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

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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.

1. Introduction

Spiders (Araneae) represent a megadiverse order of arthropods, with over 51,000 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 et al., 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 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,

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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 from Baltic amber (Wunderlich, 2004), and three extant species are currently recognized: *Mastigusa arietina* (Thorell, 1871), known from 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 and Millidge, 1953; Loksa, 1969; Tyschchenko, 1971; Wunderlich, 1986, 2004; Azarkina and Trilikauskas, 2012) or sub-species (Bristowe, 1939; Chyzer and Kulczynski, 1887; 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*. Later 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 et 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.

2. Materials and methods

2.1. 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 −20 °C 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 (Chyzer and Kulczynski, 1887; Simon, 1898a; Thorell, 1871) and the revision by Wunderlich (1986), and by comparison with type material for *M. arietina*, *M. macrophthalma* and *M. lucifuga*.

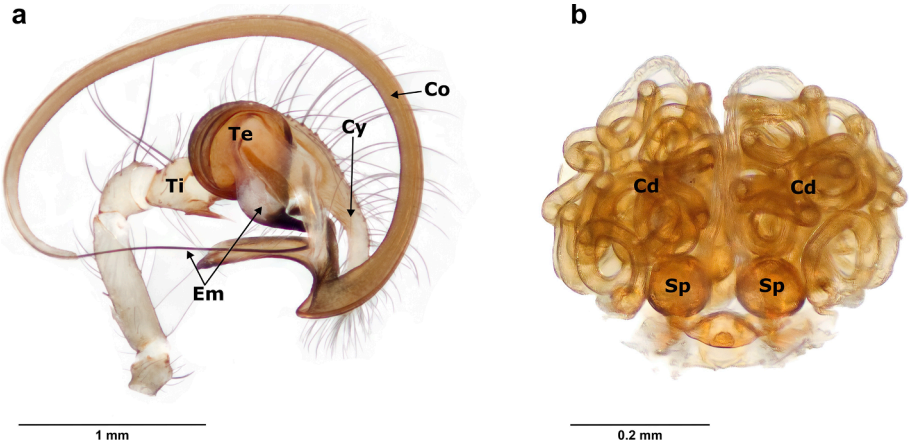

Fig. 1. Male and female genitalia of *Mastigusa arietina*. a: left male pedipalp, prolateral view. b: female genitalia, excised and cleared, ventral view. Abbreviations: Cd = copulatory ducts; Co = conductor; Cy = cymbium; Em = embolus; Sp = spermatecha; Te = tegulum; Ti = tibia. Image credits: F. Castellucci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

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.

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	6 km 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	6 km 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.

2.2. 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.

2.3. 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 (Larsson, 2014). 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 and based on 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).

2.4. 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 clock and tree models, and unlinked site models. 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.

3. Results

3.1. Marronoid phylogeny

Beside the constrained nodes, the constrained and unconstrained ML tree topologies mostly overlap (Fig. 2; Suppl. Figs. 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. Figs. 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 preferred tree (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, are 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* and *Ethobuella tuonops*. This clade is sister to dictynids (bootstrap = 74).

3.2. 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).

3.3. Divergence times

The estimated age for the diversification of the RTA clade is dated at

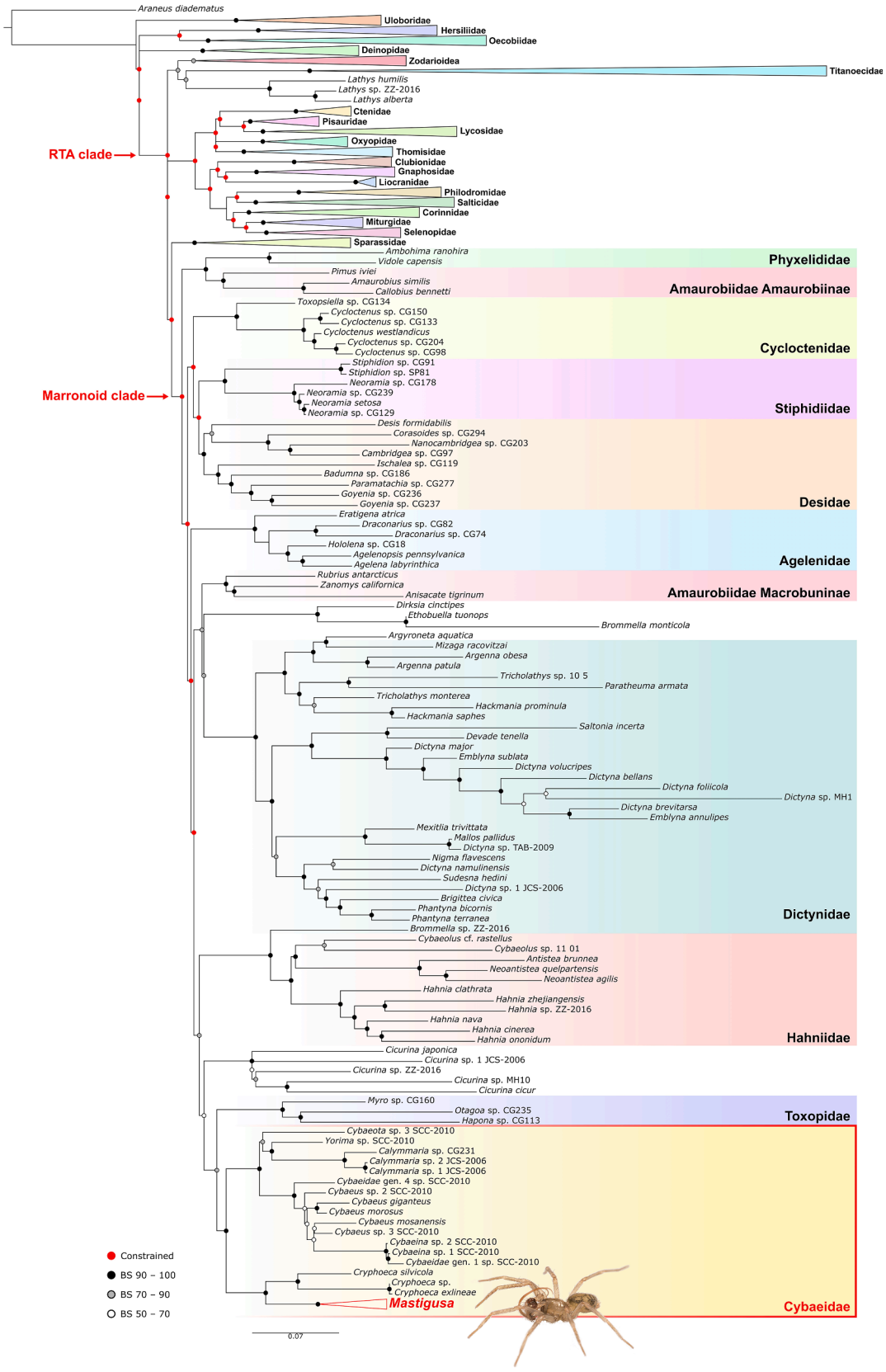

Fig. 2. Constrained Maximum Likelihood tree built using IQ-TREE with 1000 ultrafast bootstrap replicates. Non-marronoid families collapsed; *Mastigusa* clade collapsed. BS = bootstrap values. Constrained nodes evidenced in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

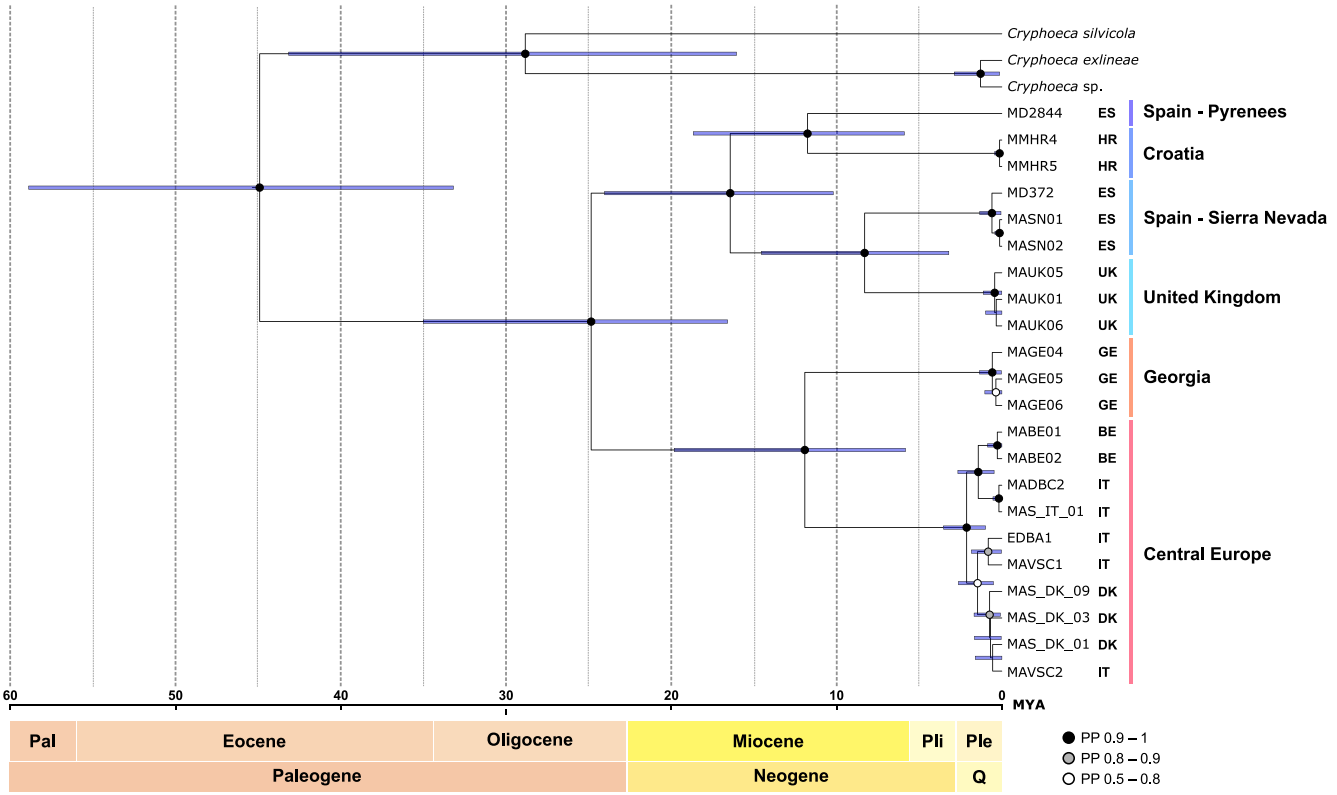

Fig. 3. Detail of the BEAST time-tree tree focusing on *Mastigusa*. Scale in million years ago. Node bars represent 95% confidence intervals. Country codes after the sample names: BE = Belgium; DK = Denmark; ES = Spain; GE = Georgia; HR = Croatia; IT = Italy; UK = United Kingdom. PP = posterior probability.

95.2 million years ago (Mya), in the Upper Cretaceous (95 % HPD = 84.7–106.2 Mya), while that of marronoid taxa is dated at 87.3 Mya, again in the Upper Cretaceous (95 % HPD = 78.7–99.7 Mya). The split between *Mastigusa* and its sister genus *Cryphoeca* is estimated at 44.9 Mya, in the Eocene (95 % HPD = 33.2–58.9 Mya). Within *Mastigusa*, the clade composed of Central European and Georgian populations diverged 24.8 Mya, in the Oligocene (95 % HPD = 16.6–35 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 = 11.9 Mya (95 % HPD = 5.8–19.8 Mya); Sierra Nevada + United Kingdom – Croatia + Pyrenees = 16.4 Mya (95 % HPD = 10.2–24 Mya); Spain – United Kingdom = 8.3 Mya (95 % HPD = 3.2–14.5 Mya); Croatia – Pyrenees = 11.8 Mya (95 % HPD = 5.9–18.7Mya) (Fig. 3).

4. Discussion

4.1. *Marronoid phylogeny and phylogenetic placement of* Mastigusa

Based on morphological data, Griswold et al. (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 moderate 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). Its 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).

4.2. 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 separate clusters, suggests continuity of gene flow between the areas, which therefore does not raise concerns regarding a possible undersampling in Central Europe. The Georgian specimens show a certain degree of genetic divergence compared to the Central European ones, from which they separated around 12 Mya, but no clear morphological differences could be identified. 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. The split between these two groups appears to have happened around 16 Mya. 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 and 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 45 Mya*,* in the Eocene, 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 late Oligocene and 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 and Nentwig, 1991; Scharff and 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 (Castellucci et al., 2022; Chyzer and Kulczynski, 1887; Kostanjšek and Kuntner, 2015). 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.

CRediT authorship contribution statement

Filippo Castellucci: Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Nikolaj Scharff:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Andrea Luchetti:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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