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Exploring the water mite fauna (Acari, Hydrachnidia) of the Madeira archipelago: DNA Barcoding reveals a remarkable species endemicity

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Abstract

Water mites represent the group with the highest degree of endemism among all macroinvertebrates living in freshwater habitats of Madeira. The water mite fauna of this archipelago has been well known for a long time, but until now they have not been molecularly investigated. So far, 25 species of water mites have been recorded, most of them endemic to Madeira. The library presented here covers 584 COI DNA barcodes grouped into 23 Barcode Index Numbers (BINs), which represent the genetic barcodes of 23 species (more than 80% of the known Madeira water mite fauna). Our study shows that COI barcode clusters generated by the Barcode of Life Data Systems (BOLD) matches to morphological identifications of specimens, with one exception in the family Lebertiidae. A large-scale comparison of the new sequences

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with those available in public databases such as BOLD confirmed the uniqueness of the genetic diversity of water mites inhabiting Madeira. *Neumania atlantida* Lundblad, 1941, a species previously synonymized with *N. uncinata* Walter, 1927, is resurrected as a valid species. Additionally, genetic data revealed that *Sperchon brevirostris* Koenike, 1895, a species common in freshwaters of Europe and Macaronesia, consists of multiple genetic lineages, one of which is restricted to Madeira. Finally, our research revealed three species new to the water mite fauna of Madeira, i.e., *Hydrachna skorikowi* Piersig, 1900, *Arrenurus bicuspidator* Berlese, 1885 and *Lebertia algeriensis* Lundblad, 1942. The latter species, found to be common in the running waters of the island, may be the first species of water mite documented as potentially, if not invasive, then non-indigenous in freshwater ecosystems of Madeira.

Key words: Macaronesia, DNA barcoding, islands, invasive species, endemism

Introduction

Water mites (Hydrachnidia) is the most diverse and numerous group of arachnids in freshwater, inhabiting a wide range of habitats, including lotic, lentic, temporary and interstitial waters (Davids *et al.* 2007). To date, almost 7500 species have been described, grouped into 550 genera (Smit 2020), mostly based on morphology. However, in recent years, the use of the DNA barcode fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene has been shown to have great potential in the identification of water mites, overcoming the limitations of the often-time-consuming morphological approach. The successful application of the "barcoding" approach is based on the comparison of the obtained sequences with public libraries containing sequences with known species identification (Hebert *et al.* 2003), the quality of which primarily depends on the morphological identification of vouchers by an expert taxonomist. Therefore, it seems that the quality and accessibility of the vouchers are the "Achilles heel" of the DNA barcoding system and its successful implementation in species identification.

The Madeira archipelago, with an area of 736.75 km^2 (Raposeiro *et al.* 2022), located in the Atlantic Ocean, between latitudes 32°24' and 33°07'N and longitudes 16°16' and 17°16'W, is part of Macaronesia which consists of four main archipelagos (Azores, Canary Islands, Cape Verde and Madeira). The dense hydrographic system on the Madeira Island includes approximately 126 watersheds and 200 streams (Marques 1994), most of them have a short course, often characterized by torrential and/or seasonal flows (Hughes 2003). In contrast the other islands from the archipelago (i.e., Porto Santo Island and the Desertas Islands) are characterized by the scarcity of freshwater habitats (Melo *et al.* 2020).

The water mite fauna of Madeira Island has been well known since the early 20th century. In 1942, Olav Lundblad published a comprehensive monograph on Madeiran water mites with a detailed description of all the species that were found to live on this island (Lundblad 1942). Lundblad listed a total of 25 species of water mites, of which 24 were originally described from Madeira (Lundblad 1941, 1942). All of them, except *Sperchon brevirostris* Koenike, 1895, and *Parathyas barbigera* (Viets, 1908), can be considered endemic to Madeira, making water mites one of the groups with the highest degree of endemism among all macroinvertebrates living on this island (Hughes 2006; Raposeiro *et al.* 2022). The presence of *Sperchon brevirostris* and *Parathyas barbigera* merits further attention and requires DNA barcoding material from their type localities. For example, *Parathyas incerta* (Lundblad, 1941) was originally described by Lundblad (1942) from Madeira based on a single deutonymph but was later synonymized with *Parathyas barbigera*, a species considered to have a Holarctic distribution (Di Sabatino *et al.* 2010). If the barcoding data of *P. incerta* from its locus typicus reveals significant genetic divergence, this could be a reason for resurrecting *P. incerta*.

The most diverse genus is *Torrenticola* Piersig, 1896 with 10 species, followed by *Atractides* Koch, 1837 with four species. The last two genera, together with the genus *Lebertia* Neuman, 1880, are the most common genera occurring in the running waters of Madeira (Lundblad 1942). On the other hand, no water mite species were known from other islands of the Madeira archipelago.

This study presents a DNA barcode reference library for the water mite fauna of Madeira. The library presented here covers 584 COI DNA barcodes publicly available through the Barcode of Life Data Systems (BOLD) database, improving tools available for the identification of Madeiran water mite fauna species and providing a foundation for future taxonomic and biogeographic research on Madeiran water mites.

Material and methods

Specimens were collected during field expeditions across the Madeira Archipelago from 2022 to 2024 (Fig. 1). The work is based on material collected during 2022 and 2023 by the second author (A.Z.) and the material collected in 2024 in the project Biodiversity Genomics Europe (BGE). Twenty-two sites were sampled in 2022, forty-seven sites were sampled in October 2023, and seventeen sites were sampled in 2024. These sites were selected to cover a wide range of habitats (Figs. 2), mainly streams, waterfalls and springs, spanning from sea level to around 1500 meters a.s.l.

Water mites were collected with kick nets and immediately preserved in 96% ethanol for the purpose of molecular analyses. After non-destructive, whole-body DNA extraction, the specimen vouchers were stored in 96% ethanol and morphologically examined. Some of these vouchers were dissected and slide mounted in Faure's medium, while the rest was transferred to Koenike's fluid.

Most vouchers, preserved in Koenike's fluid, are stored in Institute of Marine and Environmental Sciences, University of Szczecin, Poland; with others being stored at the InBIO Barcoding Initiative reference collection (Vairão, Portugal). The slide mounted material will be deposited in the Naturalis Biodiversity Center in Leiden, the Netherlands (RMNH).

Molecular and DNA barcode analyses

Part of the molecular analysis was conducted at the Institute of Biology, University of Szczecin (IoB-UoS) the University of Florence (UNIFI), Florence, Italy, and at the University of Lodz (UniLodz), Lodz, Poland. In both cases DNA was extracted using a non-destructive protocol. At the IoB-UoS, DNA was extracted using the GeneMATRIX Tissue DNA Purification Kit (EurX Gdańsk, Poland). The cytochrome oxidase subunit I (COI) gene fragment was amplified by PCR with the universal primers LCO1490-JJ and HCO2198-JJ (Astrin & Stüben 2008). The PCR amplification was carried out in a final volume of 10 µl with 5.0 µl of DreamTaq PCR Master Mixes and 2.4 µl of nuclease-free water (Thermo Fisher Scientific Baltics UAB), 0.8 µl of 5 µM forward and reverse primers and 1 µl of total DNA. The PCR reactions were performed in a T100TM Thermal Cycler (Bio-Rad, California, USA) using the HOU 2007 program for 3 hours in 35 cycles. According to the following thermal profile: initial denaturation at 94 °C for 3 minutes, then at 94 °C—0.5-minute, annealing primers at 45 °C (for COI)—1.30 minute, elongation at 72 °C—1 minute, and final elongation at 72°C for 5 minutes. The PCR products were separated on a 1% agarose gel with Midori Green Advance DNA Stain (NIPPON Genetics EUROPE GmbH, Düren, Germany) in TBE buffer. To visualize and document the results obtained, Gel DocTM XR+ system was used (Bio-Rad, California, USA). At UniLodz, a portion of DNA extracts were transferred from IEMS-UoS. The amplification, quality control and sequencing of DNA barcoding fragment of Cytochrome oxidase subunit I gene (COI) was performed using approach presented in Srivatshan *et al.* (2021, 2024) employing Oxford Nanopore Technology. The COI fragment was amplified with primer pair LCO1490-JJ and HCO2198-JJ (Astrin & Stüben, 2008) attributed with 9bp tags (Srivatshan *et al.* 2024). The sequencing results were demultiplexed and DNA barcodes determined using ONT barcoder 2.0 (Srivatshan *et al.* 2024).

At the UNIFI, samples were digested using 95 ul of extraction buffer (100mM Tris-HCl, 5mM EDTA, 100mM NaCl, 0.5% SDS, pH 8) and 5 ul of proteinase K. Dilutions (1:10) of crude digested samples were used as template for the amplification of the mitochondrial cytochrome c oxidase subunit I (COI). Amplicons were amplified and barcoded in a single-step PCR using a cocktail of two barcoded primer pairs, namely Folmer primers (LCO1490, HC02198; Folmer *et al.* 1994) and Lep primers (LepF1, LepR1; Hebert *et al.* 2004). PCR was performed using the Kapa3G Plant PCR Kit according to the manufacturer's protocol and with the following thermal profile: initial denaturation step of 3 min at 94 °C, 35 cycles of 20 s at 95 °C, annealing for 15 s at 52 °C and extension for 30 s at 72 °C, and a final extension for 1 min at 72 °C. Amplicons were checked on a 1.2% agarose gel and pooled in a single tube. The amplicon mix was used to prepare a PacBio library with the SMRTbell prep kit 3.0 according to the manufacturer's protocol. The library was sequenced on an 8M ZMW SMRT cell on a PacBio Sequel IIe platform.

Raw reads were demultiplexed using the Pacific Biosciences SMRT Link software. Consensus sequences were generated with the PacBio Amplicon Analysis (pbaa) tool. Primer trimming, translation and stop codon checking were performed using Geneious Prime 2024.0.1.

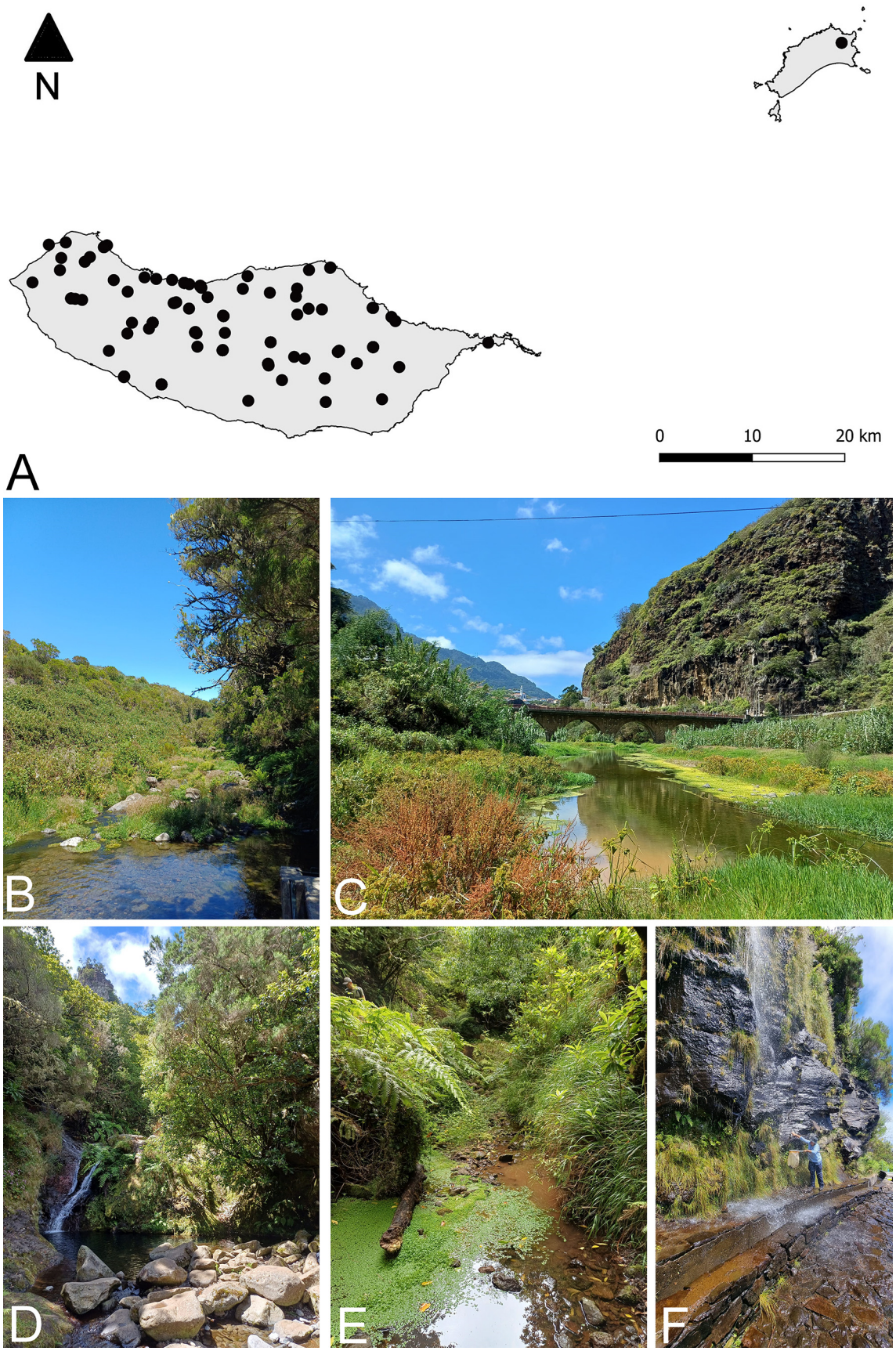

FIGURE 1. A Map of Madeira Archipelago with marked sampling sites. B–F Photographs of selected sampling sites: B—Rabaçal; C—Faial stream; D—Ribeiro frio waterfal; E—Levada da Serra do Faial; F—Levada da Serra waterfal. Photos by D. Girao (B–E) and P. Raposeiro (F).

Consensus sequences were made available in the BOLD database (Ratnasingham & Hebert 2007), and the Barcode Index Numbers (BIN) for every sequence were retrieved and analyzed. Relevant voucher information, photos, and newly generated DNA barcodes are publicly accessible through the Dataset "DS-MADWM - Contribution to the knowledge on DNA barcodes of water mites of Madeira" (http://dx.doi.org/10.5883/DS-MADWM).

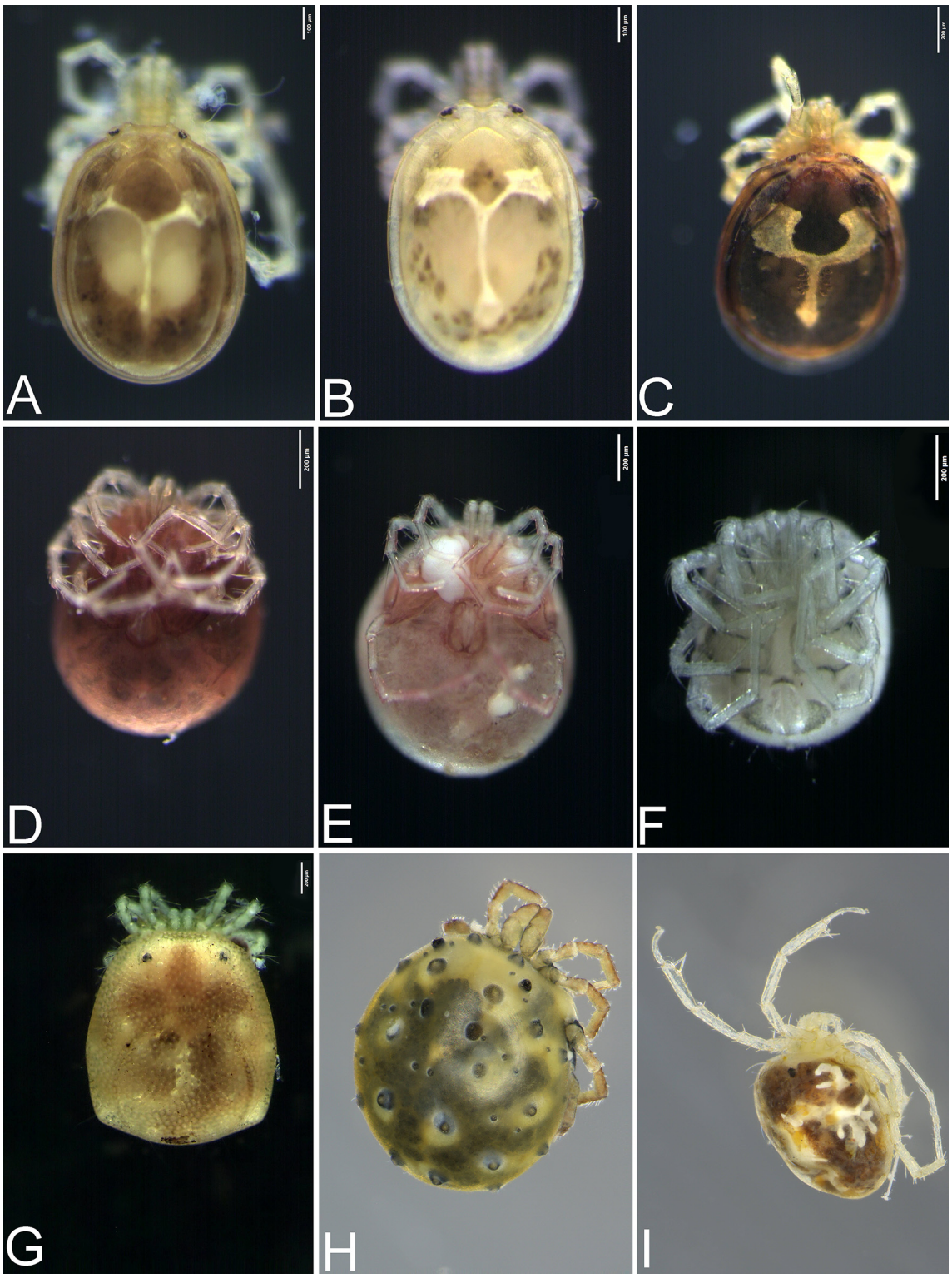

FIGURE 2. Photographs of selected water mite species. A—*Torrenticola pharyngealis*, Levada da Serra; B—*T. elliptiformis*, Paul da Serra; C—*T. nesiotes*, Levada da Serra do Faial. D—*Lebertia madericola*, Levada da Serra do Faial. E—*Limnesia atlantica*, Levada da Serra do Faial. F—*Neumania atlantida*, Levada da Serra do Faial. G—*Arrenurus bicuspidator*, Porto Santo. H—*Sperchon brevirostris*, São Jorge. I—*Atractides macaronensis*, Faja da Ovelha. Photos by D. Girao.

Sequence alignments were performed using MUSCLE (Edgar 2004). Intra- and interspecific genetic distances were calculated based on the Kimura 2-parameter model (K2P; Kimura 1980) using MEGA11 (Tamura *et al.* 2021). Additionally, the sequence data were analyzed using the Assemble Species by Automatic Partitioning (ASAP) method (Puillandre *et al.* 2012). We used the online ASAP version (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings and p-distance model.

Results

In this study, a total of 584 water mite specimens from Madeira were successfully sequenced. The most sequence-rich family was Torrenticolidae with 250 sequences (42.8% of total; 10 BINs), followed by Hygrobatidae with 149 sequences (25.5%; 3 BINs), and Lebertiidae with 143 sequences (24.5%; 3 BINs). Some families were rare, such as Hydryphantidae and Unionicolidae, represented by a single sequence each and a corresponding single BIN. One deutonymph, identified to the genus level only as *Torrenticola* sp., clusters in a separate BIN (BOLD:AGH8050).

All sequences of cytochrome c oxidase I (COI) DNA barcodes are 658 base pairs (bp) long. The average nucleotide composition of the sequences is 32.20% thymine (T), 21.47% cytosine (C), 32.12% adenine (A) and 14.20% guanine (G), for a total GC content of 35.68% for the COI barcode fragment analyzed. The Neighbour-Joining tree of all analyzed water mite species from Madeira Island based on Kimura 2-parameter distances is available in Pešić *et al.* (2025b, https://data.mendeley.com/datasets/gzswc7g76h/1)

TABLE 1. List of species that were collected and DNA barcoded within this project. ([N] indicates a new BIN that contains only sequences from this study; * indicates first DNA barcodes for the species in this study). BOLD data presented here was last accessed on 20 Feb. 2025.

Family	Species	BOLD BIN
Hydrachnidiae Leach, 1815	*Hydrachna skorikowi* Piersig, 1900	BOLD:ACS0797
Hydryphantidae Piersig, 1896	**Trichothyas* (*Lundbladia*) *rutae* (Lundblad, 1941)	[N]BOLD:AGI4694
Lebertiidae Thor, 1900	*Lebertia* (*Mixolebertia*) *madericola* (Lundblad, 1942)	BOLD:AEB4193
	Lebertia (*Mixolebertia*) *maderigena* (Lundblad, 1942)	BOLD:AEB4193
	Lebertia (*Pilolebertia*) *algeriensis* Lundblad, 1942	BOLD:AEJ1488
Sperchontidae Thor, 1900	*Sperchon* (*Sperchon*) *brevirostris* Koenike, 1895	[N]BOLD:AGH8322
Torrenticolidae Piersig, 1902	**Torrenticola* (*Torrenticola*) *affinis* (Lundblad, 1941)	[N]BOLD:AGH8051
	**Torrenticola* (*Torrenticola*) *crassa* (Lundblad, 1941)	[N]BOLD:AGH8054
	**Torrenticola* (*Torrenticola*) *crassirostris* (Lundblad, 1941)	[N]BOLD:AGK1761
	**Torrenticola* (*Torrenticola*) *elliptiformis* (Lundblad, 1941)	[N]BOLD:AGH8000
	**Torrenticola* (*Torrenticola*) *insulicola* (Lundblad, 1941)	[N]BOLD:AGH8055
	**Torrenticola* (*Torrenticola*) *maderensis* (Lundblad, 1941)	[N]BOLD:AGH8048
	**Torrenticola* (*Torrenticola*) *nesiotes* (Lundblad, 1941)	[N]BOLD:AGH8052
	**Torrenticola* (*Torrenticola*) *pharyngealis* (Lundblad, 1941)	[N]BOLD:AGH8049
	**Torrenticola* (*Torrenticola*) *rotunda* (Lundblad, 1941)	[N]BOLD:AGH8047
	Torrenticola sp. (nymph)	[N]BOLD:AGH8050
Hygrobatidae Koch, 1842	**Atractides* (*Atractides*) *insulanus* (Lundblad, 1941)	[N]BOLD:AGJ7338
	**Atractides* (*Atractides*) *macaronensis* (Lundblad, 1941)	[N]BOLD:AGH8053
	**Atractides* (*Polymegapus*) *rutae* (Lundblad, 1941)	[N]BOLD:AGH7996
Limnesiidae Thor, 1900	**Limnesia* (*Limnesia*) *atlantica* Lundblad, 1941	[N]BOLD:AGH7997
Unionicolidae Oudemans, 1909	**Neumania atlantida* Lundblad, 1941	[N]BOLD:AGK6979
Aturidae Thor, 1900	**Aturus atlantis* Lundblad, 1941	[N]BOLD:AGH8056
Arrenuridae Thor, 1900	**Arrenurus* (*Arrenurus*) *autochthonus* Lundblad, 1941	[N]BOLD:AGJ7455
	Arrenurus (*Arrenurus*) *bicuspidator* Berlese, 1885	BOLD:ACS0403

Interspecific p-distances within the genus *Torrenticola*, represented by ten species in our dataset, varied from 3.52% between the pair *T. elliptiformis* and *T. affinis* to 10.29% between the pair *T. crassa* and *T. maderensis*. In the genera *Lebertia* and *Atractides* (each represented by three species) interspecific p-distances within a genus varied from 1.19% (between the pair *L. madericola* and *L. maderigena*) to 11.79% (between the pair *L. algeriensis* and *L. maderigena*), and from 11.88% (between the pair *A. macaronensis* and *A. insulanus*) to 23.87% (between the pair *A. rutae* and *A. insulanus*), respectively. Intraspecific genetic p-distances in most species were less than 1%, except for *Atractides rutae* (1.03%). Three species were represented by a single specimen in the dataset, and, for this reason, no intraspecific distance is calculated.

The resulting sequences clustered into 23 BINs, with 19 BINs (82.6%) being unique and deposited for the first time in BOLD. The RESL (OTU) analyses assigned sequences into 23 OTUs. All BINs, except one (BOLD: AEB4193; 49 records), were concordant and 3 BINs were represented by a single sequence. Our study provided the first DNA barcodes for sixteen species endemic from Madeira (Table 1). The BINs generated by BOLD clustered together sequences that closely agree with the morphological identifications of the specimens, with only one exception in the family Lebertiidae (*L. madericola*/*L. maderigena*).

Discussion

Diversity of water mites of Madeira

Our study provides the first reference library of DNA barcodes for water mites from Madeira. The collected water mites represent 23 species out of 28 known for Madeira, three of which are reported for the first time in this study. We recorded more than 80% of the Madeiran water mite fauna. Five species previously known from Madeira, i.e., *Parathyas barbigera* (Viets, 1908), *Torrenticola mandibularis* (Lundblad, 1941), *Thyopsis maderensis* Lundblad, 1941, *Atractides maderensis* (Lundblad, 1941), and *A. hystricipes* (Lundblad, 1941) are not represented in this work.

Neumania atlantida Lundblad, 1941, a species originally described from Madeira (Lundblad 1941, 1942), has been placed into synonymy of *N. uncinata* Walter, 1927 by Pešić *et al.* (2007). The latter species which is widely distributed species in the Mediterranean region is represented by three BINs in the BOLD database, two of them (BOLD:AFV0253, BOLD:AFV0269) were recently described from mainland Portugal (Pešić *et al.* 2024), while the third one (BOLD:AER9267), includes specimens of *N. uncinata* from Sardinia (see Pešić & Goldschmidt 2023). The specimen from Madeira, here attributed to *N. atlantida*, forms a unique BIN BOLD:AGK6979. The p-distance between the Madeiran BIN and their closest neighbor, BOLD:AER9267, which includes specimens of *N. uncinata* from Sardinia, was estimated at 11.7%, indicating that *N. atlantida* belongs to a distinct species. Following the original description, *N. atlantida* differs in character state of the fourth coxae, with lateral extensions near leg insertions not hook-shaped as in *N. uncinata* (see Lundblad 1942). Therefore, based on this molecular and morphological evidence, synonymization of the latter species with *N. uncinata* needs to be rejected and *N. atlantida* is resurrected as a valid species.

Examining molecular diversity at the larger scale, we found more cases of species represented by multiple BINs. Such an example is *Sperchon brevirostris* Koenike, 1895, a species common in many types of running waters in the Western Palearctic (Di Sabatino *et al.* 2010), which includes at least five BINs available in the BOLD database. In this study, we found one, BOLD:AGH8322, which includes only specimens from Madeira and another BIN (BOLD:AGK2886) which includes specimens from the São Miguel Island, in the Azores Archipelago, from where this species was originally described (Koenike 1895). The p-distance between the Madeira and Azores clades was estimated at 4.49%, which indicates the genetic isolation of these two insular populations.

The three remaining BINs of *S. brevirostris* are present in continental Europe, BOLD:ACP6107 in Norway and Germany, BOLD:AFD0340 known from Norway, Germany and Montenegro, while the third BIN (BOLD:AED3857) is known only from Montenegro and North Macedonia. The applied ASAP procedure identified four molecular operational taxonomic units (MOTU) within the available dataset of *S. brevirostris* sequences, grouping sequences of BOLD:ACP6107 and BOLD:AFD0340, and separating the COI sequence of *S. brevirostris* from Azores (BOLD: AGK2886) and Madeira (BOLD:AGH8322) (Fig. 3). These results might indicate that *S. brevirostris* is an endemic species restricted to the Azores, while Madeira is populated with its sister species. A comprehensive taxonomic

revision is needed to clarify the relationships between European and Macaronesia lineages and other members of the *S. brevirostris* complex, ideally by integrating mitochondrial and nuclear markers within an integrative taxonomic framework.

Moreover, this study added three new species to the known water mite fauna of Madeira: *Hydrachna skorikowi* Piersig, 1900, *Arrenurus bicuspidator* Berlese, 1885, and *Lebertia algeriensis* Lundblad, 1942. The examined specimens keyed to *H. skorikowi* following Davids *et al.* (2010), cluster within BOLD:ACS0797, which in addition to the specimens used in this study, includes one specimen of an unidentified *Hydrachna* sp. from Morocco and one specimen of *H. skorikowi* from the Netherlands, available in the BOLD database.

Two females of *Arrenurus bicuspidator* collected from Porto Santo, an island located about 43 kilometres northeast of Madeira Island, clustered within BOLD:ACS0403. This BIN includes specimens collected from a large geographic area, from Norway to South Africa and from the Netherlands to Kyrgyzstan, morphologically assigned to *A. bicuspidator*, or *A. radiatus* Piersig, 1894, but also specimens from Portugal identified by Pešić *et al.* (2024) as *A. szalayi* Lundblad, 1954, an endemic species known only from mainland Portugal. As emphasized by Pešić *et al.* (2024), additional research, possibly including an analysis of both nuclear and mitochondrial markers, is needed to understand factors that led to a lack of genetic differentiation between the abovementioned morphologically different species.

The Madeiran specimens of *Lebertia algeriensis* molecularly analyzed in this study match the description of this species following Gerecke (2009). Genetic data indicates that all examined specimens clustered within BOLD: AEJ1488. In addition to specimens used in this study, the BIN includes three specimens from Slovakia and one specimen from Greece, publicly available in the BOLD database.

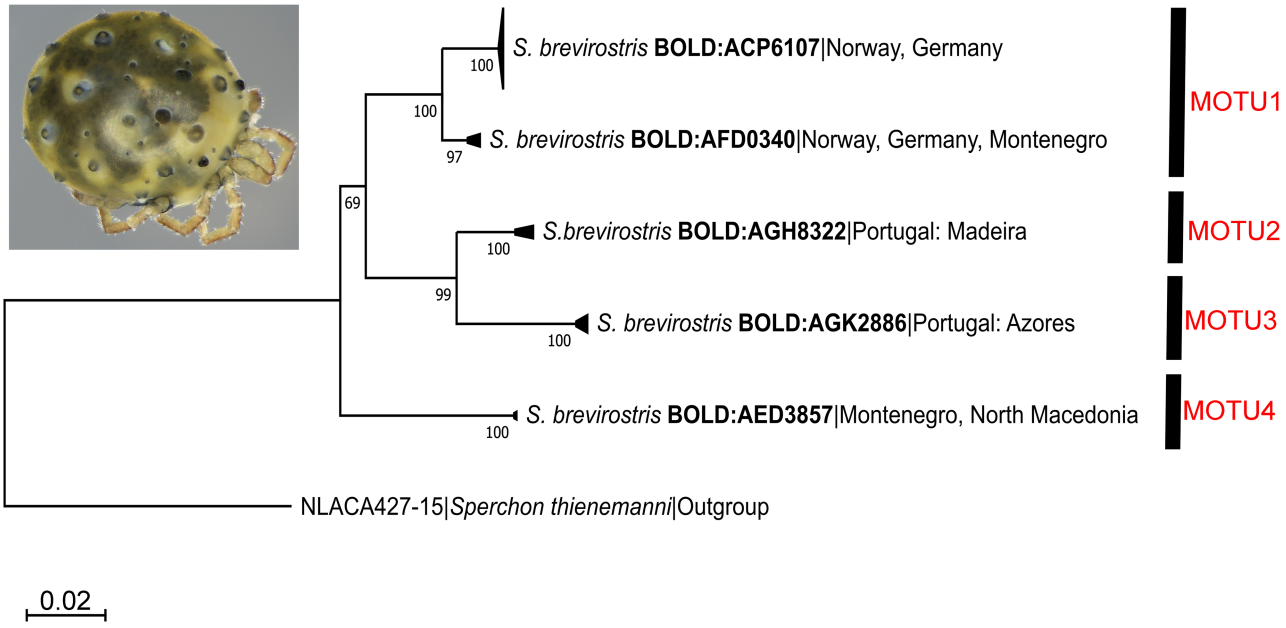

FIGURE 3. Neighbor–Joining tree of the *Sperchon brevirostris* (in inset) complex obtained from 53 nucleotide COI sequences (listed in Table 2). *Sperchon thienemanni* Koenike, 1907, from the Netherlands was used as outgroup. There was a total of 669 positions in the final dataset. Bootstrap values > 50% from 1000 bootstrap replicates on branches. The results of species delimitation by ASAP procedure are indicated by vertical bars. BINs are based on the DNA barcode analysis from 20 Feb. 2025.

Match and mismatch between morphological and barcoding data in the Madeiran water mites

In recent years, the DNA barcode technique has proven to be an effective tool for identifying water mites (e.g. Pešić *et al.* 2017, 2020, 2021, 2023, 2025a; Tyukosova *et al.* 2022). However, some studies (e.g. García-Jiménez *et al.* 2017) revealed mismatches between morphological and molecular data in water mites, warning of the danger of rigid delimitation of species based on arbitrary molecular distances.

TABLE 2. List of specimens of *Sperchon brevirostris* complex used for building Fig. 3.

Sample ID	Process ID	BIN	Country
MARB UIB 578	MARBN1065-23	BOLD:ACP6107	Norway
MARB UIB 786	MARBN1178-23		Norway
MARB UIB 953	MARBN965-23		Norway
MARBN939-23	MARB UIB 1307		Norway
MARBN923-23	MARB UIB 1291		Norway
MARBN826-23	MARB UIB 1099		Norway
MARBN819-23	MARB UIB 1092		Norway
MARBN769-23	MARB UIB 1042		Norway
MARBN768-23	MARB UIB 1041		Norway
MARBN731-23	MARB UIB 1194		Norway
MARBN692-23	MARB UIB 1155		Norway
MARBN691-23	MARB UIB 1154		Norway
MARBN681-23	MARB UIB 1144		Norway
MARBN680-23	MARB UIB 1143		Norway
HYDCA419	MMHYD283-20		Norway
HYDCA97	HYDCA097-16		Germany
HYDCA96	HYDCA096-16		Germany
HYDCA98	HYDCA098-16		Germany
EBAI-Hyd06	EBAHY006-16		Norway
EBAI-Hyd05	EBAHY005-16		Norway
EBAI-Hyd04	EBAHY004-16		Norway
EBAI-Hyd01	EBAHY001-16		Norway
EBAI-Hyd02	EBAHY002-16		Norway
HYDCA121	HYDCA121-18		Norway
MARB UIB 1093	MARBN820-23		Norway
MARB UIB 1193	MARBN730-23		Norway
EBAI-Hyd03	EBAHY003-16		Norway
HYDCA113	HYDCA113-18		Norway
MARB UIB 1098	MARBN825-23		Norway
MARB UIB 1151	MARBN688-23		Norway
MARBN851-23	MARB UIB 1124		Norway
MARBN884-23	MARB UIB 1252		Norway
MARBN885-23	MARB UIB 1253		Norway
CCDB38233 D07	DCCDB043-21	BOLD:AFD0340	Montenegro
MARB UIB 1039	MARBN766-23		Norway
MARB UIB 787	MARBN1179-23		Norway
MARB UIB 1290	MARBN922-23		Norway
HYDCA099-16	HYDCA99		Germany
CCDB38233 A11	DCCDB011-21	BOLD:AED3857	Montenegro
DCCDB044-21	CCDB38233 D08		Montenegro
8. ME2019 4 B7	DNAEC018-20		North Macedonia

.....continued on the next page

TABLE 2. (Continued)

Sample ID	Process ID	BIN	Country
BGE 00584 H05	BBIOP3224-24	BOLD:AGK2886	Portugal (Azores)
BGE 00584 H09	BBIOP3228-24		Portugal (Azores)
BGE 00584 H06	BBIOP3225-24		Portugal (Azores)
BGE 00584 H03	BBIOP3222-24		Portugal (Azores)
BGE 00584 H08	BBIOP3227-24		Portugal (Azores)
BGE 00584 H07	BBIOP3226-24		Portugal (Azores)
BGE 00584 H10	BBIOP3229-24		Portugal (Azores)
K91_18	HYDMD162-24	BOLD:AGH8322	Portugal (Madeira)
K88_31	HYDMD031-24		Portugal (Madeira)
K90_12	HYDMD108-24		Portugal (Madeira)
K93_4	HYDMD244-24		Portugal (Madeira)
K99_31	HYDMD559-24		Portugal (Madeira)

Our research provided 23 BINs, of which 19 were new to BOLD. The BINs generated by BOLD grouped the sequences that match the morphological identifications of the specimens, with only one exception in the families Lebertidae (*L. madericola*/*L. maderigena*) and Arrenuridae (*A. bicuspidator*), respectively.

In the family Lebertiidae, the sequences of specimens identified as *L. madericola* and *L. maderigena* are grouped in one BIN (BOLD:AEB4193), indicating that DNA barcodes might not differentiate between these two species. García-Jiménez *et al.* (2017), who barcoded specimens of the two abovementioned *Lebertia* species from Madeira, estimated the genetic distance between them at 1.11% K2P. This distance, as emphasized by later authors, in the recent "barcode culture", would probably lead to the conclusion that the populations of these two species belong to the same MOTU (Molecular Operational Taxonomic Unit—hypothetical species). However, using the coalescent model, the latter authors proved that *Lebertia maderigena* and *L. madericola* represent two independent lines of evolution, which result was also confirmed by Poisson tree analysis with multiple rates (see García-Jiménez *et al.* 2017). In their study, García-Jiménez *et al.* (2017) estimated the common ancestor of the two endemic Madeiran *Lebertia* species at about 4.6–5.2 million years ago (Brehm *et al.* 2003; Prada & Serralheiro 2000).

Gerecke (2009) hypothesized that both species, *L. maderigena* and *L. madericola*, probably evolved from a *Pilolebertia* ancestor that reduced swimming setation during the colonization of running waters on the island. Species of the latter subgenus show great similarity with the two endemic Madeiran species in the palp setation (third palpal segment with 5 medial setae, fourth palpal segment with dorsal hair-like setae concentrated in the distal quarter). A palp of nearly identical shape and dimensions, as noted by Gerecke (2009), can be found in *L.* (*Pilolebertia*) *inaequalis* (Koch, 1837). The close relationship between the two endemic Madeiran species (belonging to subgenus *Mixolebertia*) and the species of subgenus *Pilolebertia* is confirmed by the p-distance estimated at 6.96% to the nearest neighboring BIN, BOLD:AEF2742, which includes two sequences of specimens from Montenegro morphologically assigned to *L. inaequali*s.

According to the evolutionary scenario proposed by Gerecke (2009), after genetic isolation, *L. maderigena* and *L. madericola* independently developed a set of adaptations that allowed them to colonize the running waters of the island. These adaptations included the development of stronger leg setation and strengthening the integument by sculpturing.

In the family Arrenuridae, the sequences of two females collected on the island of Porto Santo, assigned in this study to *A. bicuspidator*, cluster in BOLD:ACS0403. However, several species present in both BOLD databases (*A. radiatus*, *A. szalayi*) cluster in the same BIN. To understand the factors that cause the lack of genetic differentiation between the three morphologically distinct species, further research is needed, preferably using a combination of nuclear and mitochondrial markers (Pešić *et al.* 2024).

Regardless of the mismatches described above, our study showed that COI barcode clusters computed by BOLD Systems can be a successful tool in identifying Madeiran water mites at the species level. In this study, we achieved more than 85% success in matching BINs (19 out of 22) to species generated in the morphological identification of specimens.

Lebertia algeriensis in Madeira—the first documented invasive species of water mites?

In his extensive work on Madeiran water mites, Lundblad (1942) lists only two species of the genus *Lebertia*, *L. maderigena* and *L. madericola*, inhabiting running waters on the island. During his field research in Madeira, Lundblad collected a total of 271 specimens, 92 from *L. maderigena* and 179 from *L. madericola* at 15 localities. This material, deposited in the Stockholm Natural History Museum, has recently been verified by García-Jiménez *et al.* (2017). The latter authors collected new material of *Lebertia*, which included only two abovementioned endemic species, at the three different localities during 2012 and 2013. Harry Smit (Pers. communication) collected water mites on Madeira in June 2006. There were 18 localities with *Lebertia madericola* and 2 with *L. maderigena*, but no *L. algeriensis* (H. Smit, unpublished data). In this study, in addition to the two previously known endemic species of the genus, we collected and barcoded one more species, *L. algeriensis*, which was not known to occur in Madeira until now. The latter species, with 92 new barcodes, is the most common *Lebertia* species in our material. Genetic data revealed that Madeiran specimens of *L. algeriensis* belong to the same BIN which also includes specimens from Greece and Slovakia.

Given that *L. algeriensis* (or any other species of *Pilolebertia*) was not found during Lundblad's and Smit's intensive surveys of the water mite fauna of Madeira, it seems reasonable to assume that *L. algeriensis* colonized Madeira recently, within the last eighty years, or even the last 15 years. Our study revealed the occurrence of this species in different watersheds of Madeira, where it has since become common, suggesting the invasive potential of this species. Available data suggests that *L. algeriensis* may be the first species of water mite documented as potentially, if not invasive, non-indigenous in the freshwater ecosystems of Madeira.

Elucidating how this species arrived in the freshwater ecosystems of Madeira requires additional information on the distribution and ecology of this species, which is still scarce. It is possible that this species was accidentally introduced by natural vectors such as their insect hosts. The presence of *L. algeriensis* in geographically distant regions (Greece, Slovakia, Madeira) suggests a broad distribution, potentially facilitated by anthropogenic or natural dispersal mechanisms. Given the relatively low diversity and high endemicity of Madeiran water mite fauna, we cannot exclude that the presence and spread of *L. algeriensis* over time could, if not already, lead to a decline in the richness and abundance of water mites in the invaded ecosystems. The presence of sequences of this species (and the corresponding BIN) in public DNA barcode libraries allows efficient monitoring of this species in Madeira's watersheds, but also in other regions (which include a large geographical area from Greece and Slovakia to Madeira) where this species has already been detected. New genetic data could help in tracing the invasion pathways of this species and identifying the origin of the population(s) now present on the island.

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