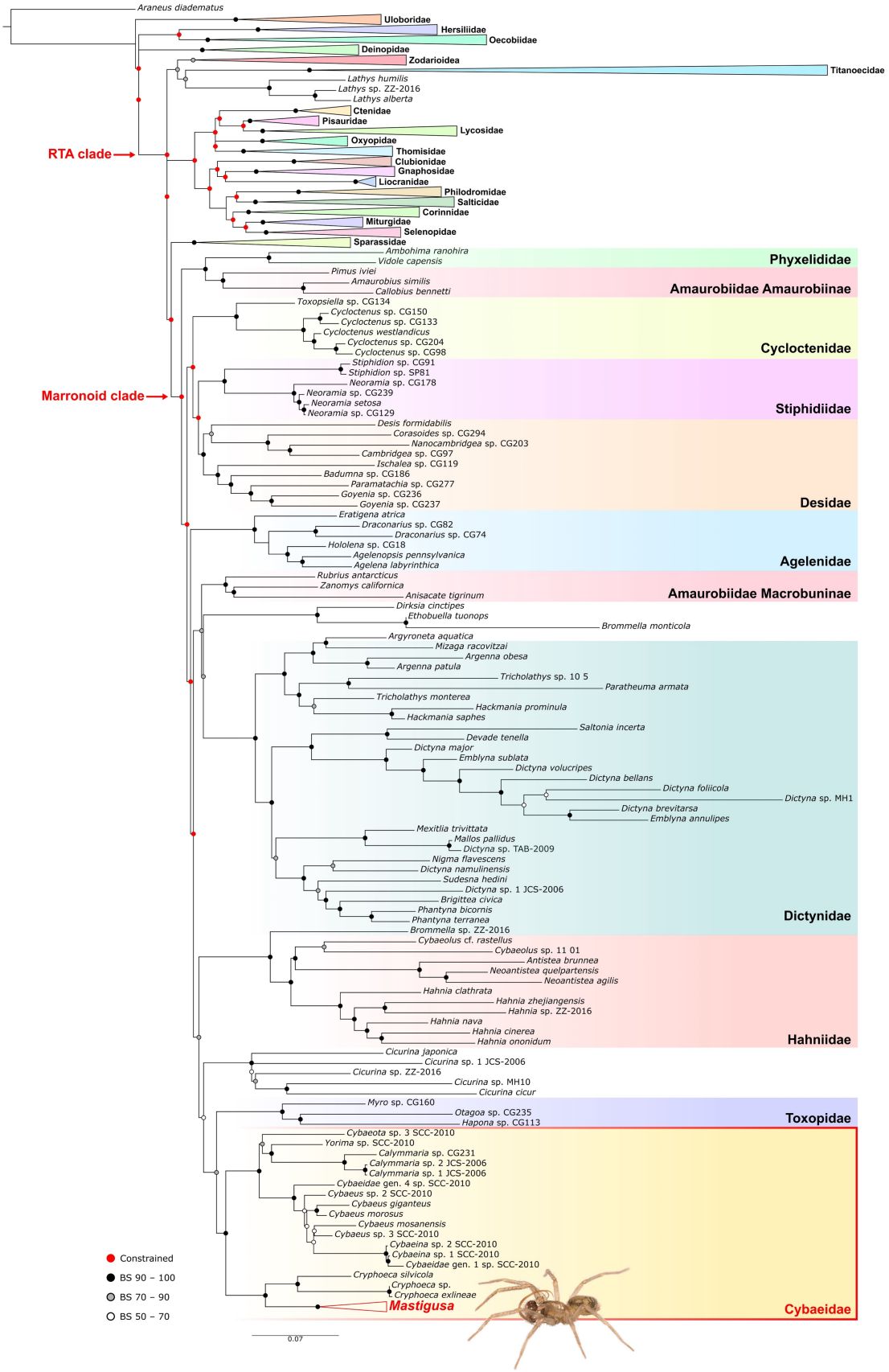


Figure 2. Constrained Maximum Likelihood tree built using IQ-TREE with 1000 ultrafast bootstrap replicates. Non-marronoid families collapsed; *Mastigusa* clade collapsed. BS = bootstrap values. Constrained nodes evidence in red.

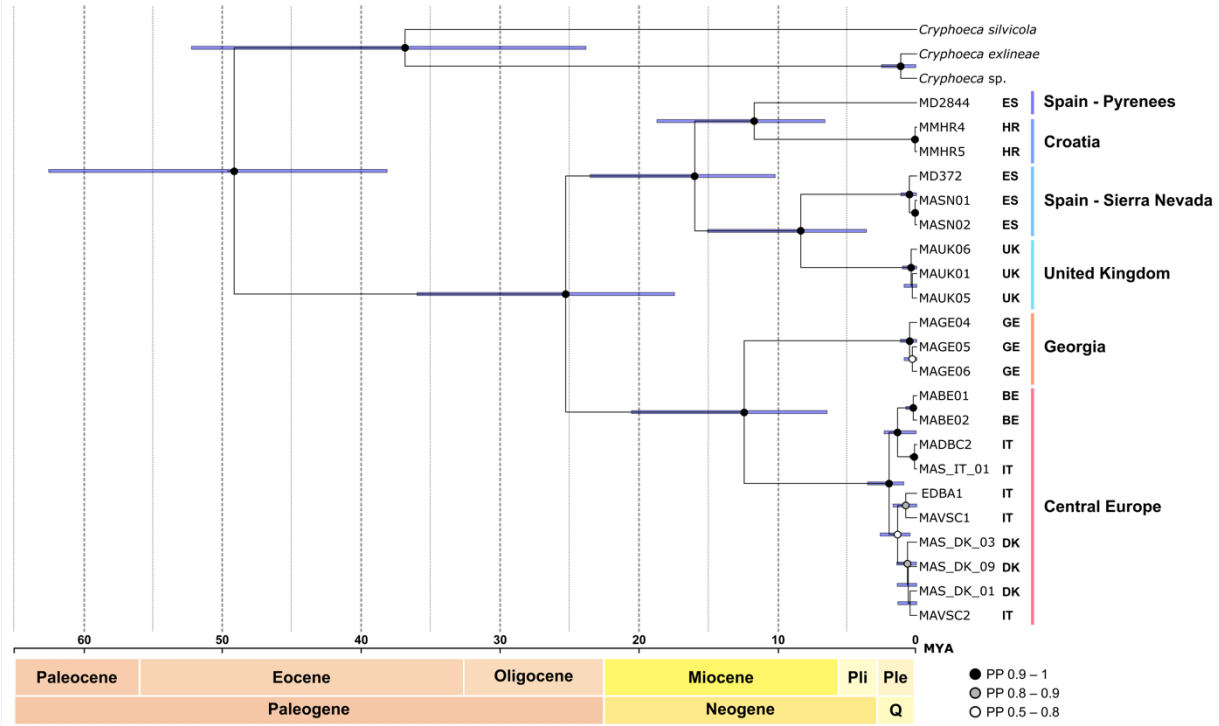


Figure 3. Detail of the BEAST time-tree tree focusing on *Mastigusa*. Scale in million years ago. Node bars represent 95% confidence intervals. Country codes after the sample names. Country codes: BE=Belgium; DK=Denmark; ES=Spain; GE=Georgia; HR=Croatia; IT=Italy; UK=United Kingdom. PP=posterior probability.

1.10. SUPPLEMENTARY TABLES

Gene	Marker	Sequence	Source	Annealing T
12S	12S-ai	AAACTAGGATTAGATACCCTATTAT	Köcher et al. (1989)	50°C
	12S-bi	AAGAGCGACGGGCGATGTGT	Köcher et al. (1989)	50°C
16S	16S-A	CGCCTGTTTATCAAAAACAT	Palumbi et al. (1991)	55°C
	16S-B	CTCCGGTTTGAACTCAGATCA	Palumbi et al. (1991)	55°C
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer at al. (1994)	48-52°C
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer at al. (1994)	48-52°C
H3	aF	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. (1998)	54°C
	aR	ATATCCTTRGGCATRATRGTGAC	Colgan et al. (1998)	54°C
18S	18S-1F	TACCTGGTTGATCCTGCCAGTAG	Giribet et al. (1996)	50°C
	18S-5R	CTTGGCAAATGCTTTCGC	Giribet et al. (1996)	50°C
	18S-3F	GTTCGATTCCGGAGAGGGA	Giribet et al. (1996)	50°C
	18S-bi	GAGTCTCGTTCGTTATCGGA	Whiting et al. (1997)	50°C
	18S-a2.0	ATGGTTGCAAAGCTGAAA	Whiting et al. (1997)	50°C
	18S-9R	GATCCTTCCGCAGGTTCACCTAC	Giribet et al. (1996)	50°C
28S	28S-Rd1a	CCCSCGTAAYTTAGGCATAT	Crandall et al. (2000), modification of Van der Auwera et al. (1994): primer 4	50°C
	28S-Rd4b	CCTTGGTCCGTGTTTCAAGAC	Crandall et al. (2000), modification of Van der Auwera et al. (1994): primer 10	50°C
	28S-Rd3.2a	AGTACGTGAAACCGTTCASGGGT	Wheeler laboratory fide Whiting (2002)	50°C
	28S-B	TCGGAAGGAACCAGCTACTA	Whiting et al. (1997)	50°C
	28S-A	GACCCGTCTTGAAGCACG	Whiting et al. (1997), modification of Nunn et al. (1996)	50°C
	28S-Bout	CCCACAGCGCCAGTTCTGCTTACC	Wheeler laboratory fide Hovmöller et al. (2002)	50°C
	28S-Rd4.8a	ACCTATTCTCAAACTTTAAATGG	Wheeler laboratory fide Whiting (2002)	52°C
	28S-Rd7b1	GACTTCCCTTACCTACAT	Wheeler laboratory fide Whiting (2002)	52°C

Supplementary Table 1. List of primers used for DNA amplification and sequencing with annealing temperatures and references.

Species	Code	12S	16S	18S	28S	COI	H3
Cryphoeca silvicola	CSBAR		OP964778	OP964464	OP964486	OP956113	OP957329
Mastigusa arietina	MABE02			OP964477	OP964498	OP956091	OP957328
Mastigusa arietina	MABE01			OP964478	OP964497	OP956111	OP957307
Mastigusa arietina	MAS_DK_01	OQ085108		OP964476	OP964499	OP956093	OP957310
Mastigusa arietina	MAS_DK_03	OQ085099		OP964483	OP964493	OP956106	OP957322
Mastigusa arietina	MAS_DK_09	OQ085106		OP964474	OP964494	OP956112	OP957321
Mastigusa arietina	MAGE04			OP964479	OP964489	OP956108	OP957326
Mastigusa arietina	MAGE05			OP964475	OP964491	OP956109	OP957327
Mastigusa arietina	MAGE06			OP964472	OP964490	OP956110	OP957324
Mastigusa arietina	MAVSC1			OP964485	OP964500	OP956097	OP957315
Mastigusa arietina	MAVSC2			OP964482	OP964501	OP956098	OP957316
Mastigusa arietina	EDBA1	OQ085107		OP964484	OP964495	OP956102	OP957313
Mastigusa arietina	MADBC2			OP964481	OP964496	OP956092	OP957325
Mastigusa arietina	MAS_IT_01	OQ085098		OP964465	OP964492	OP956094	OP957320
Mastigusa macrophthalma	MMHR4	OQ085104	OP964780	OP964469	OP964488	OP956100	OP957311
Mastigusa macrophthalma	MMHR5	OQ085105	OP964781		OP964487	OP956101	OP957312
Mastigusa sp.	MD2844	OQ085103	OP964779	OP964466	OP964508	OP956095	OP957309
Mastigusa sp.	MD372	OQ085101		OP964467	OP964505	OP956096	OP957314
Mastigusa sp.	MASN01	OQ085100	OP964782	OP964480	OP964506	OP956105	OP957308
Mastigusa sp.	MASN02	OQ085102	OP964783	OP964468	OP964507	OP956107	OP957323
Mastigusa sp.	MAUK01			OP964470	OP964503	OP956099	OP957317
Mastigusa sp.	MAUK05			OP964473	OP964502	OP956103	OP957319
Mastigusa sp.	MAUK06			OP964471	OP964504	OP956104	OP957318

Supplementary Table 2. Accession numbers for sequences produced in the present work.

Family	Species	12S	16S	18S	28S	COI	H3
Agelenidae	*Agelena labyrinthica*		JN816565	JN816781	JN816991	JN817199	
Agelenidae	*Agelenopsis pennsylvanica*	KY015266	KY015697	KY016263	KY016881	KY017545	KY018083
Agelenidae	*Draconarius* sp. ARACG000074	KY015267	KY015698	KY016264	KY016882	KY017546	KY018084
Agelenidae	*Draconarius* sp. ARACG000082	KY015268	KY015699	KY016265	KY016883	KY017547	KY018085
Agelenidae	*Eratigena atrica*	KY015269	KY015700	KY016266	KY016884	KY017548	KY018086
Agelenidae	*Hololena* sp. ARACG000018	KY015270	KY015701	KY016267	KY016885	KY017549	KY018087
Amaurobiidae	*Amaurobius similis*	KY015271	KY015703	KY016269	KY016887	KY017551	KY018089
Amaurobiidae	*Anisacate tigrinum*	KY015274	KY015706	KY016272	KY016890	KY017554	KY018091
Amaurobiidae	*Callobius bennetti*	KY015272	KY015704	KY016270	KY016888	KY017552	GQ119403
Amaurobiidae	*Pimus iviei*	KY015273	KY015705	KY016271	KY016889	KY017553	KY018090
Amaurobiidae	*Rubrius antarcticus*	KY015278	KY015712	KY016280	KY016897	KY017560	KY018096
Amaurobiidae	*Zanomys californica*	KY015279	KY015714	KY016282	KY016899	FJ949023	FJ949060
Araneidae	*Araneus diadematus*	KY015302	KY015735	KY016315	KY016926	KY017584	KY018113
Clubionidae	*Clubiona terrestris*	KY015325	KY015764	KY016342	KY016955		
Clubionidae	*Pristidia prima*	KY015327		KY016344	KY016957	KY017610	KY018138
Corinnidae	*Falconina gracilis*	KY015331	KY015775	KY016358	KY016971	KY017622	
Corinnidae	*Graptartia tropicalis*	KY015332	KY015776	KY016359	KY016972	KY017623	KY018152
Corinnidae	*Nyssus* cf. *coloripes* MR669		KY015778	KY016361	KY016974	KY017624	
Corinnidae	*Paradiestus penicillatus*	KY015333	KY015780	KY016363	KY016976	KY017626	KY018155
Corinnidae	cf. *Myrmecium* sp. Ecuador MR433		KY015771	KY016351	KY016966	KY017617	KY018146
Ctenidae	*Ctenus crulsi*	KY015337	KY015785	KY016369	KY016982	KY017633	KY018160
Ctenidae	*Phoneutria fera*	KY015340		KY016373	KY016986	KY017637	KY018164
Cybaeidae	*Calymmaria* sp. ARACG000231	KY015350	KY015802	KY016382	KY017004	KY017652	KY018167
Cybaeidae	*Calymmaria* sp. 1 JCS-2006			DQ628666 DQ628739	DQ628666	DQ628611	DQ628638
Cybaeidae	*Calymmaria* sp. 2 JCS-2006			DQ628703 DQ628740	DQ628667	DQ628612.	DQ628639
Cybaeidae	*Cryphoeca exlineae*			MN590054	MN590084	KP653069	MN590107
Cybaeidae	*Cryphoeca* sp. 1 JCS-2006			DQ628708 DQ628614	DQ628672	DQ628614	
Cybaeidae	*Cybaeina* sp. 1 SCC-2010			HM576631	HM576647		HM576666
Cybaeidae	*Cybaeina* sp. 2 SCC-2010			HM576632	HM576648		HM576667
Cybaeidae	*Cybaeota* sp. 3 SCC-2010			HM576629	HM576645		HM576664

Cybaeidae	*Cybaeus giganteus*	KY015351	KY015803	KY016383	KY017005	KY017653	KY018168
Cybaeidae	*Cybaeus morosus*			DQ628744 DQ628707	DQ628671	FJ263792	DQ628641
Cybaeidae	*Cybaeus mosanensis*		JN816569	JN816785		JN817203	
Cybaeidae	*Cybaeus* sp. 2 SCC-2010			HM576639	HM576654		HM576673
Cybaeidae	*Cybaeus* sp. 3 SCC-2010			HM576636	HM576651		HM576670
Cybaeidae	*Dirksia cinctipes*			MN590055	MN590085	MF812163	MN590109
Cybaeidae	*Ethobuella tuonops*			MN590056		KM839061	MN590110
Cybaeidae	*Yorima* sp. SCC-2010			HM576634	HM576650		HM576668
Cybaeidae	Cybaeidae Gen 1 sp. SCC-2010			HM576630	HM576646		HM576665
Cybaeidae	Cybaeidae Gen 4 sp. SCC-2010			HM576637	HM576652		HM576671
Cyclocteniidae	*Cycloctenus* sp. ARACG000098	KY015355	KY015807	KY016387	KY017009	KY017657	KY018172
Cyclocteniidae	*Cycloctenus* sp. ARACG000133	KY015352	KY015804	KY016384	KY017006	KY017654	KY018169
Cyclocteniidae	*Cycloctenus* sp. ARACG000150	KY015353	KY015805	KY016385	KY017007	KY017655	KY018170
Cyclocteniidae	*Cycloctenus* sp. ARACG000204	KY015354	KY015806	KY016386	KY017008	KY017656	KY018171
Cyclocteniidae	*Cycloctenus westlandicus*	KY015356	KY015808	KY016388	KY017010	KY017658	KY018173
Cyclocteniidae	*Toxopsiella* sp. CG134		KY015812	KY016392	KY017014	KY017662	KY018177
Deinopidae	*Deinopis spinosa*	KY015360	KY015818	KY016396	KY017020	KY017668	
Deinopidae	*Deinopis* sp. GH4	KY015359	KY015816	KY016394	KY017018	KY017666	
Deinopidae	*Deinopis* sp. SP68		KY015817	KY016395	KY017019	KY017667	KY018180
Deinopidae	*Menneus camelus*	KY015361	KC849122	KY016397	KY017021	KY017669	KY018181
Deinopidae	*Menneus capensis*	KY015362	KY015819		KY017022	KY017670	
Desidae	*Badumna* sp. ARACG000186	KY015365	KY015824	KY016402	KY017026	KY017675	KY018186
Desidae	*Cambridgea* sp. ARACG000097	KY015367	KY015827	KY016406	KY017030	KY017679	KY018188
Desidae	*Corasoides* sp. ARACG000294	KY015370	KY015830	KY016409	KY017033	KY017682	KY018191
Desidae	*Desis formidabilis*	KY015371	KY015831	KY016410	KY017034	KY017683	KY018192
Desidae	*Goyenia* sp. ARACG000236	KY015372	KY015832	KY016411	KY017035	KY017684	KY018193
Desidae	*Goyenia* sp. ARACG000237	KY015373	KY015833	KY016412	KY017036	KY017685	KY018194
Desidae	*Ischalea* sp. ARACG000119	KY015374	KY015834	KY016413	KY017037	KY017686	KY018195
Desidae	*Nanocambridgea* sp. ARACG000203	KY015381	KY015837	KY016421	KY017045	KY017692	KY018203
Desidae	*Paramatachia* sp. ARACG000277	KY015383	KY015839	KY016423	KY017047	KY017694	KY018205
Dictynidae	*Argenna obesa*			MN590060	MN590088	KP646247	MN590115
Dictynidae	*Argenna patula*			KR074002 KR073978	KR074028	KR074054	KR074080

Dictynidae	*Argyroneta aquatica*		KY015842	KY016427	KY017051	KY017697	KY018206
Dictynidae	*Brigittea civica*			MN590058	MN590086		MN590112
Dictynidae	*Brommella monticola*			MN590059	MN590087	KP657230	MN590113
Dictynidae	*Brommella* sp. ZZ-2016			KR074007 KR073983	KR074033	KR074059.	KR074085
Dictynidae	*Devade tenella*			KR074010 KR073986	KR074036	KR074062	KR074088
Dictynidae	*Dictyna bellans*			MN590063	MN590090	KP651399	MN590118
Dictynidae	*Dictyna brevitarsa*			MN590064	MN590091	KT706865.	MN590119
Dictynidae	*Dictyna foliicola*	AF466988		KR074011 KR073987	KR074037	KR074063	KR074089
Dictynidae	*Dictyna major*	KY015387		KR073988 KR074012	KR074038	KR074064	KR074090
Dictynidae	*Dictyna namuliensis*			KR074013 KR073989	KR074039	KR074065	KR074091
Dictynidae	*Dictyna volucripes*			MN590066	MN590092	KT708818	MN590121
Dictynidae	*Dictyna* sp. ARAMH000001			KY016429	KY017054 KY017053	KY017699	
Dictynidae	*Dictyna* sp. TAB-2009		FJ607452	FJ607487	FJ607526	FJ607561	FJ607600
Dictynidae	*Dictyna* sp. 1 JCS-2006			DQ628709 DQ628746	DQ628673	DQ628615	
Dictynidae	*Emblyna annulipes*			MN590067	MN590093	KT705497	MN590122
Dictynidae	*Emblyna sublata*			MN590068		KT708437	MN590123
Dictynidae	*Hackmania prominula*			MN590069	MN590094	KP656539	MN590124
Dictynidae	*Hackmania saphes*			MN590070	MN590095	KM830608	MN590126
Dictynidae	*Lathys alberta*			DQ628749 DQ628712	DQ628676	DQ628616	DQ628643
Dictynidae	*Lathys humilis*			MN590073	MN590097	MG044282	MN590128
Dictynidae	*Lathys* sp. ZZ-2016			KR074016 KR073992	KR074042	KR074068	KR074094
Dictynidae	*Mallos pallidus*	KY015388	KY015844	KY016430	KY017055	KY017700	
Dictynidae	*Mexitlia trivittata*		FJ607462	FJ607499	FJ607537	FJ607573	FJ607611
Dictynidae	*Mizaga racovitzai*			MN590074	MN590098		MN590129

Dictynidae	*Nigma flavescens*			MN590075	MN590099		MN590130
Dictynidae	*Paratheuma armata*			DQ628747 DQ628710	DQ628674	DQ411405	
Dictynidae	*Phantyna bicornis*			MN590076	MN590100	JN308669	MN590131
Dictynidae	*Phantyna terranea*			MN590077	MN590101	MG042614	MN590132
Dictynidae	*Saltonia incerta*		KY015845	KY016431	KY017056	KY017701	KY018207
Dictynidae	*Sudesna hedini*			KR074021	KR074047	KR074073	KR074099
Dictynidae	*Tricholathys monterea*			MN590078	MN590102		MN590133
Dictynidae	*Tricholathys* sp. 10_5			FJ948938 FJ948896		FJ949020	FJ949057
Gnaphosidae	*Molycria* sp. MR670	KY015537	KY016077	KY016659	KY017312	KY017884	KY018389
Gnaphosidae	*Zelotes* sp. MR525	KY015422	KY015895	KY016475	KY017111	KY017739	KY018249
Hahniidae	*Antistea brunnea*			MN590079	MN590103	KM839029	MN590134
Hahniidae	*Cicurina cicur*			MN590080	MN590104		MN590135
Hahniidae	*Cicurina japonica*		JN816573	JN816789	JN816997	JN817207	
Hahniidae	*Cicurina* sp. ARAMH000010	KY015427	KY015900	KY016480	KY017116	KY017744	
Hahniidae	*Cicurina* sp. ZZ-2016			KR074008 KR073984	KR074034	KR074060	KR074086
Hahniidae	*Cicurina* sp. 1 JCS-2006			DQ628705 DQ628742	DQ628669	DQ628613	DQ628640
Hahniidae	*Cybaeolus* cf. *rastellus*		KY015901	KY016481	KY017117	KY017745	KY018252
Hahniidae	*Cybaeolus* sp. 11_01			FJ948910 FJ948868	FJ948952	FJ948992	FJ949031
Hahniidae	*Hahnia cinerea*			MN590081	MN590105	KM824967	MN590136
Hahniidae	*Hahnia clathrata*			FJ948923 FJ948881	FJ948965	FJ949005	FJ949043
Hahniidae	*Hahnia nava*	KY015428	KY015902	KY016483		MT607760	KY018254
Hahniidae	*Hahnia ononidum*			MN590082	MN590106	KP656850	MN590137
Hahniidae	*Hahnia zhejiangensis*			KR074015 KR073991	KR074041	KR074067	KR074093
Hahniidae	*Hahnia* sp. ZZ-2016			KR074014 KR073990	KR074040	KR074066	KR074092

Hahniidae	*Neoantistea agilis*			DQ628751 DQ628714	DQ628678	HQ979361	DQ628644
Hahniidae	*Neoantistea quelpartensis*		JN816572	JN816788	JN816996	JN817206	
Hersiliidae	*Hersilia sericea*	KY015429	KY015903	KY016484	KY017119	KY017746	KY018255
Hersiliidae	*Hersiliola macullulata*			FJ948883 FJ948925	FJ948967	FJ949007	FJ949045
Hersiliidae	*Iviraiva argentina*		KY015904	KY016485	KY017121 KY017120		KY018256
Liocranidae	*Apostenus californicus*		KY015934	KY016512	KY017154	KY017775	KY018279
Liocranidae	*Apostenus* sp. MR20	KY015453	KY015935	KY016513	KY017155		KY018280
Lycosidae	*Allocosa* sp. PS114	KY015460	KY015943	KY016522	KY017164	KY017781	KY018281
Lycosidae	*Arctosa kwangreungensis*	DQ019767	JN816547	JN816763	JN816973	JN817181	
Miturgidae	*Miturga lineata*	KY015473	KY015969	KY016551	KY017199 KY017198	KY017796	KY018308
Miturgidae	*Zora spinimana*	KY015477	KY015975	KY016557	KY017205	KY017800	KY018314
Oecobiidae	*Oecobius* sp. TAB-2009		FJ607466	FJ607505	FJ607540	FJ607579	FJ607617
Oecobiidae	*Uroctea durandi*		KY015996	KY016568	KY017227	KY017819	KY018328
Oecobiidae	*Uroctea* sp. CG285	KY015488	KY015997	KY016569	KY017228	KY017820	KY018329
Oxyopidae	*Hamataliwa* sp. SP20	KY015496	KY016010	KY016586	KY017243	KY017829	KY018335
Oxyopidae	*Oxyopes salticus*		KY016011	KY016587	KY017244	KY017830	
Oxyopidae	Oxyopidae sp. Myanmar PS6	KY015497	KY016012	KY016588	KY017245	KY017831	KY018336
Penestomidae	*Penestomus egazini*	KY015502	KY016027	KY016603	KY017259	KY017842	KY018346
Philodromidae	*Tibellus oblongus*		KY016031	KY016609	KY017263	KY017846	KY018352
Philodromidae	*Thanatus formicinus*	KY015504		KY016606	KY017261	KY017843	KY018349
Philodromidae	*Titanebo mexicanus*	KY015505	KY016032	KY016610	KY017264	KY017847	KY018353
Phyxelididiae	*Ambohina ranohira*	KY015519	KY016052	KY016629	KY017284	KY017863	KY018369
Phyxelididiae	*Vidole capensis*	KY015522	KY016054	KY016634	KY017289	KY017867	FJ949059
Pisauridae	*Dolomedes tenebrosus*	KY015526	KY016061	KY016642	KY017297	KY017873	KY018377
Pisauridae	*Pisaurina mira*	KY015531	KY016067	KY016650	KY017304	KY017877	KY018382
Salticidae	*Carrhotus* sp. WM10	KY015544	KY016086	KY016670	KY017322	KY017891	KY018398
Salticidae	*Lyssomanes viridis*	KY015549	KY016094	KY016677	KY017329	KY017897	KY018406
Salticidae	*Portia* sp. SP32	KY015553	KY016098	KY016681	KY017334	KY017899	KY018411
Salticidae	*Salticus scenicus*	KY015554	KY016099	KY016682	KY017335	KY017900	KY018412

Salticidae	*Sittisax ranieri*		KY016100	KY016683	KY017336	KY017901	KY018413
Selenopidae	*Anyphops barbertonensis*		KY016105	KY016691	KY017344	KY017906	KY018417
Selenopidae	*Anyphops* sp. SP44	KY015559	KY016106	KY016692	KY017345	KY017907	KY018418
Selenopidae	*Garcorops madagascar*		HM575625			HM575917	HM576226
Selenopidae	*Hovops* sp. MR47	KY015560	KY016107	KY016693	KY017346	KY017908	KY018419
Selenopidae	*Selenops cocheleti*		KY016108	KY016694	KY017347	KY017909	KY018420
Selenopidae	*Selenops insularis*	KY015561	KY016109	KY016695	KY017348	KY017910	KY018421
Selenopidae	*Selenops nesophilus*			KY016696	KY017349	KY017911	KY018422
Sparassidae	*Caayguara album*	KY015569	KY016116		KY017359 KY017358	KY017918	KY018427
Sparassidae	*Delena cancerides*		KF372684		KF372774	KF442797	KF442867
Sparassidae	*Eusparassus* sp. ARAMR000103	KY015570	KY016117	KY016704	KY017360	KY017919	KY018428
Sparassidae	*Heteropoda venatoria*	KY015571	KY016118	KY016705	KY017361	KY017920	KY018429
Sparassidae	*Isopeda parnabyi*	KY015572	KY016119	KY016706	KY017362	KY017921	KY018430
Sparassidae	*Neostasina* sp. MR164-MR350	KY015573		KY016707	KY017363	KY017922	
Sparassidae	*Neostasina* sp. MR351			KY016708	KY017364	KY017923	KY018431
Sparassidae	*Polybetes pythagoricus*	KY015574		KY016709	KY017365	KY017924	KY018432
Sparassidae	*Pseudopoda* sp. ARAMR000532	KY015575	KY016121	KY016711	KY017367		KY018433
Sparassidae	*Sinopoda* sp. ARACG000070	KY015576	KY016123	KY016712	KY017369	KY017926	KY018435
Stiphidiidae	*Neoramia setosa*	KY015584	KY016129	KY016719	KY017378	KY017936	KY018442
Stiphidiidae	*Neoramia* sp. ARACG000129	KY015585	KY016130	KY016720	KY017379	KY017937	KY018443
Stiphidiidae	*Neoramia* sp. ARACG000178	KY015586	KY016131	KY016721	KY017380	KY017938	KY018444
Stiphidiidae	*Neoramia* sp. ARACG000239	KY015587	KY016132	KY016722	KY017381	KY017939	KY018445
Stiphidiidae	*Stiphidion* sp. ARACG000091	KY015589	KY016134	KY016725	KY017384	KY017942	KY018446
Stiphidiidae	*Stiphidion* sp. ARASP000081	KY015590	KY016135	KY016726	KY017385	KY017943	KY018447
Thomisidae	*Aphantochilus* sp. MR186	KY015620	KY016171	KY016764	KY017424	KY017976	KY018476
Thomisidae	*Epicadus heterogaster*	KY015629	KY016181	KY016776	KY017436	KY017987	
Thomisidae	*Misumenops nepenthicola*			EF418996	EF419029	EF419094	EF419123
Thomisidae	*Stephanopoides sexmaculata*	KY015635	KY016186	KY016783	KY017443	KY017994	KY018492
Thomisidae	*Tmarus holmbergi*	KY015639	KY016190	KY016792	KY017452	KY018004	KY018501
Titanoecidae	*Pandava sarasvati*	KY015643	KY016194	KY016796	KY017456	KY018009	KY018505
Titanoecidae	*Titanoeca* sp. CG64	KY015644	KY016195	KY016797	KY017457	KY018010	
Toxopidae	*Hapona* sp. ARACG000113		KY016197	KY016799	KY017459	KY018012	KY018506
Toxopidae	*Myro* sp. ARACG000160	KY015646	KY016201	KY016803	KY017463	KY018015	KY018509

Toxopidae	*Otagoa* sp. ARACG000235	KY015647	KY016203	KY016804	KY017465	KY018017	KY018510
Uloboridae	*Octonoba sinensis*	AF466983	DQ200050	JN816668	JN816880	JN817080	
Uloboridae	*Uloborus diversus*		FJ525362	FJ525399	FJ525380	FJ525329	FJ525345
Uloboridae	*Uloborus glomosus*	KY015664		KY016833	KY017495	HQ979221	EU003340
Zodariidae	*Diores femoralis*		KY016239	KY016851	KY017514	KY018057	KY018546
Zodariidae	*Zodarion italicum*	KY015684	KY016251	KY016866	KY017530	KY018069	KY018559

Supplementary Table 3. List of taxa included in the phylogenetic analyses with GenBank accession numbers.

Partition	Model
12S	TIM2+F+G4
16S	GTR+F+R5
18S	TNe+R6
28S	TNe+R5
COI, 1st position; COI, 2nd position	GTR+F+I+G4
COI, 3rd position	HKY+F+R5
H3, 1st position	JC+R4
H3, 2nd position	TVMe+R5
H3, 3rd position	GTR+F+G4

Supplementary Table 4. Best partitioning scheme and substitution models identified by ModelFinder.

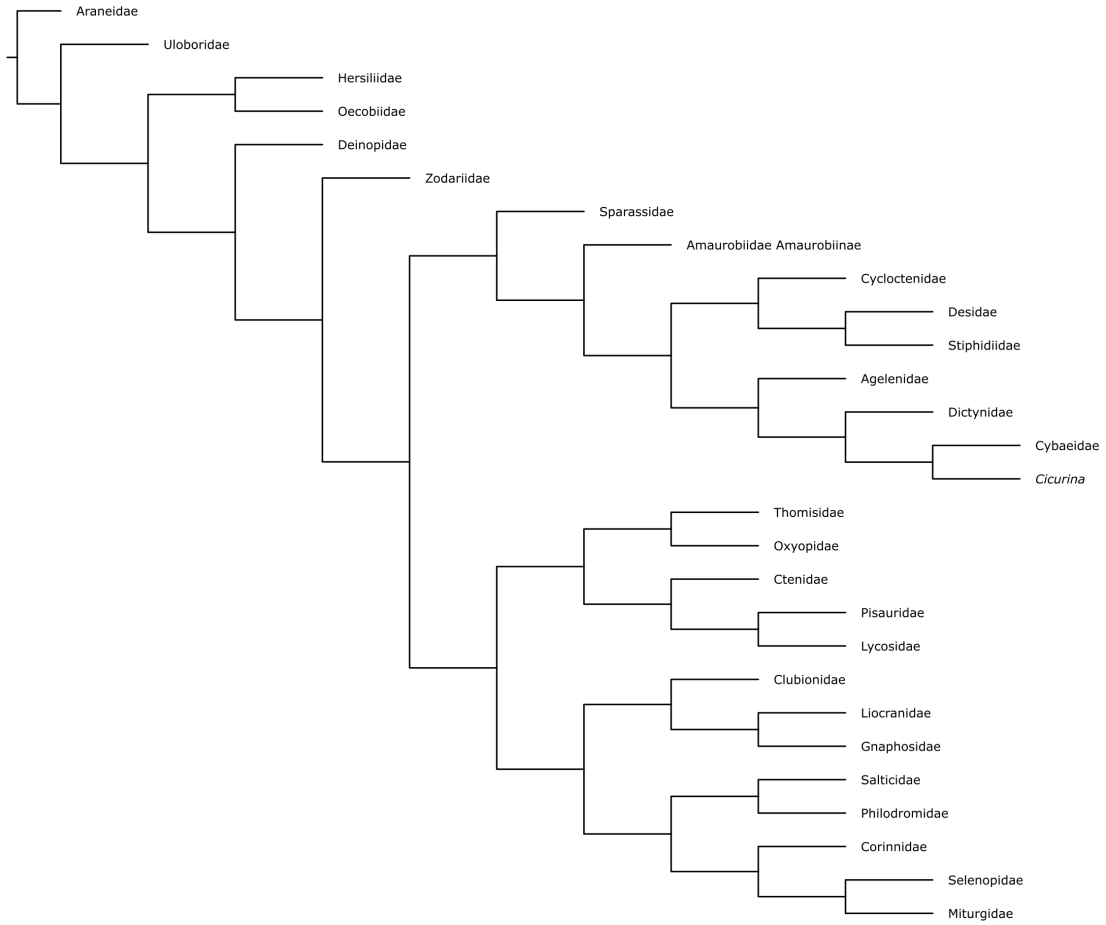
Number in time-tree	Fossils	Assignable to	Offset	Alpha	Reference
1	*Zamilia aculeopectens*/*Burmesiola cretacea*	Oecobiidae stem/Hersiliidae stem	98.17	1.09941	Magalhaes et al. 2020
2	*Oxyopes succini*/*Siphax* cf. *megacephalus*	Oxyopidae stem/Thomisidae stem	43	1.0478	Magalhaes et al. 2020
3	*Selenops* sp. indet.	Selenopidae stem	53	1.056	Magalhaes et al. 2020
4	*Almolinus ligula*	Salticidae crown	43	1.0478	Magalhaes et al. 2020

Supplementary table 5. Fossils used for calibration in the time-tree inference. Offset and Mean in million years ago.

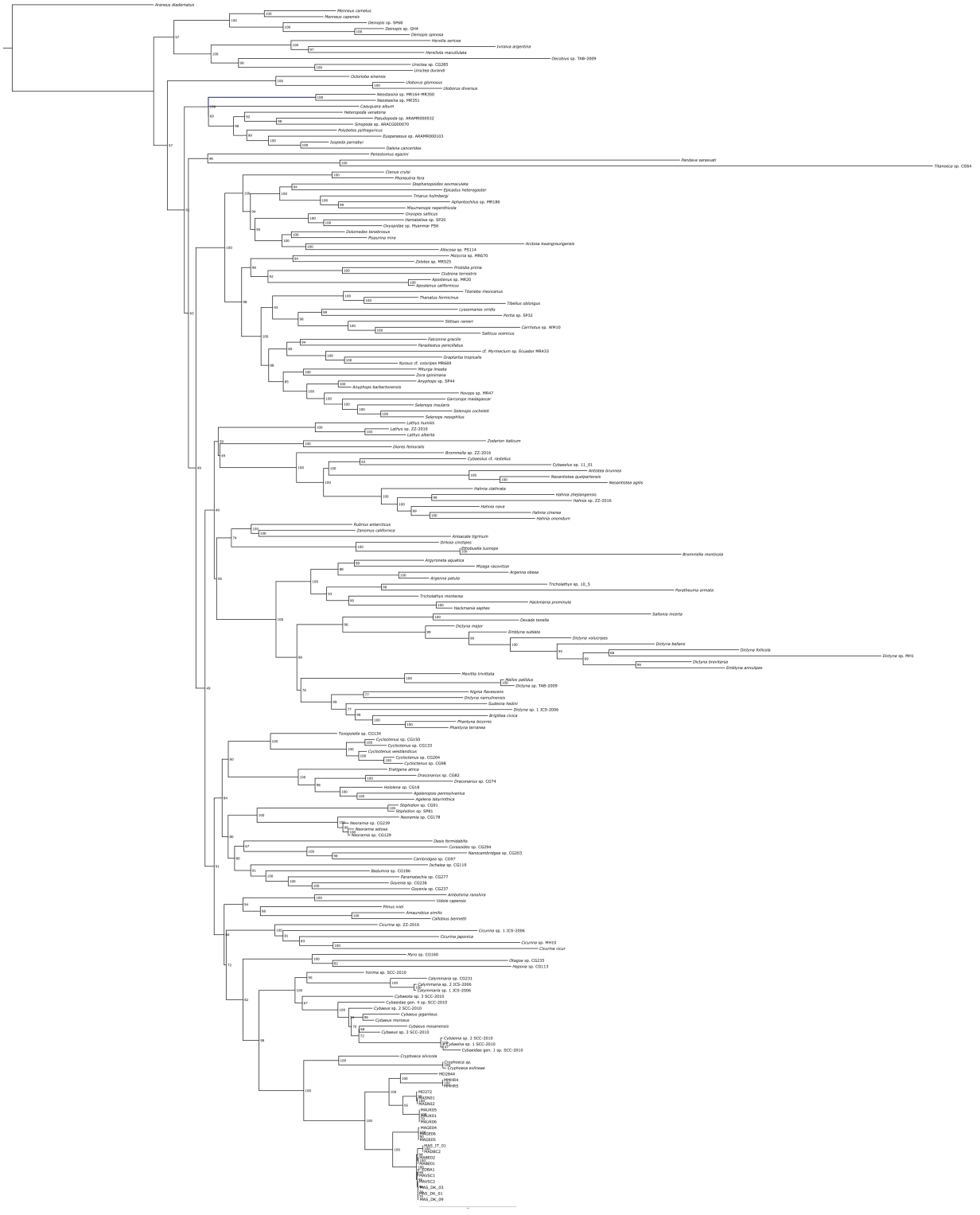
Tree	logL	deltaL	bp-RELL	p-KH	p-SH	c-ELW	p-AU	p-AU
Unconstrained	-86719,16177	0	0.92 +	0.921 +	1 +	0.919 +	0.919 +	0.919 +
Constrained	-86756,45175	37,29	0.0795 +	0.0792 +	0.0792 +	0.0807 +	0.0808 +	0.0808 +

Supplementary table 6. Topology tests of the constrained Maximum Likelihood topology vs the unconstrained one performed on IQ-TREE. deltaL: logL difference from the maximal logl in the set; bp-RELL: bootstrap proportion using RELL method; p-KH: p-value of one-sided Kishino-Hasegawa test; p-SH: p-value of Shimodara-Hasegawa test; c-ELW: Expected Likelihood Weight; p-AU: p-value of approximately unbiased test. Plus signs denote the 95% confidence sets. Minus signs denote significant exclusions. All tests performed 10000 resampling using the RELL method.

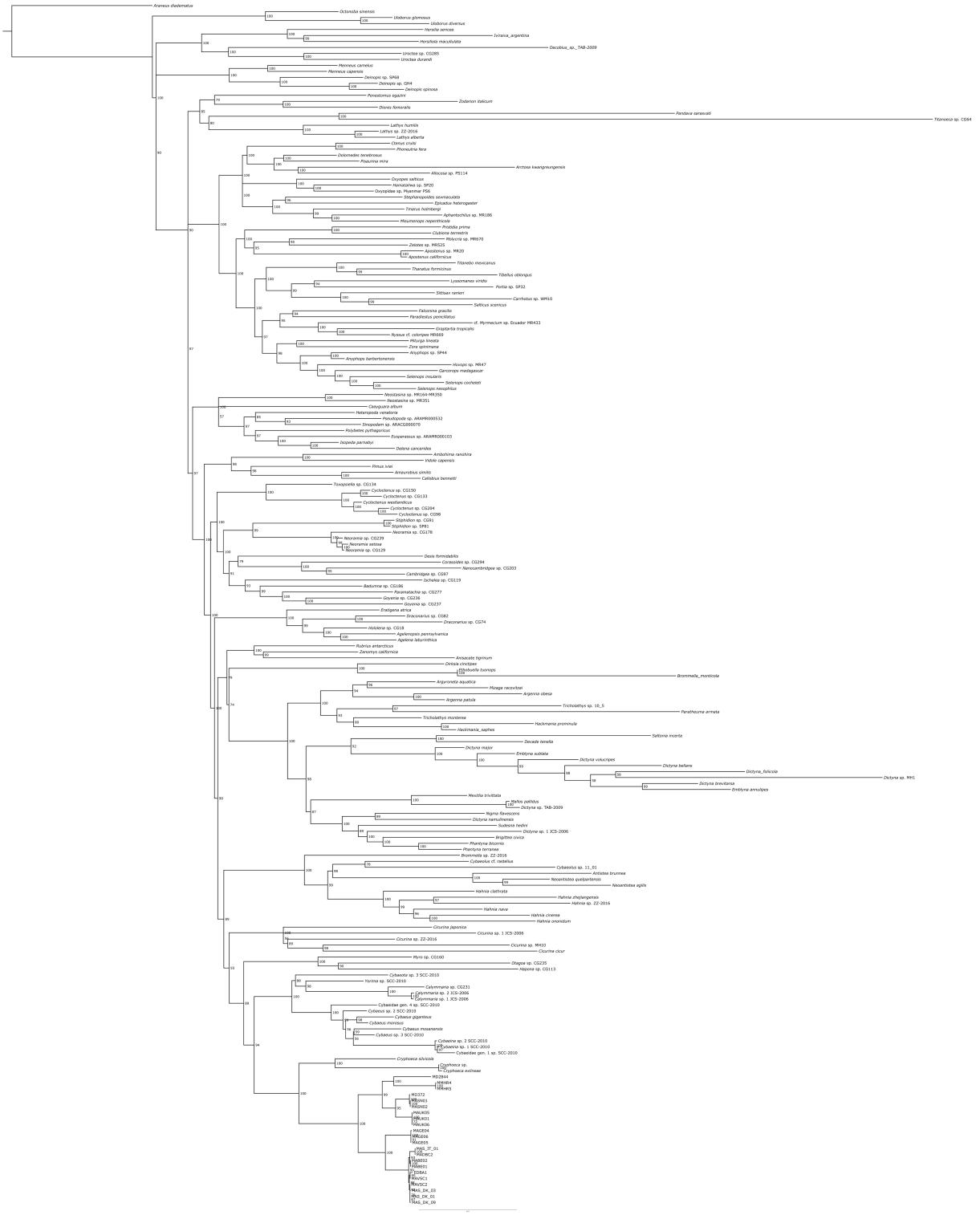
1.11. SUPPLEMENTARY FIGURES



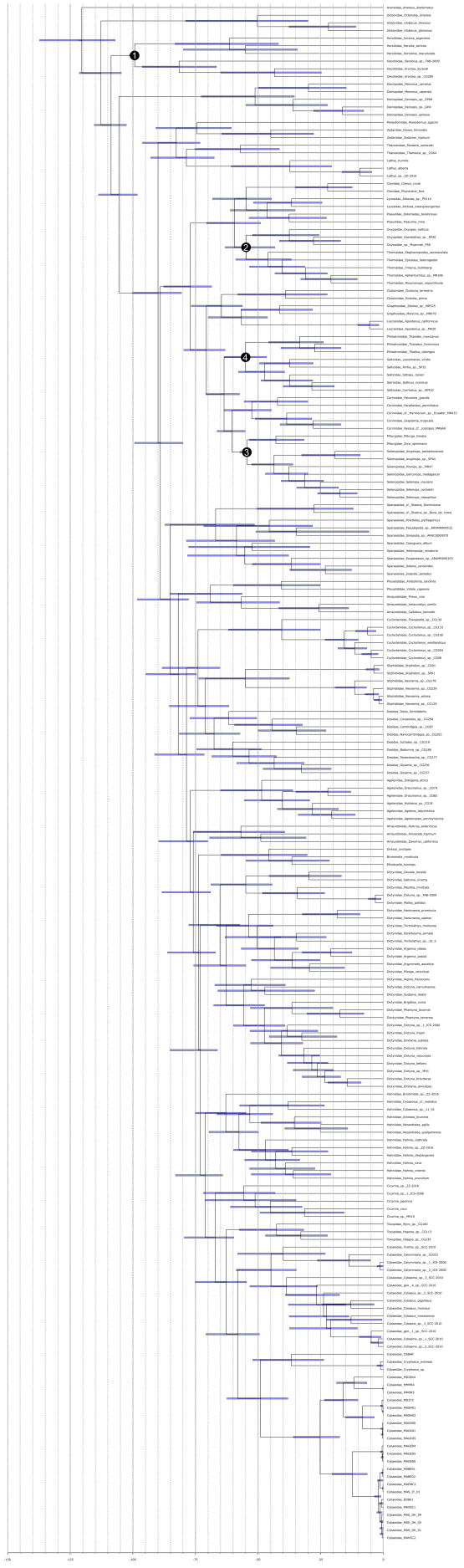
Supplementary Figure 1. Backbone tree used in the constrained Maximum Likelihood analysis.



Supplementary Figure 2. Unconstrained Maximum Likelihood tree built using IQ-TREE with 1000 ultrafast bootstrap replicates.



Supplementary Figure 3. Constrained Maximum Likelihood tree built using IQ-TREE with 1000 ultrafast bootstrap replicates.


1
2
3
4
-150
-125
-100
-75
-50
-25
0

Supplementary Figure 4. Bayesian phylogeny and divergence time estimates built in BEAST. Scale in million years. Node bars represent 95% confidence intervals. Calibration points are identified by numbers (see Suppl. Tab. 5).

2. Taxonomic revision of the spider genus *Mastigusa* (Araneae, Cybaeidae) based on morphological and molecular data

Filippo Castellucci, Andrea Luchetti and Nikolaj Scharff

2.1. ABSTRACT

Mastigusa Menge, 1854 is a genus of small palearctic spiders recently moved to the family Cybaeidae after the first inclusion of the genus in a phylogenetic matrix. Three species are currently recognized: *M. arietina* (Thorell, 1870), *M. lucifuga* (Simon, 1898) and *M. macrophthalma* (Kulczyński, 1897). Their status and delimitation, though, has always been problematic due to inconsistency in the characters used to discriminate them, leading to great confusion about their identity and distribution. We present a detailed morphological redescription of the genus and a taxonomic revision of the species it includes by the combined use of morphological data and molecular species-delimitation techniques based on the mitochondrial COI gene. The status of the three currently described species has been re-evaluated and a new species is described from the Iberian Peninsula, North Africa, and the United Kingdom: *M. raimondi* sp. n. The distribution of *Mastigusa* species is updated based on the novel taxonomical considerations and comments on the natural history and ecological differences observed in the different species are provided.

Keywords: integrative taxonomy; species delimitation; DNA barcoding; phylogenetics; morphology

2.2. INTRODUCTION

The spider genus *Mastigusa* Menge, 1854 currently holds extant as well as fossil entelegyne species. The somatic morphology of these small spiders (3 - 4.5 mm in body length) is quite ordinary (Fig. 1), while the morphology of the genitalia is remarkable. Males of *Mastigusa* have extremely large and quite bizarre looking pedipalps which are in some instances almost the length of the entire body (Roberts 1995). In the first extant species described, *Mastigusa arietina* (Thorell, 1871), the specific epithet refers to the "ram-like" conductor of the male pedipalp (from Latin *aries* = "ram") (Fig. 2) which is characteristic for the genus. The females have similarly complex internal genitalia with long and winding copulatory ducts (Fig. 3). Nonetheless, little is known about the reproductive biology of *Mastigusa* and the function of such extreme genital modifications is unknown. The genus is distributed in the Palearctic, being currently known from Europe, North Africa, and Asia (World Spider Catalog 2022) and shows a great ecological variability across its distribution range, with free-living, cave-dwelling and myrmecophilic populations (Castellucci et al. 2022). The taxonomic history of the genus is quite intricate. It was first established by Menge in 1856 based on a specimen preserved in Baltic amber (type species *Mastigusa acuminata* Menge, 1854) with no extant representatives known at that time; therefore, the genus was considered extinct. A few years later, the first extant species was discovered by Westring (1861), who collected specimens in a *Formica rufa* Linnaeus, 1758 ant nest in Sweden and wrongly identified them as *Hahnia pratensis* (C.L. Koch, 1841) (now *Antistea elegans* (Blackwall, 1841)). Based on Westring's description of the male palp of the specimen found and a re-examination of Westring's specimens from Sweden, Thorell (1871) pointed out that such specimens had unusual genitalia, very different from *H. pratensis*, therefore belonging to a different species. He suggested that Westring's specimens belonged to a new species in the newly described genus *Cryphoeca* Thorell, 1870 and re-described them as *Cryphoeca arietina* Thorell, 1871. In 1875, Simon described *Cicurina impudica* Simon, 1875 and suggested a close relationship to Thorell's *C. arietina*, placing both species in the genus *Cicurina*, since both specimens show anterior eyes in a straight row, while species in the genus *Cryphoeca* have anterior eyes in a bent row (Simon 1875). Twenty-two years later, in 1897, Chyzer & Kulczynski synonymized *C. impudica* with *C. arietina*, transferring the species to the

genus *Tuberta* Simon 1884 and described *Tuberta arietina macrophthalma* Kulczyński, in Chyzer & Kulczyński, 1897, from nowadays Croatia and Slovakia, as a subspecies of *Tuberta arietina*. According to Chyzer & Kulczynski (1897), these specimens only differ from *Tuberta arietina* by small morphological differences such as 1) a posterior row of eyes that is not procurved, 2) posterior median eyes larger than anterior median eyes and 3) small differences in the shape of the male palp. Chyzer & Kulczynski (1897) did not consider these morphological differences important enough to consider such specimens as a separate species, hence the description as a sub-species of *T. arietina*. A year later, Simon (1898a) transferred the two species to the genus *Tetrilus* Simon 1886 and described the new species *Tetrilus lucifuga* (Simon, 1898), which is only known from the type specimen, an adult female collected in the eastern French Pyrenees and never recorded again after its description. In the same year, in his *Histoire Naturelle des Araignées*, Simon did not mention the subspecies *Tetrilus arietina macrophthalma* (Simon 1898b) but in 1937 listed the subspecies as a species, *Tetrilus macrophthalmus* (Chryzer & Kulczyski, 1897) (Simon 1937). This acceptance of *T. macrophthalmus* as a separate species was followed by Locket and Milledge 1953, Loksa (1969), Tyschchenko (1971), Wunderlich (1986, 2004) and Azarkina and Trilikauskas (2012). Other authors considered *T. macrophthalmus* as a subspecies of *T. arietinus* (Bristowe 1939, Roberts 1985), as formerly suggested by Chyzer & Kulczynski (1897). In 1986, Wunderlich transferred *T. arietinus*, *T. lucifugus* and *T. macrophthalmus* to the genus *Mastigusa*, until then only known from Baltic amber fossils, based on the peculiar shape of the male pedipalp. Wunderlich revised the status of the three species including a list of morphological characters to be used to diagnose and differentiate them. While he recognized *M. lucifuga* as a species, he did not rule out the possibility to synonymize it with *M. arietina*, since the *M. lucifuga* male is unknown and the only differences that the *M. lucifuga* type shows in respect to *M. arietina* is in the dimension of the posterior median eyes, a character for which he observed a degree of variation in the latter species. He further revised the characters discriminating *M. arietina* and *M. macrophthalma,* as 1) the number of teeth in the posterior row of the chelicerae, 2) the size of posterior median eyes compared to anterior median eyes, 3) the relative distance between posterior median eyes, 4) the largest diameter of the conductor, 5) the ratio between diameter of the conductor and

length of the prosoma (Wunderlich 1986). Several authors, though, did not consider the differences proposed by Wunderlich reliable enough to discriminate *M. arietina* and *M. macrophthalma* (Klausen, 1974; Heimer and Nentwig, 1991; Scharff & Gudik-Sørensen, 2006). Despite the ambiguity regarding their status, all three species (*M. arietina*, *M. lucifuga* and *M. macrophthalma*) are currently considered valid (World Spider Catalog 2022). Doubts concerning the delimitation and identification of *M. arietina* and *M. macrophthalma* further led to confusion about their actual distribution range. The World Spider Catalog (2022) reports *M. arietina* as distributed in Europe, Algeria, Russia and Iran, while *M. macrophthalma* as distributed in Hungary, Balkans and, perhaps, Caucasus and Russia. The taxonomic uncertainty that has always surrounded *Mastigusa* species calls for a modern revision of the genus, which is the aim of the present work. The familiar placement of *Mastigusa* has been just as intricate as the delimitation of the included species, with alternative placements in the families Agelenidae (Simon 1875, 1898; Wunderlich 1986; Heimer and Nentwig 1991), Hahniidae (Lehtinen 1967; Palmgreen 1977; WSC 2022) and Dictynidae (Wunderlich 1994, 2004; Almquist 2006). A recent, comprehensive phylogenetic analysis, aimed at identifying the family to which *Mastigusa* belongs, placed the genus in a well-supported sister relationship with the genus *Cryphoeca* Thorell, 1870 (**Chapter 1**). Moreover, different *Mastigusa* populations were included in the analysis and six distinct genetic lineages were identified (Fig. 4).

In the present work we present a taxonomic revision of the genus, based on morphological characters and DNA barcoding species delimitation analysis. Moreover, we also revise the characters used for species diagnosis.

2.3. MATERIALS AND METHODS

2.3.1 Material acquisition

Fresh *Mastigusa* material was collected during fieldwork sessions in Europe (Italy, Denmark, Spain and Croatia) between 2018 and 2021, as reported in **Chapter 1**. Additional specimens from Spain, the United Kingdom, Belgium, Finland and Georgia were provided by fellow arachnologists. Specimens collected for molecular work were stored in 95% ethanol at -20 ºC prior to DNA extraction. Material for morphological examination, including type material for the three currently described species, was

acquired from the public museum collections and from private arachnological collections. For a list of the collections with abbreviations used in the text see Table 1.

2.3.2. DNA extraction, amplification, and sequencing

DNA was extracted from leg tissue using the NucleoSpin® DNA Insect kit (Macherey-Nagel) following instructions by the manufacturer. A partial fragment of the cytochrome *c* oxidase subunit 1 (COI) gene was amplified via PCR using the primer couple LCO1490-HCO2198 (Folmer et al. 1994) following the protocols from Wheeler et al. (2017). PCR products were screened on a 1% agarose gel via electrophoresis and purified prior to sequencing using the ExoSAP-IT Product Cleanup Reagent (Thermo Fisher Scientific). Sanger sequencing on the forward and reverse strands was performed at Macrogen Europe (Amsterdam, The Netherlands). Chromatograms were inspected using the software SeqTrace v.0.9.0 (Stucky 2012), and potential contaminants were screened using BLASTn (Zhang and Madden 1997) as implemented on NCBI. Sequences produced in this work were submitted to GenBank (accession numbers XXXX-YYYY).

2.3.3. Molecular species delimitation

COI sequences produced were combined in a dataset with sequences from previous works (Crespo et al. 2018; **Chapter 1**) and from the Bold Systems barcode database (https://www.boldsystems.org) and aligned using MAFFT v.7.503 (Katoh and Standley 2013) with the L-INS-i algorithm. Sixty-two *Mastigusa* terminals from nine countries were included, a full list of the specimens, with provenance, is reported in Suppl. Tab. 1. The COI dataset was used for molecular species delimitation using different approaches, as ASAP (assembling species by automatic partitioning) (Puillandre et al. 2019), PTP and mPTP (Poisson tree processes approaches) (Zhang et al. 2013; Kapli et al. 2017) and GMYC (general mixed Yule coalescent) (Pons et al. 2006). ASAP was carried out using Jukes-Cantor genetic distances on the online implementation available at https://bioinfo.mnhn.fr/abi/public/asap/#. PTP and mPTP were implemented using an IQ-TREE maximum likelihood tree as an input. This was built by partitioning the dataset in the three codon positions of the COI gene and by selecting the best fitting evolutionary models for the three partitions with ModelFinder (Kalyaanamoorthy et al. 2017), as

implemented in the IQ-TREE v1.6.12 software (Nguyen et al. 2015). Phylogenetic reconstruction using the maximum likelihood method was performed with IQ-TREE v1.6.12 with 1000 replicates of UltraFast bootstrap (Minh et al. 2013), and three *Cryphoeca* terminals were chosen as outgroups, based on the results from **Chapter 1**. For PTP, two runs with 500,000 generations each were computed with thinning every 100 generations and a burn-in 0f 20%; convergence between the two runs was assessed by comparing the likelihood trace plots. Both PTP and mPTP were used via their online implementations, available respectively at https://species.h-its.org and https://mptp.h-its.org/#. GMYC analysis with single threshold was run on an ultrametric tree generated with BEAST v2.6.7 (Bouckaert et al. 2014). This was built implementing a strict molecular clock and a coalescent process for speciation, as these parameters are known to be more conservative and minimize type I errors (Monaghan et al. 2009; Bidegaray-Batista et al. 2014). The analysis was run for 10 million generations with sampling every 5000 generations. Adequate ESS values (>200) were assessed using Tracer v1.7.2 (Rambaut et al. 2018). A maximum clade credibility tree was using Treeannotator v2.6.7 with a 20% burn-in. The GYMC analysis was carried out on the online implementation available at https://species.h-its.org/gmyc/. Genetic distances within and between morphospecies and the groups identified by molecular species delimitation methods were measured using the Jukes Cantor model with the MegaX software (Kamur et al. 2018).

2.3.4. Morphological examination

Specimens were examined using a Leica M205A stereomicroscope equipped with a Leica DFC450C camera and measured using the Leica Application Suite v3.6 software. Photographs were taken with a BK+ Imaging System from Visionary Digital equipped with a Canon EOS 7D reflex camera. SEM pictures were acquired with a Jeol JSM 6335F microscope. The material used for SEM imagery was dehydrated by submersion in ethanol solutions with increasing concentration (80%, 85%, 90% and 96%) for 30 minutes and subsequently critical point dried using a Baltec CPD-030 critical point dryer. SEM preparations were then coated with a Platinum-Palladium alloy using a Jeol JFK-2300HR high resolution coater for a duration of 160 seconds. Identification to the species level was carried out following original species descriptions (Thorell 1871; Chyzer and

Kulczynski 1897; Simon 1898a) and the taxonomic revision by Wunderlich (1986). Type material for the three described species was examined. All measurements are in millimeters. Morphological abbreviations used in the text are reported in Table 2.

2.4. RESULTS

2.4.1. Molecular species delimitation

The number of hypothetical species recovered by the different species delimitation methods ranged between 3 and 8 (Fig. 5). All methods were congruent in identifying as a potential species the Central-Northern European clade (Italy + Denmark + Belgium + Norway). The Finnish and Georgian populations were recovered as a different species by mPTP, while they were split in two species by the other methods. Specimens from Spain, the United Kingdom and Croatia were recovered as a single species by mPTP, while they were split into 3, 4 or 5 species by the other methods, as follows. ASAP: 3 species (United Kingdom + Spain – Sierra Nevada; Croatia; Pyrenees). PTP: 5 species (United Kingdom; Spain – Sierra Nevada 1; Spain – Sierra Nevada 2; Croatia; Pyrenees). GMYC: 4 species (United Kingdom; Spain – Sierra Nevada; Croatia; Pyrenees). The number of potential species recovered by the different methods is generally higher than the morphospecies identified. *Mastigusa arietina* populations from Central and Northern Europe (Norway, Denmark, Belgium, and Italy) show a 1% withing group mean genetic distance on the COI. Populations from Finland and Georgia show a relevant degree of divergence from this group (7% and 10% respectively). *Mastigusa macrophthalma* specimens from Croatia show a 15% divergence from *M. arietina* and an 11% divergence with *M. raimondi* sp. n. The latter species, shows a 12% divergence with *M. arietina*, Within *M. raimondi,* populations from the United Kingdom show a 7% divergence with Spanish populations. The single specimen from Pyrenees included in the analysis shows a 10% divergence with *M. raimondi*.

2.4.2. Taxonomy

Family Cybaeidae Banks, 1892

Genus *Mastigusa* Menge, 1854

Type species. †*Mastigusa acuminata* Menge, 1854.

Diagnosis. Male. Pedipalp with extremely large conductor with dorsal process expanding frontally, dorsally, and posteriorly forming a ram horn-like structure as large or larger than the prosoma and a ventral process expanding retro-laterally and posteriorly. Extremely long whip-like embolus held in a groove of the conductor, entering it from its posterior end and moving forward forming a loop (Fig. 2). Female genitalia showing extremely long and tangled, non-symmetrical fertilization ducts (Fig. 3).

Description. Extant species. Total length 1.68-3.83. Carapace length 1.06-1.87, carapace width 0.77-1.53; carapace oval, not prolonged around chelicerae and with cephalic part not elevated. Carapace pale-yellow to brown, with darker cephalic region and 6 more or less defined slightly darker bands radiating from the fovea to the lateral margins of the carapace, each of the bands present one to a few setae. Several setae between and behind eyes and in a line from fovea to the eyes, all pointing forward. Clypeus 0.06-0.23 high; clypeus with few setae pointing upwards (Fig. 6). AME 0.03-0.08; ALE 0.04-0.11; PME 0.05-0.1; PLE 0.06-0.11; AME-PME 0.04-0.1; ALE-PME 0.03-0.08; PLE-PME 0.03-0.09; PME-PME 0.04-0.12; PLE-PLE 0.23-0.39; ALE-0.10-0.22; anterior eyes on a weakly recurved to straight line, posterior eyes on a procurved to almost straight line. Black coloration surrounding the eyes can be more or less accentuated. Labium wider than long, 0.19-0.35 wide, 0.09-0.25 long; labium pale-yellow to brown. Sternum longer than wide, 0.51-0.97 wide, 0.59-1.10 long; sternum pale-yellow to brown with sparse setae. Chelicerae pale-yellow to brown; thick setae on promargin of rear furrow. 3-6 teeth on retrolateral margin of the cheliceral furrow. Leg I total length 2.43-6.73, leg II total length 2.47-5.93, leg III total length 2.10-5.39, leg IV total length 2.85-6.95. Two ventral rows of spines (a proventral and a retroventral row) on tibia and metatarsus of legs 1 and 2. Tibia I and II: 5 spines in retroventral row, 6 spines in proventral row, an additional prolateral spine in the middle or distally on tibia; Metatarsus I and II: 3 spines in the retroventral and proventral row, 1 or 2 additional prolateral spines in the middle or distally on metatarsus (Fig. 7).

All femora, patellae, and tibiae with proximal and a distal dorsal spine. Tarsus with 3 to 4 long dorsal trichobothria and a dorsal tarsal organ distally placed. Three claws (two superior, one inferior), with ventral comb-like structures. Legs pale-yellow to brown, uniform in color. One narrow median spiracle present, lateral tracheae absent, median tracheae present and branched. Abdomen pale-yellow to dark brown, uniform or with two lines of dorsal light patches of varying dimension that fuse into a single line posteriorly. Cribellum absent; PLS clearly protruding from the abdomen in dorsal view; ALS separated by about their diameter; PLS behind the ALS; PMS clearly visible between the PLS.

Distribution. Europe, Algeria, Russia, Georgia, Iran.

Male palp morphology. The tibia in extant *Mastigusa* species shows two apophyses. A characteristic retro-lateral ventral apophysis (RTA) expands superiorly and terminates with a hooked tip (Fig. 2) The tip of the RTA can be thinner or ticker in different species. The ventral apophysis (VTA) appears flat and expands frontally. Its shape is a diagnostic character for species discrimination, and on a ventral view can show a long spike-like tip or a broader leave-like tip (Fig. 2). The cymbium is elongated and covered with a great number of setae, the longest being on the dorsal margin. The most characteristic feature of the male pedipalp in *Mastigusa* is represented by its conductor. This expands ventrally from the tegulum and bifurcates into a dorsal process expanding frontally, dorsally, and posteriorly forming a ram horn-like structure and a ventral process expanding retro-laterally and posteriorly. The whole length of the conductor is provided with a groove on the prolateral side where the embolus sits (Fig. 2). The tip of the dorsal process of the conductor can be blunt or thin, according to the species. Where the two processes meet, in lateral view, a curve is visible and is more or less accentuated in the different species. The largest diameter of the dorsal process of the conductor, its ratio with the length of the prosoma and the general shape of the loop formed by the conductor and the embolus (approximating a circular or elliptical shape) are important diagnostic characters for species determination. The embolus is extremely long and whip like. It departs from its base in a frontal direction but soon curves steeply downwards and then backwards entering the conductor groove on the tip of the dorsal process of the conductor, forming a big loop. The embolus exits the conductor on the tip of the ventral process (Fig. 2).

Female genitalia. The epigynum in *Mastigusa* is without a scape, but externally shows a blind concavity situated near the epigastric fold. Above the concavity two separate slits represent the opening of the copulatory ducts. The first part of the copulatory ducts appears membranous, and funnel shaped. From the slits, they extend anteriorly above the second part of the ducts then going around it and coming back posteriorly. The second part of the copulatory ducts appears more sclerotized and strongly coiled, forming an asymmetric characteristic structure terminating in the two spermathecae (Fig. 3).

Spinnerets. Longitudinally striated texture on ampullate spigot shaft with fingerprint pattern on spigot base; Tartipores present on ALS, PLS and PMS; cribellum absent; ALS with only two segments (intermediate segment lost); ALS with 6 PI spigots (4 in male) with rounded base margin, not possible to identify MAP; PMS with 14-21 AC spigots, two CY spigots, one nubbin and one mAP spigot located on the median to anterior section of the PMS; two CY spigots on PMS lacking in male; AC shaft on PMS and PLS of same size; no PC spigots on PMS; PLS with 13-21 AC spigots and without aggregate glands, MS or PC; apical segment of PLS conical; no enlarged setae on PLS (Fig. 8).

***Mastigusa arietina* (Thorell, 1871)** Fig. 9, 10.

Cryphoeca arietina Thorell, 1871a: 165 (Dmf).

Cicurina impudica Simon, 1875a: 24, pl. 5, f. 2 (Dm).

Cicurina arietina Simon, 1875a: 25.

Cryphoeca diversa O. Pickard-Cambridge, 1893a: 148, f. 2 (Df).

Tuberta arietina Chyzer & Kulczyński, 1897: 156, pl. 6, f. 21 (mf).

Tetrilus arietinus Simon, 1898a: 268, f. 261 (mf).

Tetrilus arietinus O. Pickard-Cambridge, 1901a: 10, pl. A, f. 2.

Cryphoeca recisa O. Pickard-Cambridge, 1908a: 123, pl. A, f. 1-3 (Df).

Tetrilus recisus Jackson, 1913: 23 (Dm).

Tetrilus arietinus Simon, 1937: 1022, 1044, f. 1592-1593 (mf).

Tetrilus macrophthalmus Simon, 1937: 1023, 1044 (presumably misidentified).

Tetrilus diversus Simon, 1937: 1044.

Tetrilus arietinus Bristowe, 1939: 168, f. 14 (f).

Tetrilus macrophthalmus Locket & Millidge, 1953: 23, f. 17A-C (mf; misidentified per Wunderlich, 1986: 69).
Tetrilus arietinus Locket & Millidge, 1953: 24, f. 17D (mf, S).
Tuberta arietina Lehtinen, 1967: 272, f. 347, 349 (Tmf from Tetrilus).
Tetrilus macrophthalmus Locket & Millidge, 1967: 182, f. 5D-E (m; misidentified per Wunderlich, 1986: 69).
Tetrillus arietinus Miller, 1971: 179, pl. XXIX, f. 19 (m).
Tetrilus arietinus Tystshenko, 1971: 163, f. 461 (m).
Tetrilus macrophthalmus Brignoli, 1971c: 116, f. 31-34 (mf; misidentified per Wunderlich, 1986: 69).
Tetrilus macrophthalmus Klausen, 1974: 192, f. 2-4 (mf; misidentified per Wunderlich, 1986: 69).
Tuberta arietina Palmgren, 1977a: 10, f. 1.25-29 (mf).
Tetrilus macrophthalmus Roberts, 1985: 166, pl. A (mf; misidentified per Wunderlich, 1995c: 463).
Mastigusa arietina Wunderlich, 1986: 69, f. 68-69, 74, 80-87 (Tmf from Tuberta).
Tuberta arietina Heimer & Nentwig, 1991: 366, f. 950 (mf).
Mastigusa macrophthalma Roberts, 1995: 251, f. (mf; misidentified per Wunderlich, 1995c: 463).
Mastigusa arietina Wunderlich, 1995c: 464, f. 12 (m).
Mastigusa macrophthalma Roberts, 1998: 268, f. (mf; misidentified per Wunderlich, 1995c: 463).
Mastigusa arietina Trotta, 2005: 159, f. 174-175 (mf).
Mastigusa arietina Almquist, 2006: 321, f. 282a-f (mf).
Mastigusa arietina Zamani et al., 2020: 582, f. 7A-C (f).

Type material. Syntypes: 1 ♂, 1 ♀ Sweden, Göteborg, Gubbero, Oct 1844, inside *Formica rufa* ant nest. Coll. N. Westring. Deposited at NHRS (Examined).

Material examined.

BELGIUM: 2 ♂, 1 ♀ Bruges, Snellegem, 51.1749°N, 3.1382°E, 16m, 2020, in *Lasius fuliginosus* nest, Parmentier leg. (NHMD). DENMARK: 1 ♀ Silkeborg (EJ), Ørnsø,

56.0003°N, 9.5215°E, 115m, 10 Nov 2018, in *Formica* sp. Nest, Pedersen leg. (NHMD); 3 ♂ Bornholm, Slotslyngen, Holmegaards mose, 55.2572°N, 14.7577°E, 100m, 16 Sept 2005, Lissner leg. (NHMD). FINLAND: 1 ♂ Tohmajärvi, 60.1900°N, 30.4000°E, 6 Oct 1967, in *Formica* nest, Saaristo leg. (ZMUT); 1 ♀ Turku, Ruissalo, 60.4383°N, 22.1967°E, 11 Jun 2009, in *Formica polyctena* nest, Härkönen leg. (ZMUT). GEORGIA: 1 ♂, 4 ♀ Didgori, W of Tbilisi, 41.78104°N, 44.67459°E, 850m, 10 Oct 2020, under rocks, dry creek in oak forest, Otto and Seropian leg. (NHMD). GERMANY: 1 ♂ Brandenburg, Liepe, 52.8500°N, 13.9000°E, 70m, 25 Sept 2001, dead wood stump in pine forest, proj88, Blick leg. (SMF); 1 ♂ Brandenburg, Golsow, ar952, 30 Aug 1994, Lei leg. (NMBE); 1 ♀ Feuchtwangen, 49.1930°N, 10.4160°E, 495m, 1 Dec 1990, pitfall trap at the edge of forest, proj2, Blick (SMF). HUNGARY: 1 ♂ Bukk (HNHM Araneae-8424); 1 ♀ Pilis (HNHM Araneae-9960). ITALY: 2 ♂, 1 ♀ Pordenone, Claut, Casera Casavento, 46.2681°N, 12.5958°E, 934m, 8 Sep 2018, in *Formica polyctena* nest, Castellucci leg. (NHMD); 1 ♂, 3 ♀ Bolzano, Cortaccia, Corona, 46.3294°N, 11.2085°E, 1195m, 23 Sep 2018, in *Formica rufa* nest, Castellucci leg. (NHMD); 1 ♀ Bolzano, Cortaccia, Corona, 46.3327°N, 11.2111°E, 1209m, 23 Sep 2018, in *Formica polyctena* nest, Castellucci leg. (NHMD). POLAND: 1 ♀ Rez. Zamczysko ad Kielce, 13 Oct 1982, Burakowski leg. (MZPW ARA031480); 1 ♀ Chełmowa Góra, 24 Mar 1983, Jędryczkowski and Staręga leg. (MZPW ARA031471). ROMANIA: 1 ♂ Cabana Buta, Retezat Mountains, Nucsoara, 9 Mar 2002, Fetykó leg. (KF). SLOVAKIA: 2 ♀ Szinnaiko = Wsch.Slowacja, Vihorlat? (MZPW ARA035125). SWITZERLAND: 2 ♂ BE, Naturhistorisches Museum Bern Ausstellung Tiere als Baumeister, Ameise, Jun 1999, Kropf leg. (NMBE ar2943). UKRAINE: 1 ♂ Crimea, Yalta, Yalta Forest Mountain Reserve, 15 Oct 2001, Khaustov leg. (MK 2460-2); 1 ♀ Crimea, Simferopol, Kyzyl-Koba Cave, Kovblyuk, Kucherenko and Zhurkov leg. (MK 2217-5).

Diagnosis. Male: the pedipalp of *M. arietina* differs from that of *M. macrophthalma* by having a conductor approximating an elliptical shape, with a larger diameter of the conductor > 1.5mm and a C/c ratio >1, while the latter species has a conductor approximating a circular shape, with a larger diameter of the conductor < 1.5mm and a C/c ratio < 1. It also differs by having a sharp tip on the RTA, while this is blunt in *M. macrophthalma*. It differs from *M. raimondi* sp. n. by having a broad tip of the conductor,

while this is thin in the latter species, and by having a spike-like tip on the VTA, while this is tick and broad in *M. raimondi* sp. n. (Fig. 9, 16).

Redescription. Male, based on MAS_IT_02 (NHMD) from Casera Casavento, Italy. Total length 3.54; carapace 1.51 long, 1.26 wide; clypeus 0.13 high; Labium 0.19 long, 0.29 wide; sternum 0.92 long, 0.87 wide; AME 0.08; ALE 0.07; PME 0.09; PLE 0.09; AME-PME 0.08; ALE-PME 0.05; PLE-PME 0.06; PME-PME 0.08; PLE-PLE 0.30; ALE-ALE 0.17; PME/AME ratio 1.12. Leg length: femur + tibia + metatarsus + tarsus; leg I 1.36+1.33+1.14+0.66 (total length 4.49), leg II 1.31+1.13+1.03+0.64 (total length 4.11), leg III 1.13+0.92+1+0.58 (total length 3.63), leg IV 1.3+1.22+1.26+0.7 (total length 4.48). Conductor diameter 2.02, C/c ratio 1.34. Carapace, labium, sternum and legs brown, abdomen dark brown with two lines of dorsal light patches that fuse into a single line of elongated patches posteriorly.

Female, based on MAS_IT_08 (NHMD) from Corona, Italy. Total length 3.38; carapace 1.38 long, 1.05 wide; clypeus 0.11 high; labium 0.15 long, 0.28 wide; sternum 0.80 long, 0.70 wide; AME 0.07; ALE 0.08; PME 0.07; PLE 0.07; AME-PME 0.05; ALE-PME 0.06; PLE-PME 0.05; PME-PME 0.08; PLE-PLE 0.29; ALE-ALE 0.16; PME/AME ratio 1. Leg length: femur + tibia + metatarsus + tarsus; leg I 1.22+1.03+0.93+0.55 (total length 3.37), leg II 1.07+0.90+0.89+0.57 (total length 3.43), leg III 1+0.74+0.83+0.5 (total length 3.07), leg IV 1.27+1.35+1.16+0.63 (total length 4.41). Coloration as in male.

Variation. Total length 2.38-3.58 (males; n=18), 2.47-3.83 (females; n=21); carapace 1.06-1.64 (males; n=18), 1.22-1.52 (females; n=21) long, 0.78-1.30 (males; n=18), 0.88-1.16 (females; n=21) wide; clypeus 0.09-0.18 (males; n=18), 0.07-0.16 (females; n=21) high; labium 0.13-0.19 (males; n=18), 0.13-0.18 (females; n=21) long, 0.23-0.29 (males; n=18), 0.21-0.29 (females; n=21) wide; sternum 0.59-0.95 (males; n=18), 0.70-0.94 (females; n=21) long, 0.51-0.87 (males; n=18), 0.66-0.88 (females; n=21) wide; AME 0.04-0.08 (males; n=18), 0.04-0.07 (females; n=21); ALE 0.05-0.09 (males; n=18), 0.06-0.09 (females; n=21); PME 0.05-0.09 (males; n=18), 0.05-0.07 (females; n=21); PLE 0.06-0.09 (males; n=18), 0.06-0.09 (females; n=21); AME-PME 0.04-0.10 (males; n=18), 0.04-0.10 (females; n=21); ALE-PME 0.05-0.08 (males; n=18), 0.03-0.08 (females; n=21); PLE-PME 0.04-0.09 (males; n=18), 0.04-0.08 (females; n=21); PME-PME 0.08-0.11 (males; n=18), 0.06-0.10 (females; n=21); PLE-PLE 0.27-0.38 (males; n=18), 0.20-

0.35 (females; n=21); ALE-ALE 0.14-0.18 (males; n=18), 0.11-0.18 (females; n=21); PME/AME ratio 0.70-1.40 (males; n=18), 0.70-1.75 (females; n=21). Leg I total length 3.53-5.39 (males; n=18), 3.09-4.16 (females; n=21); leg II total length 3.26-4.88 (males; n=18), 2.63-3.84 (females; n=21); leg III total length 2.99-4.24 (males; n=18), 2.65-3.41 (females; n=21); leg IV total length 3.78-5.24 (males; n=18), 3.04-4.99 (females; n=21). Conductor diameter 1.67-2.50 (males; n=18); C/c ratio 1.17-1.77 (males; n=18). Carapace, labium, sternum and legs pale-yellow to brown. Abdomen pale-yellow to dark brown, uniform or with two lines of dorsal light patches of varying dimension that fuse into a single line posteriorly.

Natural history. Often collected inside ant nests belonging mostly to the genera *Formica* and *Lasius*, where adults, juveniles and egg sacks can be found. Adults occasionally collected outside ant nets. No records in caves exist for this species.

Distribution. Norway, Sweden, Finland, Estonia, Denmark, Germany, France, Belgium, the Netherlands, Austria, Switzerland, Italy, Poland, Czech Republic, Slovakia, Hungary, Bulgaria, Ukraine, Romania, Russia, Turkey, Iran.

Comments. The species was re-circumscribed and diagnostic characters were revised, for details, see the Discussion section.

***Mastigusa lucifuga* (Simon, 1898)** Fig. 11.

Tetrilus lucifuga Simon, 1898a: 261, f. 259.

Tetrilus lucifuga Simon, 1898f: 9 (Df).

Mastigusa lucifuga Wunderlich, 1986: 69, f. 79 (Tf from Tuberta).

Type material. Female holotype: France, Pyrénéés-Orientales, Simon (deposited in MNHN; MNHN12976). Examined.

Diagnosis. The holotype only differs from all the other known species by showing significantly smaller PME (Fig. 11).

Redescription. Female (based on holotype). Total length 2.50; carapace 1.38 long, 1.00 wide; clypeus 0.14 high; labium 0.14 long, 0.25 wide; sternum 0.71 long, 0.69 wide; AME 0.04; ALE 0.07; PME 0.03; PLE 0.08; AME-PME 0.04; ALE-PME 0.05; PLE-PME 0.08;

PME-PME 0.10; PLE-PLE 0.28; ALE-ALE 0.13; PME/AME ratio 0.75. Carapace, labium, sternum and legs yellow, abdomen pale yellow, uniform in color (old specimen in ethanol).
Natural history. Its original description does not mention habitat or collection method.
Distribution. Only known from the Eastern Pyrenees of France. The original description did not include any specific locality.
Comments. This species is only known from the female holotype, while the male is unknown. For a discussion regarding its taxonomic status, see the Discussion section.

***Mastigusa macrophthalma* (Kulczyński, 1897)** Fig. 12, 13.
Tuberta arietina macrophthalma Kulczyński, in Chyzer & Kulczyński, 1897: 156, pl. 6, f. 21 (Dmf).
Tuberta arietina macrophthalma Gerhardt, 1921: 98, f. 9 (m).
Tetrilus macrophthalmus Loksa, 1969: 114, f. 77C-E (mf).
Mastigusa macrophthalma Wunderlich, 1986: 70, f. 75, 88-90 (Tmf from Tuberta).
Mastigusa macrophthalma Azarkina & Trilikauskas, 2012: 205, f. 2-4 (f).

Types. Syntypes: 1 ♂, 2 ♀ Croatia: Rijeka, Vrata, Mrzla Vodica, Kulczynski leg (deposited at MZPW, ARA035123). Examined.
Material examined. BOSNIA-HERZEGOVINA: 1 ♂, 1 ♀ between Konjic and Jablanica, 20 Sept 1968, Vigna leg. (MSNV). CROATIA: 1 ♀ Fuzine, forest road 6km west of Sljeme, 45.3466N°, 14.6932E°, 915m, 29 Jun 2021, under rocks, *Fagus sylvatica* forest with some *Picea abies*, Castellucci leg. (NHMD); 1 ♀ Velebit, Thaler leg. (BT A-5096). SLOVENIA: 1 ♂, 5 ♀ Loz, ESE Postonja, 600m, 14 Oct 1990, under rocks in mixed forest (*Fagus, Acer, Picea*), Gasparo leg. (FG 1011); 2 ♂, 1 ♀ Between Girosuplje and Turjak, 450m, 10 Sept 1991, under rocks in *Fagus sylvatica* forest with some *Picea abies*, Gasparo leg. (FG 1099); 5 ♀ Podgrad, 570m, 4 Apr 1992, under rocks in *Quercus* forest with some *Pinus*, Gasparo leg. (FG 1151); 2 ♀ Mozelj, Kočevje, 500m, 11 May 1993, under rocks in mixed forest, Gasparo leg. (FG 1220); 2 ♂, 1 ♀ Lok 5: PROTOKOL gozd, 200m SZZ od vrha Pleš pri Semiču (nad cesto), 520m, 27-28 Jul 2001, Tabor leg. (RK); 1 ♀ Savica Komna, Inl. Alpe, 700m, Jul 1986, MG. TK leg. (RK).

Diagnosis. Generally smaller than *M. arietina* and *M. raimondi* sp. n. Male: the conductor differs from that of *M. arietina* and *M. raimondi* sp. n. by approximating a circular shape, while this is approximating an elliptical shape in the other two species. The conductor also differs from that of *M. raimondi* sp. n. by having a broad tip, that is instead thin in the latter; the base of the ventral process of the conductor is less arched if compared to the other two species. The larger diameter of conductor is < 1.5mm and C/c ratio is <1, while in both *M. arietina* and *M. raimondi* sp. n. the larger diameter of the conductor is >1.5mm and the C/c ratio is >1; the VTA tip is spike-like, as in *M. arietina*, and differs from that of *M. raimondi* sp. n. which is broad and tick; the RTA tip is broad, differing from that of the other two species which is sharp (Fig. 12, 16). Female: the sclerotized part of the copulatory ducts is shorter and less coiled if compared to the other two species (Fig. 13).
Redescription. Male (based on MMSI01 (FG) from Loz, Slovenia). Total length 2.75; carapace 1.23 long, 0.97 wide; clypeus 0.08 high; Labium 0.13 long, 0.20 wide; sternum 0.73 long, 0.69 wide; AME 0.03; ALE 0.06; PME 0.08; PLE 0.09; AME-PME 0.05; ALE-PME 0.05; PLE-PME 0.04; PME-PME 0.06; PLE-PLE 0.27; ALE-ALE 0.10; PME/AME ratio 2.66. Leg length: femur + tibia + metatarsus + tarsus; leg I 1.08+1.04+0.90+0.57 (total length 3.59), leg II 1.02+0.85+0.74+0.53 (total length 3.14), leg III 0.91+0.72+0.76+0.52 (total length 2.91), leg IV 1.16+1.07+0.93+0.71 (total length 3.87). Conductor diameter 1.02, C/c ratio 0.83. Carapace, labium, sternum and legs yellow, abdomen yellow without markings.
Female (based on MMSI02 (FG) from Loz, Slovenia). Total length 3.08; carapace 1.21 long, 0.84 wide; clypeus 0.08 high; labium 0.16 long, 0.19 wide; sternum 0.76 long, 0.67 wide; AME 0.04; ALE 0.08; PME 0.07; PLE 0.07; AME-PME 0.06; ALE-PME 0.05; PLE-PME 0.04; PME-PME 0.07; PLE-PLE 0.26; ALE-ALE 0.12; PME/AME ratio 1.75. Leg length: femur + tibia + metatarsus + tarsus; leg I 1.00+0.85+0.70+0.45 (total length 3.00), leg II 0.95+0.74+0.64+0.44 (total length 2.77), leg III 0.81+0.61+0.60+0.41 (total length 2.43), leg IV 1.02+0.97+0.90+0.52 (total length 3.41). Carapace, labium, sternum and legs yellow, abdomen light brown with two lines of dorsal light patches that fuse into a single line of elongated patches posteriorly.
Variation. Total length 2.03-2.75 (males; n=7), 1.68-3.62 (females; n=20); carapace 1.13-1.23 (males; n=7), 1.09-1.33 (females; n=20) long, 0.90-1.04 (males; n=7), 0.77-1.06

(females; n=20) wide; clypaeus 0.06-0.13 (males; n=7), 0.06-0.11 (females; n=20) high; labium 0.09-0.13 (males; n=7), 0.09-0.16 (females; n=20) long, 0.19-0.21 (males; n=7), 0.17-0.24 (females; n=20) wide; sternum 0.67-0.84 (males; n=7), 0.66-0.81 (females; n=20) long, 0.65-0.82 (males; n=7), 0.6-0.72 (females; n=20) wide; AME 0.03-0.05 (males; n=7), 0.03-0.05 (females; n=20); ALE 0.04-0.09 (males; n=7), 0.04-0.09 (females; n=20); PME 0.06-0.08 (males; n=7), 0.06-0.09 (females; n=20); PLE 0.07-0.09 (males; n=7), 0.07-0.10 (females; n=20); AME-PME 0.05-0.09 (males; n=7), 0.04-0.08 (females; n=20); ALE-PME 0.03-0.05 (males; n=7), 0.03-0.06 (females; n=20); PLE-PME 0.03-0.05 (males; n=7), 0.03-0.06 (females; n=20); PME-PME 0.05-0.06 (males; n=7), 0.04-0.08 (females; n=20); PLE-PLE 0.24-0.30 (males; n=7), 0.24-0.30 (females; n=20); ALE-ALE 0.10-0.14 (males; n=7), 0.11-0.14 (females; n=20); PME/AME ratio 1.40-2.66 (males; n=7), 1.50-2.33 (females; n=20). Leg I total length 3.50-3.83 (males; n=7), 2.43-3.48 (females; n=20); leg II total length 3.07-3.41 (males; n=7), 2.47-3.28 (females; n=20); leg III total length 2.74-3.1 (males; n=7), 2.10-2.95 (females; n=20); leg IV total length 3.70-3.98 (males; n=7), 2.85-3.86 (females; n=20). Conductor diameter 1.02-1.14 (males; n=7); C/c ratio 0.83-0.96 (males; n=7). Carapace, labium, sternum and legs pale-yellow to brown. Abdomen pale-yellow to dark brown, uniform or with two lines of dorsal light patches of varying dimension that fuse into a single line posteriorly.

Natural history. Collected under rocks. No records exist for this species being collected inside ant nests or in caves.

Distribution. Slovenia, Croatia, Bosnia-Herzegovina.

Comments. The species was re-circumscribed and diagnostic characters were revised, for details see the Discussion section.

***Mastigusa raimondi* sp. n.** Fig. 14, 15.

Types. Holotype: 1 ♂ Cueva de las Ventanas, Piñar, Granada, 37.4418°N, 3.4283°W, 1009m, 9 Dec 1983, in cave, Ribera leg. **Paratype:** 1 ♀ Cueva de las Ventanas, Piñar, Granada, 37.4418°N, 3.4283°W, 1009m, 9 Dec 1983, in cave, Ribera leg. Holotype and paratype deposited in the Carles Ribera collection at University of Barcelona (CR2102/85). Examined.

Material examined. ALGERIA: 1 ♂ Lac Mouzaia, Blida, 36.3665°N, 2.6923°E, 1200m, among rocks 15 meters from a lake, 14 May 1988, Bosmans leg. (RB); 1 ♂ Massif du Djurdjura, Tikjda, Tigounatine, Bouira, 36.4479°N, 4.1295°E, 1460m, Oct 1987-Jun 1988, pitfall trap in *Cedrus* forest, Bosmans leg. (RB). PORTUGAL: 1 ♂ Bezerra, Porto de Mos, 39.5485°N, 8.8461°W, 420m, 10 Feb 2006, Crespo leg. (LC); 1 ♂ Gruta do Escoural, Montemor-o-Novo, 38.5437°N, 8.1377°W, 350m, 22 May 1980, in cave, Machado leg. (CR Reg. n. 2157, fl. 87); 2 ♀ Parque Nacional Peneda Gereas, Albergaria, 41.7950°N, 8.1366°W, 660m, 4-11 Jun 2005, Cardoso leg. (NHMD). SPAIN: 1 ♂ Soportujar, Granada, 36.9615°N, 3.4188°W, 1787m, 31 May-14 Jun 2013, pitfall trap in white oak forest, Crespo et al. leg. (MA); 1 ♂ Sola de Boi, Lleida, 42.5496°N, 0.8725°E, 1760m, 15-29 Jun 2013, pitfall trap in white oak forest, Crespo et al. leg. (MA); 2 ♀ Soportujar, Granada, 36.9689°N, 3.4116W°, 1811m, 19 Jul 2021, under logs in pine forest with white oak, Castellucci leg. (NHMD); 1 ♂ Cueva del Hundidero – Gato, Montejaque, Malaga, 36.7289°N, 5.2368°W, 460m, 20 Apr 1973, in cave, Escola leg. (CR Reg. n. 1819, fl. 1); 1 ♂ Cueva de las Ventanas, Piñar, Granada, 37.4418°N, 3.4283°W, 1009m, 9 Jul 1983, in cave, Ribera leg. (CR Reg. n. 2157, fl. 87); 1 ♂, 2 ♀ Cueva de las Ventanas, Piñar, Granada, 37.4418°N, 3.4283°W, 1009m, 9 Dec 1983, in cave, Ribera leg. (CR Reg. n. 2102, fl. 85); 1 ♂ Cueva Janet, Llaveria, Tarragona, 41.018°N, 0.740°E, 670m, 23 Jan 1977, in cave, Ribera leg. (CR Reg. n. 1823, fl. 73); 1 ♂ Cueva Santa, Altura, Valencia, 39.8426°N, 0.6142°W, 842m, 20 Feb 2010, in cave; 1 ♂ Margalef, Terragona, 41.2843°N, 0.7532°E, 365m, 20 Apr 1967, Escola leg. (CR Reg. n. 1821, fl. 73); 1 ♀ Cueva del conejo, Hermita la Rogativa, Moratalla, Murcia, 38.1479°N, 2.2282°W, 1220m, 7 Apr 1983, in cave, Ribera leg.(CR Reg. n. 2092, fl. 3). UNITED KINGDOM: 2 ♂, 2 ♀ Dunsford Wood, SX79268899, 50.6881°N, 3.7106°W, 130m, 28 Dec 2021, under oak log in woodland, Gallon leg. (RG); 1 ♂, 1 ♀ Sherwood Forest, SK62716803, 53.2056°N, 1.0625°W, 69m, 5 Oct 2021, sieving cavity humus and dry red rot within hollow ancient oak 10290, Gallon leg. (RG).

Diagnosis. Male: the conductor is approximating an elliptical shape, as in *M. arietina,* but differs from having a thin tip, which is tick in the latter; the larger conductor diameter is > 1.5 mm and the C/c ratio is > 1, as in *M. arietina*, differing from *M. macrophthalma* where the diameter is <1.5 and the C/c ratio is <1; the VTA has a tick and broad tip, differing

from the other two species which show a spike-like tip; the RTA has a sharp tip. as in *M. arietina*, and differs from that of *M. macrophthalma* which has a blunt tip (Fig. 14, 16).

Description. Male holotype. Total length 3.78; carapace 1.87 long, 1.53 wide; clypeus 0.23 high; Labium 0.20 long, 0.31 wide; sternum 1.08 long, 0.97 wide; AME 0.07; ALE 0.07; PME 0.08; PLE 0.08; AME-PME 0.05; ALE-PME 0.05; PLE-PME 0.09; PME-PME 0.10; PLE-PLE 0.35; ALE-ALE 0.19; PME/AME ratio 1.14. Leg length: femur + tibia + metatarsus + tarsus; leg I 2.08+1.99+1.74+0.92 (total length 6.73), leg II 1.87+1.61+1.47+0.85 (total length 5.80), leg III 1.72+1.41+1.46+0.8 (total length 5.39), leg IV 2.22+1.87+2.06+1.1 (total length 7.25). Conductor diameter 2.48, C/c ratio 1.33. Carapace, labium, sternum and legs yellow, abdomen light brown with no markings.

Female paratype (2102/85/3). Total length 3.68; carapace 1.55 long, 1.15 wide; clypeus 0.15 high; labium 0.17 long, 0.27 wide; sternum 0.83 long, 0.78 wide; AME 0.06; ALE 0.08; PME 0.07; PLE 0.10; AME-PME 0.07; ALE-PME 0.06; PLE-PME 0.08; PME-PME 0.09; PLE-PLE 0.34; ALE-ALE 0.15; PME/AME ratio 1.16. Leg length: femur + tibia + metatarsus + tarsus; leg I 1.40+1.32+1.14+0.63 (total length 4.49), leg II 1.33+1.20+1.08+0.51 (total length 4.12), leg III 1.24+1.01+1.16+0.66 (total length 4.07), leg IV 1.64+1.47+1.56+0.79 (total length 5.46). Coloration as in male.

Variation. Total length 2.27-3.84 (males; n=15), 2.86-3.53 (females; n=8); carapace 1.36-1.87 (males; n=15), 1.23-1.57 (females; n=20) long, 1.00-1.53 (males; n=15), 0.83-1.13 (females; n=8) wide; clypeus 0.11-0.23 (males; n=15), 0.08-0.15 (females; n=8) high; labium 0.14-0.25 (males; n=15), 0.14-0.19 (females; n=8) long, 0.24-0.35 (males; n=15), 0.21-0.26 (females; n=8) wide; sternum 0.66-1.00 (males; n=15), 0.76-0.89 (females; n=8) long, 0.70-0.97 (males; n=15), 0.7-0.81 (females; n=8) wide; AME 0.05-0.08 (males; n=15), 0.05-0.07 (females; n=8); ALE 0.06-0.11 (males; n=15), 0.07-0.1 (females; n=8); PME 0.06-0.10 (males; n=15), 0.07-0.08 (females; n=8); PLE 0.07-0.11 (males; n=15), 0.07-0.08 (females; n=8); AME-PME 0.05-0.08 (males; n=15), 0-06-0.08 (females; n=8); ALE-PME 0.04-0.08 (males; n=15), 0.04-0.08 (females; n=8); PLE-PME 0.05-0.09 (males; n=15), 0.05-0.07 (females; n=8); PME-PME 0.06-0.12 (males; n=15), 0.07-0.09 (females; n=8); PLE-PLE 0.31-0.39 (males; n=15), 0.27-0.33 (females; n=8); ALE-ALE 0.14-0.22 (males; n=15), 0.14-0.16 (females; n=8); PME/AME ratio 1.00-1.8 (males; n=15), 1.14-1.60 (females; n=8). Leg I total length 4.56-6.73 (males; n=15), 3.42-4.10

(females; n=8); leg II total length 3.91-5.93 (males; n=15), 3.23-4.16 (females; n=8); leg III total length 3.60-5.39 (males; n=15), 2.74-3.74 (females; n=8); leg IV total length 4.47-7.25 (males; n=15), 3.83-4.90 (females; n=8). Conductor diameter 2.22-2.72 (males; n=15); C/c ratio 1.33-1.66 (males; n=15). Carapace, labium, sternum and legs pale-yellow to brown. Abdomen pale-yellow to dark brown, uniform or with two lines of dorsal light patches of varying dimension that fuse into a single line posteriorly.

Natural history. Collected under rocks and logs, in ant nests of *Lasius* and *Formica* and in caves. This is the only *Mastigusa* species that is known to inhabit caves. This could be a response to the elevated temperatures that it faces in the southern limits of its distribution range in Southern Iberian Peninsula and Algeria, where it cannot move to greater altitudes. Free-living *M. raimondi* populations were in fact found in Southern Spain, on the Sierra Nevada massif, but always above 1700 meters above sea level. The low tolerance of high temperatures is probably a limit to the distribution of other *Mastigusa* species, like *M. arietina*, which is generally found in montane ecosystems in the southern limits of its distribution range, like the Italian Alps, where the species was collected between 934 and 2080 meters above sea level (Castellucci et al. 2022). Myrmecophilic populations are known from the United Kingdom, while they are not known from the Iberian Peninsula or Africa.

Distribution. Spain, Portugal, United Kingdom, Algeria.

Comments. The Iberian populations have always been considered as belonging to *M. arietina*, while in the United Kingdom both *M. arietina* and *M. macrophthalma* were reported to be present, but identification was only based on one character – whether specimens had smaller or larger PME. The clear differences in the male genital morphology of these populations with respect to *M. arietina* and *M. macrophthalma* were never used for identification.

2.4.3. Key to extant Mastigusa *species*

1 PME less than half of PLE, in the Eastern Pyrenees of France……***M. lucifuga***

– PME not less than half of PLE…….……**2**

2 Male: conductor approximating a circular shape (Fig. 12), largest diameter of the conductor < 1.5 mm, C/c ratio < 1……..……..***M. macrophthalma***

– Male: conductor approximating an elliptical shape (Fig. 9, 14), largest diameter of the conductor > 1.5 mm, C/c ratio > 1……..……..……..…..**3**

3 Male: tip of conductor broad, VTA tip spike-like (Fig. 9, 16) ……..….....***M. arietina***

– Male: tip of conductor thin, VTA tip broad (Fig. 14, 16) ……..……..***M. raimondi***

2.5. DISCUSSION

2.5.1. Discussion of the familiar placement of Mastigusa

The suggested inclusion of the genus *Mastigusa* in the family Cybaeidae from **Chapter 1**, as redefined by Wheeler et al. 2017 based on molecular data, is supported by the morphological comparison of male genitalia with its sister genus *Cryphoeca*, also in Cybaeidae. In *Cryphoeca,* the conductor is also elongated and bent backwards and presents a furrow where the embolus sits. The embolus and conductor are forming a similar loop to what can be found, in an extreme form, in *Mastigusa* (Fig. 17). The previous placement of *Mastigusa* in Hahniidae was only based on its presumed close relation with the genus *Cicurina* Menge, 1871. In **Chapter 1**, though, we demonstrated that *Mastigusa* is closer to *Cryphoeca*, by for the first time including all three genera in a molecular phylogenetic matrix.

2.5.2. Morphological discrimination of Mastigusa *species*

Great confusion existed over the identity of the three previously described species *M. arietina*, *M. macrophthalma* and *M. lucifuga*, due to most authors trying to separate them by only relying on the relative dimension of PME and PLE. The only character that differentiates the female holotype of *M. lucifuga* from specimens of the other species is significantly smaller PME (Fig. 11). Wunderlich in 1986 did not rule out a synonymy between these species and *M. arietina*, given the fact that only a single female specimen was known. Great variation in the dimension of PME can be observed both in *M. arietina* and *M. raimondi* sp. n., and without more material from the type locality of *M. lucifuga* we cannot confirm that such smaller PME are typical from the populations in the Eastern Pyrenees or if they represent a peculiarity of the single specimen. The single other specimen from Pyrenees that we observed, a male collected in Central Pyrenees of Spain, does not show the extremely reduced PME. Its genital morphology is compatible

with *M. raimondi* sp. n., but its genetically distinct from it (Fig. 4). Since the male of *M. lucifuga* is unknown and the type locality “Eastern Pyrenees” is somewhat generic, it is impossible at the moment to rule out the synonymy of *M. lucifuga* with *M. arietina* or *M. raimondi*.

Concerning the distinction between *M. arietina* and *M. macrophthalma*, several authors relied on *M. arietina* showing PME smaller than PLE while *M. macrophthalma* showing PME as big as PLE, and on the distance between PME, with them being separated by more than their diameter in *M. arietina* and by less than their diameter in *M. macrophthalma*. Already Wunderlich in 1986 noticed though a degree of variation in the relative dimension of PME and PLE in *M. arietina*, and for this reason he did not consider eye characters as diagnostic, relying instead on characters in the male genitalia and chelicerae to discriminate the two species. Our examination of a larger number of specimens belonging to *M. arietina, M. macrophthalma* and *M. raimondi,* confirmed the great degree of variation in the eye dimension in *M. arietina*, similar to what can also be observed in *M. raimondi*, and revealed a degree of variation (even if to a smaller extent) also in *M. macrophthalma*, with a certain degree of overlap in eye characteristics between species. Considering the great variability in eye dimensions that exist within the genus, and in accordance with Wunderlich (1986), we do not consider eyes a reliable character to discriminate species and suggest the use of male genital morphology for identification.

2.5.3. Female morphology

While male genitalia are useful to discriminate the different species within the genus *Mastigusa*, the complex female genitalia are not as useful. Wunderlich (1986) could not find any appreciable difference in female genitalia of the three species *M. arietina*, *M. macrophthalma* and *M. lucifuga*. We still could not observe differences in female genitalia of *M. arietina*, *M. lucifuga* and the newly described *M. raimondi*, while differences can be observed in the genitalia of *M. macrophthalma*, which appear smaller and presenting fertilization ducts that are less coiled if compared to the other species (Fig. 13).

2.5.4. Genetic variability

Mastigusa species show interesting genetic patterns across their distribution range. *Mastigusa arietina* populations in Central and Northern Europe (Italy, Belgium, Denmark, and Norway) show little variability on the mitochondrial COI (around 1% within group distance), while populations from Finland and Georgia show a relevant degree of divergence from the others (7% and 10% respectively), even if no clear morphological differences can be observed. A wider geographical sampling in the eastern limits of the distribution range of *M. arietina* could help in understanding the drivers leading to this genetic pattern. *Mastigusa macrophthalma* shows a 15% divergence on the COI with *M. arietina* and an 11% with *M. raimondi* sp. n. In the phylogenetic analysis from **Chapter 1**, this species appears to form a monophyletic clade *M. raimondi* and is found in sister relationship with the uncertain specimen from the Pyrenees. *Mastigusa raimondi* shows a 12% divergence on the COI with *M. arietina* and an 11% with *M. macrophathalma*. Within this species we can also observe a degree of genetic diversification, with populations from the United Kingdom showing a 7% divergence on the COI with the Spanish populations. The single specimen from the Pyrenees that was included in the molecular analyses (MD2844, an adult male) is morphologically compatible with *M. raimondi* but appears to be genetically distinct (10% divergence) and in the phylogeny from **Chapter 1** appears to be more closely related to *M. macrophthalma* than to *M. raimondi.* This conflict between morphology and molecular data is quite interesting and the analysis of more material from the Pyrenees and adjacent areas could help in understanding its nature.

2.5.5. Natural history

Differences exist in the ecology of *M. arietina*, *M. macrophthalma* and *M. raimondi* sp. n., and their tendency to assume free-living, cave dwelling or myrmecophile lifestyles. *Mastigusa raimondi* appears to be the more plastic, with known populations assuming all three lifestyles. Cave dwelling populations are known from the southern limits of its distribution range, in Southern Iberian Peninsula and Northern Algeria, while myrmecophile populations are known from the northern limits of its distribution range, in the United Kingdom, where it associates with ants belonging to the genera *Formica* and

Lasius. The distribution of these populations suggests a role of climate in pushing towards one lifestyle or the other (**Chapter 3**). *Mastigusa arietina* is known mostly from myrmecophile populations, with few free-living and no cave records. This species, as the myrmecophile *M. raimondi* populations, also lives mostly in association with *Formica* and *Lasius* (Castellucci et al. 2022, **Chapter 4**). *Mastigusa macrophthalma* is only known from free-living populations, even if it lives in sympatry with different host ant species for *M. arietina* and *M. raimondi* and inhabits highly karstic areas (**Chapter 3**).

Mastigusa lays flat and discoidal white egg sacs that attaches to rigid supports, often in groups. These could be represented by pieces of wood inside ant nests for myrmecophile populations or to the underside of logs and rocks for the free-living ones. Egg sacs contain 3 to 5 eggs each (Fig. 18).

2.6. CONCLUSIONS

An integrative approach was required for addressing the complex taxonomic status of the species belonging to the genus *Mastigusa*, dominated by problematic delimitation and unreliable diagnostic characters. The aid of molecular data was fundamental in combination with morphology to re-circumscribe known species and identify an undescribed species. Given the strong ecological plasticity that can be observed within the genus, a clear understanding of the species boundaries and distribution will now allow comparative studies of forces leading to the very different lifestyles of individual species, such as myrmecophily and the adaptation to live in caves.

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2.9. TABLES

NHRS	Naturhistoriska riksmuseet, Srtockholm, Sweden
NHMD	Natural History Museum of Denmark, Copenhagen, Denmark
ZMUT	University of Turku, Zoological Museum, Turku, Finland
SMF	Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt-am-Main, Germany
NMBE	Naturhistorische Museums, Bern, Switzerland
HNHM	Hungarian Natural History Museum, Budapest, Hungary
MZPW	Polish Academy of Science, Museum and Institute of Zoology, Warsaw, Poland
KF	Kinga Fetykó private collection, Romania
MK	Mykola Kovblyuk private collection, Ukraine
MSNV	Museo Civico di Storia Naturale, Verona, Italy
BT	Barbara Thaler-Knoflac private collection, Austria
FG	Fulvio Gasparo private collection, Italy
RK	Rok Kostanjsek, University of Ljubljana, Slovenia
RB	Robert Bosmans private collection, Belgium
LC	Loius Crespo private collection, Spain
CR	Carles Ribera, University of Barcelona, Spain
MA	Miquel Arnedo, University of Barcelona, Spain
RG	Richard Gallon private collection, United Kingdom

Table 1. List of the institutions mentioned in this work with abbreviations used in the text.

AME	Anterior median eyes
ALE	Anterior lateral eyes
PME	Posterior median eyes
PLE	Posterior lateral eyes
ALS	Anterior lateral spinnerets
PLS	Posterior lateral spinnerets
PMS	Posterior median spinnerets
C/c	Conductor diameter / cephalothorax length ratio
RTA	Retrolateral tibial apophysis
RTAt	Tip of the retrolateral tibial apophysis
VTA	Ventral tibial apophysis
VTAt	Tip of the ventral tibial apophysis
C	Conductor
CdP	Conductor - dorsal process
CvP	Conductor - ventral process
Cym	Cymbium
E	Embolus
Eb	Embolus base
Ti	Tibia
Te	Tegulum
MAP	Major ampullate spigots
mAP	Minor ampullate spigots
AC	Aciniform spigots
CY	Cylindrical spigots
PC	Paracribellar spigots
MS	Modified spigots
N	Nubbin

Table 2. Morphological abbreviations used in the text.

2.10. FIGURES

Figure 1. *Mastigusa* habitus. Alive *M. arietina* male from Denmark. (Photo by Filippo Castellucci)

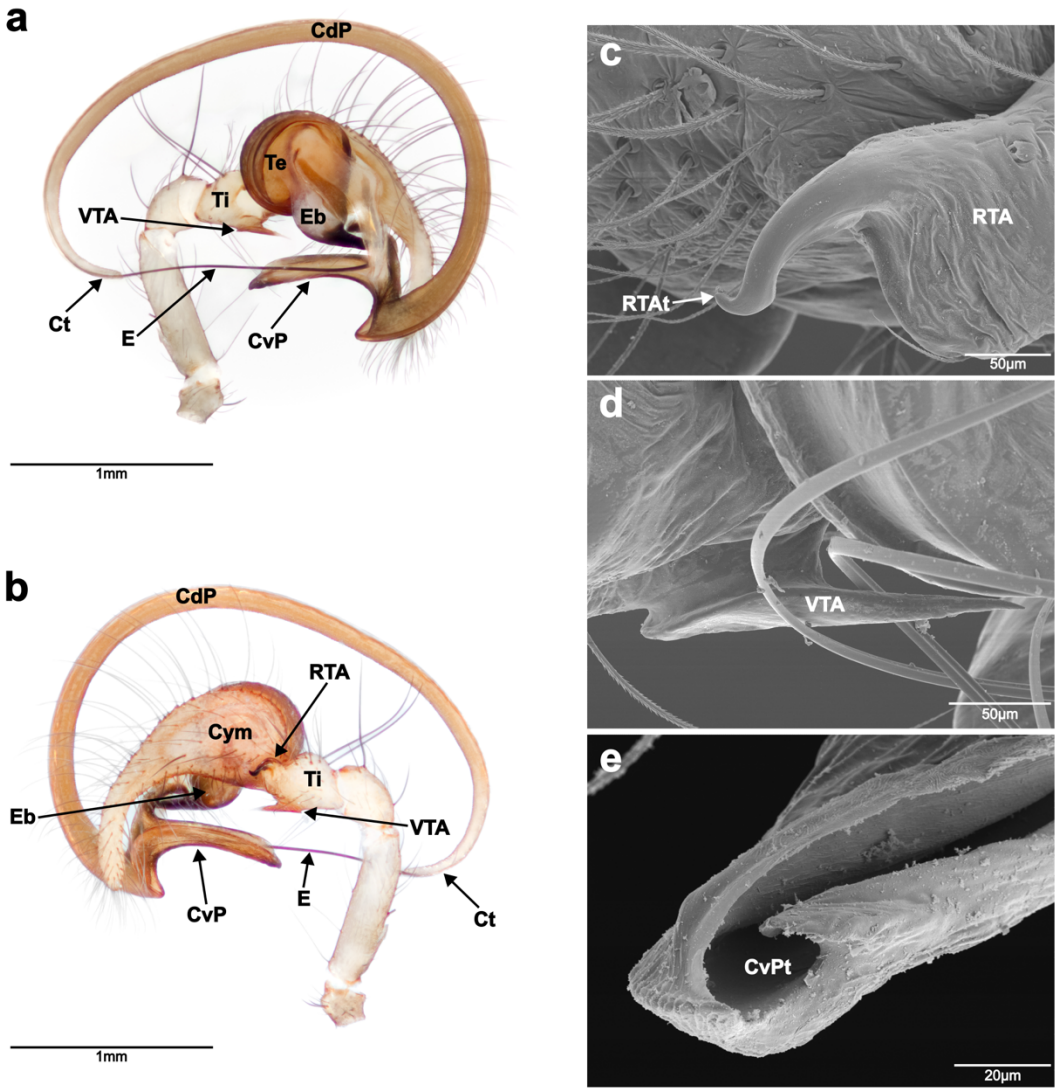

Figure 2. Male pedipalp morphology in *Mastigusa*. a: left pedipalp of *M. arietina* from Italy, prolateral view; b: retrolateral view of the same pedipalp as a; c: left pedipalp of *M. arietina* from Denmark, SEM detail of the RTA in retrolateral view (by Rasmus Jensen); d: SEM detail of the VTA of the same palp as c, prolateral view; e: left pedipalp of *M. macrophthalma* from Slovenia, SEM detail of the tip of the ventral process of the conductor. Abbreviations: CdP=conductor dorsal process; CvP=conductor ventral process; CvPt= tip of the conductor ventral process; Ct=conductor tip; E=embolus; Eb=embolus base; Te=tegulum; Ti=tibia; VTA=ventral tibial apophysis; RTA=retrolateral tibial apophysis; RTAt=tip of the retrolateral tibial apophysis.

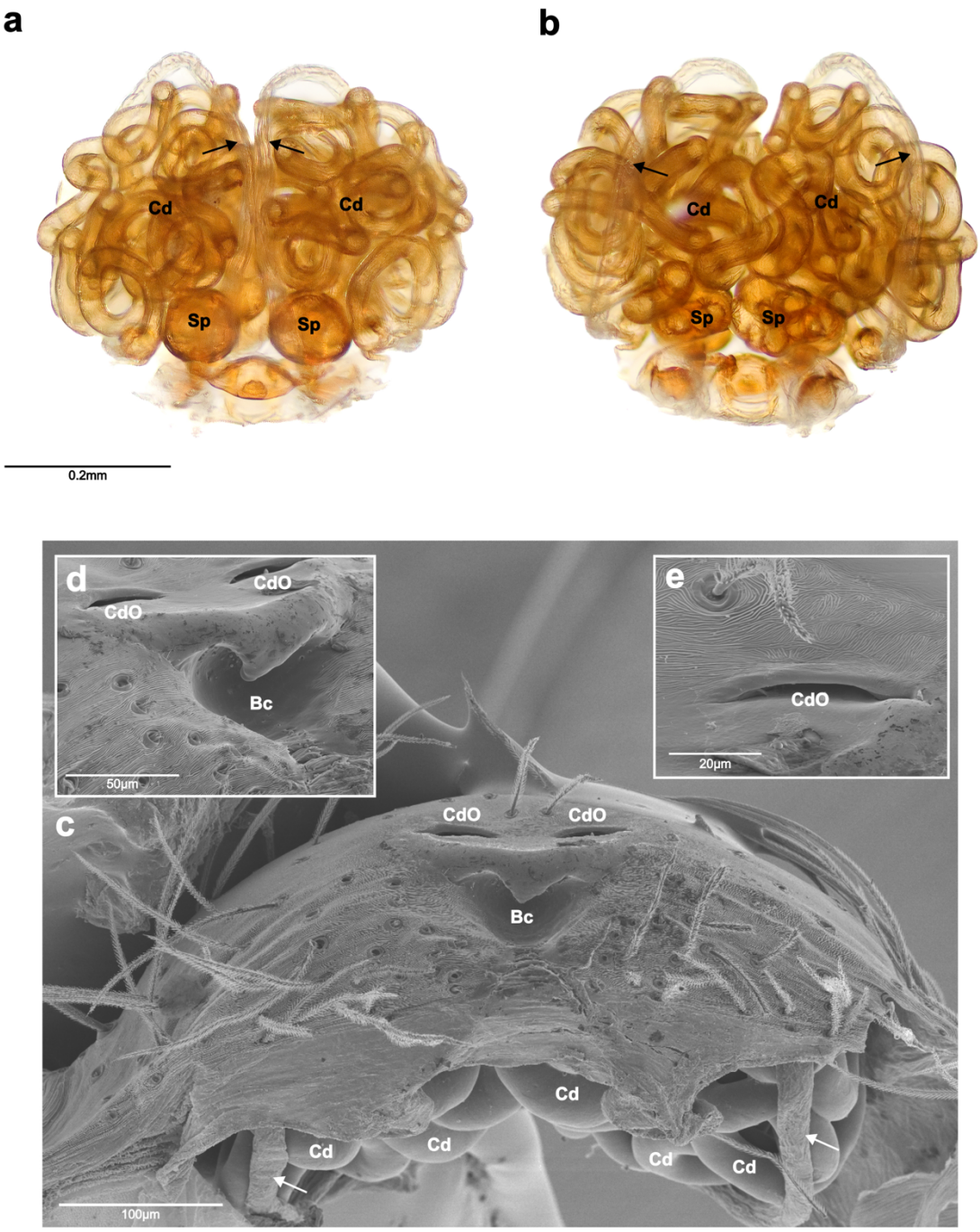

Figure 3. Female genitalia morphology in *Mastigusa*. *Mastigusa arietina* from Denmark. a: vulva, ventral view; b: vulva, dorsal view; c: epigyne, posterior view at the SEM; d: detail of blind concavity at the SEM; e: detail of one of the copulatory duct openings at the SEM. Abbreviations: Cd=copulatory ducts; Sp=spermatechae; Bc=blind concavity; CdO=copulatory duct opening. Arrows in a and b pointing to the first, membranous part of the copulatory ducts. (Photos by Rasmus Jensen).

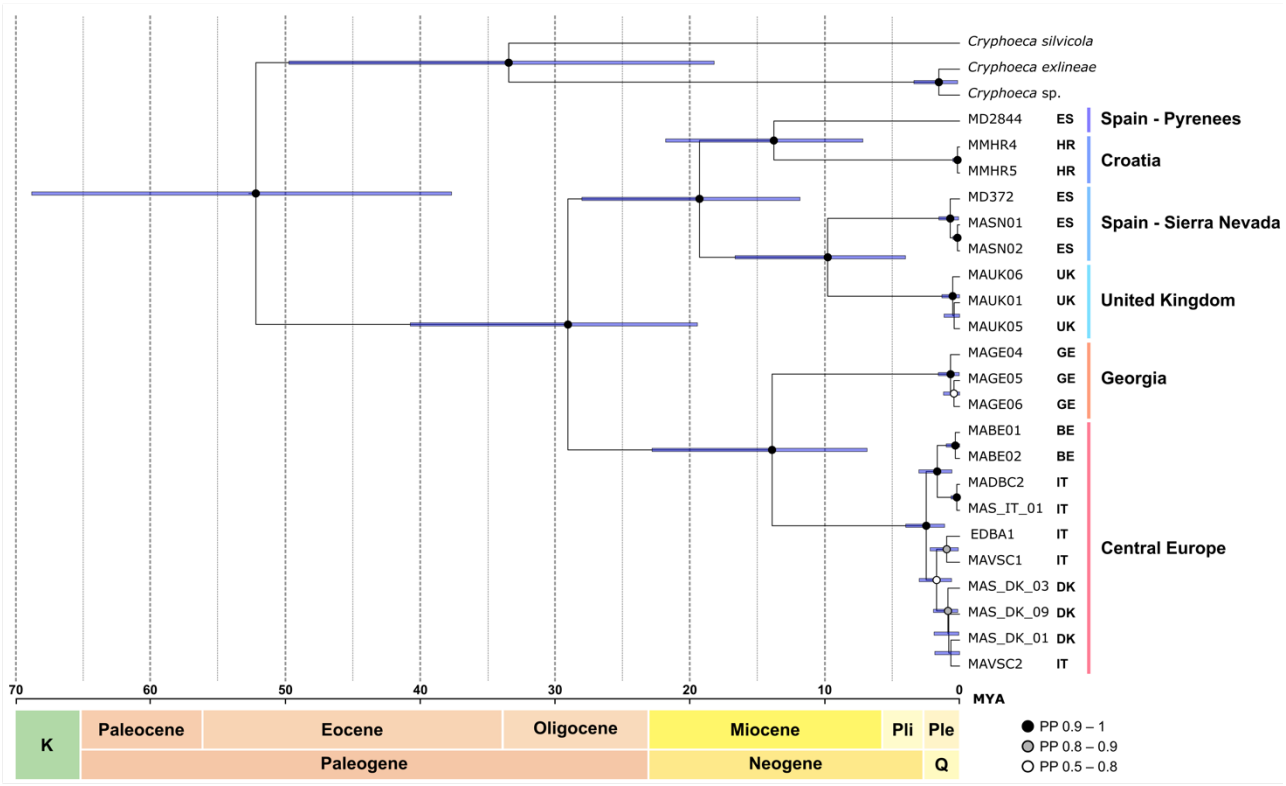

Figure 4. Time-calibrated molecular phylogeny of *Mastigusa* from Chapter 2. Scale in million years ago. Node bars represent 95% confidence intervals. Country codes after the sample names. Country codes: BE=Belgium; DK=Denmark; ES=Spain; GE=Georgia; HR=Croatia; IT=Italy; UK=United Kingdom. PP=posterior probability.

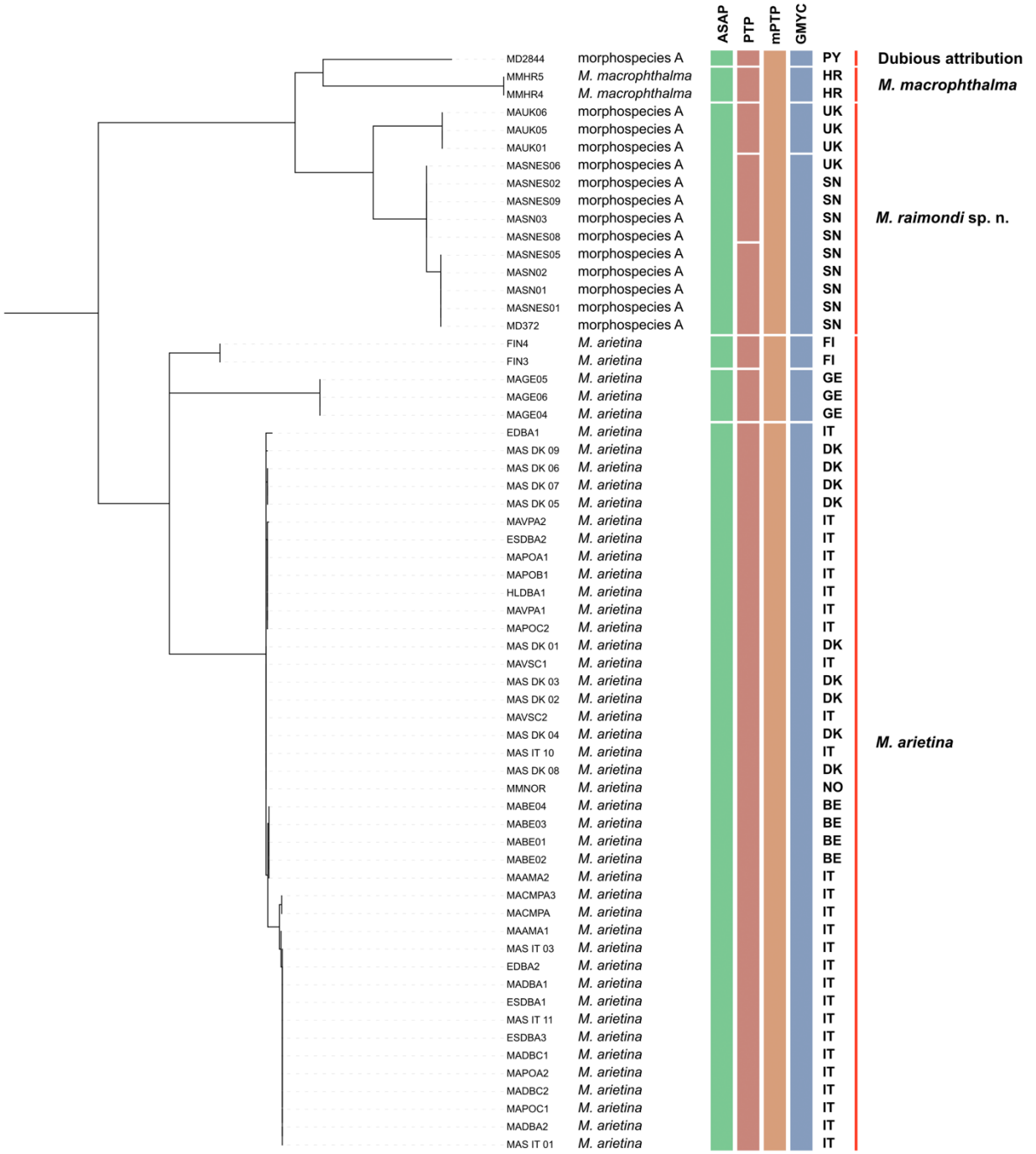

Figure 5. Summary of the results from the species delimitation analyses with the four different approaches. Tree topology from the maximum likelihood tree used for PTP and mPTP, nodes with bootstrap values below 60 collapsed. Morphological attribution after terminal name. Red lines on the right identify our species hypothesis. Country codes: BE=Belgium; DK=Denmark; FI=Finland; GE=Georgia; HR=Croatia; IT=Italy; NO=Norway; PY=Spain-Pyrenees; SN=Spain-Sierra Nevada; UK=United Kingdom.

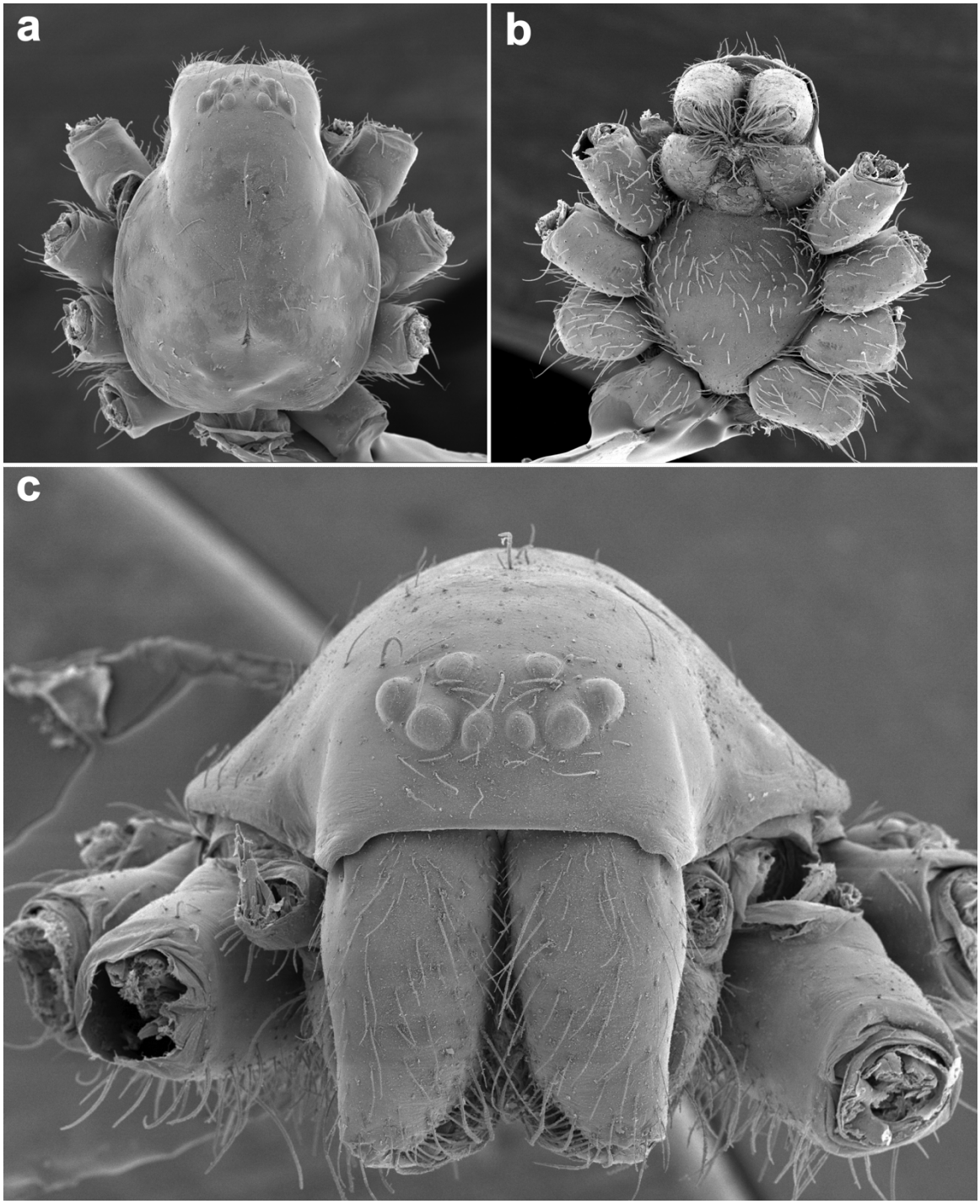

Figure 6. *Mastigusa* prosoma. Female *M. raimondi* from Spain. A: dorsal view; b: ventral view; c: frontal view. (SEM images by Rasmus Jensen).

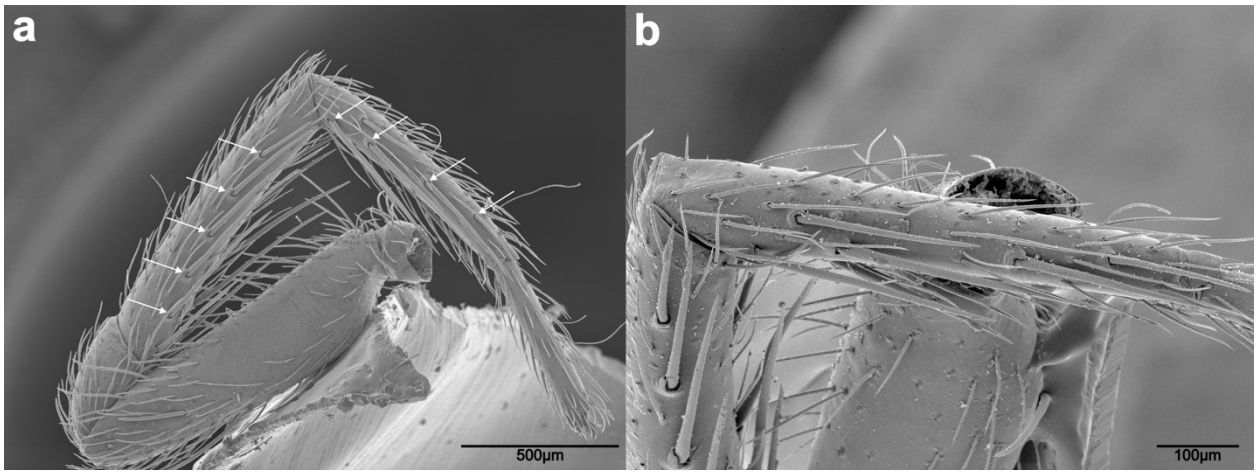

Figure 7. *Mastigusa* legs I and II. a: leg 1 of *M. arietina* from Denmark; b: leg II of *M. macrophthalma* from Slovenia. White arrows indicate the base of the spines on the ventral side of tibia and metatarsus. (SEM images by Rasmus Jensen).

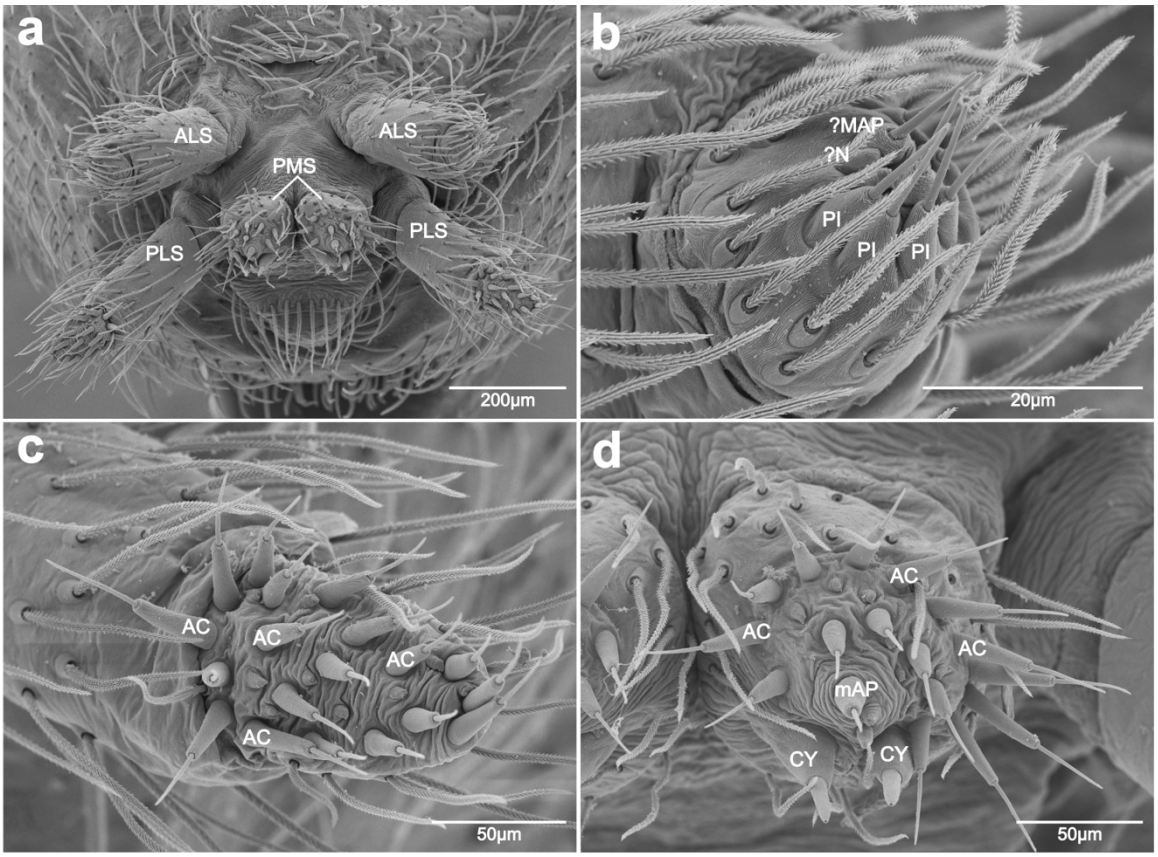

Figure 8. Spinnerets in *Mastigusa*; female *M. arietina* from Denmark. a: spinnerets in ventral view; b: anterior lateral spinnerets; c: posterior lateral spinnerets; d: posterior median spinnerets. Abbreviations: ALS=anterior lateral spinnerets; PLS=posterior lateral spinnerets; PMS=posterior median spinnerets; MAP=major ampullate spigot; N=nubbin; PI=piriform spigot; AC=aciniform spigot; CY=cylindrical spigot. (SEM images by Rasmus Jensen).

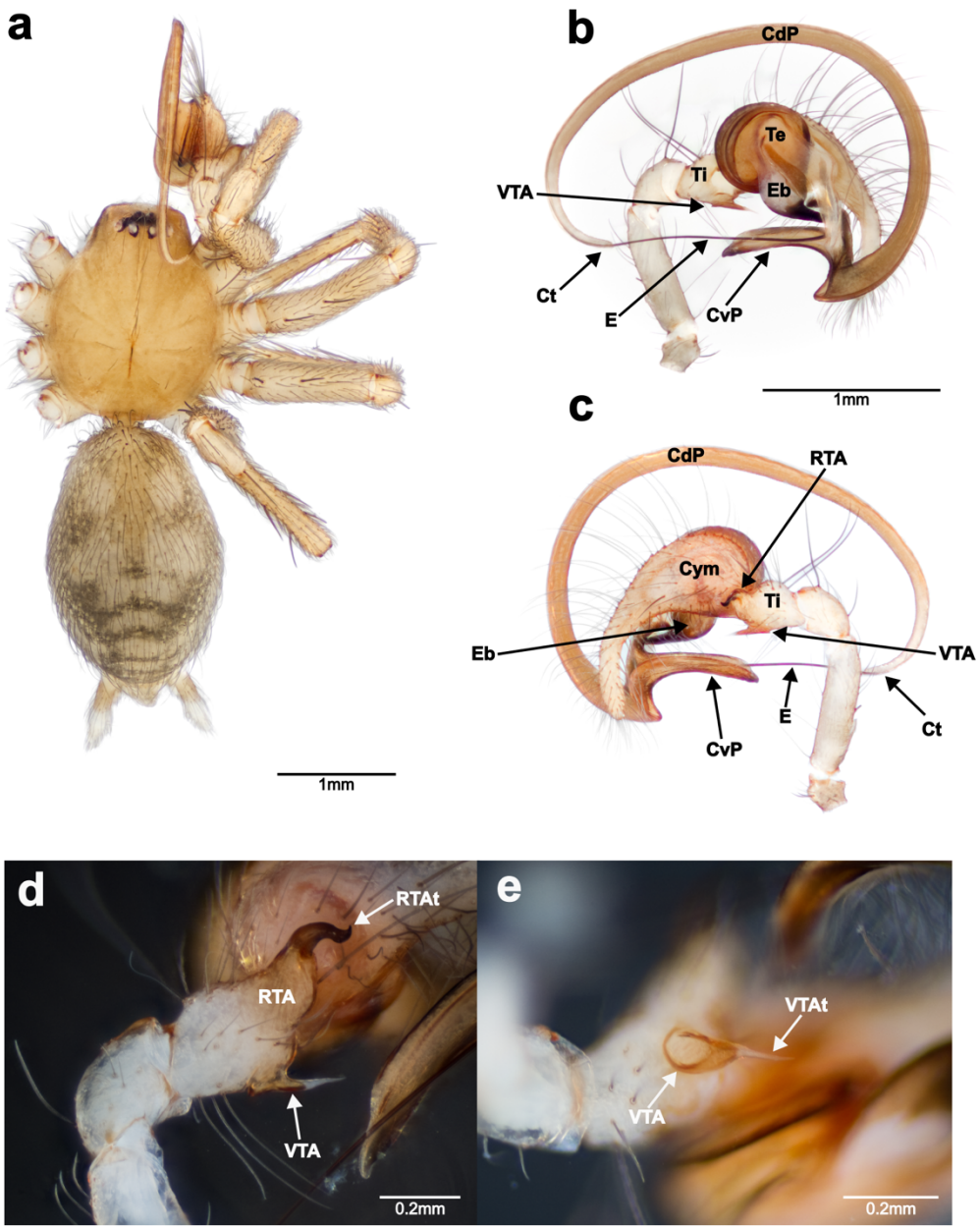

Figure 9. Male morphology in *M. arietina*, individual from Italy. a: habitus; b: left pedipalp, prolateral view; c: left pedipalp, retrolateral view; d: right pedipalp, detail of the tibia in retrolateral view; e: right pedipalp, detail of the ventral tibial apophysis in ventral view. Abbreviations: CdP=conductor dorsal process; CvP=conductor ventral process; Ct=conductor tip; E=embolus; Eb=embolus base; Te=tegulum; Ti=tibia; VTA=ventral tibial apophysis; RTA=retrolateral tibial apophysis; RTAt=tip of the retrolateral tibial apophysis; VTAt=tip of the ventral tibial apophysis.

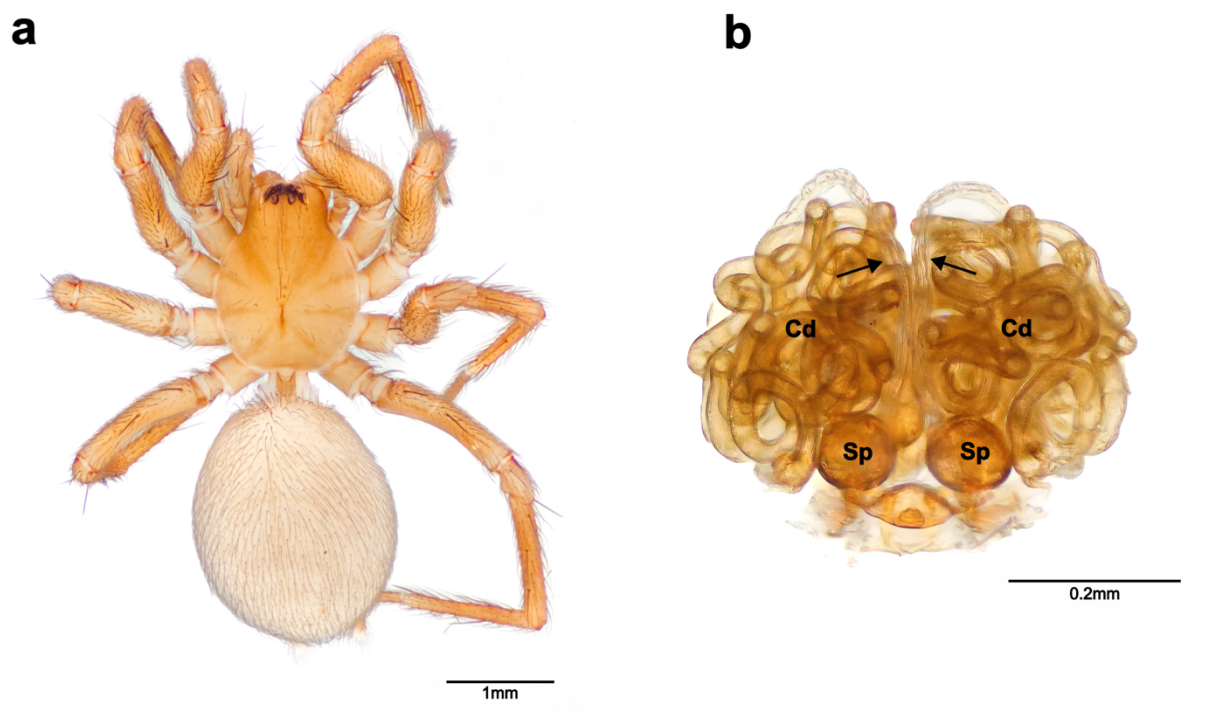

Figure 10. Female morphology in *M. arietina*, individual from Italy. a: habitus; b: vulva. Abbreviations: Cd=copulatory ducts; Sp=spermetechae. Arrows in b pointing to the first, membranous part of the copulatory ducts. (Photo b by Rasmus Jensen).

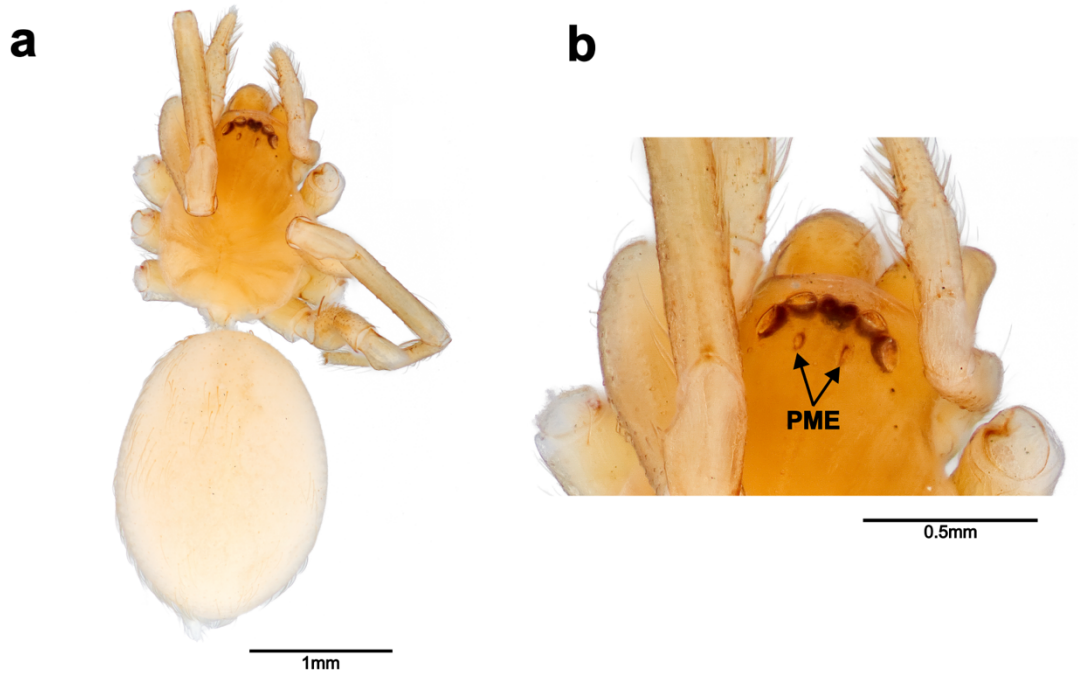

Figure 11. Holotype of *M. lucifuga*. a: habitus; b: detail of the eyes. Abbreviations: PME=posterior median eyes. (Photos by Rasmus Jensen).

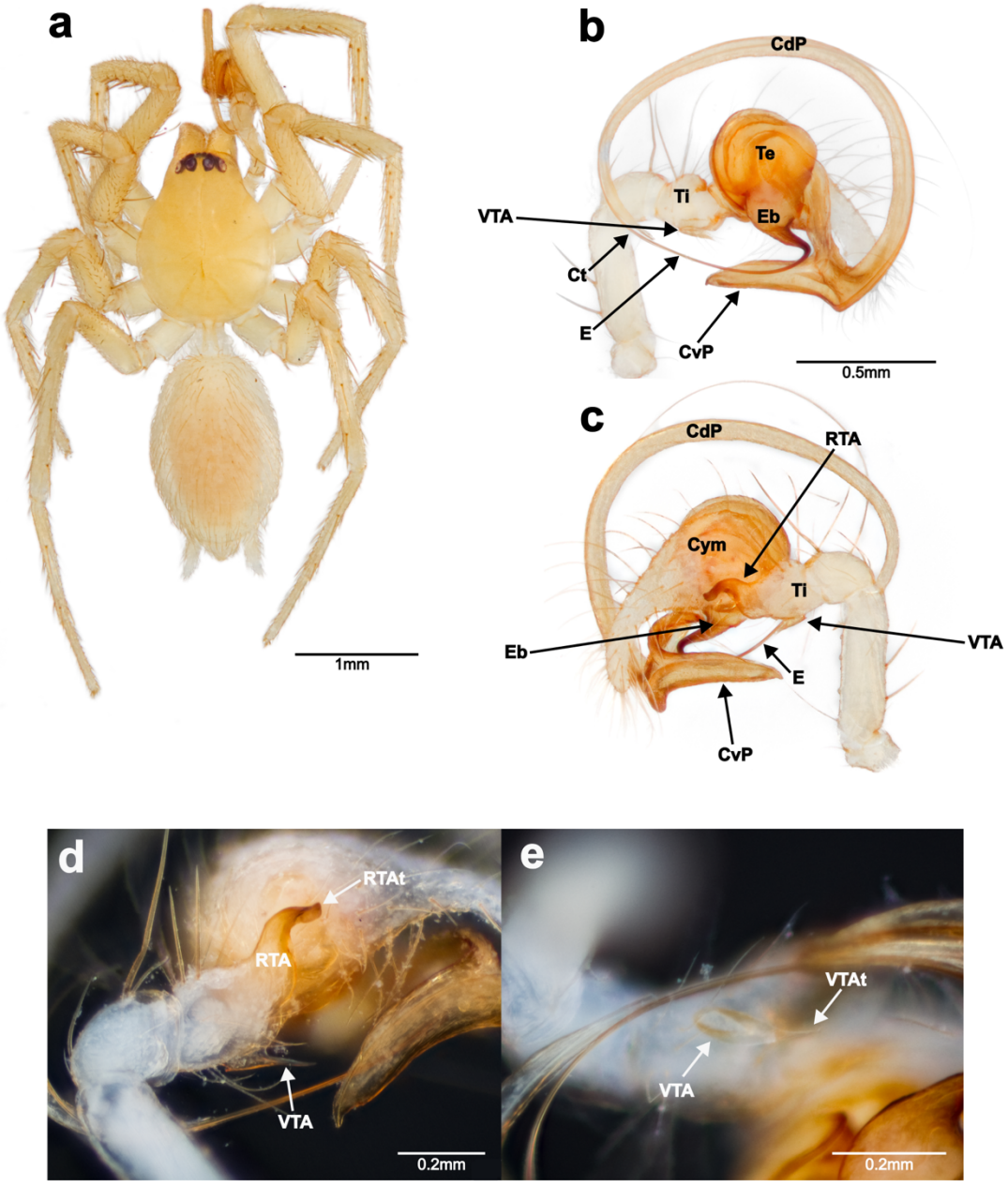

Figure 12. Male morphology in *M. macrophthalma*, individual from Slovenia. a: habitus; b: left pedipalp, prolateral view; c: left pedipalp, retrolateral view; d: right pedipalp, detail of the tibia in retrolateral view; e: right pedipalp, detail of the ventral tibial apophysis in ventral view. Abbreviations: CdP=conductor dorsal process; CvP=conductor ventral process; Ct=conductor tip; E=embolus; Eb=embolus base; Te=tegulum; Ti=tibia; VTA=ventral tibial apophysis; RTA=retrolateral tibial apophysis; RTAt=tip of the retrolateral tibial apophysis; VTAt=tip of the ventral tibial apophysis.

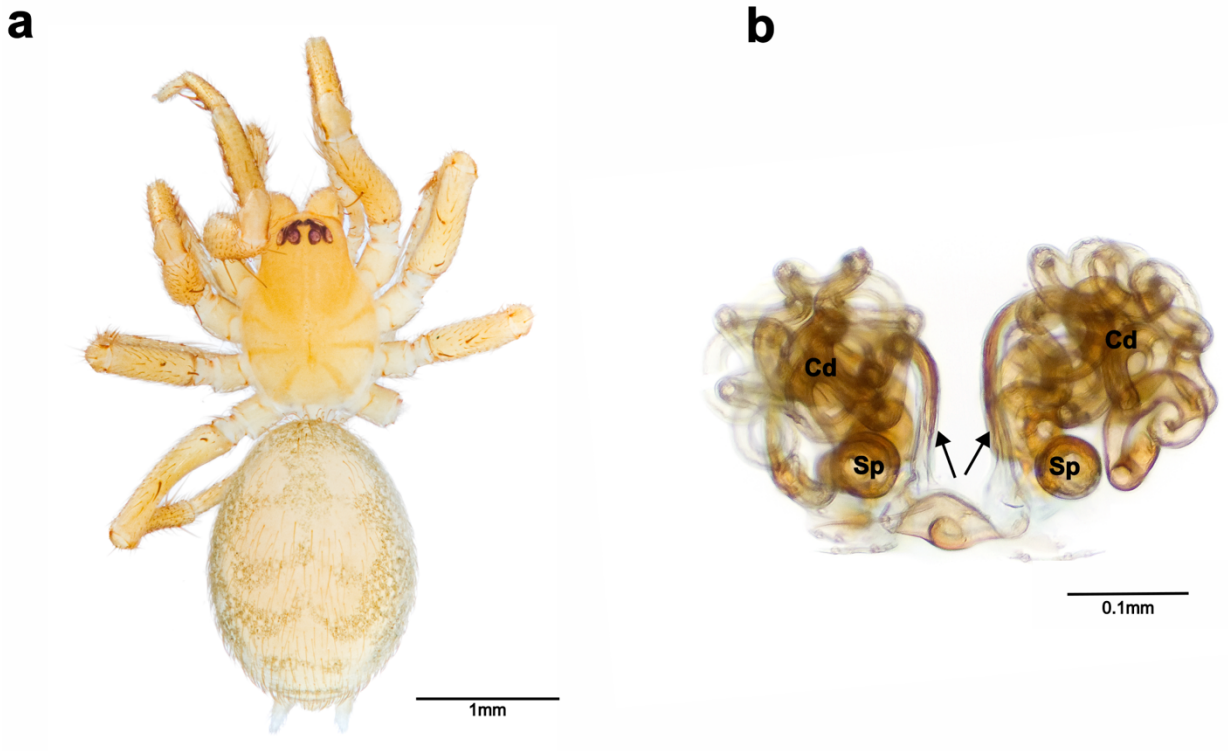

Figure 13. Female morphology in *M. macrophthalma*, individual from Slovenia. a: habitus; b: vulva. Abbreviations: Cd=copulatory ducts; Sp=spermetechae. Arrows in b pointing to the first, membranous part of the copulatory ducts. (Photo b by Rasmus Jensen).

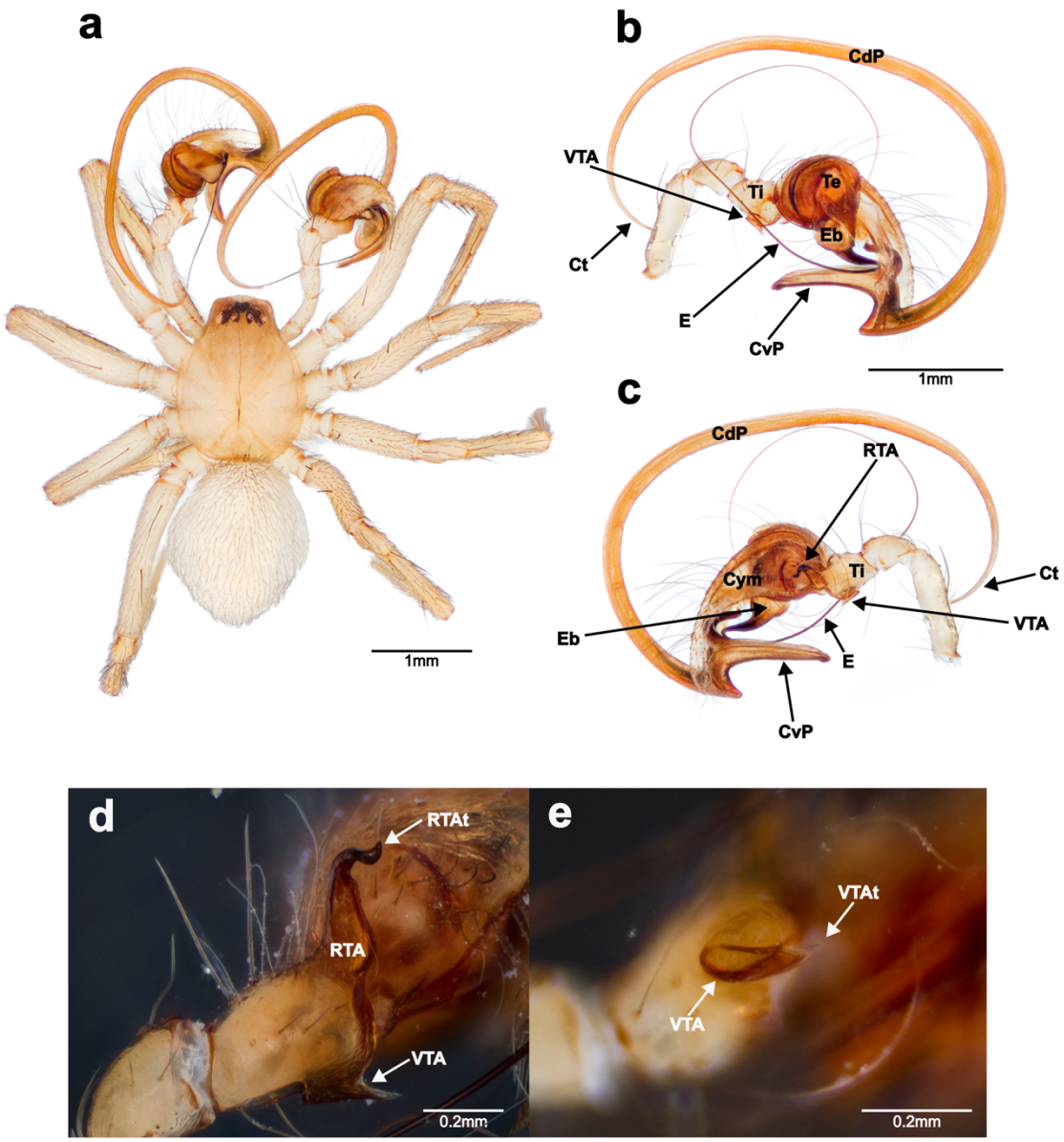

Figure 14. Male morphology in *M. raimondi* sp. n. a: habitus, individual from the United Kingdom; b: left pedipalp, prolateral view; c: left pedipalp, retrolateral view; d: right pedipalp, detail of the tibia in retrolateral view; e: right pedipalp, detail of the ventral tibial apophysis in ventral view. b-e, individual from Spain. Abbreviations: CdP=conductor dorsal process; CvP=conductor ventral process; Ct=conductor tip; E=embolus; Eb=embolus base; Te=tegulum; Ti=tibia; VTA=ventral tibial apophysis; RTA=retrolateral tibial apophysis; RTAt=tip of the retrolateral tibial apophysis; VTAt=tip of the ventral tibial apophysis.

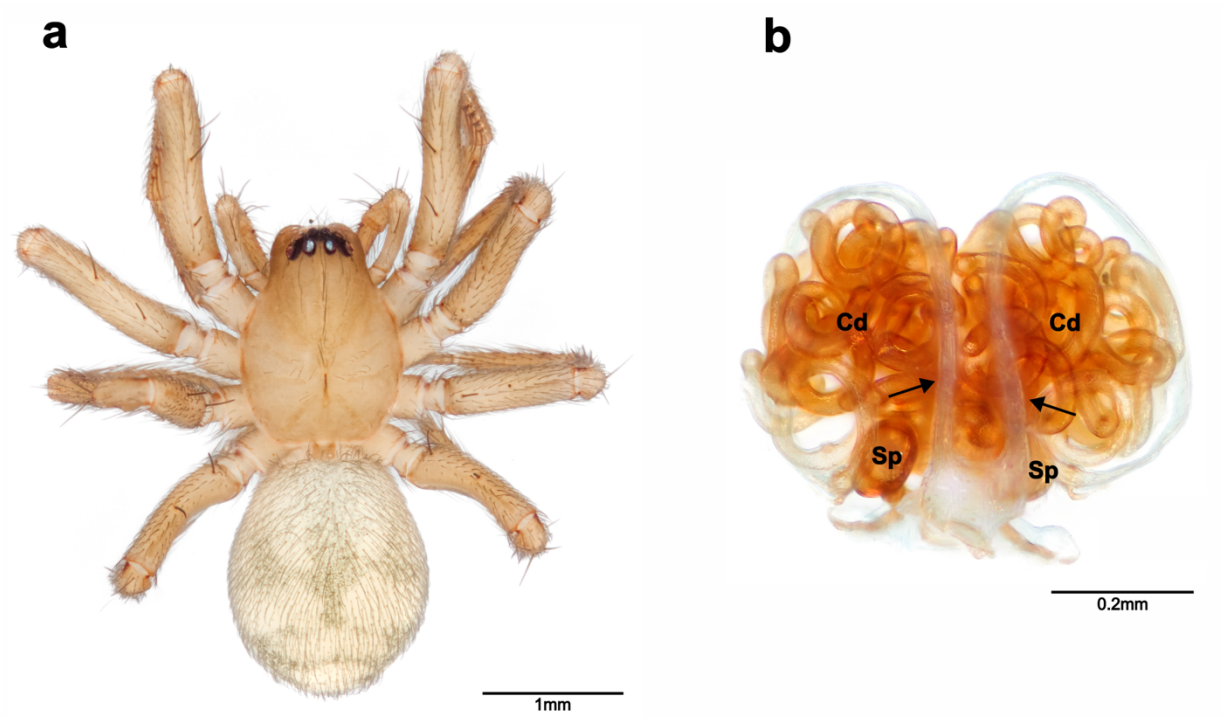

Figure 15. Female morphology in *M. raimondi* sp. n. a: habitus, individual from the United Kingdom; b: vulva, individual from Spain. Abbreviations: Cd=copulatory ducts; Sp=spermetechae. Arrows in b pointing to the first, membranous part of the copulatory ducts. (Photo b by Rasmus Jensen).

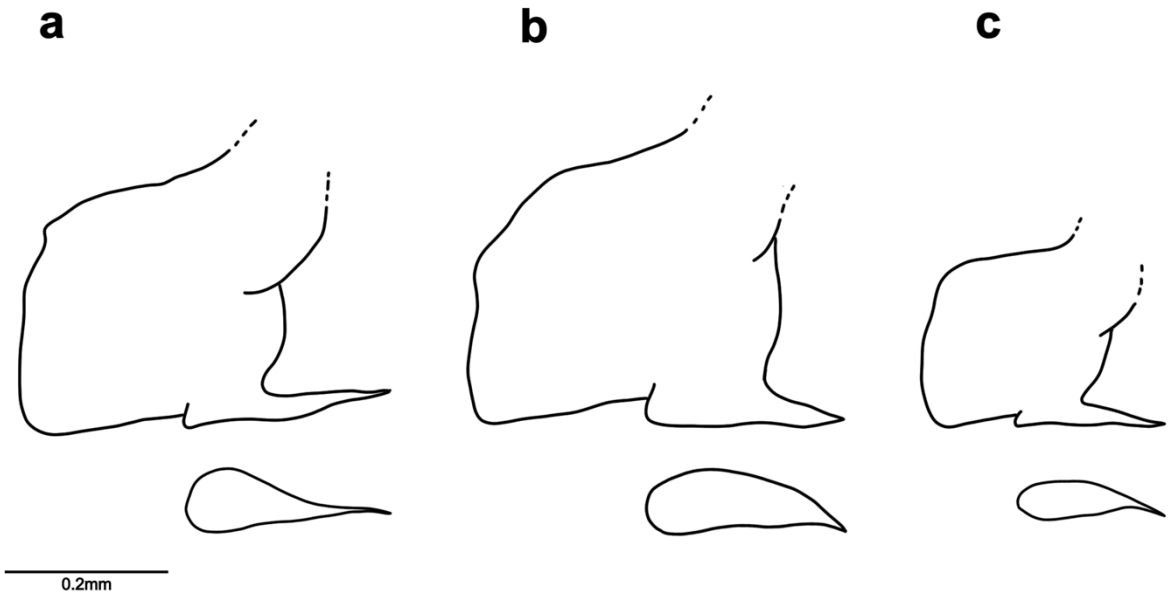

Figure 16. Schematic drawings of the right pedipalp tibia in retrolateral view for *Mastigusa* species, emphasizing the shape of the ventral tibial apophysis; on the bottom the ventral tibial apophysis outline in ventral view. a: *M. arietina*; b: *M. raimondi* sp. n.; c: *M. macrophthalma*.

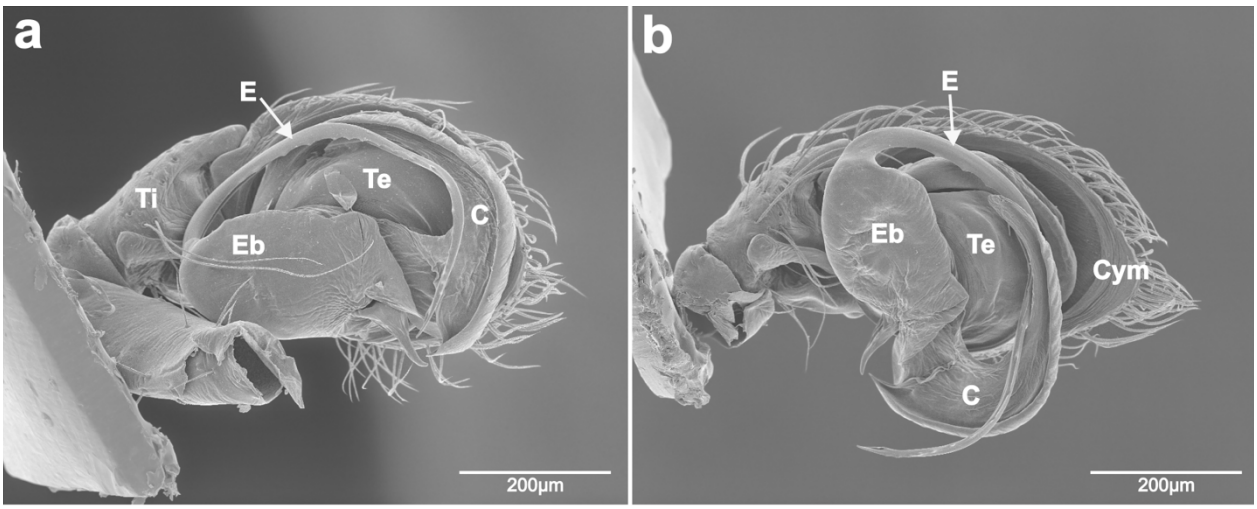

Figure 17. Male pedipalp morphology in *Cryphoeca silvicola* (Cybaeidae) from Denmark at the SEM. a: unexpanded left pedipalp; b: expanded left pedipalp. Abbreviations: C=conductor; E=embolus; Eb=embolus base; Te=tegulum; Ti=tibia.

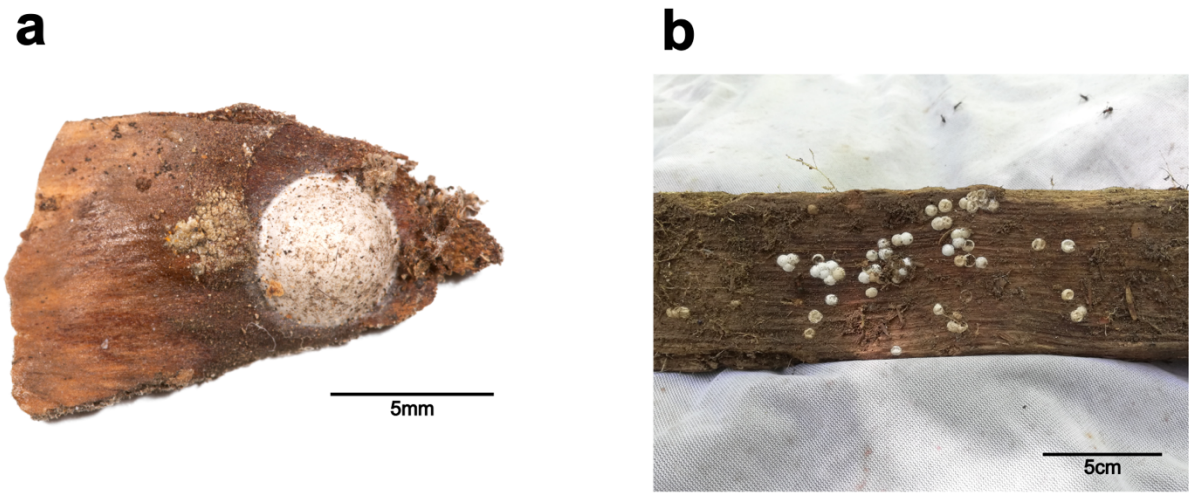

Figure 18. *Mastigusa arietina* egg sacs. a: single egg sac attached to a piece of bark, found inside a *Formica* sp. mound nest in Denmark; b: multiple egg sacs attached to a pole buried inside a *Formica* sp. mound nest in Italy. (Picture a by Filippo Castellucci, picture b by Andrea Piccinini).

2.11. SUPPLEMENTARY TABLES

Code	Nation	Locality	Habitat	Collecting date	Lat	Lon	Elevation (m a.s.l.)	Legit	Source
MABE01	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MABE02	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MABE03	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MABE04	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MMHR4	HR	6km west of Sljeme, Funzine	Under stones, *Fagus sylvatica* forest with some *Picea abies*	29/06/21	45°20.79500'	014°41.59333'	915	Castellucci F.	This study
MMHR5	HR	6km west of Sljeme, Funzine	Under stones, *Fagus sylvatica* forest with some *Picea abies*	29/06/21	45°20.79500'	014°41.59333'	915	Castellucci F.	This study
MAS_DK_01	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_02	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_03	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_04	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_05	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	10/11/18	56°09.01800'	9°31.39200'	53	Pedersen J.	This study
MAS_DK_06	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	11/11/18	56°09.01800'	9°31.39200'	54	Pedersen J.	This study
MAS_DK_07	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	12/11/18	56°09.01800'	9°31.39200'	55	Pedersen J.	This study
MAS_DK_08	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	13/11/18	56°09.01800'	9°31.39200'	56	Pedersen J.	This study
MAS_DK_09	DK	Tokkekøb Hegn, Lillerød	In *Formica* sp. nest, broad leaf forest with some conifers	12/04/18	55°53.24886'	012°23.16618'	60	Castellucci F.	This study
MAGE04	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.	This study
MAGE05	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.	This study
MAGE06	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.	This study
MAVSC1	IT	Chabod trail, Valsavarenche	In *Formica* sp. nest, *Larix decidua* forest with scarce *Pinus cimbra* and *Picea abies*	08/07/20	45°32.59620'	07°13.33200'	2024	Castellucci F.	This study

MAVSC2	IT	Chabod trail, Valsavarenche	In *Formica* sp. nest, *Larix decidua* forest with scarce *Pinus cimbra* and *Picea abies*	08/07/20	45°32.59620'	07°13.33200'	2024	Castellucci F.	This study
EDBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
EDBA2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
MADBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
MADBA2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
MADBC2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.46940'	012°09.25550'	1477	Castellucci F.	This study
ESDBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
ESDBA2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
ESDBA3	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
HLDBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
MAVPA1	IT	Around Roner Alm, Luson	In *Formica aquilonia* nest, in *Picea abies* forest with *Pinus sylvestris*	26/06/20	46°46.83600'	11°44.48400'	1841	Castellucci F.	This study
MAVPA2	IT	Around Roner Alm, Luson	In *Formica aquilonia* nest, in *Picea abies* forest with *Pinus sylvestris*	26/06/20	46°46.83600'	11°44.48400'	1841	Castellucci F.	This study
MAPOA1	IT	Val Chedul, Selva di Val Gardena	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	24/06/20	46°33.76200'	11°46.84200'	1781	Castellucci F.	This study
MAPOB1	IT	Val Chedul, Selva di Val Gardena	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	25/06/20	46°33.78000'	11°46.81800'	1760	Castellucci F.	This study
MAPOC1	IT	Col Raiser, Santa Cristina	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	25/06/20	46°35.19600'	11°44.96400'	2058	Castellucci F.	This study
MAPOC2	IT	Col Raiser, Santa Cristina	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	26/06/20	46°35.19600'	11°44.96400'	2059	Castellucci F.	This study
MAS_IT_01	IT	Casera Casavento, Claut	In *Formica* sp. nest, *Picea abies* forest	08/09/18	46°16.08600'	012°35.74800'	934	Castellucci F.	This study
MAS_IT_03	IT	Casera Casavento, Claut	In *Formica* sp. nest, *Picea abies* forest	08/09/18	46°16.08600'	012°35.74800'	934	Castellucci F.	This study
MAS_IT_10	IT	Corona, Cortaccia	In *Formica* sp. nest, *Picea abies* forest	23/09/18	46°19.76400'	11°12.51000'	1195	Castellucci F.	This study
MAS_IT_11	IT	Corona, Cortaccia	In *Formica* sp. nest, *Picea abies* forest	23/09/18	46°19.76400'	11°12.51000'	1195	Castellucci F.	This study
MAAMA1	IT	Gias delle Mosche, Valdieri	In *Formica lugubris* nest in *Picea abies* forest with scarce *Larix decidua* and *Fagus sylvatica*	12/07/20	44°10.96200'	7°16.31400'	1703	Castellucci F.	This study

MAAMA2	IT	Gias delle Mosche, Valdieri	In *Formica lugubris* nest in *Picea abies* forest with scarce *Larix decidua* and *Fagus sylvatica*	12/07/20	44°10.96200'	7°16.31400'	1703	Castellucci F.	This study
MACMPA	IT	Campigna, Santa Sofia	In *Formica paralugubris* nest in *Abies alba* forest	17/06/21	43°52.20084'	11°44.25150'	1220	Castellucci F.	This study
MACMPA3	IT	Campigna, Santa Sofia	In *Formica paralugubris* nest in *Abies alba* forest	17/06/21	43°52.20084'	11°44.25150'	1220	Castellucci F.	This study
MD2844	ES	Sola de Boi, Lleida	In pitfall trap, white oak forest	15-29/6/13	42°32.97480'	000°52.35240'	1760	Crespo L. et al.	Crespo et al. 2018
MD372	ES	Soportujar, Granada	In pitfall trap, white oak forest	31/5/13-14/6/13	36°57.69060'	003°25.12860'	1787	Crespo L. et al.	Crespo et al. 2018
MASN01	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASN02	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASN03	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASNES01	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASNES02	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASNES05	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASNES06	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASNES08	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASNES09	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
FIN3	FI	Kuopio, Lippumäki	In *Formica aquilonia* nest	07/11/21	62°50.52233'	27°38.69917'	102	Sorvari J.	This study
FIN4	FI	Kuopio, Lippumäki	In *Formica lugubris* nest	29/11/21	62°50.52350'	27°38.55317'	100	Sorvari J.	This study
MMNOR	NO	Skjaak, Oppland, Norway	NA	21/06/14	61°48.36000'	7°42.24000'	750	Fjellberg A.	Bold Systems, BOLD:ACR9918
MAUK01	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	003°42.63333'	130	Gallon R.	This study
MAUK05	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	003°42.63333'	130	Gallon R.	This study
MAUK06	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	003°42.63333'	130	Gallon R.	This study

Supplementary Table 1. List of the specimens included in the COI analysis with collecting information.

4. Phylogeography and ecological plasticity in the spider genus *Mastigusa* (Araneae, Cybaeidae)

Filippo Castellucci, Nikolaj Scharff and Andrea Luchetti

4.1. ABSTRACT

The palearctic spider genus *Mastigusa* shows great ecological variability with free-living, cave dwelling and myrmecophile populations known. A recent taxonomic revision of the genus made it possible to track down the distribution of such lifestyles on a phylogeny making a comparative study between species possible. We studied how genetic diversity is distributed among different *Mastigusa* populations in a phylogeographic framework, using molecular phylogenetics and haplotype network analysis, and applied ecological niche modelling techniques to build potential distribution models and evaluate ecological differences between three *Mastigusa* species: *M. arietina* (Thorell, 1870), *M. macrophthalma* (Kulczynski, 1897) and *M. raimondi* (Castellucci et al., in prep). *Mastigiusa* species showed significant intraspecific genetic variability across their distribution range, with strong phylogeographical patterns. The potential distribution models show minimum overlap, indicating that the three species have significantly different ecological requirements, compatible with their parapatric distribution. *M. raimondi* appears to be the more ecologically plastic of the three, being the only species for which cave dwelling populations are known. These are found in the southern margins of its distribution range, while myrmecophile populations are only known in the northern margins, suggesting an effect of climate in pushing toward a myrmecophile or cave dwelling lifestyle.

Keywords: ecological niche modelling; ENM; phylogenetics; DNA barcoding; haplotypes

4.2. INTRODUCTION

Mastigusa Menge, 1854 is a genus of small entelegyne spiders belonging to the family Cybaeidae with a Palearctic distribution. The genus had a troubled taxonomic history with uncertainties regarding its phylogenetic placement and the number and circumscription of the species it includes (**Chapters 1, 2**). These issues were addressed with the first molecular phylogenetic analyses focusing on *Mastigusa* that led to a taxonomic revision of the genus (**Chapters 1, 2**). Four species are currently recognized: *M. arietina* (Thorell, 1871), found in Central and Northern Europe and in the eastern countries up to Russia and Iran, *M. macrophthalma* (Kulczynski, 1897), recorded in Slovenia, Croatia and Bosnia-Herzegovina, *M. lucifuga* (Simon, 1898) from the French Pyrenees and *M. raimondi* Castellucci et al. in prep, recorded from Spain, Portugal, the United Kingdom and Algeria (**Chapter 2**). The genus shows an interesting ecological plasticity, with historically known free-living, cave dwelling and myrmecophile populations. Myrmecophile *Mastigusa* populations were observed in Central and Northern Europe (Westring 1861; Palmgren 1976; Roberts 1985; Heimer & Nentwig 1991; Scharff&Gudik-Sørensen 2006; Aakra et al. 2016; Parmentier et al. 2016; Castellucci et al. 2022), with the presence of adult males and females, juveniles and egg sacks, indicating that these spiders can spend their whole life cycle inside ant nests. The main ant hosts of *Mastigusa* are red wood ants belonging to the *Formica rufa* species group, including *F. aquilonia* Yarrow, 1955, *F. lugubris* Zetterstedt, 1838, *F. paralugubris* Seifert, 1996, *F. polyctena* Foerster, 1850 and *F. rufa* Linnaeus, 1761. These spiders have also been collected inside nests of the non-red wood ant *Formica* species *F. fusca* Linnaeus, 1758, of different other Formicinae species belonging to the genus *Lasius* Fabricius, 1804, as *L. fuliginosus* (Latreille, 1798), *L. alienus* (Foerster, 1850), and *L. brunneus* (Latreille, 1798), and of the Myrmicinae *Messor muticus* (Nylander, 1849) and *Tetramorium caespitum* Linnaeus, 1758 (Castellucci et al. 2022, **Chapter 4**). Cave dwelling *Mastigusa* populations have only been recorded from the Iberian Peninsula and Algeria (Simon 1898; Simon 1913; Fage 1931). The taxonomic uncertainties regarding the status of the different *Mastigusa* species and their true distribution always made it difficult to link the different lifestyles to taxonomical, geographical or climatic factors, but with the recent taxonomic revision of

the genus it is now possible to map these ecological traits on a geographic and taxonomic scale to better understand their distribution and evolution. The first molecular phylogenetic analysis including on *Mastigusa* was carried out by using three mitochondrial (COI, 12S, 16S) and three nuclear markers (H3, 18S, 28S) including specimens from six countries, and revealed the presence of six distinct genetic lineages within the genus (**Chapter 1**). *Mastigusa arietina* was found to be subdivided into two clades, one from Central and Northern Europe (Northern Italy, Belgium and Denmark) and an eastern clade represented by specimens from Georgia; *Mastigusa raimondi* included a Spanish clade and a British one. The other two lineages identified represented *M. macrophthalma* from Croatia and a single specimen of dubious attribution from the Spanish Pyrenees, whose morphology fits with *M. raimondi* but did not cluster with the other specimens of the species in the phylogenetic tree, being found in sister group relationship with *M. macrophthalma* (Fig. 1) (**Chapters 1, 2**).

4.3. MATERIALS AND METHODS

4.3.1. Matrix assembly, phylogenetic and haplotypes analyses

The matrix was built using the COI dataset used for the species delimitation analysis from (**Chapter 3**) with the addition of three *Cryphoeca* terminals, chosen as outgroups based on the results of the phylogenetic placement of the genus *Mastigusa* from **Chapter 1**. Sixty-two *Mastigusa* sequences were included in the analyses, representing nine countries. A list of the specimens included in the matrix is reported in Suppl. Tab. 1. Sequences were aligned using MAFFT v.7.503 (Katoh and Standley 2013) with the L-INS-i algorithm. The dataset was partitioned in the three codon positions of the COI gene. The selection of best fitting evolutionary models for the three partitions was performed with ModelFinder (Kalyaanamoorthy et al. 2017), as implemented in the IQ-TREE v1.6.12 software (Nguyen et al. 2015). Phylogenetic reconstruction using the maximum likelihood method was performed with IQ-TREE v1.6.12 with 1000 replicates of UltraFast bootstrap (Minh et al. 2013), while Bayesian Inference phylogenetic reconstruction was carried out using MrBayes v3.2.7 (Ronquist et al. 2012) with two runs including 10 million generations each, with sampling every 1000 trees. Convergence of the two runs was assessed using Tracer v1.7 (Rambaut et al. 2018). Haplotypes were retrieved from the same COI dataset

using the R package “haplotypes” and a haplotype network was built for the two main claes identified by the phylogenetic analyses to understand the geographic distribution of the different mitochondrial variants. Median joining haplotype networks were built using the software PopART (Leigh and Bryant 2015). Genetic diversity within the two main clades was estimated using the haplotype diversity (H) and nucleotide diversity (π) statistics (Nei 1987). These were computed using the R package “pegas”.

4.3.2. Study on the ecology of Mastigusa *populations*

Information on the lifestyles of the different *Mastigusa* populations analyzed was retrieved from literature records, from collecting information accompanying collection material, when available, and from direct observations during field surveys focused on collecting *Mastigusa* specimens in different European countries, such as Italy, Denmark, Croatia and Spain (**Chapter 1, 2, 4**).

4.3.3. Ecological niche modelling and niche equivalency

Potential distribution models for the three *Mastigusa* species *M.* arietina, *M. macrophthalma* and *M. raimondi* were built using MaxEnt v3.4.4 (Phillips et al. 2004, 2006; Phillips and Dudík 2008) with a random test percentage of 20. *Mastigusa lucifuga* was not considered since it is only known from the type specimen and was never recoded again after its original description. The 19 standard bioclimatic layers and the elevation layer from the WorldClim version 2.1 database (https://www.worldclim.org) were used for the model reconstructions at the 2.5 arc resolution. The used layers were: BIO1 = Annual Mean Temperature; BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp)); BIO3 = Isothermality (BIO2/BIO7) (×100); BIO4 = Temperature Seasonality (standard deviation ×100); BIO5 = Max Temperature of Warmest Month; BIO6 = Min Temperature of Coldest Month; BIO7 = Temperature Annual Range (BIO5-BIO6); BIO8 = Mean Temperature of Wettest Quarter; BIO9 = Mean Temperature of Driest Quarter; BIO10 = Mean Temperature of Warmest Quarter; BIO11 = Mean Temperature of Coldest Quarter; BIO12 = Annual Precipitation; BIO13 = Precipitation of Wettest Month; BIO14 = Precipitation of Driest Month; BIO15 = Precipitation Seasonality (Coefficient of Variation); BIO16 = Precipitation of Wettest Quarter; BIO17 = Precipitation of Driest Quarter; BIO18

= Precipitation of Warmest Quarter; BIO19 = Precipitation of Coldest Quarter; elev = Elevation (SRTM). Models were computed on the Western Palearctic region, in an area covering longitudes from 28°W to 78°E and latitudes from 19°N to 78°N, by clipping the bioclimatic layers using the R package “raster”. Correlation coefficients were measured for each couple of environmental variables, again using the R package “raster” (Suppl. Tab. 2) and highly correlated variables ($|r| \geqslant 0.9$) were excluded, except for one representative layer. After this process 11 variables were retained: BIO1, BIO2, BIO3, BIO7, BIO8, BIO9, BIO12, BIO15, BIO18, BIO19 and elev. Three niche identity analyses were performed to measure the degree of similarity of the potential distribution models of each couple of species. The function *idtest()* from the R package “ENMTools” was used with 100 randomized pseudoreplicates and 10000 background points, with a critical percentage of 95%.

4.4. RESULTS

4.4.1. Phylogenetic analysis

The maximum likelihood and Bayesian approaches recovered a topology that is in line with the multilocus analysis from (**Chapter 1**) (Fig. 2, Suppl. Fig. 1). Two main clades can be observed, one composed of specimens from Italy, Denmark, Belgium, Norway, Finland and Georgia (bootstrap=80, posterior probability=1), the other by specimens from Spain, Croatia and the United Kingdom (bootstrap=94, posterior probability=1). In the first clade, we observe a strongly supported Central and Northern European clade composed by specimens from Italy, Denmark, Belgium and Norway (bootstrap=99, posterior probability=1), in sister relationship with a weakly supported clade composed by specimens from Finland and Georgia (bootstrap=53) in the maximum likelihood tree. The latter clade is not recovered in the Bayesian tree, and the Central-Northern European, Finnish and Georgian clades form a polytomy. In the second clade, we see a cluster composed of specimens from Spain (Sierra Nevada) and the United Kingdom (bootstrap=68, posterior probability=1) in sister group relationship to a clade composed of specimens from Croatia and a single specimen from the Pyrenees (bootstrap=71) in the maximum likelihood. This latter clade is not supported in the Bayesian tree.

4.4.2. Haplotype analysis

Seventeen different COI haplotypes were retrieved, twelve for *M. arietina*, one for *M. macrophthalma*, three for *M. raimondi* and one from the single specimen of difficult attribution from the Pyrenees (Table 1). No shared haplotypes were found between different species. The haplotype network for *M. arietina* shows a clear distinction between a Central and Northern European haplogroup, with haplotypes from Norway, Denmark, Belgium and Italy; the two haplotypes from Finland and Georgia appear to be significantly distinct from the Central and Northern Europe haplogroup and from each other (Fig. 3). The greater number of haplotypes was sampled in Italy, where eight haplotypes were observed (haplotypes 3, 4, 5, 6, 7, 8, 9, 12), six of them being private (unique for the country), namely, haplotypes 3, 4, 5, 7, 8, 9. Denmark follows with three haplotypes (haplotypes 10, 11, 12), two of which private (haplotypes 10, 11), whereas for Belgium and Norway a single haplotype for each was observed (haplotypes 6 and 12, respectively). Haplotype 12 is shared between Denmark, Italy and Norway, while haplotype 6 is shared between Italy and Belgium. In the network built on the remaining specimens the two *M. raimondi* haplotypes from the Sierra Nevada massif of Spain (haplotypes 16, 17) appear to show a degree of divergence from the *M. raimondi* haplotype from the United Kingdom (haplotype 15) (Fig. 3). The single *M. macrophthalma* haplotype (haplotype 13) and the haplotype from the enigmatic specimen from Pyrenees (haplotype 14) appear to be strongly divergent from the *M. raimondi* haplotypes and from each other (Fig. 3). Attribution of each specimen to the 17 haplotypes is reported in Suppl. Tab. 3. A large genetic diversity was measured for the two main clades, with haplotype diversity (H) being 1 for both. On the other hand, nucleotide diversity was low for both, with $\pi = 0.0328$ for *M. arietina* and $\pi = 0.0776$ for the *M. macrophthalma* + *M. raimondi* clade.

4.4.3. Ecology of Mastigusa *populations*

Free-living, cave dwelling and myrmecophile populations are known to exist for *M. raimondi*. Free-living populations are known from literature in the United Kingdom (Donisthorpe 1908, 1927; Jackson 1913; Bristowe 1939; Locket and Millidge 1953) and were directly observed in Southern Spain during this study; adult specimens were

collected in pit-fall traps in mountainous areas of Algeria, indicating probable free-living populations in the area (specimens observed during this study). The free-living Spanish populations observed were observed above 1800m on the mountain massif of the Sierra Nevada, in Southern Spain. Several adult and juvenile specimens, and egg-sacks, were found under logs half-embedded in the ground, where conditions were significantly more humid than the surroundings (Fig. 4). The collection material from Spain and Portugal that we examined added several records to the known literature record of cave dwelling populations in Algeria and Spain (Table 2). Cave populations are known from latitudes below 41.018°N. The other species show less ecological variability, with *M. arietina* showing mostly myrmecophile populations and rare free-living ones and *M. macrophthalma* being known only from free-living populations. No cave records exist for these species. Myrmecophile *M. arietina* populations were observed mostly inside the big mound nests of *Formica rufa* species group ants (Fig. 5). A detailed overview of the host range observed in myrmecophile populations from the Italian Alps is found in **Chapter 4**. No ecological information exists on *M. lucifuga*, as this species is only known from the holotype, a female collected in the late 1800 and never recorded again, and no information on the collecting method used or habitat is given for the type specimen (**Chapter 2**).

4.4.4. Ecological niche modelling

The potential distribution models obtained for *M. arietina*, *M. macrophthalma* and *M. raimondi* are summarized in Fig. 6. The suitable conditions for the presence of *M. arietina*, which appears to be the most cold-adapted species, are found in Central and Northern Europe and in the main mountain massifs of Southern and Eastern Europe, as the Apennines, Carpathians, Balkans and Caucasus (AUC = 0.955). The environmental variables that contributed the most to the *M. arietina* model were temperature annual range (BIO7), mean temperature of driest quarter (BIO9) and elevation (elev). *Mastigusa macrophthalma* shows a more restricted potential distribution range, with high probabilities in the areas of its actual documented presence (Slovenia, Croatia, Bosnia-Herzegovina), in some areas of the central alps and Central Europe, the Pyrenees and the eastern coast of the Black Sea (AUC = 0.992). The environmental variables that

contributed the most to its model were precipitation of warmest quarter (BIO18), annual precipitation (BIO12) and mean diurnal range (BIO2). *Mastigusa raimondi* appears to be suited to warmer climates than the other species, with high probabilities in the Iberian Peninsula, Southern France, the northern regions of Morocco, Algeria and Tunisia, the Southern Balkans and Turkey (AUC = 0.975). The environmental variables that contributed the most to its model were precipitation of warmest quarter (BIO18), mean temperature of wettest quarter (BIO8) and isothermality (BIO3). The ecological niche models for the three species appear to be significantly distinct, with little general overlap, compatible with their parapatric distribution and indicating significantly small habitat overlap. This was further confirmed by the niche identity analyses, where in all the three niche comparison tests (*M. arietina* vs. *M. macrophthalma*, *M. arietina* vs. *M. raimondi*, *M. macrophthalma* vs. *M. raimondi*) the null hypothesis of niche identity was rejected with p-values < 0.05 (Suppl. Tab. 4).

4.5. DISCUSSION

The geographical distribution of the main *Mastigusa* clades is rather interesting. The first big clade is composed by *M. arietina* and shows a genetically homogeneous sub-clade composed of populations from Central and Northern Europe (Italy, Belgium, Denmark and Norway), and an eastern sub-clade represented by populations from Georgia and Southern Finland, even if little supported. The proximity of the Central and Northern European haplotypes and the presence of shared haplotypes between the Italian Alps and Norther Europe suggests an ongoing gene flow between these areas. The ecological niche model and actual distribution of this species indicates that *M. arietina* is the more cold-adapted of the three. This fits with the strong myrmecophile tendency observed in this species, that could be driven by the necessity of avoiding cold temperatures looking for more stable and protected microclimatic conditions. A wider sampling including more eastern countries could help in understanding the drivers of the genetic divergence observed in the eastern populations included in our analyses (Georgia and Finland).

The second big *Mastigusa* clade is represented by *M. raimondi*, *M. macrophthalma* and the dubious specimen from the Spanish Pyrenees. While *M. raimondi* has a wider distribution range, spanning north to the United Kingdom and South to Northern Algeria,

M. macrophthalma is relegated to a relatively small area between Slovenia, Croatia, and Bosnia-Herzegovina. The limited distribution range of *M. macrophthalma* could be determined by its narrower ecological niche, as can be deduced from the limited potential distribution model obtained for this species. The absence of known records of myrmecophile *M. macrophthalma* populations could depend on the fact that it occupies generally warmer areas than *M. arietina,* being subjected to warmer minimum temperatures that do not generate pressure towards the acquisition of a myrmecophile lifestyle. *Mastigusa raimondi* appears to be the more adapted to warm climates, with a known distribution reaching lower latitudes in respect to the other species. It also seems to be the more ecologically plastic, showing free-living, myrmecophile and cave-dwelling populations. The latter are only found in this species, even though both *M. arietina* and *M. macrophthalma* are known to inhabit highly carsic areas as the Western Alps and the Classic Karst of Slovenia and Croatia. Cave dwelling *M. raimondi* populations are only known in the southern parts of its distribution range, below 41.018°N, in Southern Iberian Peninsula and Northern Algeria. The interesting habit of colonizing caves could be a response to the high temperatures that can be registered in these areas. On the other hand, myrmecophile populations of *M. raimondi* are only known from the United Kingdom, representing the northernmost limit of its distribution range. A colder climate in these areas could be the factor leading to the acquisition of a myrmecophile lifestyle, similarly to what happens for *M. arietina*. Studies on the cuticular hydrocarbons of *Mastigusa* indicate that this species is likely avoiding attacks by the ants using a strategy known as “chemical insignificance” (Leonir et al. 2001; Witte et al. 2008; Lenoir et al. 2013), where the amount of cuticular hydrocarbons is reduced to a minimum, opposite to more demanding strategies like chemical mimicry, where the cuticular hydrocarbon profile of host ants is mimicked (Parmentier et al. 2017). Chemical insignificance is a more flexible strategy that allows the myrmecophile to live with different host species, as observed for *Mastigusa*, and could be the reason for the ecological plasticity observed in these spiders. The phylogenetic relationships between the three species confirm the picture obtained in **Chapter 1**, with *M. macrophthalma* being closer to *M. raimondi* than to *M. arietina*. This is rather interesting considering that *M. macrophthalma* and *M. raimondi* show an allopatric distribution and are separated by areas of presence of *M. arietina*. This

distribution pattern could suggest a phylogeographic history shaped by fragmentation, isolation, and dispersal events. The fragmentation of an ancestral population widely distributed in Europe could be at the origin of *M. macrophthalma* and *M. raimondi*. The two diverging populations could have become relegated to the Iberian Peninsula and the Dinaric area due to climatic constraints. A later colonization event from the east could explain the presence of *M. arietina* filling the distribution gaps between the other two species.

4.6. ACKNOWLEDGMENTS

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4.8. TABLES

Haplotype	Species	Country
1	*M. arietina*	Georgia
2	*M. arietina*	Finland
3	*M. arietina*	Italy
4	*M. arietina*	Italy
5	*M. arietina*	Italy
6	*M. arietina*	Italy
7	*M. arietina*	Italy, Belgium
8	*M. arietina*	Italy
9	*M. arietina*	Italy
10	*M. arietina*	Denmark
11	*M. arietina*	Denmark
12	*M. arietina*	Denmark, Italy
13	*M. macrophthalma*	Croatia
14	*M.* sp.	Spain - Pyrenees
15	*M. raimondi*	United Kingdom
16	*M. raimondi*	Spain - Sierra Nevada
17	*M. raimondi*	Spain - Sierra Nevada

Table 1. COI haplotypes retrieved with the R package “haplotypes” with species attribution and courtiers in which they were found.

State	Locality	Latitude	Logitude	Elevation (m a.s.l.)	Source
Algeria	Beni Add Aïn Fezza Cave, Aïn Fezza	34.8530°N	1.2062°W	1130	Simon 1898, 1913
Spain	Southern Spain	Unknown	Unknown	Unknown	Fage 1931
Spain	Cueva de las Ventanas, Piñar, Granada	37.4418°N	3.4283°W	1009	This work
Spain	Cueva del Hundidero – Gato, Montejaque, Malaga	36.7289°N	5.2368°W	460	This work
Spain	Cueva Janet, Llaveria, Tarragona	41.018°N	0.7400°E	670	This work
Spain	Cueva Santa, Altura, Valencia	39.8426°N	0.6142°W	842	This work
Spain	Cueva del conejo, Hermita la Rogativa, Murcia	38.1479°N	2.2282°W	1220	This work
Portugal	Gruta do Escoural, Montemor-o-Novo	38.5437°N	8.1377°W	350	This work

Table 2. Cave records for *M. raimondi* with source for the information.

4.9. FIGURES

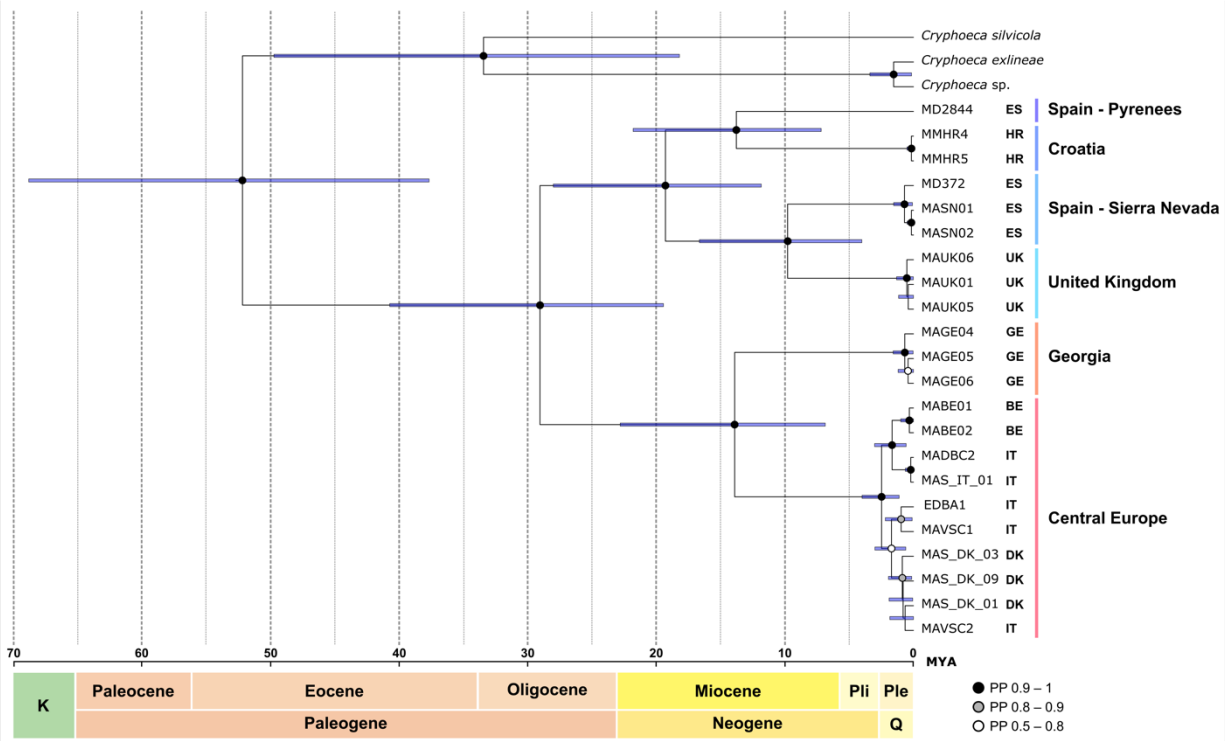

Figure 1. Detail of the BEAST time-tree tree from Chapter 1 focusing on *Mastigusa*. Scale in millions of years. Node bars represent 95% confidence intervals. Country codes after the sample names. Country codes: BE = Belgium; DK = Denmark; ES = Spain; GE = Georgia; HR = Croatia; IT = Italy; UK = United Kingdom. PP = posterior probability.

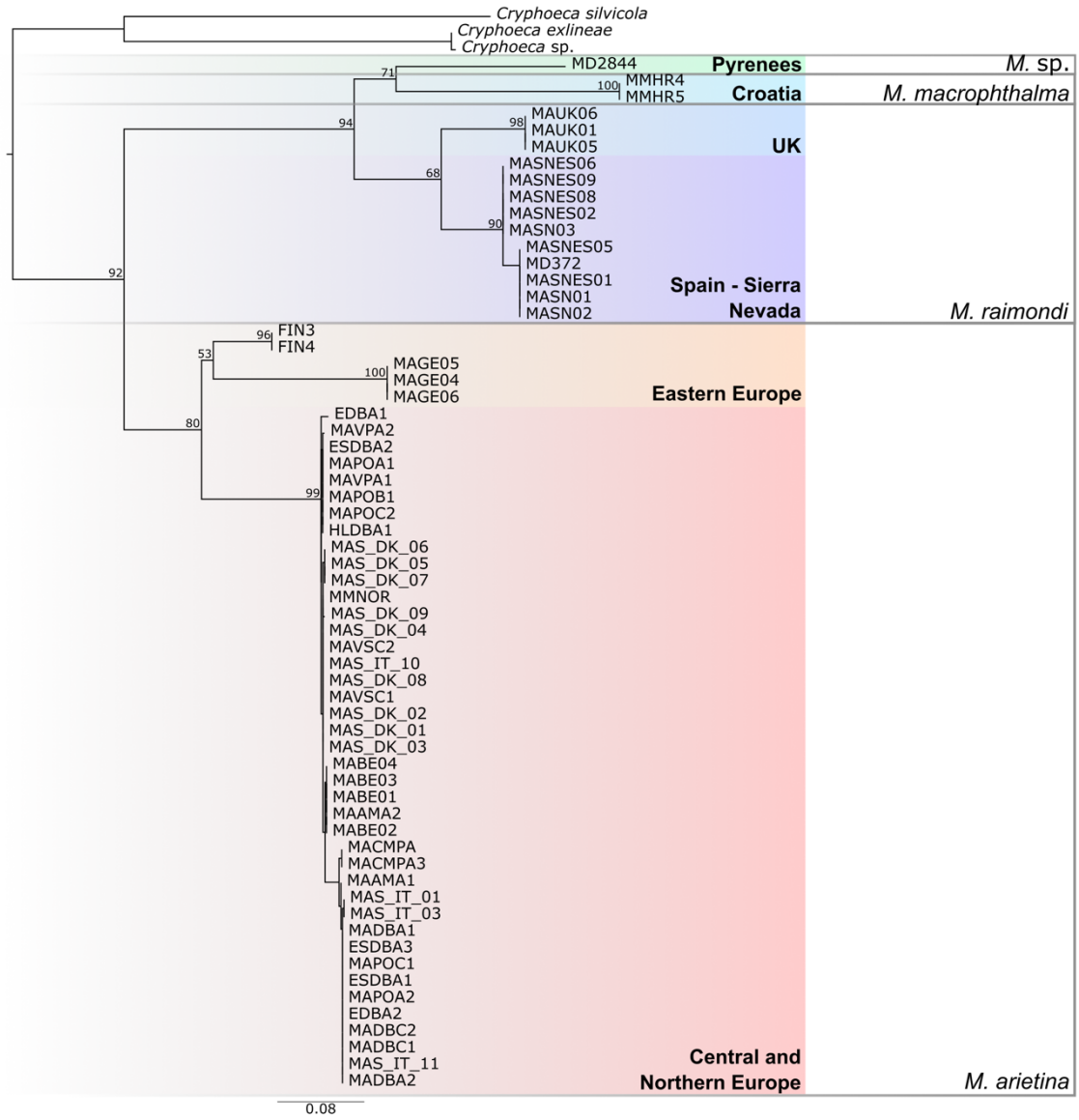

Figure 2. Maximum likelihood tree built on the COI dataset using IQ-TREE with geographical information for each clade and species attribution.

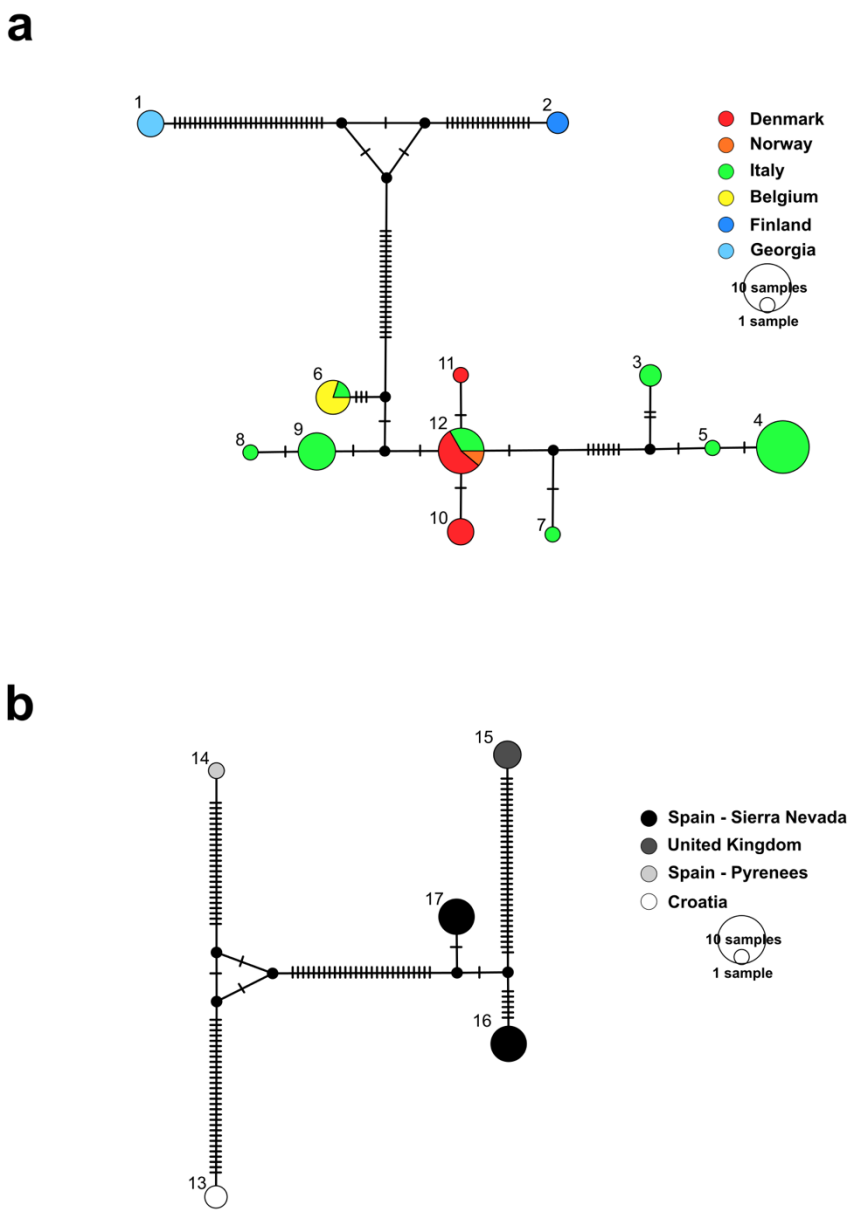

Figure 3. Haplotype networks built on the COI dataset using PopART. a: *Mastigusa arietina* network; b: *Mastigusa macrophthalma* and *M. raimondi* network.

Figure 4. Habitat of free-living *M. raimondi* populations on the Sierra Nevada massif.

Figure 5. Mound nests of *Formica rufa* species group ants where myrmecophile *M. arietina* populations were sampled.

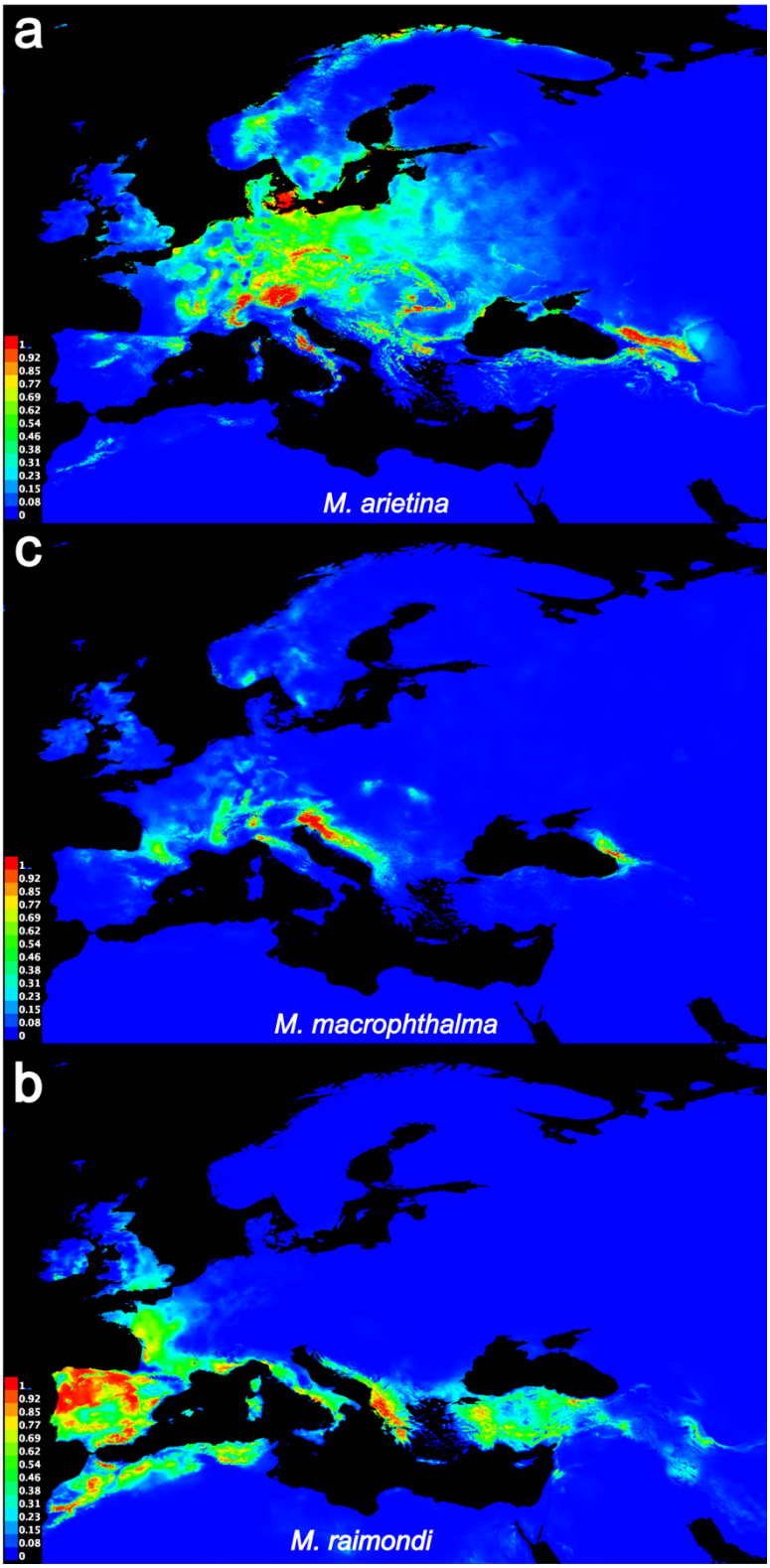

Figure 6. Ecological niche models for *M. arietina*, *M. macrophthalma* and *M. raimondi* built using MaxEnt.

4.10. SUPPLEMENTARY TABLES

Code	Nation	Locality	Habitat	Collecting date	Lat	Lon	Elevation (m a.s.l.)	Legit	Source
MABE01	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MABE02	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MABE03	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MABE04	BE	Snellegem, Brugge	In *Lasius fuliginosus* nest, decidous forest patch on sandy soil	2020	51°10.49500'	003°08.29167'	16	Parmentier T.	This study
MMHR4	HR	6km west of Sljeme, Funzine	Under stones, *Fagus sylvatica* forest with some *Picea abies*	29/06/21	45°20.79500'	014°41.59333'	915	Castellucci F.	This study
MMHR5	HR	6km west of Sljeme, Funzine	Under stones, *Fagus sylvatica* forest with some *Picea abies*	29/06/21	45°20.79500'	014°41.59333'	915	Castellucci F.	This study
MAS_DK_01	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_02	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_03	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_04	DK	Gribskov, Hillerød	In *Formica* sp. nest, broad leaf forest with some conifers	20/05/18	55°58.98300'	012°17.69300'	60	Scharff N.	This study
MAS_DK_05	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	10/11/18	56°09.01800'	9°31.39200'	53	Pedersen J.	This study
MAS_DK_06	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	11/11/18	56°09.01800'	9°31.39200'	54	Pedersen J.	This study
MAS_DK_07	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	12/11/18	56°09.01800'	9°31.39200'	55	Pedersen J.	This study
MAS_DK_08	DK	Ørnsoø, Silkeborg	In *Formica* sp. nest	13/11/18	56°09.01800'	9°31.39200'	56	Pedersen J.	This study
MAS_DK_09	DK	Tokkekøb Hegn, Lillerød	In *Formica* sp. nest, broad leaf forest with some conifers	12/04/18	55°53.24886'	012°23.16618'	60	Castellucci F.	This study
MAGE04	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.	This study
MAGE05	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.	This study
MAGE06	GE	Didgori, Tibilisi	Under rocks, montane, dry creek in oak forest	10/10/20	41°46.86240'	044°40.47540'	850	Seropian A. & Otto S.	This study
MAVSC1	IT	Chabod trail, Valsavarenche	In *Formica* sp. nest, *Larix decidua* forest with scarce *Pinus cimbra* and *Picea abies*	08/07/20	45°32.59620'	07°13.33200'	2024	Castellucci F.	This study
MAVSC2	IT	Chabod trail, Valsavarenche	In *Formica* sp. nest, *Larix decidua* forest with scarce *Pinus cimbra* and *Picea abies*	08/07/20	45°32.59620'	07°13.33200'	2024	Castellucci F.	This study
EDBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
EDBA2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
MADBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study

MADBA2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
MADBC2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.46940'	012°09.25550'	1477	Castellucci F.	This study
ESDBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
ESDBA2	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
ESDBA3	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
HLDBA1	IT	Val Pramper, Forni di Zoldo	In *Formica* sp. nest, *Picea abies* forest with some *Larix decidua*	06/08/20	46°18.79932'	012°09.47328'	1433	Castellucci F.	This study
MAVPA1	IT	Around Roner Alm, Luson	In *Formica aquilonia* nest, in *Picea abies* forest with *Pinus sylvestris*	26/06/20	46°46.83600'	11°44.48400'	1841	Castellucci F.	This study
MAVPA2	IT	Around Roner Alm, Luson	In *Formica aquilonia* nest, in *Picea abies* forest with *Pinus sylvestris*	26/06/20	46°46.83600'	11°44.48400'	1841	Castellucci F.	This study
MAPOA1	IT	Val Chedul, Selva di Val Gardena	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	24/06/20	46°33.76200'	11°46.84200'	1781	Castellucci F.	This study
MAPOB1	IT	Val Chedul, Selva di Val Gardena	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	25/06/20	46°33.78000'	11°46.81800'	1760	Castellucci F.	This study
MAPOC1	IT	Col Raiser, Santa Cristina	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	25/06/20	46°35.19600'	11°44.96400'	2058	Castellucci F.	This study
MAPOC2	IT	Col Raiser, Santa Cristina	In F*ormica aquilonia* nest in *Pinus cimbra* forest with *Picea abies* and *Larix decidua*	26/06/20	46°35.19600'	11°44.96400'	2059	Castellucci F.	This study
MAS_IT_01	IT	Casera Casavento, Claut	In *Formica* sp. nest, *Picea abies* forest	08/09/18	46°16.08600'	012°35.74800'	934	Castellucci F.	This study
MAS_IT_03	IT	Casera Casavento, Claut	In *Formica* sp. nest, *Picea abies* forest	08/09/18	46°16.08600'	012°35.74800'	934	Castellucci F.	This study
MAS_IT_10	IT	Corona, Cortaccia	In *Formica* sp. nest, *Picea abies* forest	23/09/18	46°19.76400'	11°12.51000'	1195	Castellucci F.	This study
MAS_IT_11	IT	Corona, Cortaccia	In *Formica* sp. nest, *Picea abies* forest	23/09/18	46°19.76400'	11°12.51000'	1195	Castellucci F.	This study
MAAMA1	IT	Gias delle Mosche, Valdieri	In *Formica lugubris* nest in *Picea abies* forest with scarce *Larix decidua* and *Fagus sylvatica*	12/07/20	44°10.96200'	7°16.31400'	1703	Castellucci F.	This study
MAAMA2	IT	Gias delle Mosche, Valdieri	In *Formica lugubris* nest in *Picea abies* forest with scarce *Larix decidua* and *Fagus sylvatica*	12/07/20	44°10.96200'	7°16.31400'	1703	Castellucci F.	This study
MACMPA	IT	Campigna, Santa Sofia	In *Formica paralugubris* nest in *Abies alba* forest	17/06/21	43°52.20084'	11°44.25150'	1220	Castellucci F.	This study
MACMPA3	IT	Campigna, Santa Sofia	In *Formica paralugubris* nest in *Abies alba* forest	17/06/21	43°52.20084'	11°44.25150'	1220	Castellucci F.	This study
MD2844	ES	Sola de Boi, Lleida	In pitfall trap, white oak forest	15-29/6/13	42°32.97480'	000°52.35240'	1760	Crespo L. et al.	Crespo et al. 2018
MD372	ES	Soportujar, Granada	In pitfall trap, white oak forest	31/5/13-14/6/13	36°57.69060'	003°25.12860'	1787	Crespo L. et al.	Crespo et al. 2018
MASN01	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study
MASN02	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	003°24.69420'	1811	Castellucci F.	This study

MASN03	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.	This study
MASNES01	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.	This study
MASNES02	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.	This study
MASNES05	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.	This study
MASNES06	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.	This study
MASNES08	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.	This study
MASNES09	ES	Puente Palo, Cañar	Under logs, pine forest with white oak	19/07/21	36°58.13400'	-003°24.69420'	1811	Castellucci F.	This study
FIN3	FI	Kuopio, Lippumäki	In *Formica aquilonia* nest	07/11/21	62°50.52233'	27°38.69917'	102	Sorvari J.	This study
FIN4	FI	Kuopio, Lippumäki	In *Formica lugubris* nest	29/11/21	62°50.52350'	27°38.55317'	100	Sorvari J.	This study
MMNOR	NO	Skjaak, Oppland, Norway	NA	21/06/14	61°48.36000'	7°42.24000'	750	Fjellberg A.	Bold Systems, BOLD:ACR9918
MAUK01	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	-003°42.63333'	130	Gallon R.	This study
MAUK05	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	-003°42.63333'	130	Gallon R.	This study
MAUK06	UK	Dunsford Wood, SX79268899	Under half embedded rotten oak log, in woodland	28/12/21	50°41.28333'	-003°42.63333'	130	Gallon R.	This study

Supplementary Table 1. Specimens included in the phylogenetic analysis with collecting information.

	bio1	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19	bio2	bio3	bio4	bio5	bio6	bio7	bio8	bio9	elev
bio1	**1.00**	**0.96**	**0.97**	-0.59	-0.52	-0.59	0.63	-0.54	-0.59	-0.71	-0.36	0.80	0.87	-0.50	0.93	0.94	-0.17	0.60	0.88	0.06
bio10	**0.96**	**1.00**	0.86	-0.69	-0.61	-0.69	0.62	-0.63	-0.69	-0.76	-0.48	0.86	0.74	-0.22	**0.99**	0.81	0.12	0.61	0.84	0.01
bio11	**0.97**	0.86	**1.00**	-0.47	-0.42	-0.47	0.60	-0.43	-0.47	-0.63	-0.24	0.69	**0.92**	-0.69	0.81	**0.99**	-0.40	0.53	0.86	0.10
bio12	-0.59	-0.69	-0.47	**1.00**	0.95	**0.91**	-0.48	**0.97**	**0.93**	0.85	0.89	-0.72	-0.46	-0.08	-0.70	-0.38	-0.36	-0.41	-0.52	-0.02
bio13	-0.52	-0.61	-0.42	**0.95**	**1.00**	0.78	-0.28	**0.99**	0.80	0.77	0.88	-0.64	-0.40	-0.07	-0.62	-0.34	-0.32	-0.38	-0.45	0.05
bio14	-0.59	-0.69	-0.47	**0.91**	0.78	**1.00**	-0.62	0.80	**1.00**	0.89	0.71	-0.73	-0.46	-0.07	-0.71	-0.39	-0.36	-0.32	-0.58	-0.13
bio15	0.63	0.62	0.60	-0.48	-0.28	-0.62	**1.00**	-0.32	-0.62	-0.57	-0.26	0.61	0.59	-0.26	0.62	0.55	0.00	0.33	0.61	0.24
bio16	-0.54	-0.63	-0.43	**0.97**	**0.99**	0.80	-0.32	**1.00**	0.82	0.78	**0.90**	-0.66	-0.42	-0.07	-0.64	-0.35	-0.32	-0.40	-0.46	0.03
bio17	-0.59	-0.69	-0.47	**0.93**	0.80	**1.00**	-0.62	0.82	**1.00**	0.89	0.73	-0.74	-0.46	-0.08	-0.71	-0.39	-0.37	-0.34	-0.58	-0.12
bio18	-0.71	-0.76	-0.63	0.85	0.77	0.89	-0.57	0.78	0.89	**1.00**	0.53	-0.76	-0.61	0.13	-0.77	-0.57	-0.17	-0.21	-0.78	-0.20
bio19	-0.36	-0.48	-0.24	0.89	0.88	0.71	-0.26	**0.90**	0.73	0.53	**1.00**	-0.53	-0.23	-0.22	-0.49	-0.15	-0.42	-0.49	-0.20	0.11
bio2	0.80	0.86	0.69	-0.72	-0.64	-0.73	0.61	-0.66	-0.74	-0.76	-0.53	**1.00**	0.75	-0.10	0.89	0.60	0.30	0.46	0.72	0.27
bio3	0.87	0.74	**0.92**	-0.46	-0.40	-0.46	0.59	-0.42	-0.46	-0.61	-0.23	0.75	**1.00**	-0.70	0.72	**0.90**	-0.38	0.45	0.78	0.24
bio4	-0.50	-0.22	-0.69	-0.08	-0.07	-0.07	-0.26	-0.07	-0.08	0.13	-0.22	-0.10	-0.70	**1.00**	-0.15	-0.75	**0.91**	-0.15	-0.46	-0.17
bio5	**0.93**	**0.99**	0.81	-0.70	-0.62	-0.71	0.62	-0.64	-0.71	-0.77	-0.49	0.89	0.72	-0.15	**1.00**	0.76	0.20	0.59	0.83	0.04
bio6	**0.94**	0.81	**0.99**	-0.38	-0.34	-0.39	0.55	-0.35	-0.39	-0.57	-0.15	0.60	**0.90**	-0.75	0.76	**1.00**	-0.49	0.49	0.85	0.08
bio7	-0.17	0.12	-0.40	-0.36	-0.32	-0.36	0.00	-0.32	-0.37	-0.17	-0.42	0.30	-0.38	**0.91**	0.20	-0.49	**1.00**	0.05	-0.17	-0.05
bio8	0.60	0.61	0.53	-0.41	-0.38	-0.32	0.33	-0.40	-0.34	-0.21	-0.49	0.46	0.45	-0.15	0.59	0.49	0.05	**1.00**	0.24	-0.28
bio9	0.88	0.84	0.86	-0.52	-0.45	-0.58	0.61	-0.46	-0.58	-0.78	-0.20	0.72	0.78	-0.46	0.83	0.85	-0.17	0.24	**1.00**	0.22
elev	0.06	0.01	0.10	-0.02	0.05	-0.13	0.24	0.03	-0.12	-0.20	0.11	0.27	0.24	-0.17	0.04	0.08	-0.05	-0.28	0.22	**1.00**

Supplementary Table 2. Correlation matrix for the 20 environmental variables tested for ENM. In red values that were considered significative in identifying a strong correlation between two variables ($|r| \geqslant 0.9$).

Haplotype	Specimen	Number of specimens
1	MAGE04, MAGE05, MAGE06	3
2	FIN3, FIN4	1
3	MACMPA, MACMPA3	2
4	EDBA2, ESDBA1, ESDBA3, MADBA1, MADBA2, MADBC1, MADBC2, MAPOA2, MAPOC1, MAS_IT_01, MAS_IT_03, MAS_IT_11	12
5	MAAMA1	1
6	EDBA1	1
7	MAAMA2, MABE01, MABE02, MABE03, MABE04	5
8	MAVPA2	1
9	ESDBA2, HLDBA1, MAPOA1, MAPOB1, MAPOC2, MAVPA1	6
10	MAS_DK_05, MAS_DK_06, MAS_DK_07	3
11	MAS_DK_09	1
12	MAS_DK_01, MAS_DK_02, MAS_DK_03, MAS_DK_04, MAS_DK_08, MAS_IT_10, MAVSC1, MAVSC2	8
13	MMHR4, MMHR5	2
14	MD2844	1
15	MAUK01, MAUK05, MAUK06	3
16	MASN01, MASN02, MASNES01, MASNES05, MD372	5
17	MASN03, MASNES02, MASNES06, MASNES08, MASNES09	5

Supplementary Table 3. Attribution of each specimen to the 17 COI haplotypes retrieved.

M. arietina* vs *M. macrophthalma		
	D	I
Empirical value	0.2330	0.4916
Permuted critical value	0.3540	0.6622

M. arietina* vs *M. raimondi		
	D	I
Empirical value	0.1059	0.2866
Permuted critical value	0.5864	0.8343

M. macrophthalma* vs *M. raimondi		
	D	I
Empirical value	0.1339	0.3242
Permuted critical value	0.2708	0.5626

Supplementary Table 4. Results from the niche identity test for each couple of species.

4. New association between red wood ant species (*Formica rufa* group) and the myrmecophilic spiders *Mastigusa arietina* and *Thyreosthenius biovatus*

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4.1. ABSTRACT

Ants belonging to the *Formica rufa* species group, counting 10 representatives in Europe, are often referred to as red wood ants (RWAs). These dominant, mound building species are known to host in their nests an extremely diverse fauna of associated myrmecophilic arthropods, among which are the two W-Palaearctic spider species *Mastigusa arietina* (Thorell 1871) and *Thyreosthenius biovatus* (O. Pickard-Cambridge 1875). The actual host range of these spiders within the *Formica rufa* group is little known, due to the taxonomic uncertainties that have characterized RWAs in the past. We conducted a large-scale survey for assessing the occurrence of both spider species in association with different RWAs, with a focus on an accurate identification of the ant species. We recorded co-occurrence data for 5 European representatives of the *Formica rufa* group, and we reported for the first time on the co-occurrence of *M. arietina* with *Formica aquilonia* Yarrow 1955, *F. lugubris* Zetterstedt 1838 and *F. paralugubris* Seifert 1996, and of *T. biovatus* with *F. aquilonia*. We found no association between the rate of presence/absence of the two spiders and host ant species or sampling localities, which suggests a non-selective exploitation of RWA hosts by the two myrmecophilic spiders.

Keywords: myrmecophily, RWA, host preference, ant association, Alps.

4.2. INTRODUCTION

Ant nests represent a potentially advantageous microhabitat for other arthropods as they provide less microclimatic fluctuations if compared to the outside environment and abundant food from different sources. They also represent a well-protected environment for avoiding predator and parasitoids, with the tradeoff of having to co-exist with a great number of aggressive and territorial worker ants (Cushing 1997; Parmentier 2020). Arthropods showing some degree of association with ants can be divided into two groups: myrmecomorphs and myrmecophiles (Donisthorpe 1927; Cushing 1997). Myrmecomorphs may, in rare instances, mimic ants' morphology and/or behavior as a form of Peckhamian (aggressive) mimicry, which involves a model being predated by the mimic (McIver & Stonedhal 1993; Cushing 1997). More often, they are believed to do so as a form of Batesian mimicry, playing the role of (relatively) harmless mimics imitating a harmful species to avoid attacks from visually hunting predators or parasitoids (McIver & Stonedahl 1993; Cushing 1997; Nelson & Jackson 2006; Huang et al. 2011; Nelson & Jackson 2012). Ants are in fact often avoided by generalist predators due to their aggressiveness and frequent unpalatability, distastefulness or noxiousness to vertebrates and other invertebrates (Edmunds 1978; Hölldobler & Wilson 1990; Taniguchi et al. 2005; Nelson & Jackson 2006). Myrmecophilic arthropods live in close association with ants at varying degrees, from foraging alongside them in the periphery of the colonies up to spending their whole life cycle inside the nest (Wasmann 1894; Donisthorpe 1927; Hölldobler & Wilson 1990). Myrmecophiles manage to avoid ants' attacks by using different strategies, ranging from defensive anatomical modifications and behavioral responses to chemical adaptations with modifications of their cuticular hydrocarbon (CHC) profile (Hölldobler and Wilson 1990; Lenoir et al. 2001; Akino 2002; von Beeren et al. 2011; Parker 2016). Myrmecophily in spiders was reviewed by Cushing (1997; 2012) who reports the phenomenon in 13 out of the 129 known spider families (WSC 2022), and only in a minority of cases myrmecophilic spiders also happen to be myrmecomorphic (Cushing, 2012).

Red wood ants (*Formica rufa* group; henceforth referred to as RWAs) form a species group belonging to the genus *Formica* Linnaeus 1758 distributed across the Holarctic. The fourteen species found in the Palaearctic form a monophyletic clade (Trager 2016; Borowiec et al. 2021) and can, in some cases, hybridize, making the taxonomy of the group particularly challenging (Bernasconi et al. 2011; Seifert 2021). Ten species

occur in Europe, namely *F. aquilonia* Yarrow 1955, *F. dusmeti* Emery 1909, *F. frontalis* Santschi 1919, *F. helvetica* Seifert 2021, *F. lugubris* Zetterstedt 1838, *F. paralugubris* Seifert 1996, *F. polyctena* Foerster 1850, *F. pratensis* Retzius 1783, *F. rufa* L. 1761 and *F. truncorum* F. 1804. These ants are ecologically dominant, mound-building species that constitute a key element for ecosystem functioning in temperate and boreal forests in which they live (Figure 1) (Gosswald, 1989; Frouz et al. 2005, 2016; Stockan et al. 2016). Their mound nests support an impressive diversity of obligate and facultative guests, with 125 arthropod species reported as obligate myrmecophiles living inside or in the proximity of RWA mounds (Parmentier et al. 2014). Parmentier et al. (2017) classify RWA-associated myrmecophiles on the degree of their host specificity, with values ranging from 0 to 4, with 4 being strict specialists (only recorded with RWAs), 3 being specialists (sometimes recorded with non-RWAs), 2 being characterized by moderate specificity (recorded with RWAs but distribution in non-RWAs probably equally important) and 1 being generalists (species with a broad host spectrum). Spider species that are known to occur inside Palaearctic RWA mounds are *Mastigusa arietina* (Thorell 1871) from the family Hahniidae, *Acartauchenius scurrili*s (O. Pickard-Cambridge 1873) and *Thyreosthenius biovatus* (O. Pickard-Cambridge 1875) from the family Linyphiidae and *Phrurolithus festivus* (C. L. Koch 1835) from the family Phrurolithidae (Cushing 1997; Parmentier et al. 2014). The main ant host of *A. scurrilis* is a member of the *Tetramorium caespitum* complex, but in some instances this spider was also found in association with *Formica rufa* and *Lasius flavus* (F. 1782) (Donisthorpe 1908, 1927). *Phrurolithus festivus* is common both inside and outside of ant nests and is reported to occasionally prey on ants. This species was found in association with *F. rufa, F. sanguinea* Latreille, 1798, and different species belonging to the genus *Lasius* F. 1804 (Donisthorpe 1927; Bristowe 1941; Boevé 1992).

Mastigusa arietina is one of the three currently recognized species belonging to the genus *Mastigusa* Menge 1854 (Figure 2). It is found in Europe, Algeria, Russia, and Iran (WSC 2022). In Europe, it is recoded everywhere except for Ireland, the Balkans, Moldova, Belarus, Lithuania, and Latvia (Nentwig et al. 2022). Given the small number of scattered records registered for all the countries where this species is present, it is often considered rare all over its known range, yet targeted efforts to monitor its abundance and distribution are lacking. This species was described from a specimen collected in a *Formica rufa* mound in Sweden (Westring, 1861) and was later mostly

collected in association with ants, yet with some sporadic records of reproductive populations outside of ant nests, specifically under rocks and bark or in caves (Simon 1898; Donisthorpe 1908; Jackson 1913; Simon 1913; Fage 1931). Specimens have been observed mostly in ant nests of *F. rufa*, but also *F. polyctena,* the non-RWA *F. fusca* L. 1758*, Lasius fuliginosus* (Latreille 1798), *L. alienus* (Foerster 1850) and *L. brunneus* (Latreille 1798), *Messor muticus* (Nylander 1849) and *Tetramorium caespitum* L. 1758 (Westring 1861; Pickard-Cambridge 1900; Donisthorpe 1908; Klausen 1974, Palmgren 1977, Roberts 1995; Parmentier et al. 2015; Franc & Hemala 2020; Parmentier et al. 2020). Most of the ant-association data with RWAs, starting from Westring's original description, come from a time where the distinction of the different RWA species was not clear due to the taxonomic uncertainties regarding the *Formica rufa* group, something that first started to improve thanks to Yarrow's (1955) revision of the RWAs of the British Isles. However, the taxonomy of the RWAs in Europe has long remained unresolved, so that the actual host species is unknown for the majority of *M. arietina* records. Being recorded in association with RWAs, non-RWA *Formica* species and other ant genera, *M. arietina* could be considered as a 2 (moderate specificity) according to Parmentier's classification on host specificity. Parmentier et al. (2017) registered a low amount of cuticular hydrocarbons on the cuticle of these spiders, a trait that could help myrmecophilic arthropods in avoiding chemical detection by host ants by facilitating the integration into their colony with a strategy known as "chemical insignificance" (Leonir et al. 2001; Witte et al. 2008; Lenoir et al. 2013). In laboratory trials, *M. arietina* specimens were found to provoke strong aggressive responses in *Formica* ants, suggesting a somewhat low level of integration inside the colonies (Parmentier et al. 2016; Parmentier et al. 2018).

Thyreosthenius biovatus is one of the two species belonging to the genus *Thyreosthenius* Simon 1884 (Figure 2). It is a widespread species in Europe and Russia (WSC 2022). In Europe, the only areas where it has not been found yet are the Iberian Peninsula, southeast Europe (except for Bulgaria), Belarus and Lithuania (Nentwig et al. 2022). Similar to *M. arietina*, the few specimens recorded from its distribution range lead it to be considered a rare species. It has been recorded from ant nests of the RWA species *F. lugubris, F. polyctena*, *F. pratensis* and *F. rufa*, and the non-RWA *Formica* species *F. fusca* and *F. sanguinea* (Bösenberg 1899; Simon 1926; Bristowe 1939; Wiehle 1960; Palmgren 1976; Robinson 1998; Parmentier et al. 2014, 2015). Regarding its actual host range within the *Formica rufa* group, our

knowledge suffers from the same taxonomic problems mentioned for *M. arietina*. Being recorded both with RWAs and non-RWAs, but not with species from other genera, *T. biovatus* host specificity can be classified as a 3 (specialist). As in *M. arietina*, Parmentier et al. (2017) registered a low amount of CHCs in *T. biovatus*, hypothesizing again chemical insignificance as an integration strategy. In laboratory trials, this species showed lower levels of aggression if compared to *M. arietina*, suggesting a higher level of integration in RWA colonies (Parmentier et al. 2016; Parmentier et al. 2018). Records of adults outside ant nests are known but may be related to dispersal activity or temporary foraging away from the mounds rather than to true free-living habits, since reproduction outside of ant nests has never been observed (Bristowe 1939; Parmentier et al. 2021). This species tough shows a great mobility in the proximity of the nests, as observed by Parmentier et al (2021) who collected great number of specimens in pitfall traps placed up to 25 meters away from the mounds.

In this study, a large-scale field survey was carried out within the Alpine regions of Northern Italy to assess the occurrence of *M. arietina* and *T. biovatus* inside nests of different RWA species, with the aim of better understanding their ecology and host preferences within the *Formica rufa* group.

4.3. MATERIALS AND METHODS

4.3.1 Study area

Fieldwork was conducted between 2018 and 2020 in 10 localities in the Eastern and Western Italian Alps, based on previous records of the two spider species or given the known presence of RWAs in the area. A complete list of the sampling localities with elevation and habitat type is given in Table 1. A map showing the investigated localities is given in Figure 3**.**

4.3.2. Sample collection

The search for myrmecophilic spiders inside RWA ant hills was carried out by digging carefully by hand inside the above ground mound and collecting a couple of liters of mound material. This was then sifted on a white fabric sheet for better spotting the spiders, using an entomological sieve with an 8 x 8mm mesh. After sifting, nest material, ants and brood were carefully returned to the nest to minimize disturbance.

The presence of spiders in the nests was assessed by the collection of adult and juvenile specimens, yet for *M. arietina* also by the finding of its characteristic flat and discoidal egg-sacks, often laid on the surface of hard debris such as small logs, pinecones, or pieces of bark found inside the nest (Donisthorpe 1927). Specimens were collected by the means of entomological forceps or pooters and stored in 70% or 96% ethanol, in order to allow for an ideal preservation for both morphological examination and molecular analyses. Ant worker specimens were collected with entomological forceps from the top of the nests and stored in 70% and 96% ethanol for the same reasons as above. For each of the investigated mounds, coordinates, elevation, and habitat type were recorded. Given the location of most of the sampling sites inside protected areas, permits were obtained for the collection of both spider and ant specimens.

4.3.3. Morphological identification of spiders and ants

Spiders were examined and measured using a stereoscopic microscope equipped with a Leica DFC450C camera and Leica Application Suite v3.6 software and photographed using a BK+ Imaging System from Visionary Digital equipped with a Canon EOS 7D camera. Identification was carried out with the keys of Roberts (1987) for *T. biovatus* and the redescriptions and keys provided by Wunderlich (1986) for *M. arietina*.

Ants were examined with a stereoscopic microscope. Measurements were taken on photos by using the software ImageJ (Schneider et al. 2012) and pictures obtained with a Canon MP-E 65mm f/2.8 1–5x macro lens mounted on a Canon 1300D camera. Identification was carried out with the key provided by Seifert (2021). *Formica helvetica*, recently described based on only molecular data, was not taken into consideration due to the impossibility of identifying it morphologically and its geographic range being outside our study area.

4.4. RESULTS

A total of 26 RWA mounds were investigated at the 10 sampling sites, details and coordinates for each of the nests are reported in Supplementary Table S1. These belonged to 5 out of the 7 RWA species known to occur in the study area, namely *F. aquilonia*, *F. lugubris, F. paralugubris, F. polyctena* and *F. rufa*; no nests of *F. pratensis*

and *F. truncorum* were found in the sampling sites. Only one RWA species was observed in each sampling site, except site 3, in which *F. polyctena* and *F. rufa* were found in sympatry. The majority of investigated nests (62%) belonged to *F. aquilonia*, the other RWA species being present in a lower percentage of the nests: *F. paralugubris* = 15%, *F. polyctena* = 11%, *F. rufa* = 8%, *F. lugubris* = 4%. There was a clear geographic distinction regarding the RWA species found, with *F. aquilonia, F. polyctena* and *F. rufa* observed in the Eastern Alps sites and *F. lugubris* and *F. paralugubris* in the Western Alps sites. The presence of each RWA species in the 10 sampling sites is summarized in Table 1. The spider *Mastigusa arietina* was present in 81% of the inspected mounds and at all sampling sites (Figure 4). It was found in association with all the 5 RWA species investigated, although with different occurrence frequencies (Figure 5). It was recorded between 934 and 2058m a.s.l. in coniferous and mixed forests dominated by European spruce, larch, or pine. Adult males were collected from June to July and in September, while adult females from July to September.

The occurrence of *T. biovatus* was lower, as it was recorded in only 31% of the inspected nests and only in the Eastern Alps sites (Figure 4). This species was only recorded with 3 out of the 5 RWA species investigated, namely *F. aquilonia*, *F. polyctena* and *F. rufa* (Figure 5). It was recorded in the same habitats as *M. arietina,* yet from a narrower altitudinal range (934-1837m). Adult males were collected in June, and August to September, adult females in June and September. New distributional data generated for the two spider species in Italy can be found in Supplementary Table S2.

4.5. DISCUSSION AND CONCLUSIONS

In the present work we report about updated co-occurrence data of RWA species and myrmecophilic spiders *M. arietina* and *T. biovatus*, expanding knowledge about their ecology, ant-association, and distribution in the Italian range.

4.5.1. Host range and ecology

The geographical distribution of the different RWA species sampled was not random and reflected the actual distribution of the species over the Alpine arch area. *Formica aquilonia* is known to be present in Eastern and Central Alps, with its westernmost

limit being E°9 (Stockan et al. 2016). Considering that both sites investigated in the Western Alps are located westward that distribution limit (E°7), the observed absence of this species in the area was expected. On the contrary, *F. paralugubris* is known to be present in Western and Central Alps, with its easternmost limit being E11° (Stockan et al. 2016). Being all sites investigated in Eastern Alps located eastward E°11.2, again, the observed absence of this species in the area was expected. The other three species are more widely distributed over the Alpine arch, and their presence or absence may be related to ecological factors or local distribution patterns (Ronchetti 1963, 1965, 1966; Seifert 2021). The presence of both *F. polyctena* and *F. rufa* in site 3 agrees with known co-occurrence of the two species in Central Europe where they may form hybrid zones (Seifert 1991; Czechowski 1996; Gyllestrand et al. 2004; Bernasconi et al. 2011).

During the surveys, *M. arietina* was found in association with all the RWA species observed in the study area. This represents the first direct observation of the co-occurrence of *M. arietina* with *F. aquilonia, F. lugubris* and *F. paralugubris*. On the other hand, *T. biovatus* was found in association with only three RWA species, namely *F. rufa, F. polyctena* and *F. aquilonia*, without being observed with *F. paralugubris* and *F. lugubris.* As for the absence of observations of *T. biovatus* during our surveys in the Western Alps, this could likely be due to the smaller number of sampling localities and ant nests that were inspected as compared to those investigated in the Eastern Alps, especially when considering the known presence of this species In both Switzerland and France (Nentwig et al. 2022) or its ability to live in *F. lugubris* nests (see a record from the United Kingdom, Robinson 1998). This represents the first observation of co-occurrence of this species with *F. aquilonia.* As reported before, the lack of literature records of both species in association with *F. aquilonia*, and of records of *M. arietina* in association with *F. lugubris* and *F. paralugubris* is probably due to the taxonomic issues affecting the different RWA species in the past (Seifert 2021), leading to RWAs being identified with a generic *“Formica rufa”*. These, together with the limited knowledge about the historical and present distribution of the different RWA species in most of the countries, makes it difficult to reconcile most of the historical records to actual RWA species.

This work represents the first attempt to search for these spiders in nests of as many different RWAs as possible and with a strong focus on an accurate species-level identification of the ants, according to the modern taxonomy of the group, to get

detailed information about the host preferences of these spiders. The wide range of host species that were found in association with *M. arietina* confirms the fact that this spider shows a low host specificity, having been also recorded with ants belonging to other genera in the subfamily Formicinae, such as *Lasius,* and even species from the subfamily Myrmicinae. The new records of co-occurrence with three RWA species expand our knowledge about the ecology of this spider and its myrmecophilic habits. The observations in site 3, where spiders were collected in sympatric nests of *F. rufa* and *F. polyctena* few hundred meters apart one from the other, suggests that *M. arietina* opportunistically occupies RWA mounds based on their local availability rather than exhibiting a preference for certain species. Something similar can be observed for *T. biovatus.* The new data concerning its co-occurrence with *F. aquilonia* add new information about the ecology and host preference of this species and points out to a non-selective exploitation of the different RWA species like in *M. arietina*. The wide host range of the two spiders is also compatible with the finding of reduced levels of CHCs on their cuticle (Parmentier et al. 2017). Chemical insignificance would indeed allow both species to quickly adapt and move from one host to the other. In contrast to more complex strategies involving the imitation of the host's CHC profile, which require a high degree of specialization, chemical insignificance may facilitate dispersal and colonization of new areas.

4.5.2. Presumed rarity of M. arietina *and* T. biovatus

According to literature data, both species can be considered rare in Italy, given the low number of collected specimens and the limited number of sites from which they have been recorded (Pantini & Isaia 2019). Our findings show a different picture, as specimens were easily collected in all sampling sites for *M. arietina* and in all sampling sites in the Eastern Alps for *T. biovatus*. The suggested rarity of this species is probably an effect of biased sampling techniques, since the collection of myrmecophilic arthropods associated with RWAs requires access to the mound nest and subsequent extraction of specimens from the nest material, procedures not commonly performed during general biodiversity surveys, or the placement of pitfall traps in the proximity of the nests (Parmentier et al. 2021). By selectively investigating RWA nests both spider species appear to be more widespread and common in the study area than previously thought, as also reported for other European countries like Denmark (Scharff & Gudik-Sørensen 2006) and Britain (Donisthorpe 1927). Given the

almost continuous presence of RWAs over the Alps Mountain range it is likely that the distribution of the two species follows that of the hosts, something that could be tested also in other European countries where RWA occur.

4.5.3. Concluding remarks

Ant nests are known to host a wide range of myrmecophilic arthropod taxa. These are though poorly studied, with some noticeable exceptions in Coleoptera (Parker 2016) and Lycaenid butterflies (Fiedler 1991; Pierce et al. 2002; Casacci et al. 2019). Moreover, little is still known about their distribution, ecology, and the nature of their relationships with the host ants. A proper knowledge of myrmecophilic taxa is even more important as the great number of non-ant species living inside of RWA mound nests represent a component of biodiversity which is often overlooked and that, if not properly considered, can lead to an underestimation of the species richness of a given area. The new data produced here regarding the occurrence of *M. arietina* and *T. biovatus* in association with widely distributed RWA species and the ease with which the two species were collected in areas where their presence went unnoticed until now, clearly emphasize this. Our findings confirm how little research has been conducted on ant associates in Southern Europe, implying that the same patterns observed for the two spider species could easily apply to myrmecophilic species belonging to other arthropod taxa that await re-discovery.

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4.8. TABLES

Site	Locality	Elevation (m a.s.l.)	Habitat	RWA species
1	Gias delle Mosche, Valdieri (CN)	1703	European spruce forest with European larch and European beech	*F. lugubris*
2	Chabod trail, Valsavarenche (AO)	1890-2080	European larch forest with Swiss pine and European spruce	*F. paralugubris*
3	Corona, Cortaccia (BZ)	1195-1211	Eurpean spruce forest	*F. polyctena; F. rufa*
4	Above Hofer Alpl, Fié allo Sciliar (BZ)	1533	Pine forest with European spruce	*F. aquilonia*
5	Sella Pass, Canazei (TN)	2040	Pine forest with European spruce	*F. aquilonia*
6	Around Roner Alm, Luson (BZ)	1777-1819	European spruce forest with Baltic pine	*F. aquilonia*
7	Val Chedul, Selva di Val Gardena (BZ)	1760-1781	Swiss pine forest with European spruce and European Larch	*F. aquilonia*
8	Col Raiser, Santa Cristina (BZ)	2058	Swiss pine forest with European spruce and European larch	*F. aquilonia*
9	Val Pramper, Forni di Zoldo (BL)	1433-1477	European spruce and European larch forest	*F. aquilonia*
10	Casera Casavento, Claut (PN)	934-940	European spruce forest	*F. polyctena*

Table 1. List of sampling sites with localities, altitudinal range covered, habitat type and RWA species observed.

4.9. FIGURES

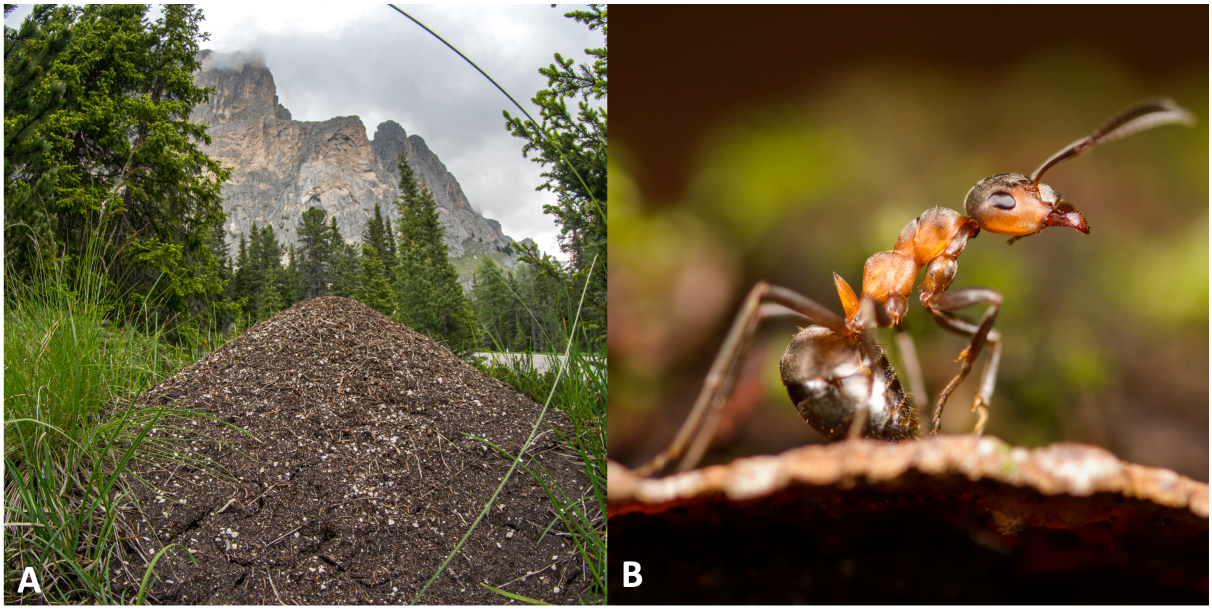

Figure 1. *Formica rufa* group. **A)** *Formica aquilonia* mound at Sella Pass (TN), Italy; **B)** *Formica polyctena* worker.

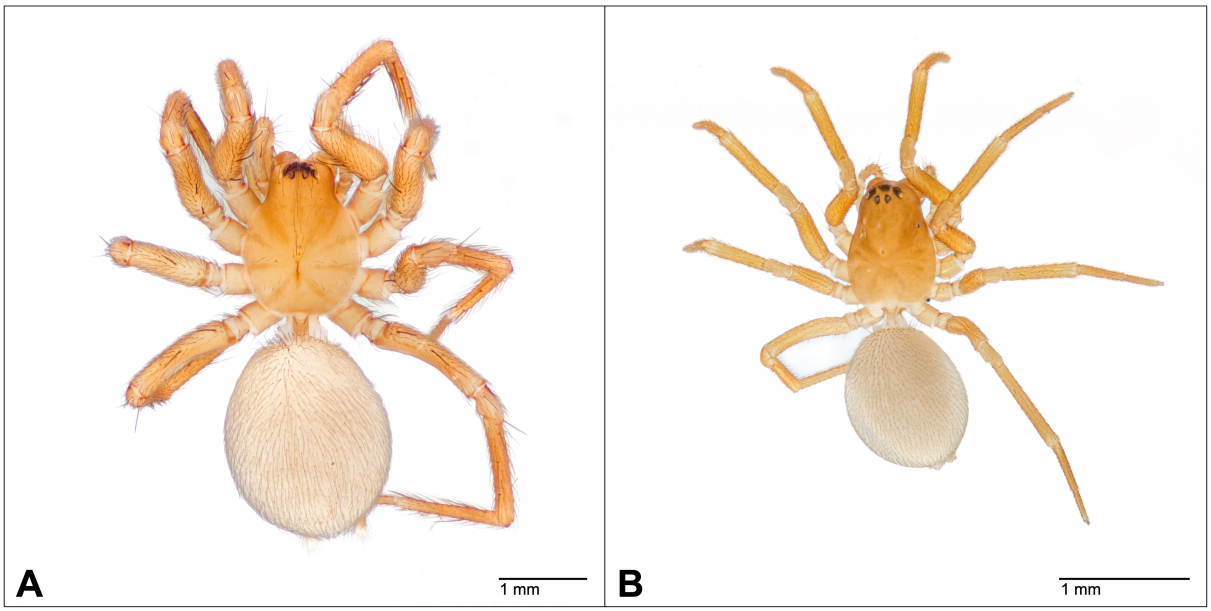

Figure 2. Myrmecophilic spiders found in mounds of RWA. **A)** *Mastigusa arietina* female, from Corona (BZ), Italy; **B)** *Thyreosthenius biovatus* female, from Corona (BZ), Italy.

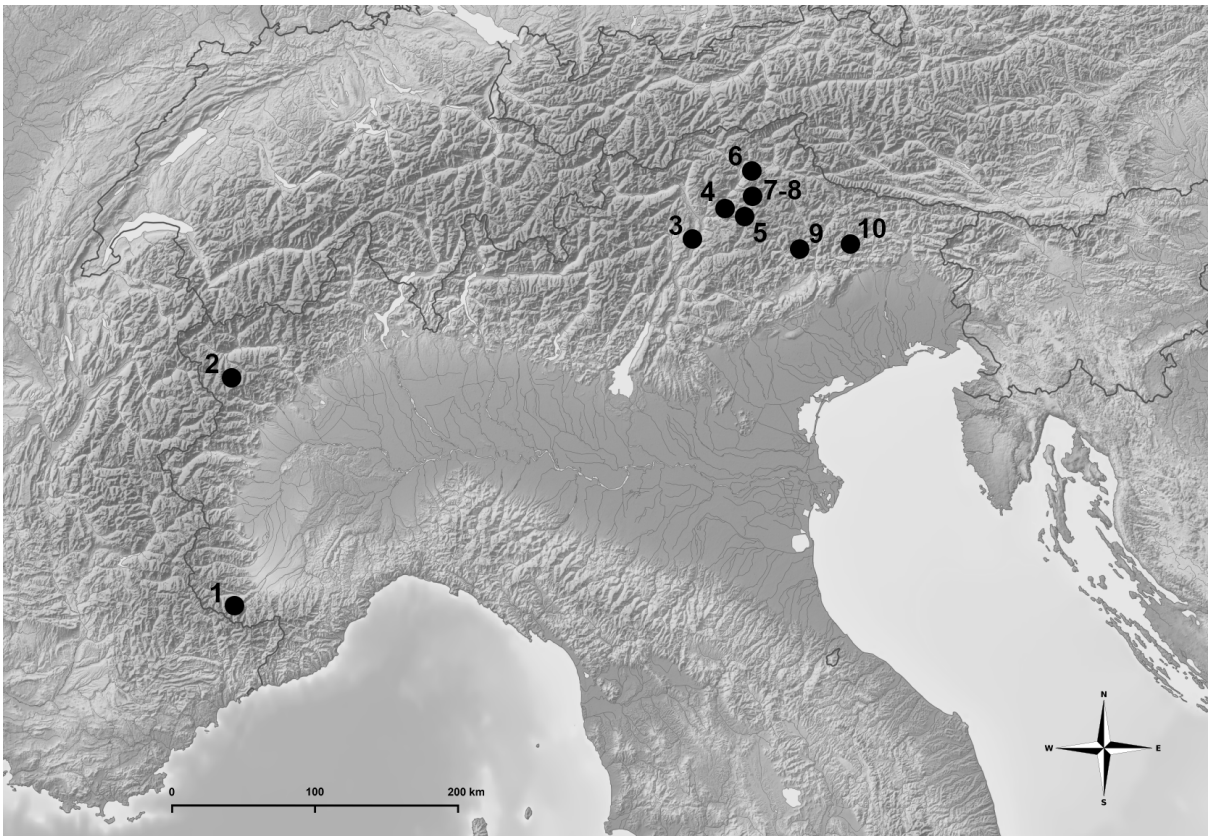

Figure 3. Map of the investigated localities in the Alps. Numbers refer to sites as reported in Table 1.

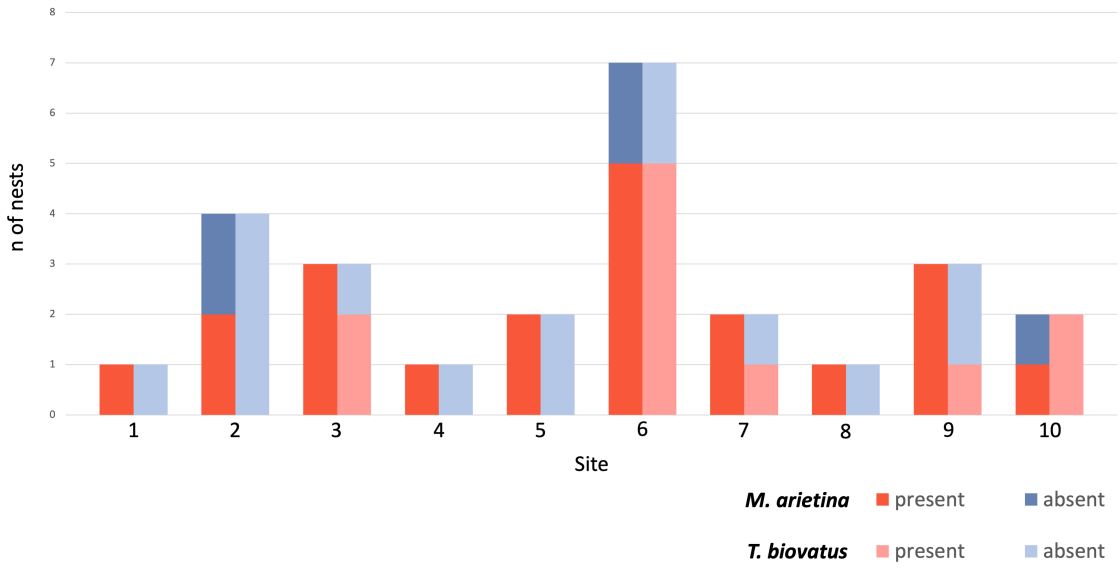

Figure 4. Number of nests with presence/absence of the two spider species for each of the 10 sampling sites. Numbers refer to sites as reported in Table 1.

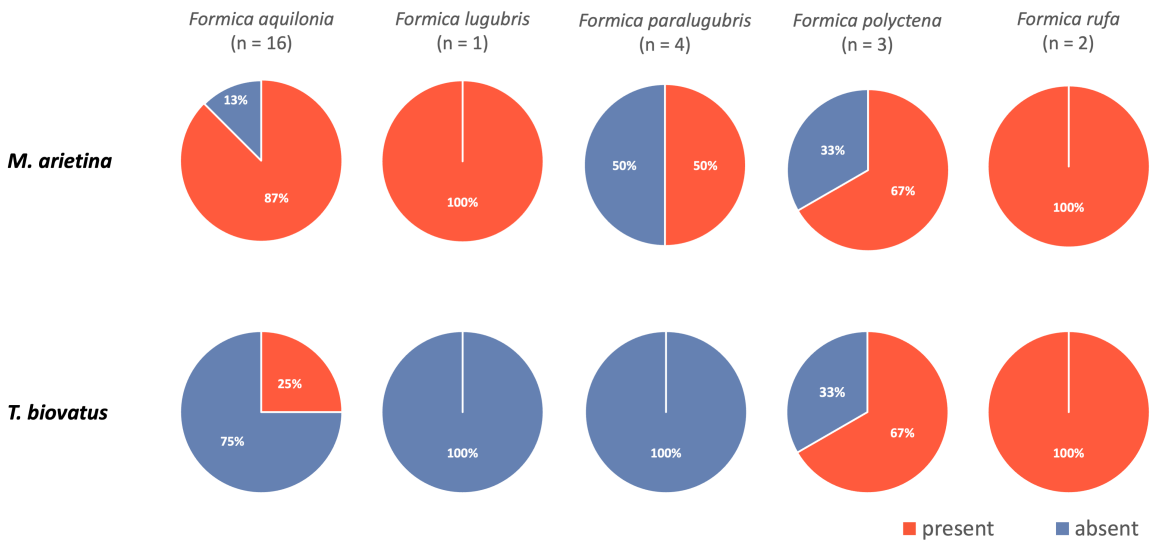

Figure 5. Ratio of presence/absence of the two spider species in the investigated mounds for each RWA species.

4.10. SUPPLEMENTARY TABLES

Nest	Site	Locality	Province	Lat.	Long.	Elevation (m a.s.l.)	Date	Ant species
AA01	3	Corona, Cortaccia	BZ	46.3294° N	011.2085° E	1195	23/09/18	*F. rufa*
AA02	3	Corona, Cortaccia	BZ	46.3329° N	011.2109° E	1211	23/09/18	*F. rufa*
AA03	3	Corona, Cortaccia	BZ	46.3327° N	011.2111° E	1209	23/09/18	*F. polyctena*
VP03	6	Around Roner Alm, Luson	BZ	46.7873° N	011.7534° E	1807	24/09/18	*F. aquilonia*
VP04	6	Around Roner Alm, Luson	BZ	46.7869° N	011.7538° E	1808	24/09/18	*F. aquilonia*
VP05	6	Around Roner Alm, Luson	BZ	46.7801° N	011.7406° E	1819	24/09/18	*F. aquilonia*
VP06	6	Around Roner Alm, Luson	BZ	46.7795° N	011.7418° E	1788	24/09/18	*F. aquilonia*
VP07	6	Around Roner Alm, Luson	BZ	46.7791° N	011.7401° E	1777	26/09/18	*F. aquilonia*
VPA	6	Around Roner Alm, Luson	BZ	46.7806° N	011.7414° E	1841	26/06/20	*F. aquilonia*
VPB	6	Around Roner Alm, Luson	BZ	46.7817° N	011.7445° E	1837	26/06/20	*F. aquilonia*
SCB	4	Above Hofer Alpl, Fié allo Sciliar	BZ	46.5053° N	011.5413° E	1533	23/06/20	*F. aquilonia*
POA	7	Val Chedul, Selva di Val Gardena	BZ	46.5627° N	011.7807° E	1781	24/06/20	*F. aquilonia*
POB	7	Val Chedul, Selva di Val Gardena	BZ	46.5630° N	011.7803° E	1760	24/06/20	*F. aquilonia*
POC	8	Col Raiser, Santa Cristina	BZ	46.5866° N	011.7494° E	2058	25/06/20	*F. aquilonia*
PSA	5	Sella Pass, Canazei	TN	46.5055° N	011.7856° E	2039	24/06/20	*F. aquilonia*
PSB	5	Sella Pass, Canazei	TN	46.5053° N	011.7854° E	2040	24/06/20	*F. aquilonia*
DF01	10	Casera Casavento, Claut	PN	46.2681° N	012.5958° E	934	08/09/18	*F. polyctena*
DF02	10	Casera Casavento, Claut	PN	46.2684° N	012.5957° E	940	08/09/18	*F. polyctena*
DBA	9	Val Pramper, Forni di Zoldo	BL	46.3133° N	012.1579° E	1433	06/08/20	*F. aquilonia*
DBB	9	Val Pramper, Forni di Zoldo	BL	46.3085° N	012.1553° E	1473	06/08/20	*F. aquilonia*
DBC	9	Val Pramper, Forni di Zoldo	BL	46.3078° N	012.1543° E	1477	06/08/20	*F. aquilonia*

VSA	2	Chabod trail, Valsavarenche	AO	45.5420° N	007.2162° E	1890	08/07/20	*F. paralugubris*
VSB	2	Chabod trail, Valsavarenche	AO	45.5432° N	007.2211° E	2080	08/07/20	*F. paralugubris*
VSC	2	Chabod trail, Valsavarenche	AO	45.5433° N	007.2222° E	2024	08/07/20	*F. paralugubris*
VSD	2	Chabod trail, Valsavarenche	AO	45.5427° N	007.2172° E	2014	10/07/20	*F. paralugubris*
AMA	1	Gias delle Mosche, Valdieri	CN	44.1827° N	007.2719° E	1703	12/07/20	*F. lugubris*

Supplementary Table 1. Locality, coordinates, elevation and date of inspection of the 26 RWA mound nests investigated.

Mastigusa arietina									
Site	**Region**	**Province**	**Locality**	**Elevation (m a.s.l.)**	**Habitat**	**Sex**	**Date**	**Legit**	**Notes**
1	Piemonte	CN	Gias delle Mosche, Valdieri	1703	*F. lugubris* nest in European spruce forest with scarce European larch and European beech	1M, 1F	12/07/20	Castellucci F.	
2	Valle d'Aosta	AO	Chabod trail, Valsavarenche	2014	*F. paralugubris* nest in European larch forest with scarce Swiss pine and European spruce	2F	10/07/20	Castellucci F.	**First record for Valle d'Aosta**
3	Trentino-Alto Adige	BZ	Corona, Cortaccia	1195 -1209	*F. rufa* and *F. polyctena* nests in Eurpean spruce forest	1M, 4F	23/09/18	Castellucci F.	
8	Trentino-Alto Adige	BZ	Col Raiser, Santa Cristina	2058	*F. aquilonia* nest in Swiss pine forest with European spruce and European larch	1M	25/06/20	Castellucci F.	
6	Trentino-Alto Adige	BZ	Around Roner Alm, Luson	1841	*F. aquilonia* nest in European spruce forest with Baltic pine	1M	26/06/20	Castellucci F.	
9	Veneto	BL	Val Pramper, Forni di Zoldo	1433 -1477	*F. aquilonia* nests in European spruce and European larch forest	2F	06/08/20	Castellucci F.	**First record for Veneto**
10	Friuli Venezia-Giulia	PN	Casera Casavento, Claut	934	*F. polyctena* nest in European spruce forest	2M, 1F	08/09/28	Castellucci F.	**First record for Friuli Venezia-Giulia**
Thyreosthenius biovatus									
Site	**Region**	**Province**	**Locality**	**Altitude (m a.s.l.)**	**Habitat**	**Sex**	**Date**	**Legit**	**Notes**
3	Trentino-Alto Adige	BZ	Corona, Cortaccia	1195 -1209	*F. rufa* and *F. polyctena* nests in Eurpean spruce forest	2M, 3F	23/09/18	Castellucci F.	
7	Trentino-Alto Adige	BZ	Val Chedul, Selva	1780	*F. aquilonia* nest in Swiss pine forest with European spruce and European larch	1M, 3F	24/06/20	Castellucci F.	
6	Trentino-Alto Adige	BZ	Around Roner Alm, Luson	1808	*F. aquilonia* nest in European spruce forest with Baltic pine	1M, 2F	24/09/18	Castellucci F.	
9	Veneto	BL	Val Pramper, Forni di Zoldo	1433	*F. aquilonia* nest in European spruce and European larch forest	1M	06/08/20	Castellucci F.	**First record for Veneto**
10	Friuli Venezia-Giulia	PN	Casera Casavento, Claut	934 -940	*F. polyctena* nests in European spruce forest	3F	08/09/28	Castellucci F.	**First record for Friuli Venezia-Giulia**

Supplementary Table 2. New distribution data for *Mastigusa arietina* and *Thyreosthenius biovatus* in Italy. Only localities for which adult specimens were collected are reported.

Conclusions

The present thesis sheds light on the taxonomy, systematics, and ecology of the spider genus *Mastigusa*.

The phylogenetic placement of the genus *Mastigusa*, previously classified in the family Hahniidae, was revised through the analysis of mitochondrial and nuclear markers, which resulted in a well-supported inclusion in the family Cybaeidae, in sister group relationship with the genus *Cryphoeca*. In the age of phylogenomics, the use of molecular markers based on Sanger sequencing is still a valuable tool for the revision of the phylogenetic placement of a genus. Sanger based markers available for spiders, indeed, while not particularly informative for deep phylogenetic relationships, have been demonstrated to perform well at the intrafamilial level. Moreover, the availability of such Sanger based markers for a considerable number of genera and species allows a precise placement and investigation of phylogenetic relationships at the family level and below. Phylogenomic and transcriptomic datasets available, on the other hand, are still limited in term of taxon sampling, with few representatives for each family, making it more difficult to get detailed information on a shallow phylogenetic level, without the extensive generation of new genomic data.

The molecular analyses leading to the phylogenetic placement of the genus also resulted in a phylogeny of *Mastigusa* populations across its distribution range. Through combining molecular phylogenetics and DNA barcoding species delimitation with a classical morphological approach, the delimitation of the extant species belonging to the genus *Mastigusa* was revised and a new species, *M. raimondi* sp. n., was described. This demonstrates how the use of an integrative approach of morphological and molecular techniques is key in answering complex taxonomical questions, especially when dealing with taxa of difficult delimitation, like *Mastigusa* species, and allows to identify and describe previously unobserved diversity.

The taxonomic revision of *Mastigusa* species was an essential step for further analyzing their distribution, phylogeography, and ecology. Being the genus represented by free-living, myrmecophile and cave-dwelling populations, the problematic delimitation of its species and the resulting uncertainty about their distribution hindered the identification of any taxonomical, geographical, or climatic patterns in the distribution of the abovementioned life traits. Molecular techniques as phylogenetics and mitochondrial

haplotype network analysis were used to describe how the genetic diversity is distributed among distinct geographic populations within the main *Mastigusa* species. As a result, diverging geographical lineages within *M. arietina* and *M. raimondi* were identified.

The ecological analyses based on data collected in the field, from literature and from museum specimens allowed to reconstruct ecological niche models for *M. arietina*, *M. macrophthalma* and *M. raimondi*. This resulted in the observation of little overlap between the models of the three species, indicating ecological specialization, as confirmed by their parapatric distribution and presence in areas characterized by different climatic conditions. This study also highlighted how some species appear to be more adaptable in respect to the microhabitat they can occupy. The acquisition of free-living, myrmecophile or cave-dwelling lifestyles appears to be linked to climate, even within the same species. *Mastigusa arietina* and *M. raimondi* populations acquire a myrmecophile lifestyle in countries of Central and Northern Europe, probably as a response to cold temperatures. *Mastigusa raimondi* populations living in the southern limits of its distribution range, on the other hand, can colonize caves, as observed in Southern Iberian Peninsula and North Africa, probably to avoid excessive warmth.

Finally, field research conducted in the Italian Alps led to new information regarding the ecology and distribution of myrmecophile *Mastigusa* populations and those of *Thyreosthenius biovatus* (Linyphiidae), another myrmecophile spider that occur in the mound nests of red wood ants belonging to the *Formica rufa* species group. As a result, *M. arietina* populations were found inhabiting ant nests of five different *Formica* species, confirming the ability of this species to exploit different hosts, selecting them due to their local availability and abundance. Eventually, these surveys showed how the two myrmecophile spider species are way more common and widely distributed than previously thought and are often overlooked, probably due to their cryptic lifestyle. Ant nests represent a microcosm that is often little considered during general biodiversity surveys, hosting a significant component of biodiversity whose consideration could lead to interesting faunistic, taxonomic and ecological discoveries.

Overall, this study increased our understanding of the diversity and ecology of a little-known spider genus, highlighting its potential as a model for studying adaptation,

ecological plasticity and the development of extreme life traits as myrmecophily and troglophily.

Side research activities

During the three years of my PhD project, I collaborated with different research groups to side research activities not directly linked to the specific focus of this thesis. These included faunistic studies focused on improving our knowledge on the diversity and distribution of Italian arachnids, studies on the diversity and evolution of ants and a mitophylogenomic investigation of the relationships among the major lineages of branchiopod crustaceans. Below are titles, bibliographic references, and abstracts of the published outputs of such research activities.

First record of Amblypygi from Italy: *Charinus ioanniticus* (Charinidae)

Bibliographic reference: Colla A., Legittimo C.M., Castellucci F., Simeon E., De Miranda, G.S. 2020. First record of Amblypygi from Italy: *Charinus ioanniticus* (Charinidae). *Arachnology* 18, 642–648.

Abstract

The arachnid order Amblypygi is recorded for the first time in Italy, with the species *Charinus ioanniticus* (Kritscher, 1959). An isolated reproductive population was found in an underground air-raid shelter dating back to World War II below the city center of Trieste. This represents the second record of this parthenogenetic species in continental Europe and also its westernmost known population.

Keywords: distribution; expansion; Kleine Berlin; parthenogenesis; Trieste; whip spiders

Is mimicry a diversification-driver in ants? Biogeography, ecology, ethology, genetics and morphology define a second West-Palaearctic *Colobopsis* species (Hymenoptera: Formicidae)

Bibliographic reference: Schifani E., Giannetti D., Csősz S., Castellucci F., Luchetti A., Castracani C., Spotti F.A., Mori A., Grasso D.A. 2021. Is mimicry a diversification-driver in ants? Biogeography, ecology, ethology, genetics and morphology define a second Palaearctic *Colobopsis* species (Hymenoptera: Formicidae). *Zoological Journal of the Linnean Society* 194, 1424–1450.

Abstract

The West-Palaearctic *Colobopsis* ant populations have long been considered a single species *(Colobopsis truncata*). We studied the diversity of this species by employing a multidisciplinary approach and combining data from our surveys, museum and private collections, and citizen science platforms. As a result, we have revealed the existence of a second species, which we describe as *Colobopsis imitans* sp. nov., distributed allopatrically from *Co. truncata* and living in the Maghreb, Sicily and southern Iberia. While the pigmentation of *Co. truncata* is reminiscent of *Dolichoderus quadripunctatus*, that of *Co. imitans* is similar to *Crematogaster scutellaris*, with which *Co. imitans* lives in close spatial association, and whose foraging trails it habitually follows, similar to *Camponotus lateralis* and other ant-mimicking ants. The isolation between *Co. imitans* and *Co. truncata* seems to have occurred relatively recently because of significant, yet not extreme, morphometric differentiation, and to mtDNA polyphyly. Both *Co. imitans* and *Co. truncata* appear to employ mimicry of an unpalatable or aggressive ant species as an important defensive strategy; this ‘choice’ of a different model species is motivated by biogeographic reasons and appears to act as a critical evolutionary driver of their diversification.

Keywords: adaptation; Batesian mimicry; citizen science; COI mtDNA; discriminant-function analysis; Mediterranean; multivariate statistics; North Africa; sibling species; speciation

Exploring mitogenome evolution in Branchiopoda (Crustacea) lineages reveals gene order rearrangements in Cladocera

Bibliographic reference: Castellucci F., Luchetti A., Mantovani B. 2022. Exploring mitogenome evolution in Branchiopoda (Crustacea) lineages reveals gene order rearrangements in Cladocera. *Scientific Reports* 12, 4931.

Abstract

The class Branchiopoda, whose origin dates back to Cambrian, includes ~ 1200 species which mainly occupy freshwater habitats. The phylogeny and systematics of the class have been debated for long time, until recent phylogenomic analyses allowed to better clarify the relationships among major clades. Based on these data, the clade Anostraca (fairy and brine shrimps) is sister to all other branchiopods, and the Notostraca (tadpole shrimps) results as sister group to Diplostraca, which includes Laevicaudata + Spinicaudata (clam shrimps) and Cladoceromorpha (water fleas + Cyclestherida). In the present analysis, thanks to an increased taxon sampling, a complex picture emerges. Most of the analyzed mitogenomes show the Pancrustacea gene order while in several other taxa they are found rearranged. These rearrangements, though, occur unevenly among taxa, most of them being found in Cladocera, and their taxonomic distribution does not agree with the phylogeny. Our data also seems to suggest the possibility of potentially homoplastic, alternative gene order within Daphniidae.

Keywords: mtDNA; phylogenomics; gene order; mitogenome

A new trans-Ionian spider species for the Italian fauna: *Habrocestum graecum* Dalmas, 1920 (Araneae, Salticidae)

Bibliographic reference: Castellucci F., Caroli M., Simeon E., Kulczycki A., Piccinini A., Luchetti A., Legittimo C.M. 2022. A new trans-Ionian spider species for the Italian fauna: *Habrocestum graecum* Dalmas, 1920 (Araneae, Salticidae). *Biogeographia* 37, a023.

Abstract

The salticid spider *Habrocestum graecum* Dalmas, 1920, until now only known from Greece, is for the first time recorded in Italy. Observations on ecology and behavior are also reported and pictures of its habitus and genitalia are provided. Furthermore, the first DNA barcode sequence for H. graecum is produced and made publicly available. The species has been observed in Puglia, in South-Eastern Italy, and a trans-Ionian dispersal pattern is most likely the cause of its presence both in Greece and Southern Italy, as reported for other taxa with similar distribution in different animal groups.

Keywords: Araneae; DNA barcoding; first record; trans-Adriatic; salticid

First records of *Anagraphis ochracea* (Araneae: Gnaphosidae) for continental Italy and Sicily with new observations on its myrmecophilous lifestyle

Bibliographic reference: Lenzini L., Castellucci F., Poso M., Kulczycki A., Simeon E., Greco G., Piccinini A., Legittimo C.M. 2022. First records of *Anagraphis ochracea* (Araneae, Gnaphosidae) for continental Italy and Sicily with new observations about its myrmecophilous lifestyle. *Arachnologische Mitteilungen / Arachnology Letters* 64, 83–92.

Abstract

In the present study we describe and discuss for the first time the peculiar myrmecophilous habits of *Anagraphis ochracea* (L. Koch, 1867) and its strong association with the ant species *Messor ibericus* Santschi, 1931. The study is based on behavioral observations carried out both in the field and in captivity, and sheds light on the lifestyle of this poorly studied and rarely observed species. We also record the presence of *A. ochracea* on continental Italy and Sicily for the first time, provide a brief overview of its taxonomic history and present photographs of adult and juvenile specimens, the egg sac and the copulatory organs of both sexes. Finally, we provide a DNA-barcode (COI) for *A. ochracea*, which is the first for the genus *Anagraphis* as well.

Keywords: ant; ant association; Arachnida; *Messor ibericus*; myrmecophily; spider; symbiosis