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An appraisal of the water mite genus *Hygrobates* Koch, 1837 from Türkiye, based on morphological and molecular data (Acariformes, Hydrachnidia, Hygrobatidae)

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Abstract

Water mites of the genus *Hygrobates* Koch, 1837 are one of the most abundant water mite species in all kinds of running and standing waters in Türkiye. In this study, DNA barcodes were recovered from 26 specimens, morphologically assigned to five *Hygrobates* species. One species of the *H. trigonicus* species-complex (*H. crypticus* sp. nov.) from Eastern Türkiye, is described as new for science. Two species, *Hygrobates thori* and *H. anatolicus* were represented by two BINs. The first COI sequences is provided for *Hygrobates anatolicus* Esen & Pešić, 2013. The current diversity of the genus *Hygrobates* in Türkiye is discussed based on molecular and morphological data.

Keywords: Water mites, DNA-barcoding, new species, taxonomy

Introduction

Water mites of the genus *Hygrobates* Koch, 1837 are among the most common water mites in different types of running and standing waters all over the Palaearctic (Pešić *et al.* 2017, 2020a). In Türkiye the genus is relatively well studied and most of the species known to inhabit Europe and the Middle East have been reported in a number of publications on Turkish water mites (e.g., Özkan 1982; Özkan *et al.* 1996; Erman & Özkan 2000; Aşçı *et al.* 2006–2007; Erman *et al.* 2006, 2008, 2010; Boyacı & Özkan 2007; Pešić *et al.* 2007, Esen *et al.* 2013, 2014). The aforementioned publications which were based exclusively on morphological identifications, registered 12 species of the genus *Hygrobates* in Türkiye, two species (i.e., *H. quanaticola* Schwoerbel & Sepasgozarian, 1976, and *H. diversiporus* Sokolow, 1927) of the polyacetabulate subgenus *Rivobates*, and ten species of the nominate subgenus, i.e., *H. anatolicus* Esen & Pešić, 2013, *H. angustipalpis* K.O. Viets, 1982, *H. bucharicus* Sokolow, 1928, *H. calliger* Piersig, 1896, *H. fluviatilis* (Strøm, 1768), *H.*

longipalpis (Hermann, 1804), *H. longiporus* Thor, 1898, *H. nigromaculatus* Lebert, 1879, *H. porrectus* Koenike, 1908, and *H. trigonicus* Koenike, 1895.

In the recent years, molecular approaches to taxonomy by using the DNA barcode technique has led to the detecting of distinct lineages among most of the known *Hygrobates* morphospecies previously often considered to have a wide distribution (e.g., *Hygrobates anatolicus* Esen & Pešić, 2013; Martin *et al.* 2010; Pešić *et al.* 2017, 2019a, b, 2020a, b, 2021). As a result of the integrative taxonomy of some widespread *Hygrobates* species (see under Discussion for an overview), several new species (e.g., *H. turcicus* Pešić, Esen & Dabert, 2017, *H. persicus* Pešić & Asadi, 2017, *H. thori* Pešić & Smit, 2022, *H. neotrigonicus* Pešić & Esen, 2022, *H. ponticus* Pešić, Esen & Mumladze, 2022) have been added to the fauna of Türkiye, challenging our current knowledge of this genus and enhancing the need of including an increased number of areas sampled and a number of individuals in further molecular analysis.

This paper aims to expand the existing COI (cytochrome c oxidase subunit I) dataset of water mites inhabiting Türkiye (available at dx.doi.org/10.5883/DS-TRIRHYD) by new sequences of the genus *Hygrobates* generated in this study, and to verify the genetic identity of the local population of widespread species in reference to those available in the publicly accessible DNA-barcode reference libraries.

Material and Methods

Water mites were collected by hand netting, sorted live in the field, and immediately preserved in 96% ethanol for further molecular analyses (see below). After DNA extraction, the specimen vouchers were stored in 96% EtOH 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 and stored in the collection of the first author. DNA sequences prepared in the course of this study were deposited in BOLD (The Barcode of Life Data System) and GenBank (see Table 1). The DNA extracts were archived in -80°C freezers at the Centre for Biodiversity Genomics (CBG; biodiversitygenomics.net).

Morphological nomenclature follows Gerecke *et al.* (2016). For explanations concerning morphology and measurements of *Hygrobates* species see Figs. 1B-D in Pešić *et al.* (2017). The genital acetabula in both sexes and the genital plate in the female were measured on both sides, therefore their dimensions are given as a range. The holotype and paratypes of the new species will be deposited in the Naturalis Biodiversity Center in Leiden (RMNH).

All measurements are given in μm . The following abbreviations are used: Ac-1=most anterior acetabulum; Cx-I–IV=first to fourth coxae; dL=dorsal length; H=height; I-L-1–6=first to sixth segments of first leg; L=length; mL=median length; P-1–5=palp segments 1 to 5; W=width.

Molecular and DNA barcode analyses

The molecular analysis was conducted at the Canadian Centre for DNA Barcoding (Guelph, Ontario, Canada (CCDB; <http://ccdb.ca/>)). The specimens were sequenced for the barcode region of COI using standard invertebrate DNA extraction (Ivanova *et al.* 2007), amplification (Ivanova and Grainger 2007a), and sequencing protocols (Ivanova & Grainger 2007b). DNA was extracted from 26 specimens of the genus *Hygrobates* listed in Table 1. For all other species, COI sequence data were taken from Pešić *et al.* (2019b, 2021) and Pešić & Esen (2022), and downloaded from the respective sequence data archives.

TABLE 1. List of newly sequenced specimens in this study.

Locality (country, name)	Lat/Long	Voucher code	BOLD/Genbank Acc. nos.	BOLD BIN
<i>Hygrobatas crypticus</i> sp. nov.				
Bingöl Province, Cicekdere stream	38.9142°N, 40.4689°E	CCDB 41823 E01	HYDME049-22/ OQ320816	
Bingöl Province, Cevrîmpinar stream	38.9092°N, 40.4744°E	CCDB 41823 E02	HYDME050-22/OP620945	
Bingöl Province, Capakcur stream	38.8936°N, 40.4772°E	CCDB 41823 E05	HYDME053-22/OP620942	BOLD:AET8584
		CCDB 41823 E07	HYDME055-22/OP620940	
		CCDB 41823 E08	HYDME056-22/OP620939	
Bingöl Province, Gens District, stream in Geyikdere Village	38.6533°N, 40.8581°E	CCDB 41823 D11	HYDME047-22/OQ413515	
		CCDB 41823 D12	HYDME048-22/OQ413516	
<i>Hygrobatas longipalpis</i> (Hermann, 1804)				
Isparta Province, Aksu District, Pazarköy, Yılanlı ponds	37.7667°N, 30.999°E	CCDB_44300_E01	HYDOC049-22/OQ320830	
		CCDB_44300_E02	HYDOC050-22/OQ320829	
		CCDB_44300_E03	HYDOC051-22/OQ320828	
		CCDB_44300_E04	HYDOC052-22/OQ320821	BOLD:AES0232
		CCDB_44300_E05	HYDOC053-22/OQ320813	
<i>Hygrobatas anatolicus</i> Esen & Pešić, 2013				
Diyarbakir Province, Kulp District, Soravang Stream	38.5825°N, 41.1228°E	CCDB_44300_C12	HYDOC036-22/OQ320815	
Bingöl Province, Gens District, stream in Geyikdere Village	38.6533°N, 40.8581°E	CCDB_44300_D08	HYDOC044-22/OQ320817	
		CCDB_44300_D10	HYDOC046-22/OQ320823	BOLD:AEY8569
		CCDB_44300_D11	HYDOC047-22/OQ320818	
Diyarbakir Province, Kulp District, Soravang Stream	38.5825°N, 41.1228°E	CCDB_44300_D01	HYDOC037-22/OQ320824	
Bingöl Province, Gens District, stream in Geyikdere Village	38.6533°N, 40.8581°E	CCDB_44300_D09	HYDOC045-22/OQ320814	BOLD:AEX2126
		CCDB_44300_D12	HYDOC048-22/OQ320820	
<i>Hygrobatas thori</i> Pešić & Smit, 2022				
Elazığ Province, Karakocan District, Bulgurcuk stream	38.94°N, 40.1256°E	CCDB_44300_D02	HYDOC038-22/OQ320833	
		CCDB_44300_D05	HYDOC041-22/OQ320832	
		CCDB_44300_D06	HYDOC042-22/OQ320831	BOLD:AEL4319
		CCDB_44300_D07	HYDOC043-22/OQ320834	
		CCDB_44300_D03	HYDOC039-22/OQ320825	BOLD:AEK7910
CCDB_44300_D04	HYDOC040-22/OQ320826			
<i>Hygrobatas turcicus</i> Pešić, Esen & Dabert, 2017				
Diyarbakir Province, Kulp District, Soravang Stream	38.5825°N, 41.1228°E	CCDB_44300_C11	HYDOC035-22/OQ320835	BOLD:ADX1364

Sequence comparisons were performed using MUSCLE alignment (Edgar 2004). Intra- and interspecific genetic distances were calculated using the Kimura 2-parameter model (K2P; Kimura 1980), using MEGA X software (Kumar *et al.* 2018). The latter software was used to calculate Maximum Likelihood (ML) trees (model selected by the BIC (Bayesian Information Criterion) implemented in MEGA X: GTR+G+I) with an initial Neighbour-Joining (NJ) tree and using the Subtree-Pruning-Regrafting - Extensive heuristic search (SPR level 5). The support for tree branches was calculated by the nonparametric bootstrap method (Felsenstein 1985) with 500 replicates and shown next to the branches. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair.

We displayed the haplotype relationship within the species through a Minimum Spanning Network using PopART software (Leigh & Bryant 2015). Additionally, the sequence data were analyzed using the Assemble Species by Automatic Partitioning (ASAP), a method designed for species partitioning using a hierarchical clustering algorithm based on the pairwise distance distribution (Puillandre *et al.* 2021). We used the online ASAP version (<https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html>) with default settings and the K2P distance model.

Results

In this study, DNA barcodes of 26 specimens, morphologically assigned to four species of the water mite genus *Hygrobates* were newly generated (Table 1). One species of the *H. trigonicus* species-complex (*H. crypticus* **sp. nov.**) from eastern Türkiye is described as new to science. Two species, *Hygrobates thori*, and *H. anatolicus* were represented by two BINs. The first COI sequences is provided for *Hygrobates anatolicus* Esen & Pešić, 2013.

We conducted Minimum Spanning Network for the species with multiple BINs in our study using ours and publicly available data from BOLD and GenBank (Fig. 1). We found geographical and phylogenetic patterns for each species. In *Hygrobates thori*, BIN AEK7910 contained three haplotypes, and individuals were distributed in Türkiye and Iran. Moreover, BINs AEL4319 (7 individuals) and AEL4320 (1 individual) were restricted to Türkiye with a single haplotype each. Within *Hygrobates anatolicus*, two BINs AEY8569, and AEX2126 were represented among seven analyzed individuals. We found that individuals from both BINs co-occur in two regions of Türkiye: Kulp and Geyikdere. *Hygrobates longipalpis* sequences were assigned to two BINs. Individuals from Türkiye are within BIN AES0232, and spatial representation of BIN ACR9783 is quite complex, as it consists of individuals from Western Europe (the Netherlands, Germany) and the Balkan Peninsula (Romania, Montenegro) (Fig. 1).

Systematics

Family Hygrobatidae Koch, 1842

Genus *Hygrobates* Koch, 1837

Subgenus *Hygrobates* s.s.

Hygrobates (Hygrobates) crypticus Pešić, Esen & Smit **sp. nov.**

Fig. 2–3

Material examined—Holotype ♂ (RMNH), sequenced (voucher code: CCDB 41823 E07), dissected and slide mounted, Türkiye, Bingöl Province, Capakcur stream, 38.8936° N, 40.4772° E, 1147 m asl., 28 May 2021 leg. Esen. Paratypes: 2♂, same data as the holotype, sequenced (Table 1); 1♀, Bingöl Province, Cevrîmpinar stream 38.9092° N, 40.4744° E, 1097 m asl., 15 May 2021, leg. Esen, sequenced (Table 1), dissected and slide mounted (voucher code: CCDB 41823 E02; RMNH); 1♂, Bingöl Province, Cicekdere stream, 38.9142° N, 40.4689° E, leg. Esen, sequenced (Table 1), dissected and slide mounted (voucher code: CCDB 41823 E01; RMNH); 1♂, 1♀, Gens District, stream in Geyikdere village, 38.6533° N, 40.8581° E, 30 August 2022, leg. Esen (sequenced; Table 1)

Diagnosis—*Morphological*: posteromedial margin of Cx-I broadly rounded; ventrodistal margin of P-3 convex; posterior margin of male genital field indented with a large medial projection; Ac-3 subtriangular; in the female pregenital sclerite with 2–3 setae located in the anterior half of the

sclerite, genital plates with an extended border of secondary sclerotization and two setae along the medial margins. *Molecular*: this lineage differs from two European *H. trigonicus* clades from Germany and Greece by 18.1% and 17.0%, respectively, and from lineage from eastern Türkiye (*H. neutrigonicus*) by 19.4% K2P for *COI*.

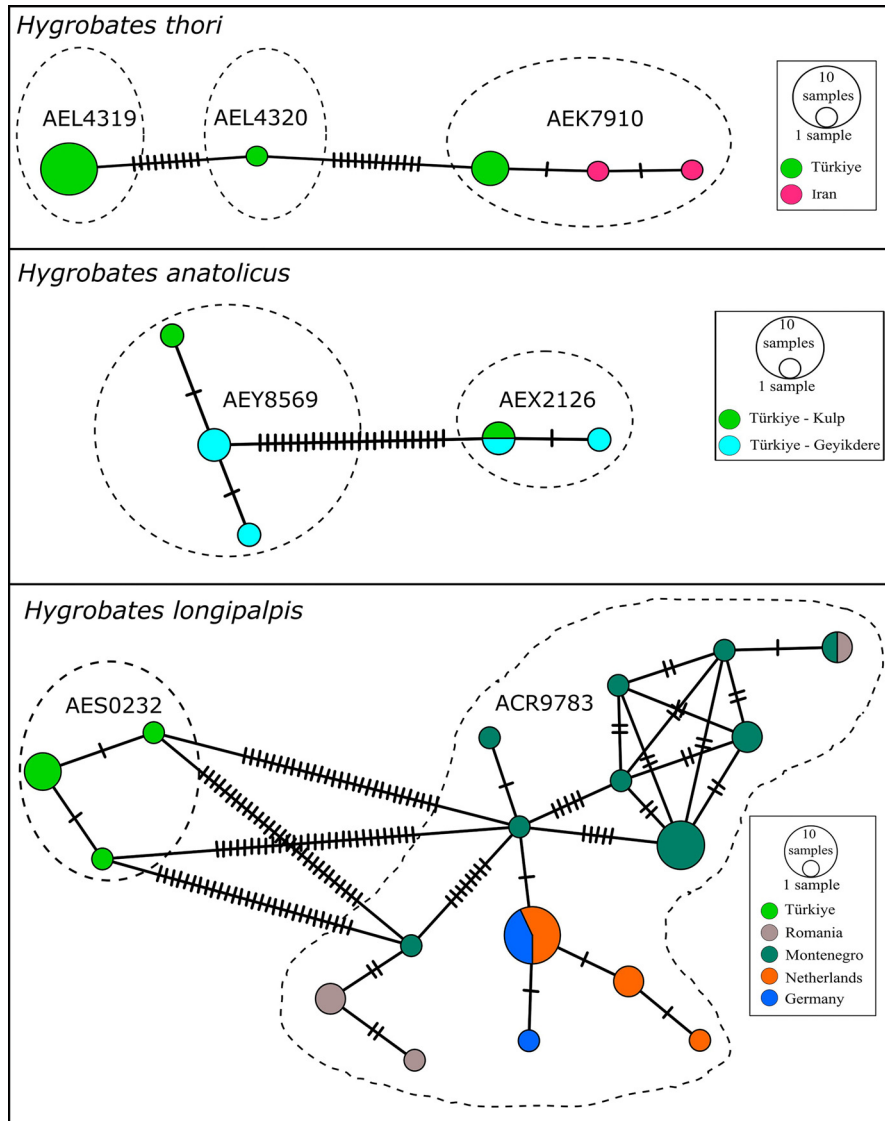


FIGURE 1. Minimum Spanning Network showing phylogenetic relationships within three *Hygrobatas* species: *H. thori* Pešić & Smit, 2021, *H. anatolicus* Esen & Pešić, 2013, and *H. longipalpis* (Hermann, 1804). Each bar represents a single mutational change, and a dashed line encircled by separate BINs. The diameter of the circles is proportional to the number of individuals in each haplotype sampled (see open circles with numbers). The final dataset of *H. thori* includes 13 sequences from Pešić *et al.* (2022a) and this study; dataset of *H. anatolicus* includes seven sequences from this study. For *H. longipalpis*, we used 26 sequences from Pešić *et al.* (2019a), five sequences from this study, and three unpublished sequences from Montenegro (deposited in BOLD and GenBank with accession numbers OQ507472, OQ507473, OQ507474).

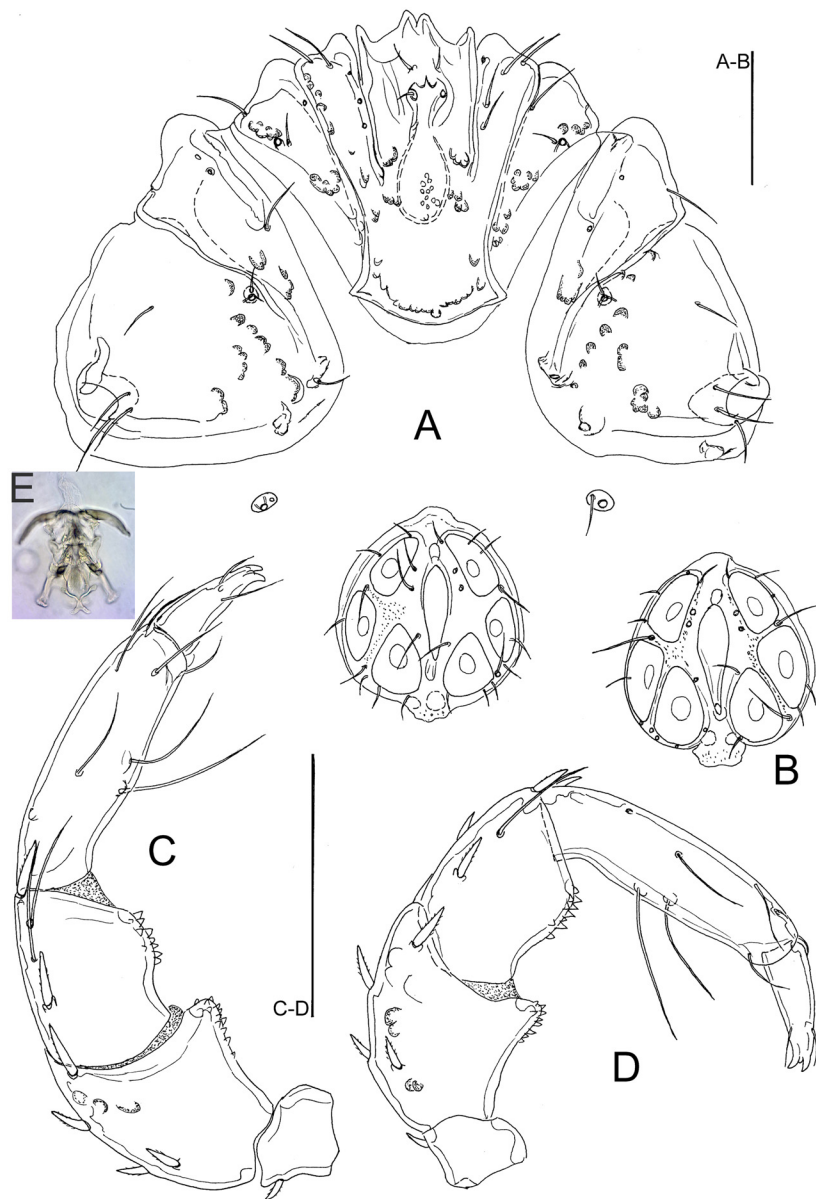


FIGURE 2. *Hygrobates crypticus* sp. nov. (A, C—♂ paratype CCDB 41823 E01; B, D—♂ holotype CCDB 41823 E07). A—coxal and genital field; B—genital field; C—palp, lateral view; D—palp, medial view; E—photograph of ejaculatory complex. Scale bars=100 μ m.

Description—General features—Colour yellow to brown. Integument dorsally striated (due to the chemical treatment during barcoding striation not visible in examined specimens, but on some dorsal patches it can be noticed). Posteromedial margin of Cx-I broadly rounded (Figs. 2A, 3A); Cx-IV subtriangular in shape, with anterior and posterior margins converging to median line (Figs. 2A, 3A). Gnathosoma anteriorly with clearly offset projections. P-2 ventral margin straight, distally forming a slightly knob-shaped projection covered by large, scattered denticles, ventral margin distally convexly slightly protruding, with large, scattered denticles covering distal half of ventral margin; P-4 ventral setae separated (Figs. 2C-D). IV-L-5 with one short distal swimming seta (Fig. 3E). *Male*—Anterior margin of genital field convexly rounded, posterior margin indented with a

large medial projection, Ac in obtuse line, Ac-3 subtriangular (Figs. 2A-B). *Female*—Genital plates sickle-shaped, with strongly concave medial margins, pregenital sclerite with 2-3 setae, genital plates with an extended border of a porose secondary sclerotization (Fig. 3A); P-4 more slender than in male.

Measurements—*Male* (holotype CCDB 41823 E07; in parentheses some measurements of paratype CCDB 41823 E01)—Idiosoma L 719 (800), W 688 (688); coxal field: L 325 (330); Cx-III W 413 (419); mL of Cx-I + gnathosoma L 244 (253); distance between lateralmost ends of caudolateral Cx-II apodemes, 112 (119); genital field L/W 163 (164)/144 (144), ratio 1.13 (1.14); L Ac 1-3: 50-55 (50-53), 63 (50-53), 56-60 (50-53). Ejaculatory complex L 166 (191).

Palp—Total L 341 (346); dL/H, dL/H ratio: P-1, 23/33, 0.71 (27/34, 0.77); P-2, 88/59, 1.48 (92/62, 1.48); P-3, 67/50, 1.34 (67/51, 1.32); P-4, 113/31, 3.59 (113/34, 3.27); P-5, 50/17, 2.91 (47/18, 2.63); P-2/P-4 ratio 0.78 (0.82). Chelicera total L 247, L basal segment 153, claw 91, L basal segment/claw ratio 1.68.

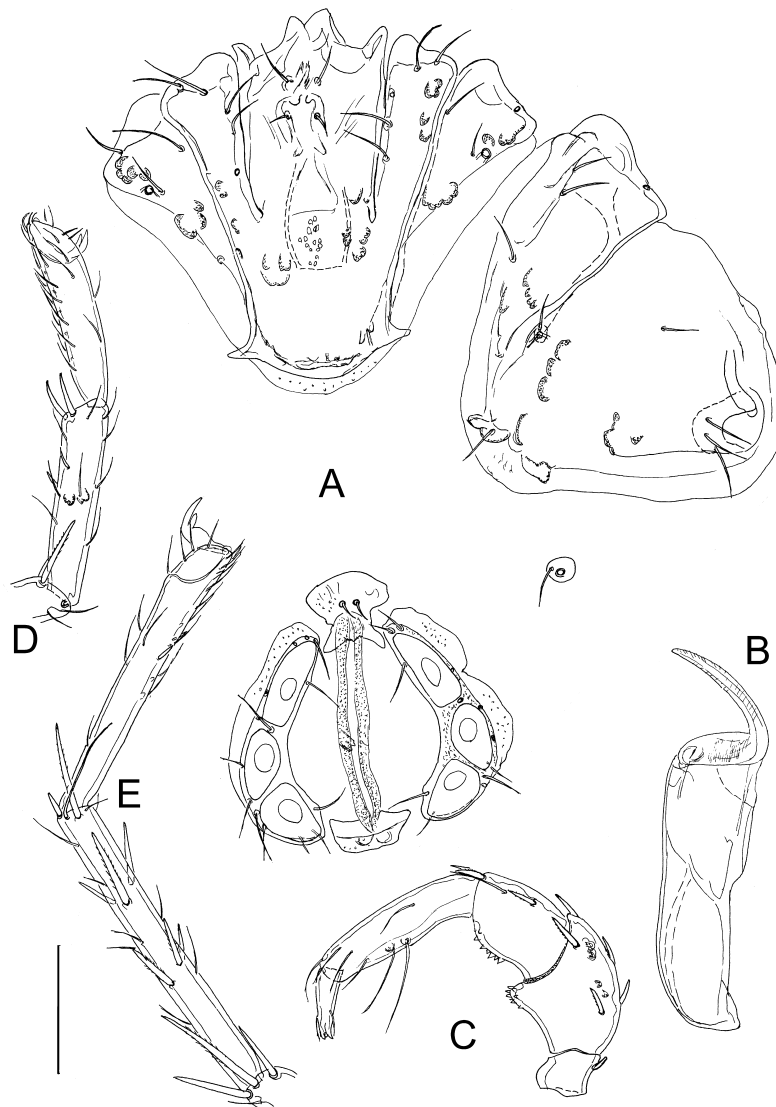


FIGURE 3. *Hygrobates crypticus* sp. nov., ♀ paratype CCDB 41823 E02. A—coxal and genital field (partial view); B—chelicera; C—palp, medial view; D—I-L-5 and 6; E—IV-L-5 and 6. Scale bar=100 µm.

Legs—dL of I-L-1-6: 53 (63), 67 (67), 84 (88), 129 (123), 139 (130), 139 (139). dL of IV-L-1-6: 116 (117), 97 (100), 141 (144), 206 (208), 216 (228), 191 (194).

Female (paratype CCDB 41823 E02)—Idiosoma L 1030, W 935; coxal field: L 384; Cx-III W 519; mL of Cx-I + gnathosoma L 291; distance between lateralmost ends of caudo-lateral Cx-II apodemes, 146; genital field L/W 220/282; genital plates L 172–175; pregenital sclerite W 66; gonopore L 175; L Ac 1-3: 70–72, 56–55, 50.

Palp—Total L 437; dL/H, dL/H ratio: P-1, 34/42, 0.81; P-2, 119/78, 1.52; P-3, 80/64, 1.25; P-4, 145/39, 3.69; P-5, 59/20, 3.1; P-2/P-4 ratio 0.82. Chelicera total L 305, L basal segment 178, claw 111, L basal segment/claw ratio 1.62.

Legs—dL of I-L-2-6: 78, 109, 164, 163, 163. dL of IV-L-1-6: 139, 125, 189, 256, 273, 244.

Etymology—From the Greek *krypto* meaning hide, in reference to possible confusion with similar and partly sympatric *H. neotrigonicus*, which is distinguishable by subtle morphological characteristics.

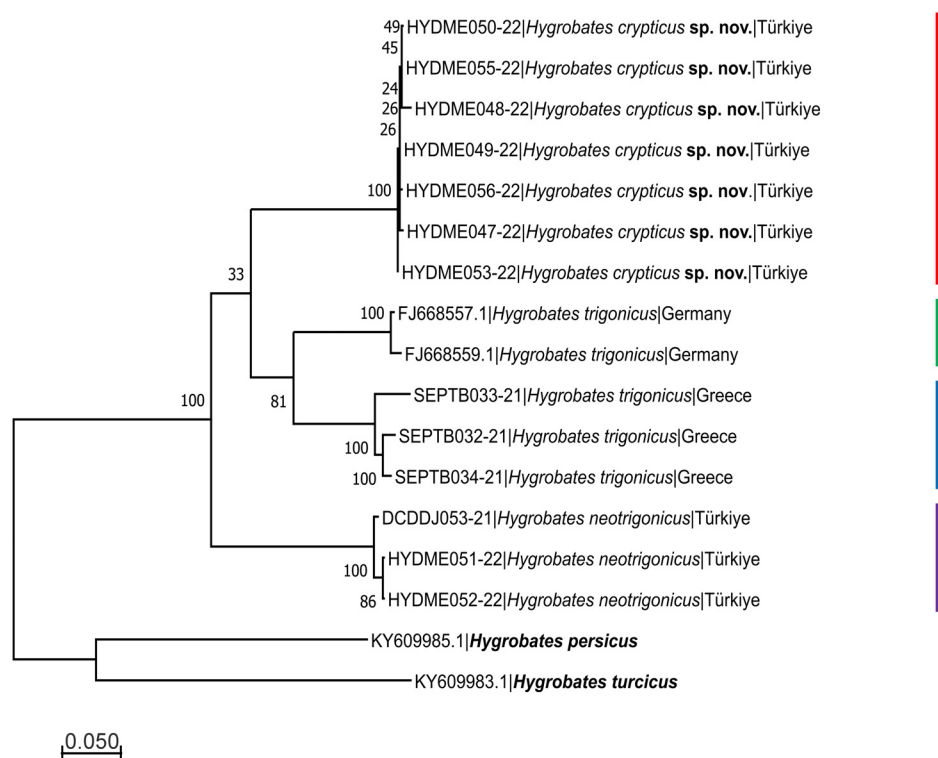


FIGURE 4. Maximum Likelihood tree (GTR+G+I model) of the *Hygrobatas trigonicus*-complex obtained from 15 nucleotide COI sequences. Outgroups are given in bold. The results of species delimitation by ASAP procedure are indicated by vertical bars.

Species delimitation using DNA-barcodes—The final alignment for species delimitation using COI sequence data comprised 662 nucleotide positions (nps) for 15 specimens of the *Hygrobatas trigonicus*-complex taken from Pešić & Esen (2022) and this study, and one specimen each of *H. prosiliens* and *H. longipalpis* as outgroups. The nucleotide sequences could be translated into amino acid sequences without any stop codons. ML analyses clustered COI sequences of analysed specimens into four maximally supported clades, three of which corresponded to the previously known members of the *H. trigonicus*-complex in Pešić & Esen (2022). The clade containing sequences of specimens from Eastern Türkiye used in this study was placed (albeit with

low support) as a sister to clade grouping sequences of two European clades from Germany and Greece, respectively (Fig. 4).

The mean genetic distance between COI sequences ranged from 13.6±1.6% between two European *H. trigonicus* clades (Germany-Clade and Greece-Clade) to 19.5±20% K2P between new species from eastern Türkiye and *H. trigonicus* Germany-Clade. These genetic distances were higher than the barcoding gap found by the ASAP method (5 to 11%) in the genetic distances among all species belonging to the *H. trigonicus*-complex (Fig. 5), which additionally supported the species status of the new species. The mean intraspecific distance within clades was relatively low, ranging from 0.29±0.12% (in *H. crypticus* sp. nov.) to 3.3±0.63% K2P in *H. trigonicus* Greece-Clade (Table 2).

Discussion—Morphologically, the new species described in this study most closely resembles to *H. neotrigonicus* Pešić & Esen, 2022, which is partially sympatric in eastern Türkiye, with syntopic populations in some locations (Capakur and Cevrimpinar streams). From the latter species, the new species can be distinguished by the ventrodiscal margin of P-3 being somewhat convexly protruding, and the posteromedial margin of Cx-I comparatively more rounded in both sexes; in the female, a small difference is found in the shape of a genital field with the postgenital sclerite bearing 2-3 setae located in the anterior part of the sclerite (four setae in the posterior part of the sclerite in *H. neotrigonicus*, see Pešić & Esen 2022, Fig. 3C), and the genital plates with an extended border of secondary sclerotization and a smaller number of setae on the medial margins. The level of COI differentiation between these two species of the *H. trigonicus* complex from eastern Türkiye was estimated to be 19.4±2.0% K2P, thus confirming the validity of the new species, also proved by the results of the ASAP procedure.

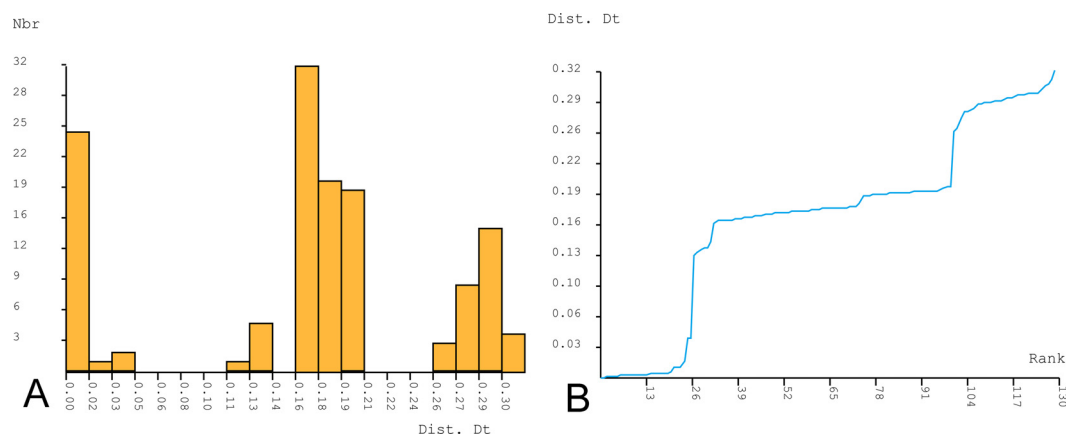


FIGURE 5. Results of ASAP analysis for COI sequences of the *Hygrobatas trigonicus*-complex. (A) Distribution of pairwise differences, (B) Ranked pairwise differences.

TABLE 2. Estimates of average genetic distance (K2P) within and between clades examined species of the *H. trigonicus*-complex sequence pairs in and between COI haplogroups are shown. Standard error estimates are shown above the diagonal.

	Intragroup	Intergroup			
		(1)	(2)	(3)	(4)
(1) <i>H. crypticus</i> sp. nov.	0.0029±0.0012		0.020	0.020	0.018
(2) <i>H. neotrigonicus</i>	0.0085±0.0031	0.194		0.022	0.019
(3) <i>H. trigonicus</i> Germany-Clade	0.0101±0.0045	0.181	0.195		0.016
(4) <i>H. trigonicus</i> Greece-Clade	0.0334±0.0063	0.170	0.171	0.136	

Phylogenetic analysis reveals that the new species is a sister species of the two other European clades of *H. trigonicus*. The taxonomic status of the European clades still remains unclear, and as emphasized by Pešić and Esen (2022) should be clarified by resolving the taxonomic status of *H. properus* Láska, 1954, a junior synonym of *H. trigonicus*, originally described on the basis of a single female from the Orava River and its tributaries in Slovakia (Láska 1954). Pešić & Esen (2022), who examined *H. trigonicus* specimens from Germany, which probably should be assigned to the nominal lineage, state that they can be distinguished by a less triangular (and more roundish) Ac-3 in both sexes. In specimens of *H. trigonicus* from Greece, the third acetabulum is more elongated, as in the species from eastern Türkiye, but they differ in the posterior margin of the male genital field, which is convex due to secondary sclerotization and without indentation (Pešić & Esen 2022).

Distribution—Known from four streams in Bingöl Province (Eastern Türkiye).

Discussion

Molecular studies conducted during the last years using DNA barcodes revealed the presence of a high degree of cryptic or pseudo-cryptic diversity within most of *Hygrobates* morphospecies in the Western Palearctic (Martin *et al.* 2010; Pešić *et al.* 2017, 2019a, 2021, 2022b; Pešić & Esen 2022), challenging the taxonomic status of species of the latter genus which have been reported for Türkiye. The available molecular data allow a critical reassessment of the current diversity of the genus *Hygrobates* in Türkiye, showing that some species frequently appeared in the faunistic list of Turkish mites such as *H. fluviatilis*, *H. calliger*, and *H. trigonicus* should be removed from the list of species occurring in Türkiye. For example, molecular studies of populations attributed to *H. fluviatilis* (Strøm, 1768), a species often reported to occur in Türkiye revealed the presence of two distinct lineages, corresponding to *H. persicus* Pešić & Asadi, 2007 and *H. turcicus* Pešić, Esen & Dabert, 2007 respectively (Pešić *et al.* 2017).

A recent study performed by Pešić *et al.* (2022a) showed that populations of *H. longiporus* Thor, 1898 morphotype in Türkiye also belong to two distinct clades, one comprising specimens from southwestern Türkiye and the Balkan and another comprising specimens from northern Iran and eastern Türkiye, described as *H. thori* Pešić & Smit, 2022. In this study, we report a new record of this species from the Bulgurcuk stream in Elazığ province. In the analysed material *H. thori* was represented by six barcoded individuals assigned to two BINs, AEL4319 (4 individuals) restricted to Türkiye and AEK7910 (2 specimens) distributed in Türkiye and Iran.

Recently, integrative studies conducted on populations attributed to *H. calliger* Piersig, 1896 and *H. trigonicus* Koenike, 1895, revealed the presence of different lineages originating in Türkiye (Pešić *et al.* 2022b; Pešić & Esen 2022). Both species are considered to have a wide distribution in the Western Palearctic (Gerecke *et al.* 2016). A recent study conducted by Pešić *et al.* (2022b) found that the *H. calliger*-complex in the Western Palearctic includes at least eight distinct lineages, one of which consists of specimens from eastern Türkiye and Georgia described as *H. ponticus* Pešić, Esen & Mumladze, 2022.

Hygrobates trigonicus is another species previously registered for Türkiye based on morphological evidence (e.g., Erman & Özkan 2000; Esen *et al.* 2014). However, Pešić and Esen (2022) recently showed that populations of specimens similar to *H. trigonicus* from eastern Türkiye represent a different lineage, described as *H. neotrigonicus* Pešić & Esen, 2022. The use of DNA barcodes allowed us to define in this study another species within the *H. trigonicus* complex, *H. crypticus* **sp. nov.** The last species is partially sympatric with *H. neotrigonicus*, with locally syntopic populations. Morphologically, these two species are very close to each other, and we can consider

them functionally cryptic species, with a high genetic distance of 19.4% K2P, indicating their possible long independent history.

An extensive assessment of morphological variability reveals that most of the characters used to distinguish species within the *H. trigonicus* complex (also in *H. fluviatilis* and *H. calliger* complexes, respectively, see Pešić *et al.* 2017, 2022b) show significant variations in the population level and are probably not suitable for providing reliable discrimination of different COI lineages. Given that morphological species delimitation can be erroneous, we suggest that the diagnosis of new species be extended by both morphological and molecular (COI) data for species delimitation, as was done in this study for *H. crypticus* **sp. nov.**

Among our results, for two species, *H. anatolicus* Esen & Pešić, 2013 and *H. thori*, the first COI data for Türkiye are given. *Hygrobates anatolicus* was originally described from a stream in Kahramanmaraş Province in eastern Türkiye (Esen *et al.* 2013). In regard to the combination of *H. nigromaculatus*-like palp (with a straight ventral margin of P-2, distally forming a right angle and a coarse lineated soft integument, *H. anatolicus* differs from all other species of the genus (Esen *et al.* 2013).

Hygrobates longipalpis (Hermann, 1804) is frequently recorded from Türkiye, both from stagnant and slow-flowing waters (Özkan 1982; Özkan *et al.* 1996; Boyacı & Özkan 2007; Aşçı *et al.* 2006–2007; Erman *et al.* 2008; Esen *et al.* 2014). A molecular study by Pešić *et al.* (2019a) of European populations of *H. longipalpis* revealed the presence of two distinct lineages, with *H. prosiliens* Koenike, 1915 occurring in standing water and *H. longipalpis* preferring slow-flowing sectors of running waters. Therefore, most records of *H. longipalpis* in Türkiye require confirmation due to the possible confusion with *H. prosiliens*, but *H. longipalpis* is likely a widespread species. In the analyzed material, *H. longipalpis* is represented by five barcoded individuals that form BIN:AES0232 which includes also one private sequence of the latter species from North Macedonia. The BIN from Türkiye differs from BIN:ACR9783, already known from different parts of Europe (Germany, the Netherlands, Romania, Montenegro) by 6.3±0.95% K2P distance. Such spatial segregation may reflect the complex evolutionary history of *H. longipalpis* in the Palearctic.

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