Hygrobates calabricus, a new species of water mite (Acariformes, Hydrachnidia, Hygrobatidae) from Italy, based on morphological and molecular evidence

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Received 16 January 2022 | Accepted by V. Pešić: 31 January 2022 | Published online 1 February 2022.

Abstract
In the present study we used an integrative taxonomic approach that combines morphological and DNA barcoding data to describe a new species of the H. nigromaculatus-complex, Hygrobates calabricus sp. nov. from Calabria, Italy. The average K2P-distance between DNA-barcode sequences of H. calabricus sp. nov. and its closest relative H. setosus Besseling, 1942 was estimated at 8.7±1.2%.

Key words: Water mites, DNA-barcoding, species delimitation, new species, running waters.

Introduction
Water mites of the genus Hygrobates Koch, 1837 have been found in all biogeographical regions except Antarctica (Pešić et al. 2017). Members of this genus are often one of the most abundant water mite species in all kind of running and standing waters all over Europe (Pešić et al. 2017). Recent integrative taxonomic studies applying DNA barcodes have revealed that most of the widely distributed European Hygrobates species which were considered to be morphologically well-defined species represent complexes of distinct (semi-cryptic) species that appear to have a more restricted distribution (e.g., Hygrobates nigromaculatus Lebert, 1879 – Martin et al. 2010; H. flaviatilis (Ström, 1768) – Pešić et al. 2017, 2019b; H. longipalpis (Hermann, 1804) – Pešić et al. 2019a; H. longiporus Thor, 1898 – Pešić et al. 2021; H. calliger Piersig, 1896 – Pešić et al. 2021).

The H. nigromaculatus species-complex includes four species: H. nigromaculatus Lebert, 1879 and H. setosus Besseling, 1942, both widely distributed in Northern and Central Europe (see Martin et al. 2010 for an overview) as well as H. lacrima Pešić, 2020 and H. limnocrenicus Pešić, 2020, both originally described from the Western Balkans (Pešić et al. 2020). The record of H. limnocrenicus from Goldersbach
NEW WATER MITE SPECIES FROM ITALY

As a result of an integrative taxonomic approach, we discovered one new species of the *H. nigromaculatus* species-complex from South Italy, which will be described in the present study.

**Material and Methods**

Water mites were collected by hand netting, sorted alive in the field, and immediately preserved in 96% ethanol. Specimens for molecular analysis were examined without dissecting under a compound microscope in ethanol, using a cavity slide with a central depression. Two specimens of the *Hygrobates nigromaculatus* complex from Italy, collected by the junior author, were sent for molecular analysis (see below). After DNA extraction, the specimen vouchers were stored in 96% EtOH and returned to the first author for morphological examination. The voucher of the successfully barcoded specimen was dissected and slide mounted in Faure’s medium (gum arabic (15 g), distilled water (25 ml), glycerine (10 ml) and chloral hydrate (25 g)), while the second specimen was transferred to Koenike’s fluid.

Morphological nomenclature follows Pešić et al. (2017; for explanations concerning morphology and measurements of *Hygrobates* species see there Figs. 1B-D). The holotype of the new species will be deposited in Naturalis Biodiversity Center in Leiden (RMNH).

All measurements are given in µm. The genital acetabula were measured on both sides, and therefore their dimensions were given as a range. The following abbreviations are used: Ac-1 to -3 = acetabula (numbered from anterior to posterior); Cx-I to -IV = coxae (numbered from anterior to posterior); dL = dorsal length; H = height; I-L-4-6 = fourth to sixth segments of first leg; L = length; mL = median length; P-1 to -5 = palp segments 1 to 5; W = width.

**Molecular analysis**

Molecular analyses was conducted at the Canadian Centre for DNA Barcoding (Guelph, Ontario, Canada; (CCDB: [http://ccdb.ca/](http://ccdb.ca/)). In the latter institution the specimens were sequenced for the barcode region of COI using standard invertebrate DNA extraction, amplification and sequencing protocols (Ivanova et al. 2007, Ivanova and Grainger 2007a, b).

For DNA-barcoding and phylogenetic analysis we used also previously published COI sequence data from Martin et al. (2010) and Pešić et al. (2020). In total, we used 59 sequences representing COI haplotypes of *Hygrobates lacrima* (1), *H. limnocrenicus* (4), *H. setosus* (n=39), *H. nigromaculatus* (n=14) and *H. calabricus* sp. nov. (n=1), with *H. longipalpis* and *H. prosiliens* as outgroup taxa (Table 1).

Sequence comparisons were performed using MUSCLE alignment (Edgar 2004). Intra- and interspecific genetic distances were calculated based on the Kimura 2-parameter model (K2P; Kimura 1980), using MEGA-X (Kumar et al. 2018). MEGAX software was used to calculate Neighbour-Joining (NJ) trees based on K2P distances (standard for barcoding studies) and pairwise deletion of missing data. The support for tree branches was calculated by the nonparametric bootstrap method (Felsenstein 1985) with 1000 replicates and shown next to the branches. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair.

In order to assess the genetic differentiation of species we used the online ASAP version ([https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html](https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html)) with default settings and the K2P distance model. The latter procedure was designated to a list of partitions of species hypotheses using genetic distances, calculated between DNA sequences and ranked by their ASAP-scores: the lower the score, the better the partition (Puillandre et al. 2021).

**Results**

**Species delimitation using DNA-barcodes**

The final alignment for species delimitation using COI sequence data comprised 670 nucleotide positions (nps) for 59 specimens of the *H. nigromaculatus*-complex as well as one *H. longipalpis* and one *H. prosiliens* specimen as outgroups (Tab. 1). The nucleotide sequences could be translated into amino acid
sequences without any stop codons. Neighbour-Joining (NJ) analysis clustered the COI sequences of the *H. nigromaculatus*-complex into five strongly supported clades (Fig. 1). The sequence representing *H. calabricus* sp. nov. is reconstructed as a sister branch of the clade grouping COI sequences found in *H. setosus*.

Table 1. List of sequenced specimens used in this study.

| Locality (country, name) | Lat/Long         | Voucher code | BOLD Acc. nos. |
|--------------------------|------------------|--------------|----------------|
| **Hygrobates setosus** Besseling, 1942 |                  |              |                |
| Netherlands, Tongerense Beek | 52.33978 N, 5.92983 E | RMNH.ACA.1255 | NLACA118-15 |
| Netherlands, Winterswik: Ratumse Beek | 51.97365 N, 6.81877 E | RMNH.ACA.1257 | NLACA119-15 |
| Netherlands, Winterswik: Ratumse Beek | 51.97365 N, 6.81877 E | RMNH.ACA.1258 | NLACA120-15 |
| Netherlands, Overdinkel: Ruenbergerbeek | 52.24428 N, 7.03811 E | RMNH.ACA.1108 | NLACA077-15 |
| Netherlands, Vaassen: Rode Beek | 52.29181 N, 5.95670 E | RMNH.ACA.467 | NLACA206-15 |
| Netherlands, Vaassen: Rode Beek | 52.29181 N, 5.95670 E | RMNH.ACA.468 | NLACA207-15 |
| Germany, Farver Au | 54.26 N, 10.80 E | PM-Hyd065 | FJ668539.1 |
| Germany, Farver Au | 54.26 N, 10.80 E | PM-Hyd066 | FJ668540.1 |
| Germany, Menhorst | 54.13 N, 10.46 E | PM-Hyd068 | FJ668541.1 |
| Germany, Menhorst | 54.13 N, 10.46 E | PM-Hyd083 | FJ668545.1 |
| Germany, Menhorst | 54.13 N, 10.46 E | PM-Hyd085 | FJ668546.1 |
| Germany, Menhorst | 54.13 N, 10.46 E | PM-Hyd086 | FJ668547.1 |
| Germany, Menhorst | 54.13 N, 10.46 E | PM-Hyd087 | FJ668548.1 |
| Germany, Glasbek | 54.04 N, 0.91 E | PM-Hyd094 | FJ668549.1 |
| Germany, Glasbek | 54.04 N, 0.91 E | PM-Hyd095 | FJ668550.1 |
| Germany, Glasbek | 54.04 N, 0.91 E | PM-Hyd096 | FJ668551.1 |
| Germany, Glasbek | 54.04 N, 0.91 E | PM-Hyd097 | FJ668552.1 |
| Germany, Moorsaalsweide | 54.25 N, 10.53 E | PM-Hyd113 | FJ668564.1 |
| Germany, Moorsaalsweide | 54.25 N, 10.53 E | PM-Hyd114 | FJ668565.1 |
| Germany, Moorsaalsweide | 54.25 N, 10.53 E | PM-Hyd115 | FJ668566.1 |
| Germany, Moorsaalsweide | 54.25 N, 10.53 E | PM-Hyd116 | FJ668567.1 |
| Germany, Moorsaalsweide | 54.25 N, 10.53 E | PM-Hyd117 | FJ668568.1 |
| Germany, Moorsaalsweide | 54.25 N, 10.53 E | PM-Hyd118 | FJ668569.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd119 | FJ668570.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd120 | FJ668571.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd121 | FJ668572.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd122 | FJ668573.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd123 | FJ668574.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd125 | FJ668575.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd126 | FJ668576.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd127 | FJ668577.1 |
| Germany, Engelau | 54.25 N, 10.54 E | PM-Hyd128 | FJ668578.1 |
| Germany, Osterau | 53.93 N, 0.997 E | PM-Hyd129 | FJ668579.1 |
| Germany, Osterau | 53.93 N, 0.997 E | PM-Hyd130 | FJ668580.1 |
| Germany, Osterau | 53.93 N, 0.997 E | PM-Hyd133 | FJ668581.1 |
| Germany, Osterau | 53.93 N, 0.997 E | PM-Hyd134 | FJ668582.1 |
| Germany, Bollingstedter Au | 54.56 N, 0.933 E | PM-Hyd139 | FJ668583.1 |
| Germany, Bollingstedter Au | 54.56 N, 0.933 E | PM-Hyd140 | FJ668584.1 |
| Germany, Bollingstedter Au | 54.56 N, 0.933 E | PM-Hyd141 | FJ668585.1 |

**Hygrobates calabricus** sp. nov.

Italy, Giganti de la Sila 39.3184 N, 16.4596 E CCDB 38392 G01 DCBDJ073-21

..continued on the next page
TABLE 1.

**Hygrobates lacrima** Pešić, 2020
Montenegro, Tara River at Mateševo 42.7897 N, 19.5383 E 27. CG2020_3_C7 DNAEC029-20

**Hygrobates limnocrenicus** Pešić, 2020
Germany, Goldersbach stream 48.5578 N, 9.0498 E CCDB 38392 A10 DCBDJ010-21
North Macedonia, Crni Drim river 41.1856 N, 20.6777 E 19. MEC2019_3.2_C2 DNAEC024-20
Montenegro, Mareza canal 42.479 N, 19.1813 E 13. M19_20_5_E4 DNAEC050-20
Montenegro, spring Vitoja 42.324 N, 19.3637 E CCDB 38560 F11 NOVMC071-21

**Hygrobates nigromaculatus** Lebert, 1879
Netherlands, Utrecht: Maarseveense Plas 52.144 05 N, 5.084 73 E RMNH.ACA.425 NLACA171-15
Netherlands, Utrecht: Maarseveense Plas 52.144 05 N, 5.084 73 E RMNH.ACA.426 NLACA172-15
Netherlands, Utrecht: Maarseveense Plas 52.144 05 N, 5.084 73 E RMNH.ACA.424 NLACA170-15
Germany, Pohlsee 54.23 N, 09.93 E PM-Hyd075 FJ668542.1
Germany, Schoehsee 54.16 N, 10.44 E PM-Hyd080 FJ668543.1
Germany, Schoe VALUES see 54.16 N, 10.44 E PM-Hyd081 FJ668544.1
Germany, Dobersdorfer See 54.31 N, 10.32 E PM-Hyd098 FJ668553.1
Germany, Dobersdorfer See 54.31 N, 10.32 E PM-Hyd099 FJ668554.1
Germany, Dobersdorfer See 54.31 N, 10.32 E PM-Hyd100 FJ668555.1
Germany, Dobersdorfer See 54.31 N, 10.32 E PM-Hyd101 FJ668556.1
Germany, Trammer See 54.17 N, 10.43 E PM-Hyd105 FJ668558.1
Germany, Trammer See 54.17 N, 10.43 E PM-Hyd109 FJ668561.1
Germany, Trammer See 54.17 N, 10.43 E PM-Hyd111 FJ668562.1
Germany, Trammer See 54.17 N, 10.43 E PM-Hyd112 FJ668563.1

OUTGROUPS

**Hygrobates prosiliens** Koenike, 1915
Netherlands, Utrecht: Maarseveense Plas 52.14405 N, 5.08473 E RMNH.ACA.436 NLACA180-15

**Hygrobates longipalpis** (Hermann, 1804)
Netherlands, Vaassen: Rode Beek 52.29181 N, 5.95670 E RMNH.ACA.568 NLACA286-15

The genetic distance between the COI sequence of *H. calabricus* sp. nov. and its closest relative, *H. setosus*, was estimated at 8.7±1.2% K2P. This value is more than fifteen times higher than the mean intraspecific differences in the COI sequence of *H. setosus* (0.52±0.13% K2P; Table 2), which additionally supported the species-status of the new clade from Italy. As only one specimen of the new species could be acquired for use in molecular analyses, intraspecific differences in COI sequence could not be investigated.

**Hygrobates limnocrenicus** was separated from *H. calabricus* sp. nov. with a genetic distance of 12.2±1.5% K2P (Table 2).

Table 2. Estimates of average genetic distance (K2P) (given as distance ± standard error) between clades (lower diagonal) and within each clade (diagonal) of examined species of the *H. nigromaculatus*-complex sequence pairs.

| Species                  | (1)       | (2)       | (3)       | (4)       | (5)       |
|--------------------------|-----------|-----------|-----------|-----------|-----------|
| (1) *H. setosus*         | 0.0052±0.0013 |         |          |          | n/c       |
| (2) *H. nigromaculatus*  | 0.177±0.018  | 0.012±0.0027 |         |          | n/c       |
| (3) *H. limnocrenicus*   | 0.126±0.015  | 0.170±0.017  | 0.0173±0.0038 |         | n/c       |
| (4) *H. lacrima*         | 0.146±0.018  | 0.159±0.017  | 0.134±0.016  | 0.144±0.018 | n/c       |
| (5) *H. calabricus* sp. nov. | 0.087±0.012  | 0.179±0.019  | 0.122±0.015  | 0.144±0.018 | n/c       |

The applied ASAP procedure identified 5 MOTUs (hypothetical species) at the threshold distance of 5.04 % (K2P) which has the best ASAP-score (1.00) within the available molecular data: *H. setosus, H. nigromaculatus, H. lacrima, H. limnocrenicus and H. calabricus* sp. nov.
Systematics

Family Hygrobatidae Koch, 1842

Genus Hygrobates Koch, 1837
Subgenus Hygrobates s.s.

Hygrobates calabricus sp. nov.
http://zoobank.org/urn:lsid:zoobank.org:act:916B3A71-6C13-4F05-A9CA-438122B48E5E
Fig. 2

Material examined — Holotype ♂ (RMNH), sequenced [BOLD Acc No.: DCBDJ073-21; voucher code: CCDB 38392 G01], dissected and slide mounted, Italy, Calabria, Sila, Giganti de la Sila, stream, pool, 39.3184 N, 16.4596 E, 1405 m asl., 22 Aug. 2018, leg. Goldschmidt. Paratype: 1♂, same data as holotype.

Diagnosis (Male) — Anterior margin of male genital field with a less pronounced medial projection, posterior margin without indentation, with a small convexly rounded projection; L of IV-L-6 proximoventral seta 38 μm.

Description: Male — Colour yellowish. Integument finely striated. Posteromedial margin of Cx-I rounded, caudo-lateral apodemes of Cx-I+II slightly developed (Fig. 2A); Cx-IV subtriangular, with a distinct nose-like protruding medial margin. Anterior margin of genital field convex, with a small medial projection, typically with irregular margin of a secondary sclerotization, posterior margin due to the secondary sclerotization without indentation, with a small centrally rounded projection, Ac in triangular arrangement (Fig. 2B). Gnathosoma anteriorly with clearly offset projections. P-2 ventral margin straight, distally forming a right angle, denticles covering distal half of ventral margin; P-3 with denticles covering distal two thirds of ventral margin; P-4 ventral setae on the same level (Figs. 2C-D).
Figure 2. *Hygrobes calabricus* sp. nov., holotype ♂, Giganti de la Sila, Italy: A – gnathosoma, coxal and genital field; B – genital field; C – palp, medial view (P-1 lacking); D – palp, lateral view (P-1 lacking); E – chelicera; F – IV-L-5 and -6 (inset: proximo and medioventral setae of IV-L-6). Scale bars = 100 µm.
Measurements — Idiosoma L 1080; coxal field: L 516; Cx-III W 677; mL of Cx-I + gnathosoma L 392; distance between lateralmost ends of caudo-lateral Cx-II apodemes, 194; genital field L/W 247/288, ratio 0.86; L Ac 1-3: 75-81, 72-78, 84-91.

Chelicera total L 402, L basal segment 266, claw 141, L basal segment/claw ratio 1.9. Palp: total L 588; dL/H, dL/H ratio: P-1, 67/31, 2.1; P-2, 151/89, 1.69; P-3, 113/77, 1.47; P-4, 189/46, 4.1 (basal H 41, dL/basal H ratio 4.7); P-5, 68/27, 2.6; P-2/P-4 ratio 0.6.

Legs: dL of I-L-1-6: 84, 115, 159, 219, 241, 234. dL of IV-L-1-6: 160, 177, 272, 378, 381, 344; L of IV-L-6 proximoventral seta 38, L of IV-L-6 medioventral seta 15.

Female — Unknown.

Etymology — Named after the (so far exclusive) occurrence of the species in Calabria (Italy).

Remarks — The phylogenetic analysis based on COI data placed Hygrobates calabricus sp. nov. as a sister clade to H. setosus, a species with a preference for running water habitats as well. The average K2P genetic distance between these two species was estimated to be 8.7±1.2% K2P indicating their genetic isolation. The results of ASAP analysis strongly supported the species status of the Hygrobates specimens collected in Calabria.

Morphologically, the male of H. calabricus sp. nov. differs from H. setosus in the posterior margin of the genital field without indentation (posterior margin indented in H. setosus) and a longer proximoventral seta on IV-L-6 (30 µm in H. setosus). From H. limnocrenicus (in parentheses) the new species differs in a narrower genital field (L/W ratio 0.8), smaller acetabula (Ac-3 >110 µm) and posterior margin of the genital field without indentation (vs. indented in H. limnocrenicus).

Distribution: Italy (so far only known from one stream in South Italy (Calabria, Fig. 3).
Acknowledgements

This study is part of the “DNA-Eco” scientific project supported by a grant of the Montenegrin Ministry of Science.

References

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high 679 throughput. *Nucleic acids research.*, 32(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39, 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Ivanova, N. V., de Waard, J. R. & Hebert, P. D. N. (2007) CCDB protocols, glass fiber plate DNA extraction. Available from: ccdb.ca/site/wp-content/uploads/2016/09/CCDB_DNA_Extraction.pdf (Accessed 20 Dec. 2021)

Ivanova, N. V. & Grainger, C. M. (2007a) CCDB protocols, COI amplification. Available from: ccdb.ca/site/wp-content/uploads/2016/09/CCDB_Amplification.pdf (Accessed 20 Dec. 2021)

Ivanova, N. V. & Grainger, C. M. (2007b) CCDB protocols, sequencing. Available from: ccdb.ca/site/wp-content/uploads/2016/09/CCDB_Sequencing.pdf (Accessed 20 Dec. 2021)

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16,111–120.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549.

Martin, P., Dabert, M. & Dabert, J. (2010) Molecular evidence for species separation in the water mite *Hygrobatides nigromaculatus* Lebert, 1879 (Acari, Hydrachnidia): evolutionary consequences of the loss of larval parasitism. *Aquatic Science*, 72, 347–360. https://doi.org/0.1007/s00027-010-0135-x

Pešić, V., Asadi, M., Cimpean, M., Dabert, M., Esen, Y., Gerecke, R., Martin, P., Savić, A., Smit, H. & Stur, E. (2017) Six species in one: Evidence of cryptic speciation in the *Hygrobatides fluviatilis* complex (Acariformes, Hydrachnidia, Hygrobatidae). *Systematic & Applied Acarology*, 22, 1327–1377. https://doi.org/10.11158/saa.22.9.4

Pešić, V., Broda, Ł., Dabert, M., Gerecke, R., Martin, P. & Smit, H. (2019a) Re-established after hundred years: Definition of *Hygrobatides prosiliens* Koeneke, 1915, based on molecular and morphological evidence, and redescription of *H. longipalpis* (Hermann, 1804) (Acariformes, Hydrachnidia, Hygrobatidae). *Systematic & Applied Acarology*, 24(8), 1490–1511. https://doi.org/10.11158/saa.24.8.10

Pešić, V., Saboori, A., Zawal, A. & Dabert, M. (2019b) Hidden but not enough: DNA barcodes reveal two new species in *Hygrobatides fluviatilis* complex from Iran (Acariformes, Hydrachnidia, Hygrobatidae). *Systematic & Applied Acarology*, 24(12), 2439–2459. http://doi.org/10.11158/saa.24.12.11

Pešić, V., Jovanović, M., Manović, A., Zawal, A., Bańkowska, A., Broda, Ł., Martin, P. & Dabert, M. (2020) Two new species from the *Hygrobatides nigromaculatus*-complex (Acariformes, Hydrachnidia, Hygrobatidae), based on morphological and molecular evidence. *Acarologia*, 60(4), 753–768. https://doi.org/10.24349/acarologia/20204400

Pešić, V., Jovanović, M., Manović, A., Karauzas, I. & Smit, H. (2021) New records of water mites from the Balkans revealed by DNA barcoding (Acari, Hydrachnidia). *Ecologica Montenegrina*, 49, 20–34. http://dx.doi.org/10.37828/em.2021.49.2

Puillandre, N., Brouillet, S. & Achaz, G. (2021) ASAP: assemble species by automatic partitioning. *Molecular Ecology Resources*, 21 (2), 609–620. https://doi.org/10.1111/1755-0998