Re-established after hundred years: Definition of Hygrobates prosiliens Koenike, 1915, based on molecular and morphological evidence, and redescription of H. longipalpis (Hermann, 1804) (Acariformes, Hydrachnidia, Hygrobatidae)

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Re-established after hundred years: Definition of *Hygrobates prosiliens* Koenike, 1915, based on molecular and morphological evidence, and redescription of *H. longipalpis* (Hermann, 1804) (Acariformes, Hydrachnidia, Hygrobatidae)

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Abstract

DNA-barcodes reveal that European populations attributed to *Hygrobates longipalpis* (Hermann, 1804), thus far considered a common inhabitant of standing and slowly flowing running waters all over the Western Palaearctic, represent two distinct lineages, both widely distributed over the continent. They are differentiated also from an ecological point of view, with specimens from standing waters ("clade I") separated from specimens collected in slowly running waters ("clade II"). Morphological examination revealed that, based on the length ratio of proximo- and distomedial setae on terminal segments of fourth legs, two morphospecies correspond to these two clades. As molecular examination of a specimen from the type locality of *H. prosiliens* Koenike, 1915 (Bremen, Heiligenrode) shows that it belongs to "clade I", populations from standing waters are attributed to that species. In view of the sketchy original description, loss of holotype and insufficient locality data of *H. longipalpis*, we arbitrarily assign to it stream-dwelling populations of "clade II" and designate a neotype.

Keywords: Europe, DNA-barcoding, species delimitation, standing waters, running waters, water mites

Introduction

In Central Europe, there are at least 745 species of water mites (Acari: Hydrachnidia) which inhabit almost all types of freshwater habitats (Gerecke *et al.* 2016). Despite recent studies revealing that water mites are well suited for assessing environmental change (Van der Hammen & Smit 1996; Gerecke & Lehmann 2005; Goldschmidt 2016; Smit 2018; Pešić *et al.* 2019), they are not commonly monitored in Europe in the sense of the water framework directive.

Difficulties in identification of water mites were always addressed as the main obstacle in including water mites in assessment of freshwater habitats. However, recent progress has widely overcome this problem by (1) publication of three Hydrachnidia volumes in the series "Süßwasserfauna von Mitteleuropa" (Davids *et al*. 2007; Di Sabatino *et al*. 2010; Gerecke *et al*.

2016) which facilitate morphological identification in this group, and (2) increasing possibilities to identify water mites using molecular tools. As shown by several integrative taxonomic studies (e.g., Martin *et al*. 2010; Stålstedt *et al*. 2013; Pešić & Smit 2016, 2017; Fisher *et al*. 2017; Pešić *et al*. 2012, 2017) applying DNA barcodes has the potential to improve the quality and effectiveness of ecological assessment by detecting cryptic species.

Water mites of the genus *Hygrobates* have been recorded from all biogeographical regions except Antarctica. Pešić *et al.* (2017) stated that a high degree of cryptic or pseudo-cryptic diversity is expected within described *Hygrobates* species. For instance, molecular studies of populations attributed to *Hygrobates fluviatilis* in traditional taxonomy, have shown that this is a species complex including six distinct lineages (Pešić *et al*. 2017). Revision of this species complex, was mostly hampered by (1) type material being lost or in too poor state for testing the validity of old names, and (2) original descriptions in too scanty quality as to allow any interpretation of the morphological characters separating the lineages established with the help of molecular tools. In view of insufficient locality data, and/or potential faunal change, additional sampling at the type localities will often be of limited help for clearing problems in nomenclature (see Pešić *et al*. 2017 for further discussion).

Up to now, *Hygrobates longipalpis* has been considered a species with a Holarctic distribution. Data from all over Europe suggested that it inhabits both standing and running waters, in the latter preferably pool sectors (Gerecke *et al*. 2016).

Jean-Frédéric Hermann (1804) described *Hygrobates longipalpis* under the name of "*Hydrarachna longipalpis*". The simple description included an illustration showing a palp with second segment bearing a ventral projection and slender distal segments (Plate IX, P, in Hermann 1804). Since then, the following 15 species were described and later proposed to represent junior synonyms of *H. longipalpis* (see Piersig 1897–1900 and K. Viets 1956): *Nesaea scapularis* Koch,1836; *Hygrobates rufifrons* Koch, 1837; *H. galbinus* Koch 1837; *H. rotundatus* Koch, 1837; *Atractides setiger* Koch, 1837; *H. iconicus* Koch, 1837; *H. varians* Koch, 1837; *H. lutescens* Koch, 1841; *H. o-nigrum* Koch, 1841; *H. v-brunneum* Koch, 1841; *Nesaea dentata* Kramer, 1875; *H. impressus* Neuman, 1880; *H. hemisphaericus* Soar, 1896; *H. prosiliens* Koenike, 1915, and *H. ruber* Marshall, 1926. As *H. longipalpis* itself, most of these species are no more represented by type material (K. Viets 1956, for *H. impressus* see Lundblad 1954, for *H. hemisphaericus* Terence Gledhill, pers. comm., for *H. ruber* Julia Snyder, pers. comm.), with the only exception of *Hygrobates prosiliens* Koenike, 1915, a species similar to *H. longipalpis* but differing in a more distinct, nose-like protruding medial margin of Cx-IV, described after a single female from the Klosterbach near Heiligenrode, Bremen, North Germany. The holotype of this species is deposited in the Swedish Museum of Natural History (SMNH), Stockholm.

In this study we applied molecular and morphological methods to analyse specimens of (former) *Hygrobates longipalpis* from several parts of Europe. As a result, the existence of two distinct clades identified by DNA-barcoding could be demonstrated. We were also able to define the most important morphological characteristics allowing to separate these two clades as separate species with standard light-microscopical techniques. As a consequence, a neotype is designated for *H. longipalpis*, and *Hygrobates prosiliens* is re-established and redescribed as a separate species. Both species are obviously widely distributed in Europe, but differ in ecological requirements, *H. longipalpis* having a preference for slow flowing parts of streams, and *H. prosiliens* bound to standing waters.

Material and Methods

Water mites were collected by hand netting, sorted from the living material in the field, and preserved in 96% ethanol for molecular analysis. After DNA extraction, exoskeletons were dissected

as described in Davids *et al*. (2007), and slide mounted in Hoyer's medium. Morphological nomenclature follows Gerecke *et al*. (2016) and Pešić *et al.* (2017); for explanations concerning morphology and measurements see Figs. 1B–D. All material, including the neotype of *H. longpalpis* and vouchers were deposited in the Naturalis Biodiversity Center in Leiden (RMNH). COI sequences prepared in the course of his study are deposited in BOLD and GenBank with accession numbers indicated in Table 1; ribosomal DNA sequences were published in GenBank under acc. nos. MN123766-770.

Composition of the material is given as males/females/deutonymphs. All measurements are given in µm. The following abbreviations are used: Ac-1 = most anterior acetabulum; Cx-I = first coxae; dL = dorsal length; Gp = genital plate; H = height; I-L-4-6 = fourth to sixth segments of first leg; L = length; mL = median length; n = number of specimens examined; P-1– P-5 = palp segments 1 to 5; pregen = pregenital sclerite; SMNH = Swedish Museum of Natural History, Stockholm; W = width.

Molecular analysis

Specimens for molecular analysis were examined without dissecting under a compound microscope in ethanol, using a cavity well slide with a central depression. After examination, they were returned into 96% ethanol. Molecular analysis was conducted in the Naturalis Biodiversity Center (NCB) Leiden and in the Molecular Biology Techniques Laboratory (MBTL) of the Adam Mickiewicz University in Poznan. For the methods used for cytochrome *c* oxidase subunit I (COI), gene amplification and sequencing see Pešić *et al*. (2017). A fragment coding for the D2 region of 28S rRNA gene from nuclear DNA was amplified using a primer set: forward Hy28SFD2 (CACGTGGTAAGCTCCACG) (developed in this study) and reverse 28SR1200 (GCATAGTTCACCATCTTTCGG) (Telford *et al.* 2003). PCR amplification and sequencing of the D2 region was carried out in the same way as in the case of COI.

Sequence and phylogenetic analyses

DNA was extracted and amplified from a total of 26 specimens of *Hygrobates longipalpis* species complex, collected in The Netherlands, Germany, Montenegro, and Romania (accession numbers in Table 1); a previously published sequence for *Hygrobates corsicus* Pešić & Smit, 2017 (RMNH.5070752) from BOLD (NLACA994-17) was used as an outgroup for COI-barcode analysis.

Sequences were aligned using MUSCLE as implemented in Geneious R11 (Biomatters Ltd.). Phylogenetic analysis for species delimitation was carried out with Neighbour-Joining (NJ) method as implemented in MEGA7 (Kumar *et al*. 2016). The support for tree branches was calculated by the nonparametric bootstrap method (Felsenstein 1985) with 1000 replicates.

Pairwise distance calculations between nucleotide sequences were computed using Kimura's 2-parameter (K2P) distance model (Kimura 1980) for all codon positions and transition/transversion ratio was calculated using MEGA7. Probability of species distinctiveness was estimated by the probability of making a correct identification under either strict or relaxed multivariate criteria [P ID(Strict) or P ID(Liberal)], reciprocal monophyly $P_{(AB)}$ (Rosenberg 2007), and Randomly Distinct $P_{(RD)}$ (Rodrigo *et al*. 2008) as implemented in Geneious R11 species delimitation plugin (Masters *et al*. 2011). Additionally, the sequence data were analyzed using the Automatic Barcode Gap Discovery (ABGD) method to delimit genetic clusters by detecting a significant gap in the pairwise distance distribution (Puillandre *et al*. 2012). We used the online ABGD version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with default settings and K2P distance model. The tree was edited in MEGA7 and further in Corel Draw X5.

Morphometric analysis

Canonical discrimination analysis (CDA) was performed in order to examine discrimination power properties of morphometric variables in discrimination between clades of the *Hygrobates longipalpis*-species group. CDA was conducted (separately for each sex) using the stepwise method. Canonical scores for each case were calculated in order to estimate the distance between individuals that were used for visualizing differences between species.

TABLE 1. List of specimens used in this study.

Locality (country, name)	Lat/Long	Voucher code	GenBank/BOLD Accession No
***Hygrobates prosiliens* (Clade I)**			
The Netherlands, Plasje langs Sipkes-pad	Am. Coord. 064.273-437.048	RMNH.ACA.977	BOLD NLACA524-15
The Netherlands, Maarsseveense Plas	Am. Coord. 134.307-461.380	RMNH.ACA.436	BOLD NLACA180-15
The Netherlands, Maarsseveense Plas	Am. Coord. 134.307-461.380	RMNH.ACA.437	BOLD NLACA181-15
The Netherlands, Maarsseveense Plas	Am. Coord. 134.307-461.380	RMNH.ACA.438	BOLD NLACA182-15
Germany, Torfkanal	3°06'30.4“N, 8°49'27.9“E	RMNH. 5012672	BOLD NLACA1237-18
Germany, Pohlsee	54.23 N, 09.93 E	PM_01_Hyd061	FJ668538
Germany, Heiligenrode	52°58'53.3" N, 8°42'24.2" E	AMU-LB202	MN123765
***Hygrobates longipalpis* (Clade II)**			
Germany, Benninger Moos	47°58'18.43"N, 10°12'18.5"E	RMNH.5012673	BOLD NLACA1238-18
Germany, Benninger Moos	47°58'31.11“N, 10°11'59.73“E	RMNH.5012626	BOLD NLACA1239-18
The Netherlands, Rode Beek	52º17.479 N, 5º58.362 E	RMNH.5091795	BOLD NLACA1205-18
The Netherlands, Rode Beek	52º17.479 N, 5º58.362 E	RMNH.5091796	BOLD NLACA1206-18
The Netherlands, Rode Beek/Nieuwe Beek	Am.coord. 193.269-478.024	RMNH.ACA.569	BOLD NLACA597-15
The Netherlands, Beek langs Julianalaan	52°17.374 N, 5°57.727 E	RMNH. 5091820	BOLD NLACA1230-18
The Netherlands, Beek langs Julianalaan	52°17.374 N, 5°57.727 E	RMNH. 5091821	BOLD NLACA1231-18
The Netherlands, Slotgracht langs Prins Bernardlaan	52°17.529 N, 5°57.411 E	RMNH.5091815	BOLD NLACA1225-18
The Netherlands Slotgracht langs Prins Bernardlaan	52°17.529 N, 5°57.411 E	RMNH.5091816	BOLD NLACA 1226-18
Norway, Slotgracht langs Prins Bernardlaan	52°17.529 N, 5°57.411 E	RMNH.5091817	BOLD NLACA1227-18
Montenegro, Vrijesko Vrelo	42°28'51.39“N, 19°8'44.55“E	RMNH.5091803	BOLD NLACA1213-18
Montenegro, Svinjiska Vrela	42°38'18.39“N, 19°0'26.54“E	RMNH.5091804	BOLD NLACA1214-18
Montenegro, Mareza	42°28'47.79“N, 19°10'55.17“E	RMNH.5091805	BOLD NLACA1215-18
Montenegro, Mareza	42°28'47.79“N, 19°10'55.17“E	RMNH.5091806	BOLD NLACA1216-18
Montenegro, Mareza	42°28'47.79“N, 19°10'55.17"E	RMNH.5091807	BOLD NLACA1217-18
Montenegro, Mareza	42°28'47.79"N, 19°10'55.17"E	RMNH.5091808	BOLD NLACA1218-18
Romania, Someșul Mic River	46.763877 N, 23.546480 E	VP-Hyd687	MN123761
Romania, Someșul Mic River	46.763877 N, 23.546480 E	VP-Hyd688	MN123762
Romania, Someșul Mic River	46.763877 N, 23.546480 E	VP-Hyd690	MN123763
Romania, Someșul Mic River	46.763877 N, 23.546480 E	VP-Hyd698	MN123764

Measurements were taken from 16 specimens genetically assigned to clade I (1♂, 3♀♀), and clade II (3♂♂, 9♀♀) (see below for molecular analysis). The 43 morphological character states measured in this study are listed in Table 3. Measurements included length of the coxal field (Cx L), width of coxal plate 3, median length of coxal plate I + gnathosoma (Cx-I mL + Gnath. L), distance between lateralmost ends of Cx-II apodemes (Cx-II apodeme. Dist.), length and width of the genital field (Gf), length of acetabula 1-3, dorsal length and height of palp segments P-1 to P-5, dorsal length of I–L (1–6) and IV–L (1–6) segments and length of posteromedial and distomedial seta on IV-L-6 and their ratio. For females, length of genital plate (Gp) and length of gonopore were included. Measurements of ejaculatory complex in males and chelicera segments in both sexes were not included into the analysis due to missing values for most analysed specimens. The software SPSS 17.0 (SPSS for Windows, Rel. 17.0.0.2008, Chicago: SPSS Inc.) was used.

TABLE 2. Standardized canonical discriminant function coefficients and functions at group centroids (group means), calculated from the "entering independents together" method for females of *H. longipalpis* species complex. Only significant values are presented, other values are non-available (n/a).

	standardized coefficients	
	Males	Females
	CDF1	CDF1
L Ac 3	-17.275	n/a
L/H P-4	n/a	-.932
L IV-L-6 proximomedial seta	17.339	1.226
	centroids	
Clade I	18.902	1.519
Clade II	-56.707	-4.558

TABLE 3. The level of significance for each of the successive discriminant functions, calculated from the 'entering independents together' method for males and females of *H. longipalpis* species complex.

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
males	0.000	7.671	2	0.022
females	0.107	20.078	2	0.000

Results

Species delimitation using DNA-barcodes

The final alignment for species delimitation using COI sequence data comprised 645 nucleotide positions (nps) of the 26 specimens listed in Table 1 and one outgroup, *H. corsicus*. The nucleotide sequences could be translated into amino acid sequences without any stop codons. In the dataset, 178 nps out of 645 were variable, and average transition to transversion ratio for all variable sites was 2.0.

Neighbour-Joining (NJ) analysis clustered COI sequences of specimens attributed to *Hygrobates longipalpis* ("*H. longpalpis* complex") into two maximally supported clades (clade I and II) (Fig. 1). In clade I was grouped, among others, the COI sequence found in a specimen collected from the *locus typicus* of *H. prosiliens*. The mean genetic distance recovered in the NJ analysis between these two clades was 16.95% (SD=1.7). The ABGD method found in the genetic distances between clades I and II a barcoding gap between 4 and 16% pairwise distances; the second gap–between 18 and 23%–was found between sequences representing *H. longipalpis* complex and the outgroup species, *H. corsicus* (Fig. 2). The probability of making a correct identification under either strict or relaxed cladistics criteria was significant: [P ID(Strict) = 0.93 (0.82, 1.0), P ID(Liberal) = (0.99 (0.93, 1.0)], supporting the species status of the two clades recovered in NJ analysis, as did the result of a $P_{(RD)}$ species delimitation test (significant, with a p value of <0.05). Also, the Rosenberg´s $P_{(AB)}$ gave a highly significant result (p=0.00000012). Nuclear DNA data supported COI-barcoding results as well: two representatives of clade I, including one from the type locality of *H. prosiliens*, shared the same D2 sequence with a length of 662 bp, while all Romanian specimens, clustering in clade II, had the same sequence fragment with a length of 664 bp that differed from clade I by 3.90% (SD 0.76%).

Canonical discriminant analysis

A discriminant function analysis of 16 specimens was 100% accurate in assigning independently identified individuals to two *a priori* defined groups. Low values of Wilks' lambda for two functions and statistically significant Chi-square for these functions (Table 3) revealed that

those two functions are significant predictors. Individual male specimens were projected on CDF I using the stepwise method, which accounted for 100% of the original variance. Also, in the analysis based on females, the first discriminant function accounted for 100% of the original variance.

TABLE 4. Measurements of females of *Hygrobates longipalpis* (clade II of molecular analysis) and *H. prosiliens* (clade I of molecular analysis).

	Hygrobates longipalpis (clade II)					*H. prosiliens* (clade I)
	The Netherlands, Roode Beek n = 1	The Netherlands, Beek Langs Julianula n = 2	Germany, Benninger Moos n = 2	Montenegro, Vrijesko Vrelo, n = 1	Montenegro, Svinjiska Vrela, n = 1	The Netherlands, Plasje langs Sipkes-pad, n = 1
Idiosoma L	1130	1380–1430	1400–1670	1560	1070	1145
Coxal field L	650	628–633	644–659	709	638	578
Cx-III W	813	775–781	806–819	838	747	763
mL Cx-I + Gnath. L	466	450–469	469–522	509	475	209
Cx-II apodem. dist.	284	219–250	275–288	266	284	234
Gf L	255	253–266	284–313	313	267	410
Gf W	344	325–353	372–428	422	350	338
Ac-1 L	106–122	109–119	119–134	131–136	144–153	84–91
Ac-2 L	109	73–109	116–128	150–153	131–134	88–91
Ac-3 L	106–108	94–103	122–138	125–134	125–127	84–88
Gp L	245–255	251–267	272–316	309–313	263–267	223–225
Gonopore L	203	209–216	209–244	241	187	209
Palp, total L	842	744–789	829–850	887	836	732
P-1 dL	53	45–50	50–53	56	53	53
P-1 H	78	64–69	64–70	72	67	66
P-2 dL	231	211–219	228–231	234	230	203
P-2 H	138	109–119	125–127	138	131	119
P-3 dL	169	138–147	163–172	184	170	139
P-3 H	123	106–113	117–125	127	116	106
P-4 dL	305	269–289	297–309	319	298	259
P-4 H	66	56–63	61	72	66	56
P-5 dL	84	81–84	88	94	85	78
P-5 H	31	28	28–31	31	30	28
P-1 dL/H ratio	0.68	0.7–0.73	0.76–0.78	0.78	0.79	0.81
P-2 dL/H ratio	1.68	1.84–1.93	1.8–1.85	1.7	1.76	1.71
P-3 dL/H ratio	1.37	1.29–1.31	1.38–1.39	1.46	1.47	1.31
P-4 dL/H ratio	4.64	4.62–4.78	4.88–5.05	4.44	4.54	4.6
P-5 dL/H ratio	2.7	2.9–3.0	2.8–3.1	3.0	2.83	2.8
dL P-2/P-4 ratio	0.76	0.76–0.78	0.75–0.77	0.73	0.77	0.78
Chelicera	503	419	523	-	519	422
I-L-1 L	121	97–100	105–109	116	125	88
I-L-2 L	175	141–163	168–172	188	169	142
I-L-3 L	300	238–270	281–288	300	278	225
I-L-4 L	375	319–353	359–381	406	366	313
I-L-5 L	406	341–359	381–391	428	384	328
I-L-6 L	334	278–306	322	344	313	290
IV-L-1 L	231	203–216	209–213	225	216	194
IV-L-2 L	256	231–244	247–250	281	241	216
IV-L-3 L	422	369–388	400–416	438	394	333
IV-L-4 L	569	488–544	556–565	631	547	463
IV-L-5 L	538	469–513	525–541	569	522	466
IV-L-6 L	431	390–434	443–447	481	438	380
L IV-L-6 prox.med. seta	100	97–114	103–119	116	91	41
L IV-L-6 dist.med. seta	62	61–72	58–60	47	47	53
L IV-L-6 prox/dist medial setae ratio	1.61	1.58–1.59	1.78–1.98	2.5	1.94	0.77

A scatter plot of the first and second canonical scores clustered all the examined males and females, respectively into two distinct groups corresponding to the two *a priori* molecularly defined groups (Fig. 3). From the canonical coefficients in Table 2 it can be concluded that the size of Ac-3 is a character state useful for discriminating males of the two clades, the L/H ratio of P-4 helps to discriminate females, and the relative L of the proximomedial seta on IV-L-6 is distinctive in both sexes.

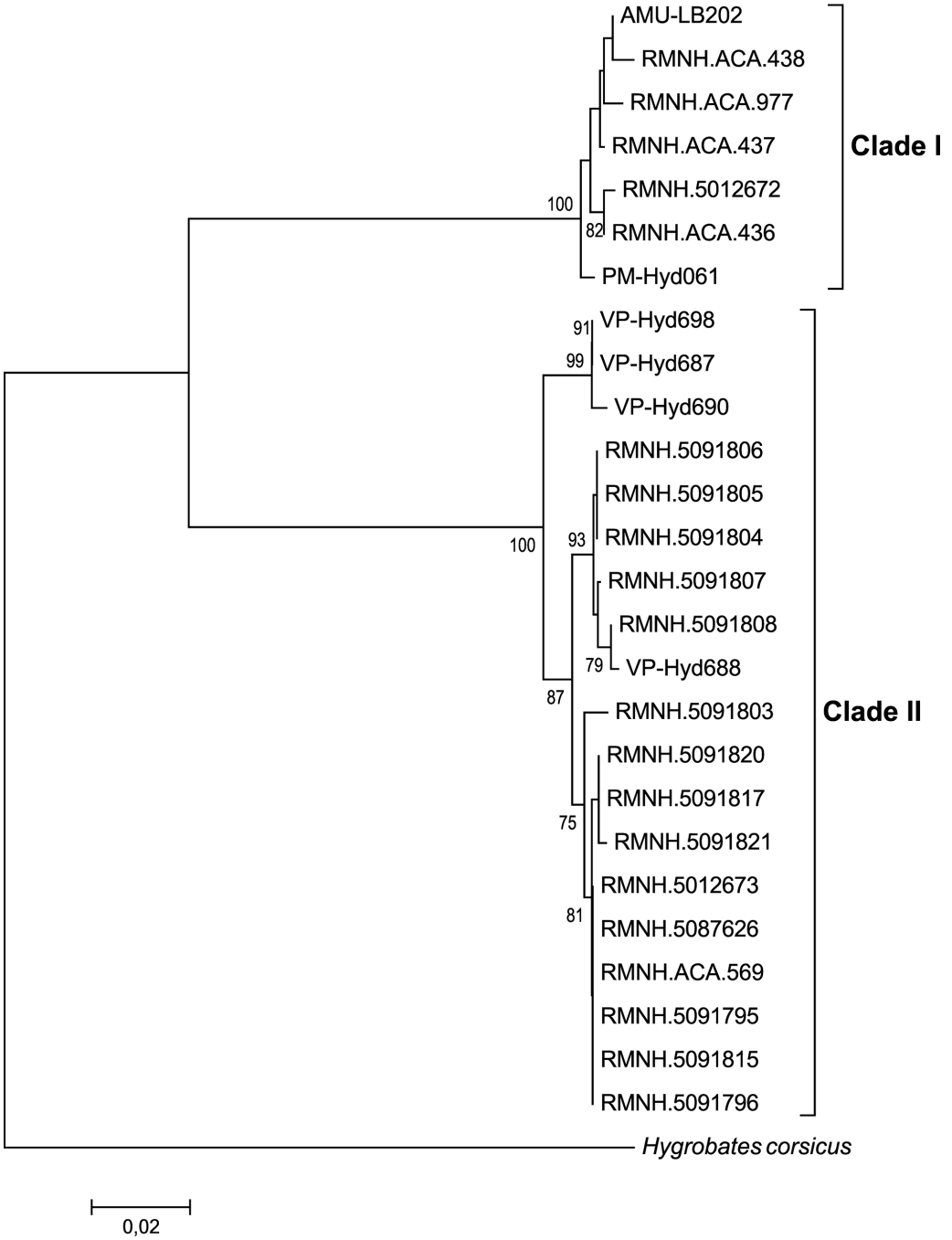

FIGURE 1. Neighbor-Joining (NJ) tree used for species-delimitation analysis. Values near branches show bootstrap support (BS); only support >75% is shown.

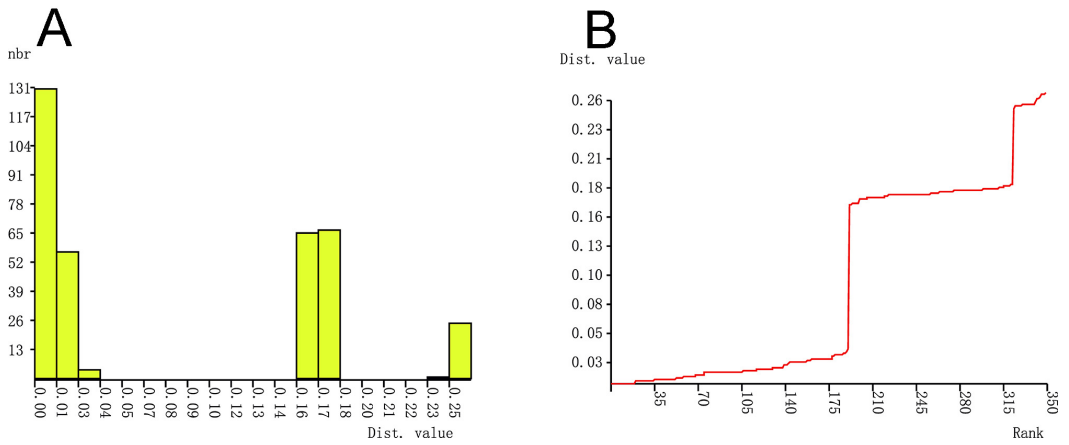

FIGURE 2. Results of Automatic Barcode Gap Discovery (ABGD) analysis for COI sequences. (A) Distribution of pairwise differences, (B) Ranked pairwise differences.

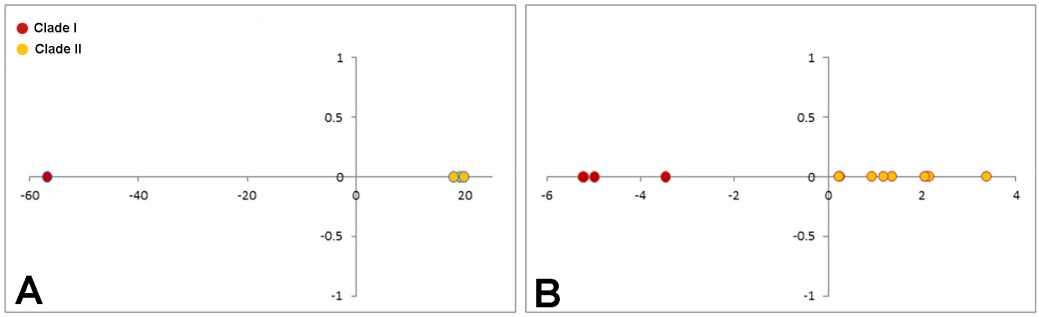

FIGURE 3. Results of multivariate analysis of morphology. Scatter plot of first two canonical discriminant functions using the 'entering independents together' method from the analysis of morphometric data for *H. longipalpis* species group specimens. A = male, B = female.

CDAs performed on 43 meristic characters in female and male specimens examined revealed that the following features significantly contribute to a morphological discrimination of these two *a priori* molecularly defined clades: ♂♂: Ac-3 L (mean [range] 97 [84–105] in clade I *vs.* 67 µm in clade II); ♀♀: P-4 L/H ratio (mean [range] 4.65 [4.4–5.1] in clade I *vs.* 4.99 [4.6–5.4] in clade II); both sexes: IV-L-6 proximomedial seta L (mean [range] clade I *vs*. clade II, ♂♂: 82 [76–86] *vs.* 45, ♀♀: 102 [69–119] *vs*. 43 [36–53] µm). It is worth mentioning that the multivariate analysis was based on a limited number of specimens, in our study assigned to one of the two clades by DNA sequencing. It is likely that at a wider geographical scale some of the abovementioned characters may be blurred by graded series of intermediate measurement values (see Table 4).

The morphological analysis reveals that the specimens corresponding to the two *a priori* molecularly defined groups can be best separated on the basis of the absolute and relative length of the two medial setae on IV-L-6. In the clade I-morphotype (here assigned to *H. prosiliens*, see below) the proximal seta is shorter (Figs. 10A–C), in the clade II-morphotype (assigned here to *H. longipalpis*, see below) it is longer than the distal seta (Fig. 10D–F).

Systematic Part
Family Hygrobatidae Koch, 1842
Genus *Hygrobates* Koch, 1837
Subgenus *Hygrobates* Koch, 1837

***Hygrobates longipalpis* (Hermann, 1804)**
Figs. 4A–E, 5A–C, 6A–F, E–F
Hygrobates longipalpis—Gerecke *et al*. 2016: 153 [in part].

Type series

Neotype male, here designated: The Netherlands: Gelderland, Vaassen, Slotgracht langs Prins Bernardlaan, 52°17.529 N, 5°57.411 E, 5 m asl., 22.vi.2017 leg. Smit (RMNH.5091817, sequenced, BOLD NLACA1227-18; slide mounted in Hoyer's liquid). Paraneotypes (same data as neotype, slide mounted in Hoyer's liquid; sequenced), one male (RMNH.5091816), one female (RMNH.5091815). The neotype is designated for the purpose of the taxonomy and clarifying the status of *H. longipalpis*.

Material examined

The Netherlands: Gelderland, Rode Beek, 52º17.479 N, 5º58.362 E, 6.ix.2011 leg. Smit, 1/1/0 (mounted, RMNH.5091795-6); Rode Beek/Nieuwe Beek, N of Vaassen, 52°17.333 N, 5°56.886 E, 14.viii.2011 leg. Smit, 0/1/0 (mounted, RMNH.ACA.569); Vaassen, Beek along Julianalaan, 52°17.374 N, 5°57.727 E, 35 m, 22.vi.2017 leg. Smit, 1/1/0 (mounted, RMNH.5091820-21). Germany: Bavaria, Memmingen, Benninger Moos, spring area SW, 47°58'18.43"N, 10°12'18.5"E, 29.v.2017 leg. Gerecke 0/1/0 (RMNH.5012673), ibid., pond at W border, 47°58'31.11"N, 10°11'59.73"E, leg. Gerecke 0/1/0 (RMNH.5012626). Montenegro: Podgorica, Tološi, canal Mareza, 42°28'47.79“N, 19°10'55.17“E, 25.iii.2017 leg. Pešić 1/3/0 (mounted, RMNH.5091805-8); Danilovgrad, Bandići, spring “Vriješko Vrelo“, 42°28'51.39“N, 19°8'44.55“E, 5.iii.2017 leg. Pešić 0/1/0 (mounted, RMNH.5091803); Danilovgrad, spring “Svinjiška Vrela“, 42°38'18.39“N, 19°0'26.54“E, 11.ii.2017 leg. Pešić 0/1/0 (mounted, RMNH.5091804).

Diagnosis

Characters of the *Hygrobates longipalpis* species complex; L/H P-4 4.4–5.0 in ♂♂, 4.2–4.7 in ♀♀; Ac large (♂♂: Ac-3 L > 90 µm, ♀♀: Ac-3 L > 100 µm); IV-L-6 proximomedial seta longer than distomedial one, L ratio 1.4–2.5 (mean 2.0 in ♂♂, 1.8 in ♀♀).

Description

Both sexes: Colour yellowish with pale to dark brown spots. Posteromedial margin of Cx-I+II projecting, tongue-shaped; Cx-IV with a distinct nose-like protruding medial margin, occasionally indented at transition to posterior margin. Acetabula rounded, in triangular arrangement. Basal segment of chelicera dorsally with a pointed projection. P-2 distoventrally protruding in a short, rounded projection covered by small denticles, P-3 with denticles covering distal two thirds of ventral margin, P-4 slightly protruding near insertions of the ventral setae, ventral setae in distal parts. Male: Anterior margin of genital field convex with a small knob-shaped medial projection, posterior margin indented, in the centre of the indentation with a small protrusion not extending beyond posterior plate margin, Ac-3 L 84–102 µm; P-4 L/H ratio 4.4–5.1 (mean 4.77). Female: Medial margin of the genital plate strongly indented in the centre, Ac-3 L 94–138 µm; P-4 more slender than in male, L/H ratio 4.2–5.1 (mean 4.65).

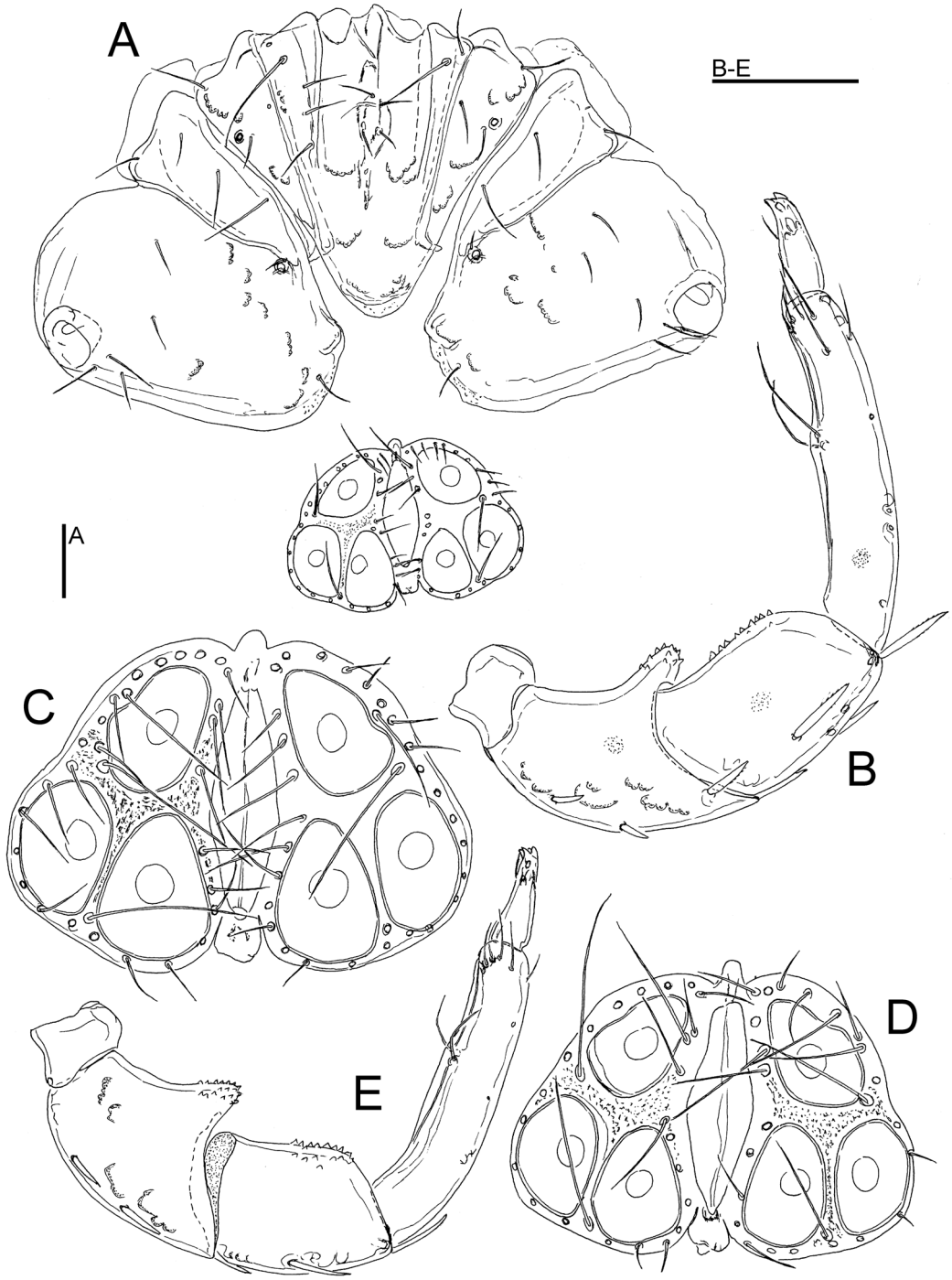

FIGURE 4. *Hygrobates longipalpis*, ♂. A–B, neotype, RMNH.5091817; C, RMNH.5091795; D–E, RMNH.5091816. A, coxal and genital field; B, palp, lateral view; C–D, genital field; E, palp, medial view. Scale bars = 100 μm.

Measurements

Male (neotype; in parentheses paraneotype, in square parentheses RMNH.5091795 from Rode Beek): Idiosoma L 1320 (1140) [1280]; coxal field: L 556 (522) [595]; Cx-III W 671 (631) [713];

mL of Cx-I + gnathosoma L 403 (369) [444]; distance between lateralmost ends of Cx-II apodemes, 189 (200) [209]; genital field L/W 228/319 (206/278) [234/319]; L Ac 1-3: 97–100, 100, 105 (81–89, 81–84, 82–84) [94, 94–97, 100–102].

Palp: total L 685 (614) [737]; dL/H: P-1, 41/59 (41/56) [44/61]; P-2, 188/100 (159/100) [200/103]; P-3, 129/97 (122/91) [150/103]; P-4, 252/50 (220/45) [263/59]; P-5, 75/28 (72/25) [80/28]; dL/H ratio: P-1, 0.68 (0.72) [0.72]; P-2, 1.88 (1.59) [1.94]; P-3, 1.34 (1.35) [1.46]; P-4, 5.03 (4.86) [4.43]; P-5, 2.67 (2.88) [2.85]; P-2/P-4 L ratio 0.75 (0.73) [0.76].

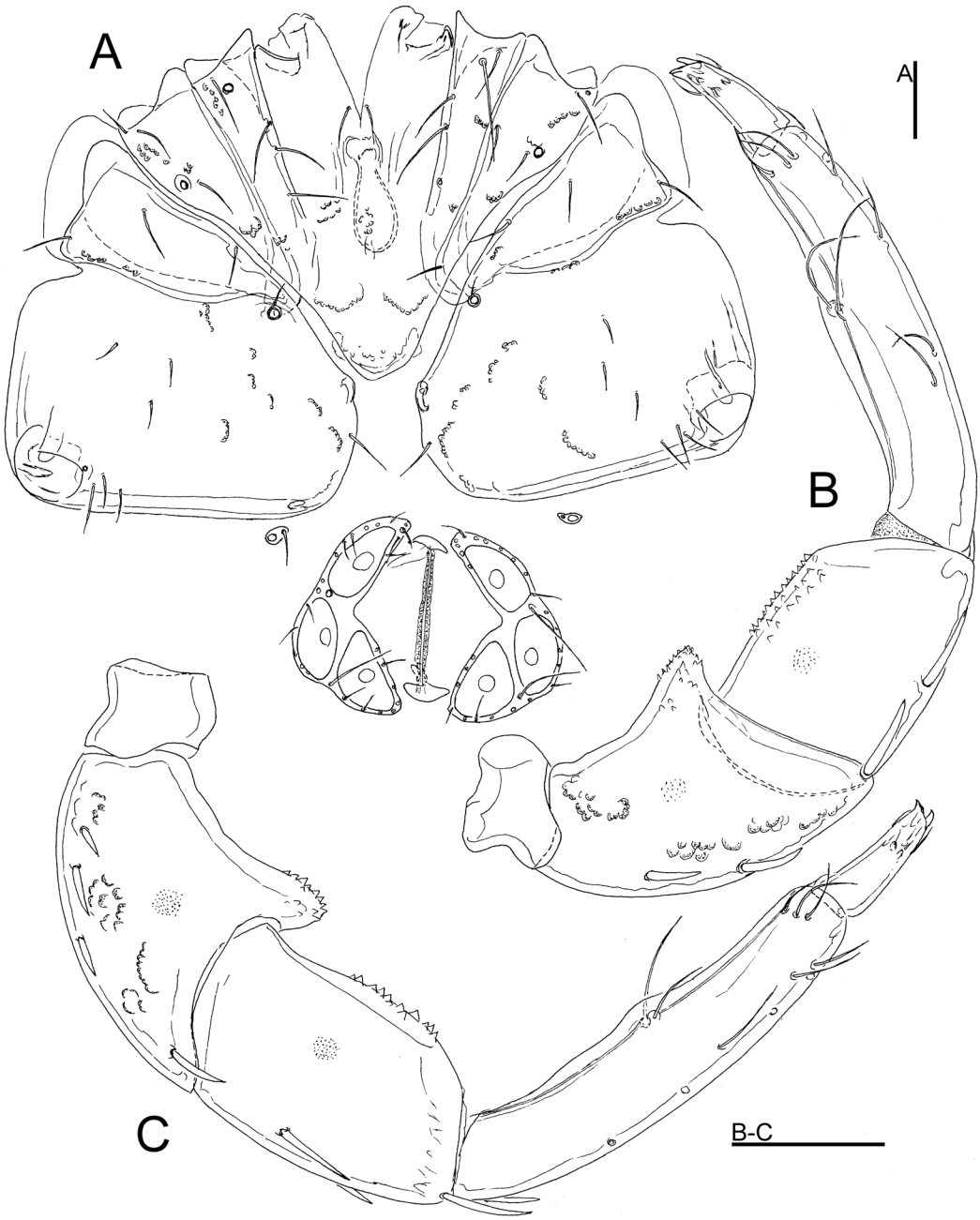

FIGURE 5. *Hygrobates longipalpis*, ♀. A–B, RMNH.5091796; C, RMNH.5091803. A, coxal and genital field; B, palp, medial view; C, palp, lateral view. Scale bars = 100 µm.

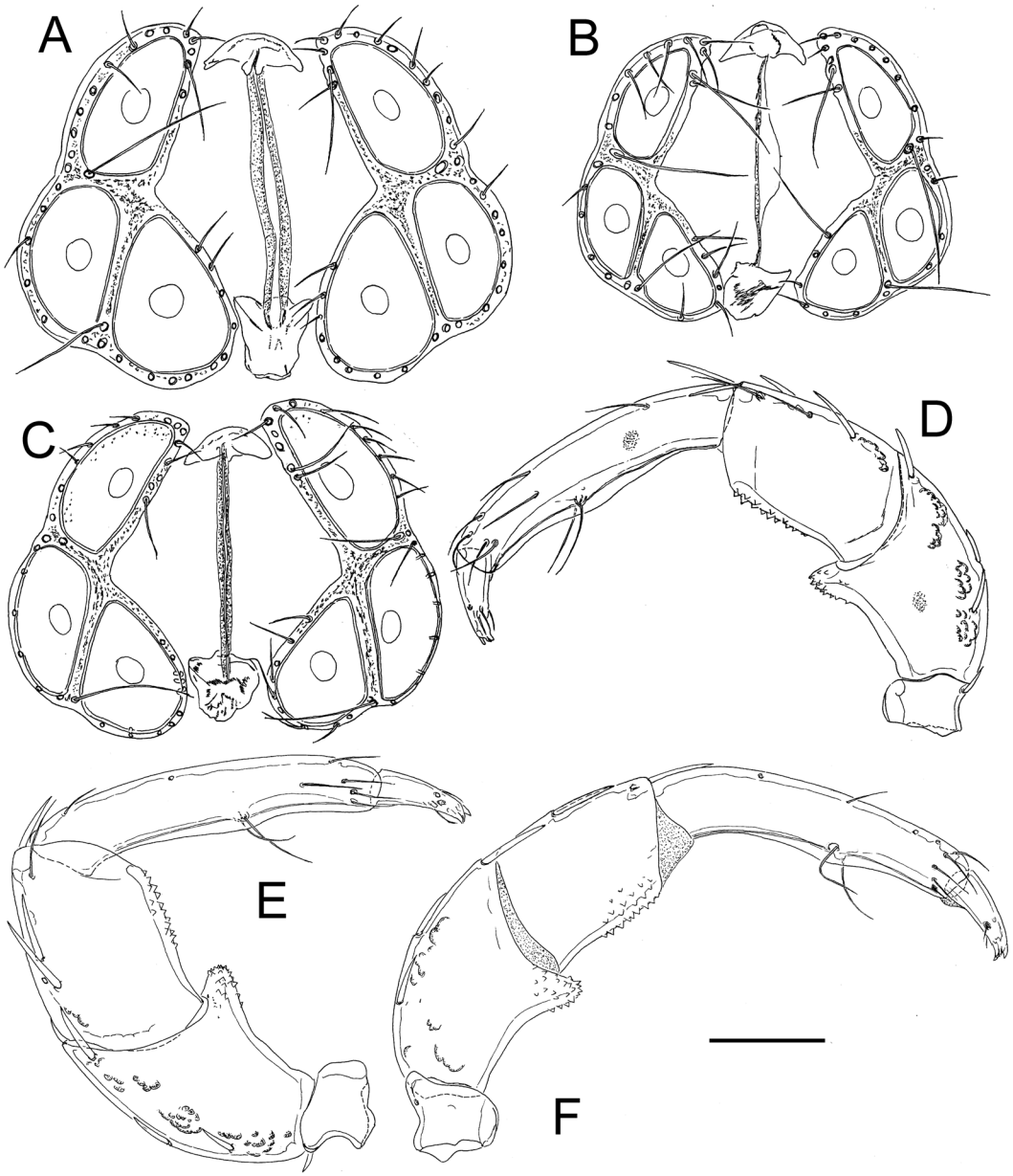

FIGURE 6. *Hygrobates longipalpis*, ♀. A–C, genital field; D–F, palp. A, E, RMNH.5012673; B, D, RMNH.ACA.815; C, F, RMNH.5091808. Scale bars = 100 µm.

Legs: dL of I-L-1-6: 97 (94) [119]; 147 (128) [150]; 225 (206) [269]; 306 (265) [363]; 324 (300) [381]; 278 (253) [313]. dL of IV-L-1-6: 188 (175) [194]; 213 (194) [238]; 331 (306) [375]; 475 (431) [531]; 450 (425) [481]; 381 (363) [400]; IV-L-6 proximomedial seta L 86 (76) [84], distomedial seta 44 (34) [45], proximomedial/distomedial seta L ratio 1.95 (2.23) [1.87].

Female (paraneotype; in parentheses RMNH.5091808 from Mareza, Montenegro): Idiosoma L 1340 (1300); coxal field: L 606 (619); Cx-III W 750 (744); mL of Cx-I + gnathosoma L 466 (459); distance between lateralmost ends of Cx-II apodemes, 259 (275); genital field L/W 259/323 (284/373); genital plate L 247-253 (281-284); gonopore L 209 (209); L Ac 1-3: 103–109, 98–100, 94 (131–141, 131–134, 119); egg maximum diameter (n = 3) 156–161.

Palp total L 758 (794); dL/H: P-1, 42/66 (59/68); P-2, 209/112 (216/122); P-3, 148/105 (150/116); P-4, 275/66 (288/61); P-5, 84/28 (81/31); dL/H ratio: P-1, 0.64 (0.87); P-2, 1.87 (1.77); P-3, 1.42 (1.3); P-4, 4.19 (4.72); P-5, 3.0 (2.6); P-2/P-4 L ratio 0.76 (0.75). Chelicera total L 447 (472).

Legs: dL of I-L-1-6: 109 (109), 156 (153), 259 (259), 333 (353), 356 (378), 305 (302). dL of IV-L-1-6: 225 (206), 228 (230), 369 (378), 525 (525), 481 (516), 413 (422); IV-L-6 proximomedial seta L 69 (112), distomedial seta 49 (59), proximomedial/distomedial seta L ratio 1.41 (1.9).

Remark

The original description of *H. longipalpis* does not allow any interpretation of the taxonomic characters proposed here. In view of the lack of type material and information on the type locality, as well as the incomplete original description, it does not make sense to discuss whether the mites described by Hermann under the name of *H. longipalpis* belonged to clades I or II. Our arbitrary decision to assign populations of clade II to *H. longipalpis* is induced by the fact that the specimen collected from the type locality of *H. prosiliens* belongs to clade I in our analysis.

Habitat

All representatives of *H. longipalpis* (clade II) examined in this study were collected from slow flowing sectors of running waters.

Distribution

Europe; due to confusion with *H. prosiliens*, the distribution is mostly unknown.

***Hygrobates prosiliens* Koenike, 1915**

Figs. 7A–D, 8A–F, 9A–E, 10A–C

Hygrobates longipalpis—Gerecke *et al*. 2016: 153 [in part], Olomski & Gerecke 2019, Van Hezewijk & Davids 1985.

Type series

Holotype ♀ *Hygrobates prosiliens* - Koenike -259-1741 - Type Heiligenrode 16.4.1915“(SMNH).

Material examined

Germany: Bremen, Torfkanal, 53°06'30.4“ N, 8°49'27.9“ E, 18.viii.2010 leg. Olomski & Gerecke, 0/1/0 (mounted, RMNH.5012672; palps lacking); Bremen, Klosterbach near Heiligenrode, stagnant reach above mill, 52°58'53.3" N, 8°42'242" E, 15.viii.2010, leg. Olomski & Gerecke, 0/0/1. The Netherlands: South-Holland, pool along Sipkes-path, Dunes of Voorne, 51°54.871 N, 4°4.110 E, 5.iv.2012 leg. Smit, 0/1/0 (mounted; RMNH.ACA.977); Utrecht, Lake Maarsseveen, 52°8.414 N, 5°5.096 E, 13.vi.2011 leg. Smit, 1/2/0 (mounted, RMNH.ACA.436-438).

Diagnosis

Characters of the *Hygrobates longipalpis* species complex (posteromedial margin of Cx-I+II projecting, tongue-shaped; basal segment of chelicerae dorsally with a pointed projection; P-2 distoventrally protruding in a short, rounded projection covered by small denticles); P-4 relatively slender, L/H ratio 5.3 in ♂♂, 4.6-5.4 in ♀♀; Ac moderately large (Ac-3 L ♂♂: < 80, ♀♀: < 90 µm); IV-L-6 proximomedial seta shorter than distomedial one, L proximomedial/distomedial seta ratio 0.7-0.9 (mean 0.8 in both sexes).

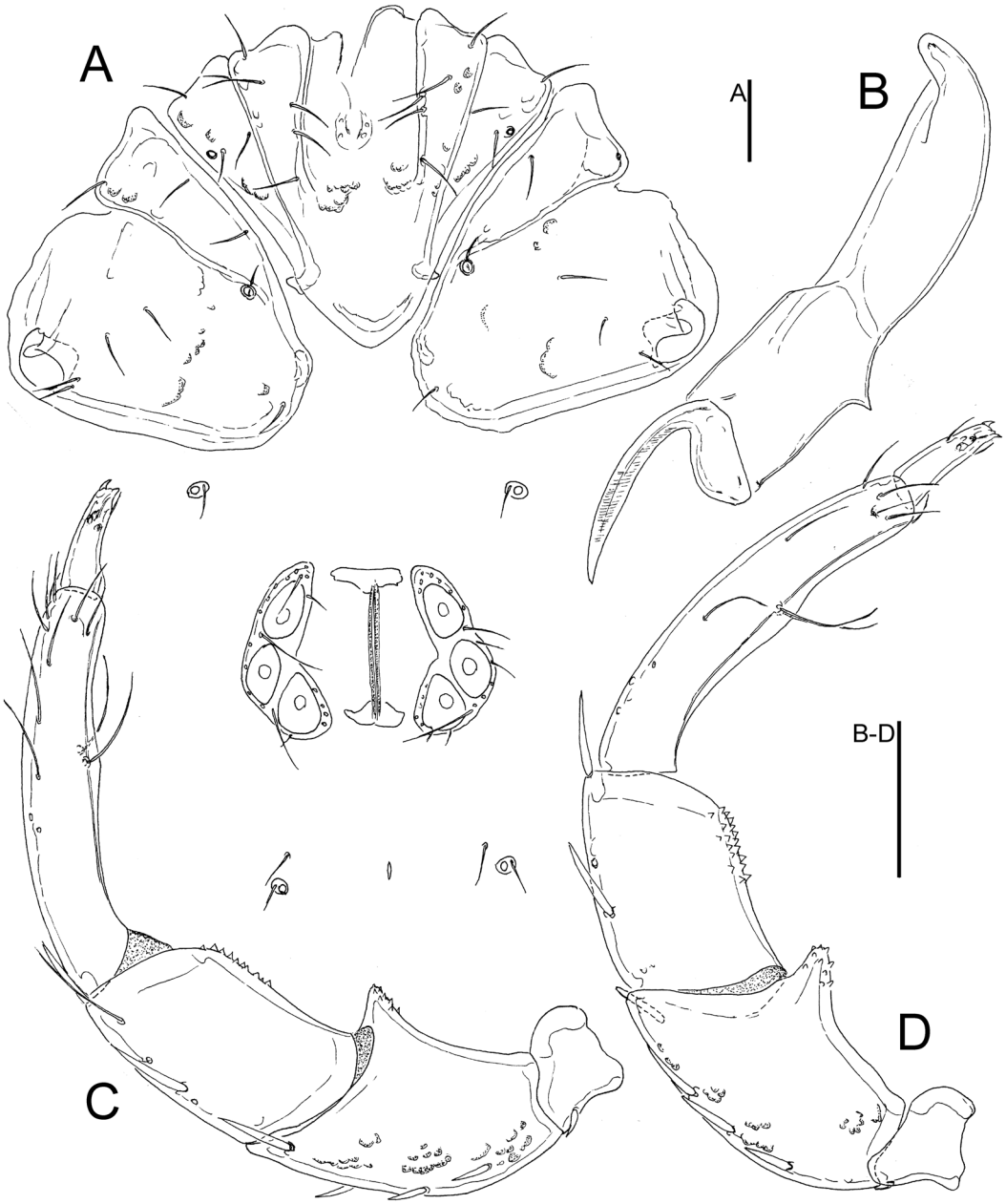

FIGURE 7. *Hygrobates prosiliens*, ♀, RMNH.ACA.437. A, coxal and genital field; B, chelicera; C, palp, lateral view; D, palp, medial view. Scale bars = 100 µm.

Description

Both sexes: Colour yellowish with pale to dark brown spots. Posteromedial margin of Cx-I+II projecting, tongue-shaped; Cx-IV with rounded or nose-like protruding medial margin, occasionally indented at transition to posterior margin. Acetabula rounded, in triangular arrangement. Basal segment of chelicerae dorsally with a pointed projection. P-2 distoventrally protruding in a short, rounded projection covered by small denticles, P-3 with denticles covering distal two thirds of ventral margin, P-4 slightly protruding near ventral setae insertions, ventral setae in distal part. Male: anterior margin of genital field convex with a small knob-shaped medial projection, posterior margin

indented, in the centre of the indentation with a small protrusion not extending beyond posterior plate margin, Ac-3 L < 80. Female: Medial genital plate margin strongly indented in the centre, Ac-3 L < 90 µm.

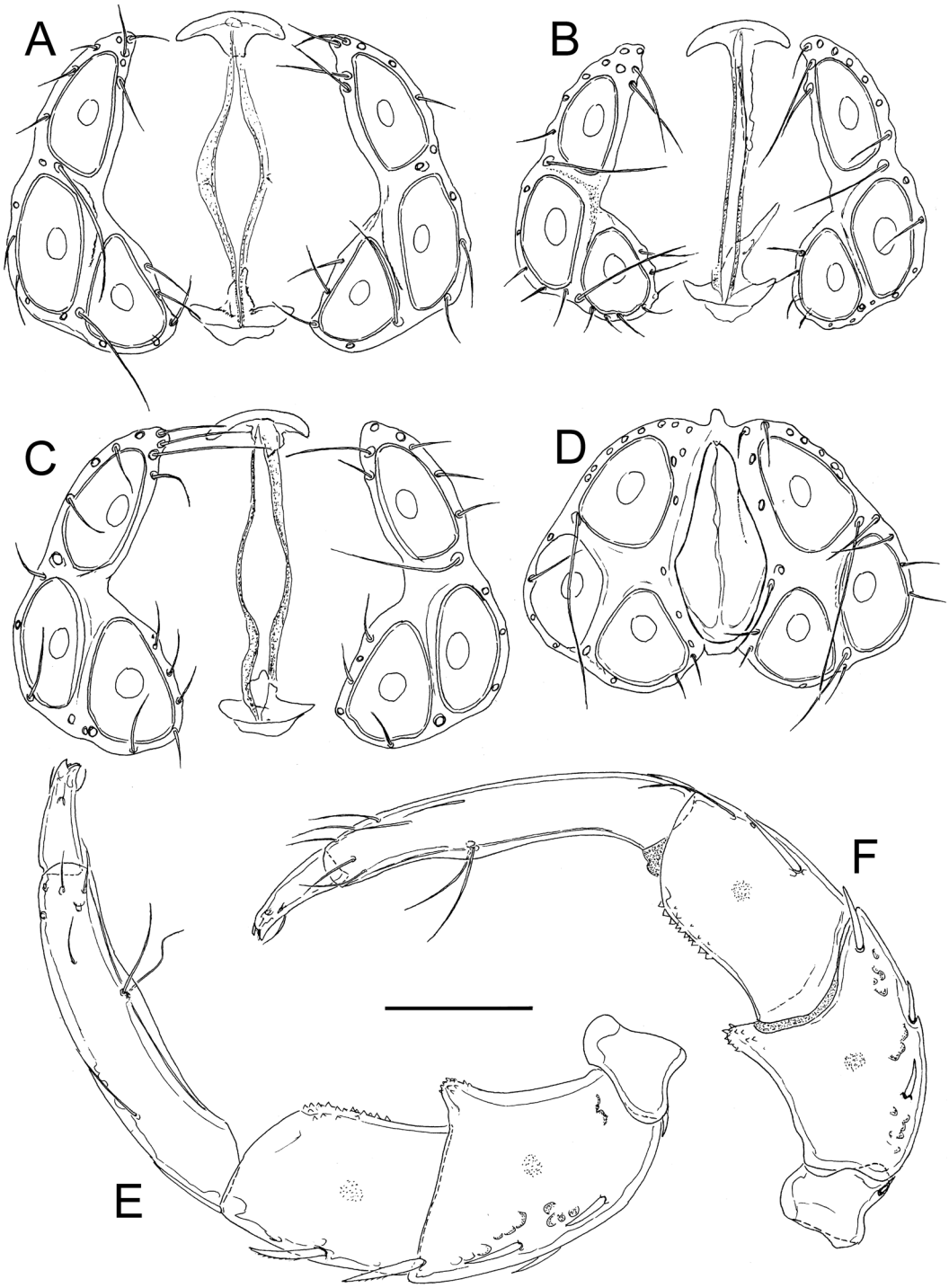

FIGURE 8. *Hygrobates prosiliens*. D, F ♂, A–C, E ♀. A–D, genital field; E–F, palp. A, RMNH.ACA.436; B–E, Torfkanal; C, E, RMNH.ACA.977; D, F, RMNH.ACA.438. Scale bars = 100 µm.

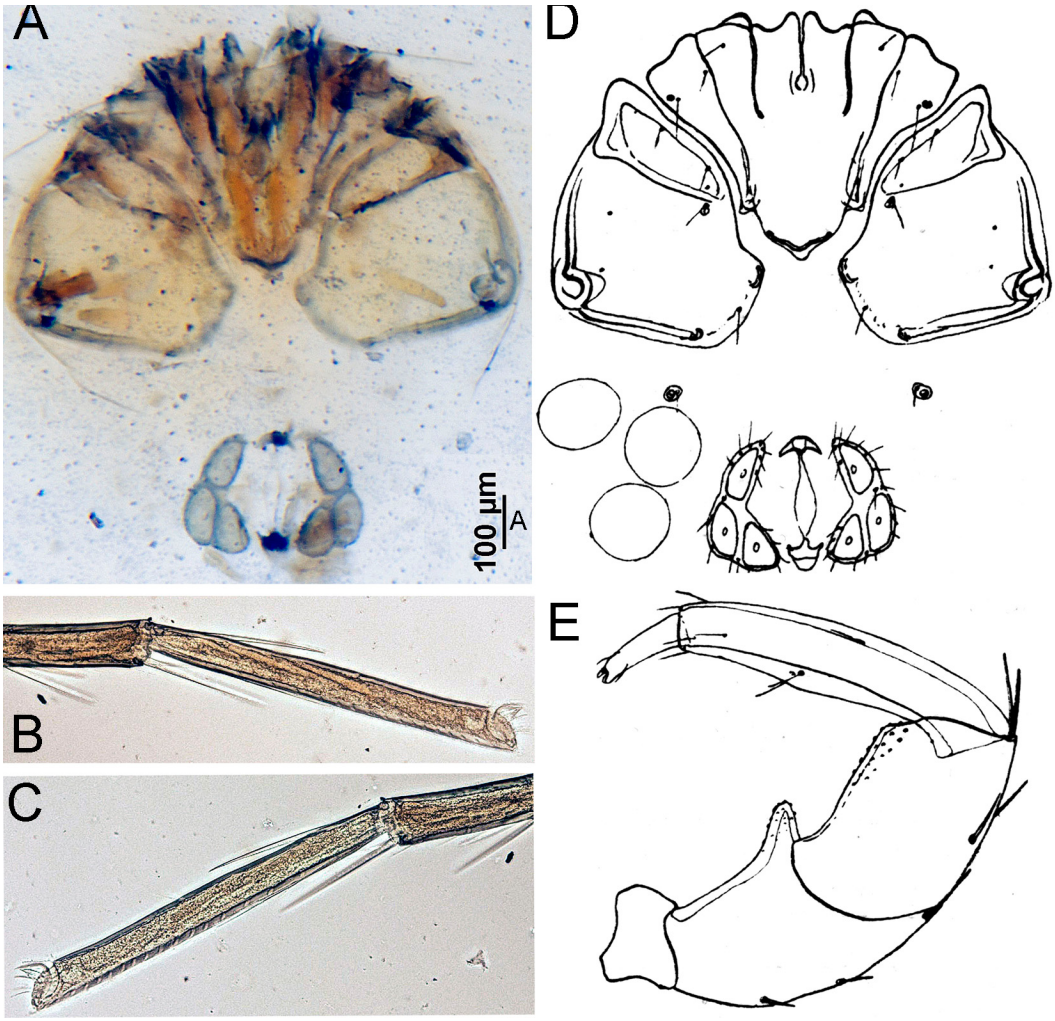

FIGURE 9. *Hygrobates prosiliens*, holotype (SMNH) (A–C photographs, D–E from K. Viets 1936): A, D, coxal and genital field; B–C, outer and inner sides of IV-L-6; E, palp. Photographs by G. Lindberg.

Measurements

Male (RMNH.ACA.438 from Maarsseveense Plas): Idiosoma L 875; coxal field: L 519; Cx-III W 645; mL of Cx-I + gnathosoma L 398; distance between lateralmost ends of Cx-II apodemes, 175; genital field L/W 194/266, ratio 0.73; L Ac 1–3: 78–84, 78–81, 64–67.

Palp: total L 706; dL/H: P-1, 50/59; P-2, 188/112; P-3, 137/95; P-4, 256/48; P-5, 75/27; dL/H ratio: P-1, 0.84; P-2, 1.68; P-3, 1.45; P-4, 5.3; P-5, 2.82; P-2/P-4 L ratio 0.73.

Legs: dL of I-L-1-6: 92, 141, 222, 297, 313, 267, IV-L-1-6: 181, 200, 313, 431, 447, 368; IV-L-6 posteromedial seta L 45, IV-L-6 distomedial seta 61, posteromedial/distomedial seta L ratio 0.74.

Female (RMNH.ACA.436–437 from Maarsseveense Plas, n = 2): Idiosoma L 1230–1380; coxal field: L 544–575; Cx-III W 686–694; mL of Cx-I + gnathosoma L 409–441; distance between lateralmost ends of Cx-II apodemes, 191–200; genital field L/W 221–224/317–323; genital plate L 219–222; gonopore L 203-209; L Ac 1-3: 78–88, 81–95, 73–78; egg maximum diameter (n = 6) 138–196 (mean 152).

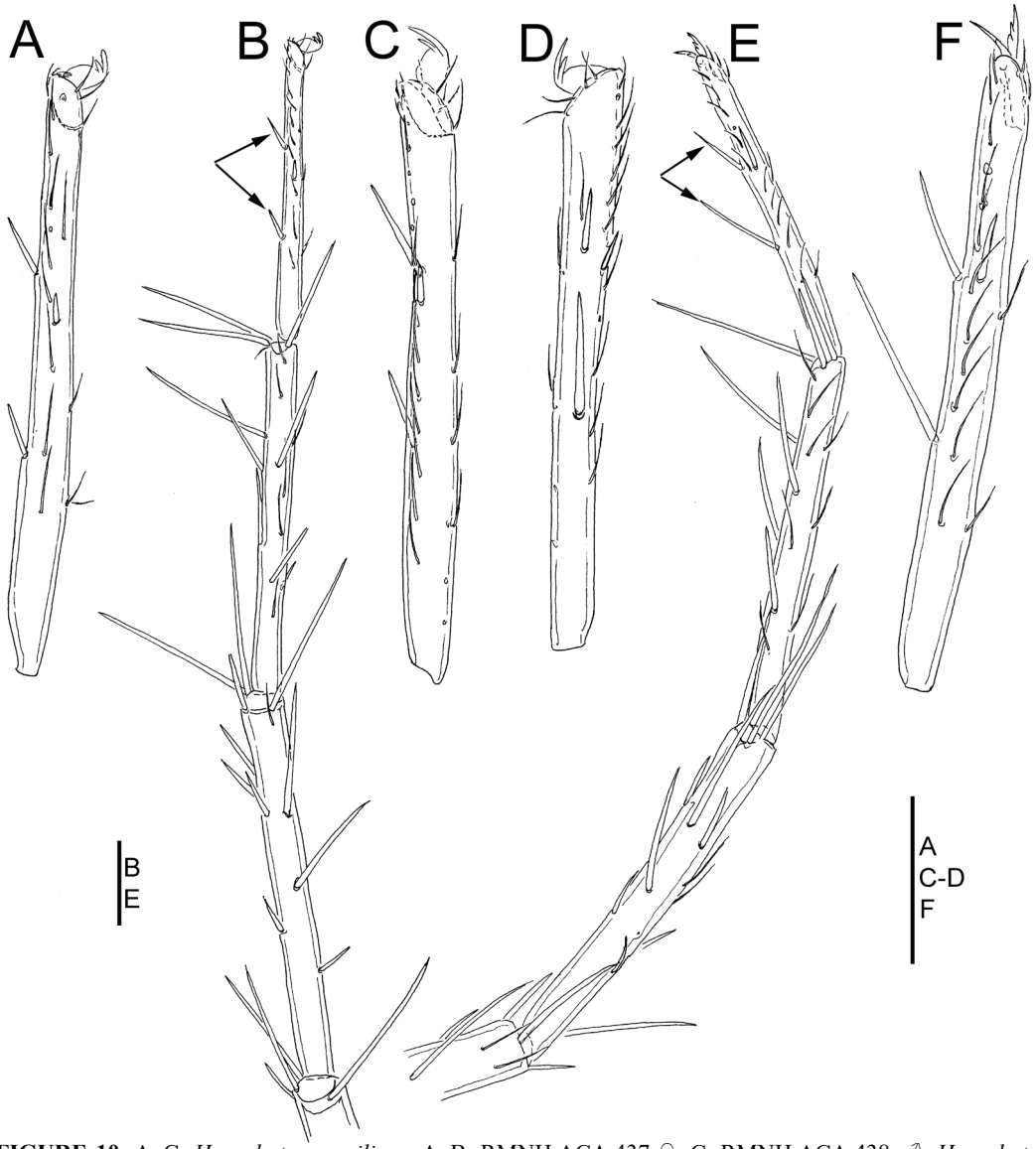

FIGURE 10. A–C, *Hygrobates prosiliens*. A–B, RMNH.ACA.437 ♀. C, RMNH.ACA.438, ♂. *Hygrobates longipalpis*. D, RMNH509816 ♂. E–F, RMNH.5091820 ♀. A, C–D, F, IV-L-6; B, F, IV-L-4-6. Scale bars = 100 μm.

Palp total L 730–783; dL/H: P-1, 50–53/59–66; P-2, 203–212/103–116; P-3, 139–156/97–103; P-4, 266–284/53; P-5, 72–78/25–28; dL/H ratio: P-1, 0.81–0.84; P-2, 1.83–1.97; P-3, 1.43–1.52; P-4, 5.0–5.36; P-5, 2.78–2.88; P-2/P-4 L ratio 0.75–0.76. Chelicera total L 416–456.

Legs: dL of I-L-1-6: 91–97, 147–153, 238–240, 313–331, 327–359, 275–300; IV-L-1-6: 181–197, 216–219, 338–348, 450–483, 428–478, 356–394; IV-L-6 proximomedial seta L 36–53, distomedial seta 42–59, proximomedial/distomedial seta L ratio 0.86–0.9.

Remark

Both, photographs (Figs. 9B–C) and light microscope observations (Gunvi Lindberg, pers. comm.) of the holotype of *H. prosiliens* did not reveal presence of medial setae on IV-L-6,

suggesting that they were lost during slide preparation. As a consequence, assignment of the type of *H. prosiliens* to one of two above-described morphotypes was not possible. However, molecular analysis of a specimen collected at the type locality of *H. prosiliens*, revealed that this specimen belongs to the clade I-morphotype.

Habitat

All representatives of *H. prosiliens* (clade I) examined in this study were collected from standing waters.

Distribution

Europe; most records require confirmation due to the confusion with *H. longipalpis* but probably widespread.

Discussion

We examined molecular and morphological evidence in order to understand the taxonomic status of populations thus far attributed to *Hygrobates longipalpis* ("*H. longipalpis* s.l."), considered a common species in the Palearctis. Both molecular and morphological analysis demonstrated the existence of two well-supported species. Standing water-dwelling populations (clade I of our molecular analysis) are attributed to *H. prosiliens*, populations from slowly flowing rivers (clade II) to *H. longipalpis*. As mentioned in the systematic part, the latter assignment is arbitrary. The main diagnostic character for separating adults of the two species in both sexes is found in the absolute and relative length of proximo- and distomedial setae on IV-L-6, a feature so far not taken in consideration for separating *Hygrobates* species.

Molecular analysis revealed that levels of COI differentiation between *H. prosiliens* and *H. longipalpis* is substantial (ca. 17%) and comparable with the genetic distance between other congeneric species pairs in Europe (e.g., *H. nigromaculatus/setosus* ca. 18%–Martin *et al*. 2010; *H. fluviatilis/arenarius* ca. 23%–Pešić *et al*. 2017).

Until now *H. longipalpis* s.l. was considered a species inhabiting both standing and flowing waters. Results of our study support the hypothesis that most or all previous records of *H. longipalpis* from standing waters in fact refer to *H. prosiliens* (clade I of our molecular analysis). This assumption should be verified (or falsified) by screening museum collections deposited under the name of *Hygrobates longipalpis* s.l.

Interestingly, the existence of more than one species included in former *H. longipalpis* s.l. was already supposed in the early time of molecular analysis, but Di Sabatino & Cicolani (1995), by allozyme comparison, could not find differences between a lake- and a spring-population. In the view of our recent results it may be possible that both of the Italian habitats (lake and limnocrenic spring) were inhabited by the same species (probably *H. prosiliens*).

In most studies on life history and/or ecology of *H. longipalpis* s.l., lake-dwelling populations were investigated, i.e., probably *H. prosiliens*. Concerning the rather detailed study of Imamura (1950) and studies from North America (Prasad & Cook 1972, Modlin & Gannon 1973), the species identity with one of the two Western Palaearctic species is hitherto unclear.

Cichocka *et al.* (2015) reported detailed data both on the reproduction of lake- and river-dwelling populations of *H. longipalpis* s.l. The females of both populations did not show significant differences in egg numbers and hatching success. Van Hezewijk & Davids (1985) reported for *H.* cf. *prosiliens* a decrease of the hatching time between eggs layed in April and July (with an increase in temperature); independently of the study season, in absence of a host, larvae died after 7 to 25 days.

There is a relatively high number of larval descriptions of *H. longipalpis* s.l. (e.g., Piersig 1896-1899; Münchberg 1935; North America: Prasad & Cook 1972), but most of these are not sufficiently detailed for comparative analyses. However, Van Hezewijk & Davids (1985) described the larval morphology and stressed some differences between their larvae deriving from a lake (= Lake Maarsseveen) and a description of larvae of a population from a Russian river (Wainstein 1980: *H.* cf. *longipalpis*). For example, caudal setae V4 of Russian larvae were markedly shorter (105 µm) than those of specimens from the Netherlands (237 µm). But Petr Tuzovskij (personal communication) stressed that Wainstein's measurements and/or descriptions are probably incorrect and comparable with the Dutch *H. longipalpis*. Thus, separation of two separate species hidden behind *H. longipalpis* s.l. may be supported by differences in larval morphology but this has to be checked by breeding larvae from *H. longipalpis*/*prosiliens* populations from Central Europe and Eastern Europe.

Also another item is remarkable and should be focused in future studies. As typical for *Hygrobates* (Smith & Oliver 1986), larval parasitism is observed also for lake-dwelling *H. longipalpis* s.l. (probably *H. prosiliens*), with Chironomidae (Diptera, Nematocera) (Kouwets & Davids 1984, Van Hezewijk & Davids 1985) and Chaoboridae (Diptera, Nematocera) (Münchberg 1935) reported as hosts. As typical in the genus, mite larvae were found attached to the host's abdomen (Smith & Oliver 1986). Loss of parasitism may be an evolutionary driver for water mite speciation (Smith 1998), as supposed for the species pair *H. setosus* (from streams, parasitic larva) and *H. nigromaculatus* (from lakes, no parasitic larva) (Martin *et al.* 2010). For stream dwelling *H. longipalpis* s.l. populations no hosts were reported–also in the study with a special focus on this topic published by Müller (2015). The question if parasitism is eventually lost in *H. longipalpis* s. str. merits particular attention. Finding (not yet reported) conspecific populations would be of special interest for better understanding differences in life cycle and ecology of both species.

Occurrence in both lakes and rivers has been reported for populations of several other water mite species. Molecular and morphological analysis of populations previously assigned to *H. nigromaculatus* s.l. demonstrated the presence of two well defined species also in that species complex, with *H. setosus* living in streams, and *H. nigromaculatus* in lakes (Martin *et al*. 2010). In a similar way, other species claimed to inhabit different types of inland waters, could represent complexes of cryptic species as well. An interesting example is *Lebertia porosa* Thor, 1900, for which Stur (2017) reports Norwegian populations attributed to this species on the base of traditional systematics to comprise 7 BINs with a mean intraspecific *p*-distance of 11.7%. *Lebertia porosa* is a species reported from many different habitat types, including standing and flowing waters, with more than 25 (sub)species considered its junior synonyms over the past 11 decennia (Gerecke 2009). If molecular methods are carefully combined with traditional morphological and ecological analysis, they are a promising tool in taxonomy, allowing to detect misunderstood divergent lineages differing in habitat preference (Weigand *et al*. 2019).

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