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Two new species from the *Hygrobates nigromaculatus*-complex (Acariformes, Hydrachnidia, Hygrobatidae), based on morphological and molecular evidence

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Original research

ABSTRACT

We analyse the taxonomic structure of the *Hygrobates nigromaculatus*-complex from the Balkan Peninsula. We describe two new species: *Hygrobates lacrima* Pešić **sp. nov.** (Montenegro) and *H. limnocrenicus* Pešić **sp. nov.** (Montenegro, North Macedonia). Although both species are morphologically similar, the average K2P-distance between DNA-barcode sequences from *H. limnocrenicus* **sp. nov.** and its closest relative *H. setosus* was 12.43% (SD = 1.47), and between *H. lacrima* **sp. nov.** and its closest relative *H. nigromaculatus* 15.87% (SD = 1.74). The new species exhibit distinct differences in terms of habitat preference: *H. lacrima* **sp. nov.** inhabits pools and shallow eddies along faster flowing waters, whereas *H. limnocrenicus* **sp. nov.** prefers deeper, fast flowing water, typically found in the outflow of a limnocrenic springs or lake outlets. The finding of these two new species suggests that efforts to investigate mites of the *H. nigromaculatus*-complex in the Balkans should be intensified.

Keywords water mites; DNA-barcoding; species delimitation; new species; running waters; Montenegro; Macedonia

Zoobank <http://zoobank.org/754BE1B0-A316-409B-8008-556CE54ED5E4>

Introduction

Water mites of the genus *Hygrobates* Koch, 1837 are often the most ubiquitous and usually the most abundant representatives of the group in different types of running and standing waters over the Palaearctic (Pešić *et al.* 2017). In terms of ecology, many species of this genus have been reported in literature to be present in both standing (lakes) and running (streams) water habitats. Such kind of bipolar habitat preference, for example has been reported for *H. nigromaculatus* Lebert, 1879 and *H. longipalpis* (Hermann, 1804), both often reported as common species in Europe living both in lakes and streams (see Martin *et al.* 2010 for a discussion). Recently, the status of these species has been questioned by the integrative studies using DNA barcodes, proving that lake and stream populations, indeed, represent morphologically and genetically

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distinct lineages (see Martin *et al.* 2010 for *H. nigromaculatus*; Pešić *et al.* 2019a for *H. longipalpis*).

Hygrobates nigromaculatus has been subject of controversial debate in taxonomy for a long time (see Martin *et al.* 2010 for an overview). The status of populations from Northern and Central Europe was resolved by a molecular study: using the DNA barcode region of the mitochondrial cytochrome *c* subunit I (COI) gene and the nuclear D2 region of 28S rRNA gene, the presence of two well-defined species could be revealed, *H. setosus* Besseling, 1942 living in streams, and *H. nigromaculatus* in lakes (Martin *et al.* 2010).

In this study we used morphological data and results of DNA-barcoding to analyse specimens of the *H. nigromaculatus* s.l. from the Balkans, with the aim to evaluate potentially cryptic species and establish the *nigromaculatus* species complex in the genus *Hygrobates*. As a result, two species new to science are described.

Materials and methods

Water mites were collected by hand netting, sorted live 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 well slide with a central depression. After DNA extraction, some specimens were dissected and slide mounted in Faure's medium.

Morphological nomenclature follows Pešić *et al.* (2017; for explanations concerning morphology and measurements of *Hygrobates* species see there Figs. 1B-D). The holotypes of the new species are deposited in Naturalis Biodiversity Center in Leiden (RMNH). DNA sequences prepared in the course of this study are published in BOLD with accession numbers indicated in Table 1.

All measurements are given in μm . The following abbreviations are used: Ac-1 = most anterior acetabulum; Cx-I = first coxae; dL = dorsal length; 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; W = width; \bar{x} = mean value.

Molecular analysis

Molecular analysis was conducted in the Department of Invertebrate Zoology and Hydrobiology, University of Łódź, Poland. For methods used for COI gene amplification and sequencing see Pešić *et al.* (2017). For this study, DNA was extracted from a total number of three specimens of genus *Hygrobates* from Montenegro and North Macedonia (Table 1).

For DNA-barcoding and phylogenetic analysis we used previously published COI sequence data from Martin *et al.* (2010). In total, we used 51 sequences representing COI haplotypes of *Hygrobates lacrima* **sp. nov.** (1), *H. limnocrenicus* **sp. nov.** (2), *H. setosus* (n=33), *H. nigromaculatus* (n=11), with *H. trigonicus* (2), *H. prosiliens* (1) and *Limnesia undulata* (1) as outgroup taxa; the latter ones were chosen following Martin *et al.* (2010).

Sequences were aligned by MUSCLE 3.8.425 algorithm as implemented in Geneious Prime 2020.1.1 (Biomatters Ltd.). Phylogenetic tree for species delimitation was constructed

Table 1 List of newly sequenced specimens used in this study. For previously published sequences of the *Hygrobates nigromaculatus*-complex see Martin *et al.* (2010).

Locality (country, name)	Lat/Long	Voucher code	BOLD Acc. nos.
<i>Hygrobates limnocrenicus</i> sp. nov.			
Montenegro, Mareza	42°28'44.01"N, 19°10'52.50"E	13. M19_20_5_E4	DNAEC051-20
North Macedonia, Struga	41°11'3.39"N, 20°40'40.88"E	19. MECD2019_3_2_C2	DNAEC024-20
<i>Hygrobates lacrima</i> sp. nov.			
Montenegro, Tara River	42°47'18.78"N, 19°32'20.71"E	27. CG2020_3_C7	DNAEC029-20

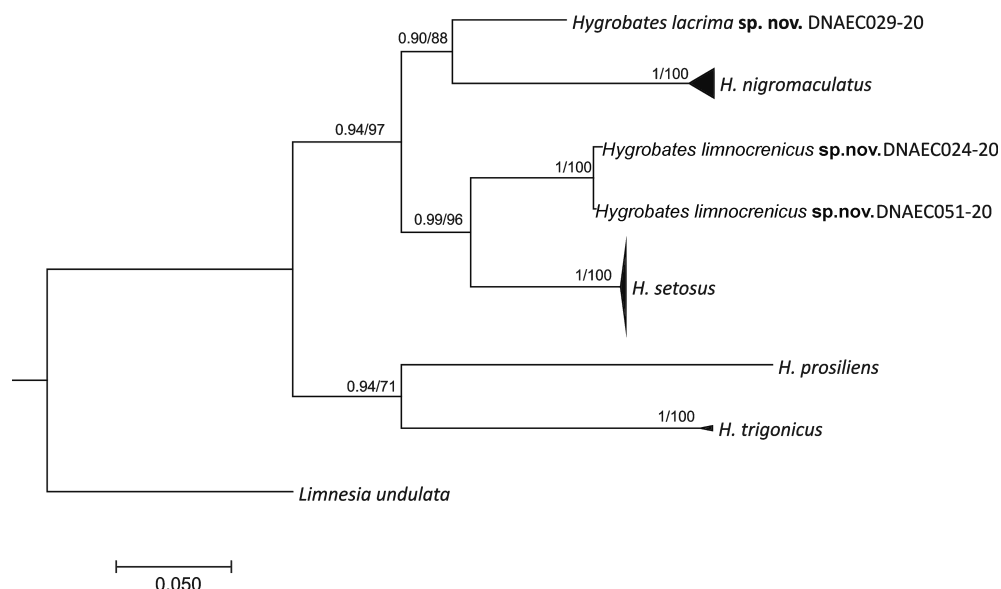


Figure 1 Phylogenetic tree (BI/FastTree) used for species-delimitation analysis. Values near branches show Bayesian posterior probability/Shimodaira-Hasegawa local supports; only values > 0.9/70 are shown.

using FastTree 2.1.11 and MrBayes, both using GTR+G model, as implemented in Geneious Prime 2020.1. Statistical supports for branches were estimated by Shimodaira-Hasegawa test (SH) (Shimodaira and Hasegawa 1999) and Bayesian posterior probability (PP). Tree was edited in MEGA7 and further in Corel Draw X5. 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 (Kumar *et al.* 2016). Additionally, sequence data were analysed 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://www.wabi.snv.jussieu.fr/public/abgd/abgdweb.html>) with default settings and K2P distance model.

Results

Species delimitation using DNA-barcodes

The final alignment for species delimitation using COI sequence data comprised 603 nucleotide positions (nps) for 51 specimens including outgroups. The nucleotide sequences could be translated into amino acid sequences without any stop codons. In the dataset, 243 nps out of 603 were variable, and average transition to transversion ratio for all variable sites was 1.7.

Molecular analysis shows that COI sequences from *Hygrobatinae* specimens collected in the Balkans form sister groups to the species previously known in the complex (Fig. 1). The sequence representing *H. lacrima* **sp. nov.** is reconstructed as a sister branch to the clade grouping COI sequences found in *H. nigromaculatus*, and two haplotypes found in *H. limnocrenicus* **sp. nov.** form a sister clade to the sequences found in *H. setosus* (Fig. 1); both relationships were recovered with strong support (0.90 PP, 88% SH and 0.99 PP, 96% SH, respectively).

The results of genetic distance analysis strongly supported the species status of the *Hygrobatinae* specimens collected in the Balkans. The genetic distance between the COI sequence of *H. lacrima* **sp. nov.** and its closest relative, *H. nigromaculatus*, was 15.87%

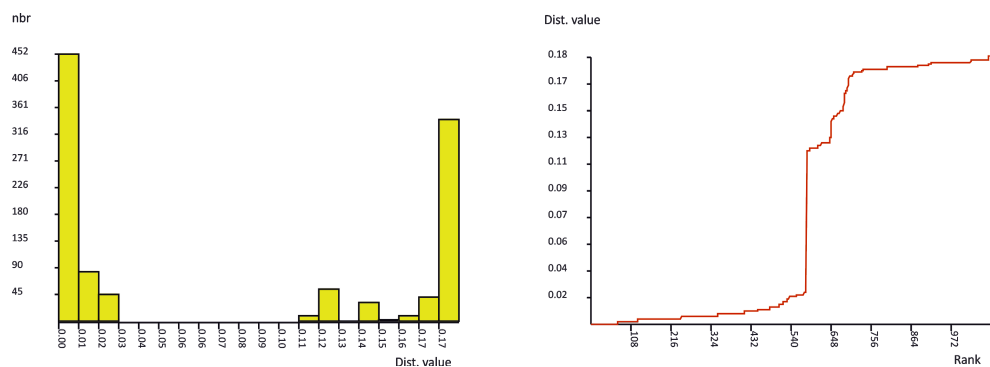


Figure 2 Results of Automatic Barcode Gap Discovery (ABGD) analysis for the COI sequences of *H. nigromaculatus* complex. (left) Distribution of pairwise differences, (right) Ranked pairwise differences.

(SD = 1.74) K2P, whereas the distance between *H. limnocrenicus* **sp. nov.** and *H. setosus* amounted to 12.43% (SD = 1.47) K2P. These distances were higher than the barcoding gap found by the ABGD method (3 to 11%) in the distances among all species belonging to the *H. nigromaculatus*-complex (Fig. 2), which additionally supported the species-status of the two new clades.

Systematics

Family Hygrobatidae Koch, 1842

Genus *Hygrobates* Koch, 1837

Subgenus *Hygrobates* s.s.

Hygrobates lacrima Pešić **sp. nov.**

Zoobank: [107B544E-3B5E-41BA-96BB-4D2F2F967F5E](https://zoobank.org/107B544E-3B5E-41BA-96BB-4D2F2F967F5E)

Figs. 3, 4, 5A-B

Material examined — Holotype ♀ [27. CG2020_3_C7], sequenced, dissected and slide mounted, Montenegro, River Tara near Mateševo, 42°47'18.78" N, 19°32'20.71" E, 26.10.2019, leg. Pešić. Paratypes: 1♂, 2♀♀ (1♂ and 1♀ partially dissected [palp, I-L and IV-L from one side slide mounted], River Tara near Trebaljevo, 42°51'47.68" N, 19°31'37.30" E, 1.9.2019, leg. Pešić.

Diagnosis — Large in size (mL of Cx-I + gnathosoma > 340, L genital plate > 210, P-4 > 170 µm); Cx-I+II apodemes protruding in both sexes, in females mediocaudal margins of Cx-IV with well-developed apodemes; anterior margin of male genital field with a bluntly pointed medial projection; L of IV-L-6 proximoventral seta ♂ < 20, ♀ < 35 µm.

Description — General feature – Colour yellowish to brown. Integument finely striated. Posteromedial margin of Cx-I rounded, caudal apodemes of Cx-I+II well-developed (Figs. 3A, 4A); Cx-IV subtriangular, with a distinct nose-like protruding medial margin. Genital field (in female holotype slightly damaged during dissection and mounting): Ac in triangular arrangement (Figs. 5A-B). 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. 3B-C, 4B).

Male – Anterior margin of genital field convex, with a small bluntly pointed medial projection, posterior margin indented, with a small central protrusion not extending beyond posterior genital plate margin (Fig. 5A).

Female – Genital plates distinctly longer than gonopore (Fig. 5B); P-4 slenderer than in male, L/H ratio 4.2-4.5.

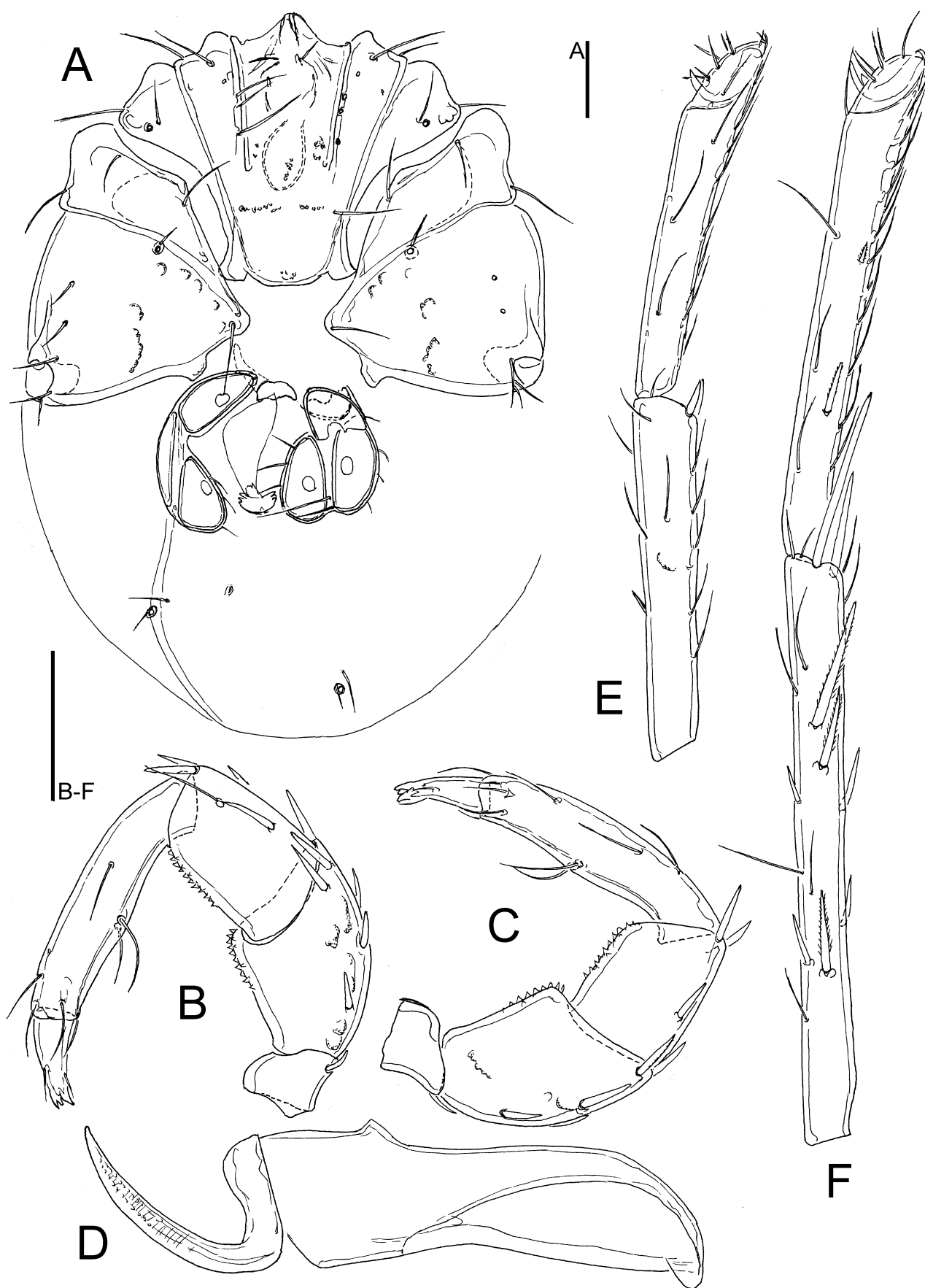


Figure 3 *Hygrobatres lacrima* sp. nov., holotype ♀, Mateševo, Montenegro: A – idiosoma, ventral view; B – palp, lateral view; C – palp, medial view; D – chelicera; E – I-L-5 and -6; F – IV-L-5 and -6. Scale bars = 100 µm.

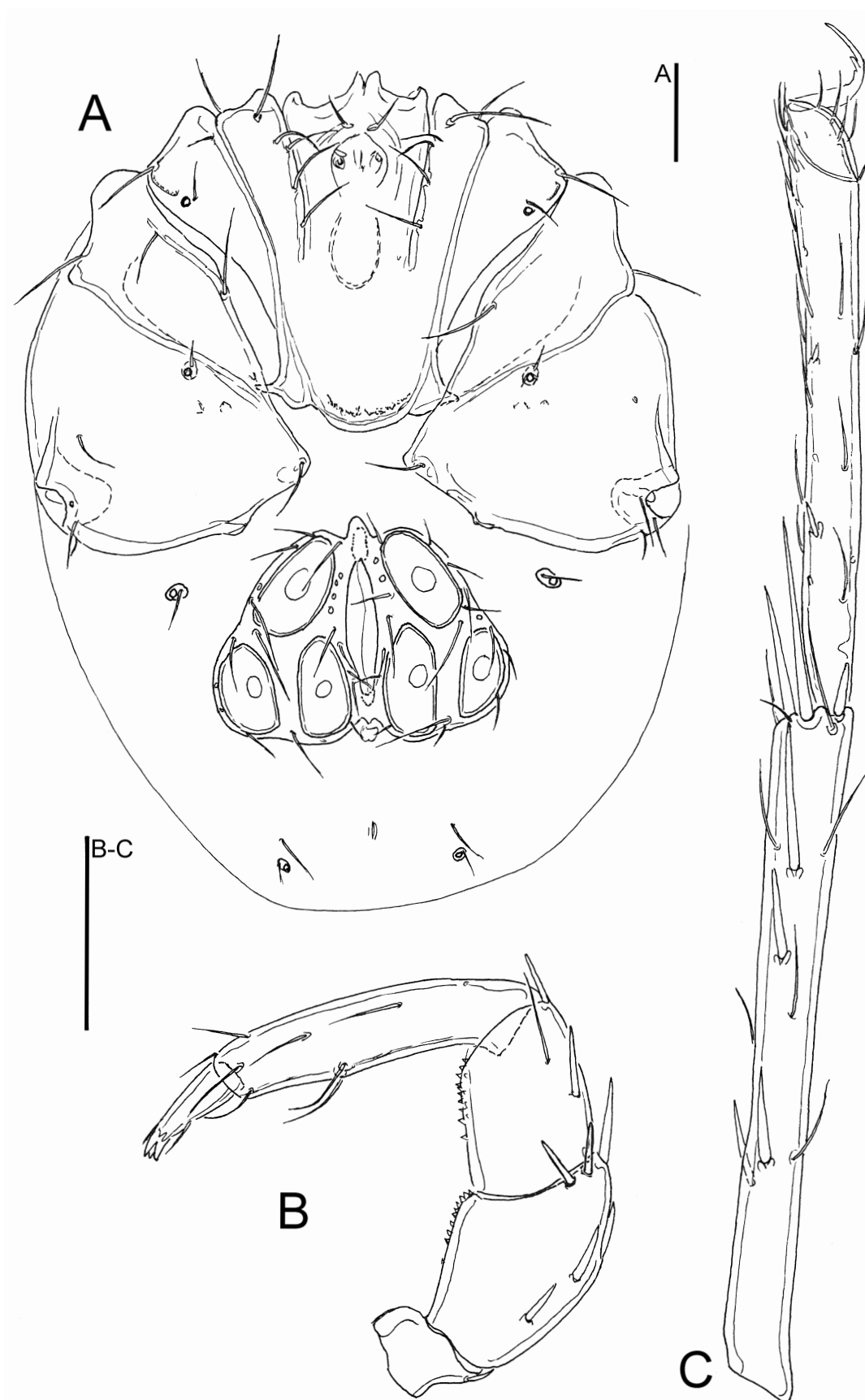


Figure 4 *Hygrobatella lacrima* sp. nov., ♂. Montenegro, Trebaljevo: A – idiosoma, ventral view; B – palp, medial view; C – IV-L-5 and -6. Scale bars = 100 µm.

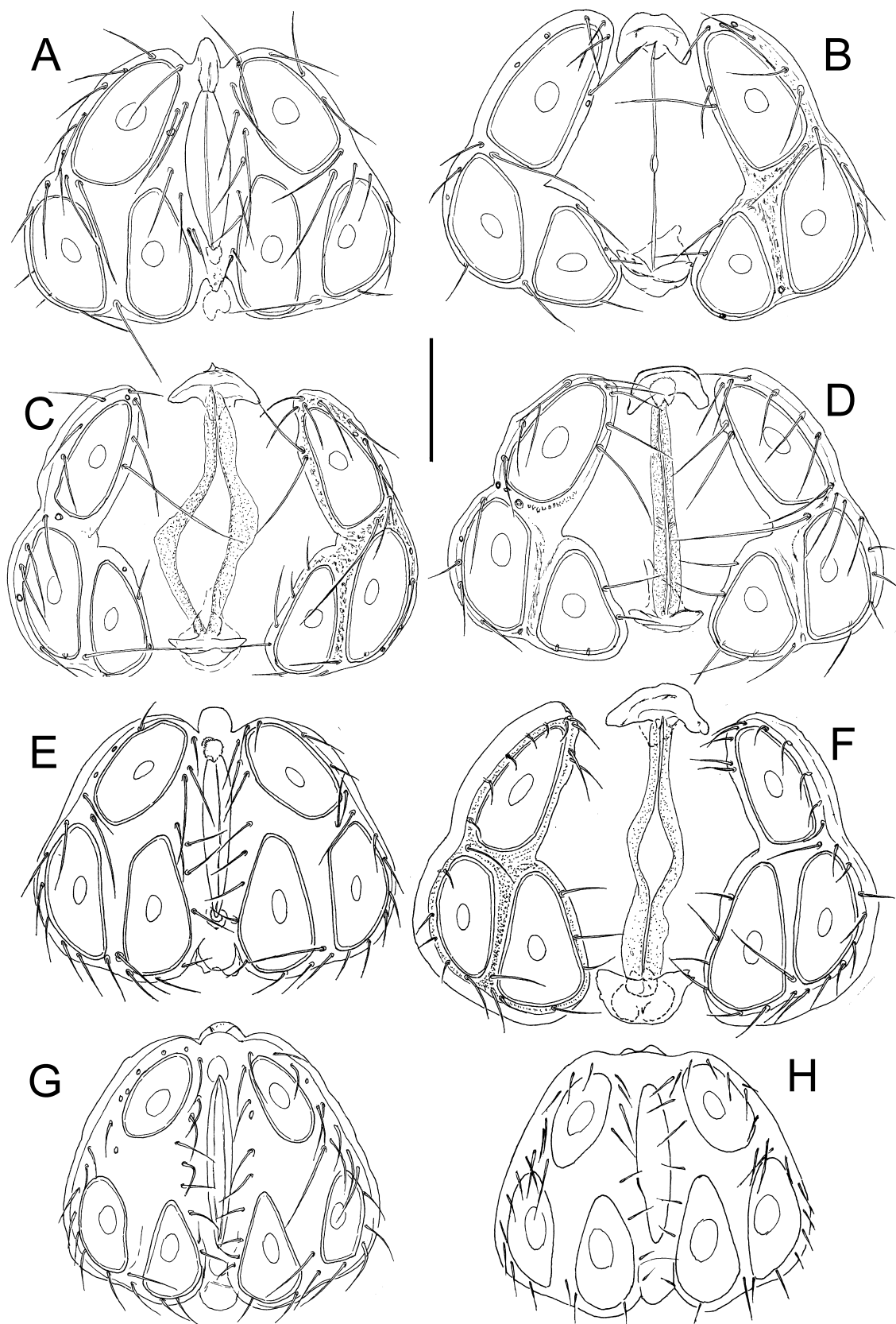


Figure 5 Genital field: A-B – *Hygrobates lacrima* sp. nov., paratypes (A, ♂; B, ♀), Trebaljevo, Montenegro; C-F – *Hygrobates limnocrenicus* sp. nov. (C-D, F, ♀; E, ♂); C, holotype, Mareza, Montenegro; D, paratype, Struga, North Macedonia; E-F, Vitoja, Montenegro; G-H – *H. setosus* Besseling, 1942, ♂, G, Farver Au, Germany; H, redrawn from Besseling (1964).

Measurements — Female (holotype; in parentheses measurements of paratype from Trebaljevo, Montenegro, n = 1)

Idiosoma – L (1025), W (944); coxal field: L 481 (506); Cx-II W 458 (485); Cx-III W 600 (653); mL of Cx-I + gnathosoma L 344 (361); distance between lateralmost ends of Cx-II apodemes, 191 (212); genital field L/W 219/294 (244/345); genital plate L 219 (241-248); gonopore L (203); L gonopore/genital plate ratio (0.82-0.84); L Ac 1-3: 119 (119-125), 128 (108-123), 95-113 (81-84).

Palp – total L 549 (623); dL/H, dL/H ratio: P-1, 44/50, 0.88 (52/53, 0.97); P-2, 147/89, 1.65 (161/98, 1.64); P-3, 99/77, 1.29 (122/82, 1.49); P-4, 192/45, 4.24 (217/48, 4.48); P-5, 67/23, 2.9 (71/29, 2.4); P-2/P-4 ratio 0.77 (0.74).

Legs – dL of I-L-1-6: 92 (95), 116 (141), 164 (184), 241 (259), 242 (272), 247 (266). dL of IV-L-1-6: 173 (188), 180 (200), 284 (328), 397 (438), 394 (441), 350 (364); L of IV-L-6 proximoventral seta 34 (23).

Male (paratype from Trebaljevo, Montenegro, n = 1)

Idiosoma – L 875, W 680; coxal field: L 477; Cx-II W 428; Cx-III W 577; mL of Cx-I + gnathosoma L 350; distance between lateralmost ends of Cx-II apodemes, 197; genital field L/W 231/300, ratio 0.77; L Ac 1-3: 106-119, 97-100, 106-115.

Palp – total L 511; dL/H, dL/H ratio: P-1, 41/47, 0.87; P-2, 134/86, 1.56; P-3, 95/70, 1.35; P-4, 177/42, 4.2; P-5, 64/22, 2.9; P-2/P-4 ratio 0.76.

Legs – dL of I-L-1-6: 78, 109, 147, 213, 231, 216. dL of IV-L-1-6: 150, 166, 263, 378, 362, 314; L of IV-L-6 proximoventral seta 18.

Etymology — “*lacrima*” (Latin): tear, in reference to the name of Tara River – The Tear of Europe.

Remarks — Based on molecular analysis, *Hygrobates lacrima* **sp. nov.** is closely related to *H. nigromaculatus*. The distance between these two species was 15.87% (SD = 1.74) K2P.

In comparison with the new species from Montenegro, *H. nigromaculatus* (data taken from Martin *et al.* 2010) generally is smaller in dimensions. In males of *H. nigromaculatus*, the length of genital plate is < 160 (vs. > 220 µm in *H. lacrima*), in females < 175 (vs. > 210 µm in *H. lacrima*); mL Cx-I + gnathosoma in males is < 280 (vs. > 340 µm in *H. lacrima*), in females < 350 µm (vs. > 340 µm in *H. lacrima*); the length of P-4 in males *H. nigromaculatus* is < 130 (vs. > 160 µm in *H. lacrima*), in females < 165 µm (vs. > 180 µm in *H. lacrima*). Moreover, the species live in different habitats: *H. nigromaculatus* in lakes, whereas *H. lacrima* inhabits pools and shallow eddies along faster flowing waters (see Figs. 9A-C). From *H. setosus* (in parentheses, based on material from Farver Au, Germany), male of new species differs in having the bluntly-pointed medial projection of the genital field and comparatively wider genital field (L/W ratio 0.83-0.9; compare Fig. 5A and 5G). Although Tuzovskij (2017) showed different types of male genital plates for *H. nigromaculatus*, we cannot exclude that he dealt with several species. Therefore, we think that the differences between the male genital plates of *H. lacrima* and other species as given in the key are justified.

Due to its larger size and habitat preference for running waters, the new species is similar to *H. limnocrenicus* **sp. nov.** (see there for further discussion).

Distribution — Montenegro; known from two localities along the middle course of the Tara River (Figs. 9A-C). These two sites were subject of a monthly monitoring survey in 2019 on the river biota of the Tara River during the Bar-Boljare highway development activities (see Pešić *et al.* 2020a). At the Mataševo site, only one specimen (which was successfully barcoded) was collected, probably due to the negative ecological impact associated with highway development activities in the immediate vicinity (for the controversy surrounding highway construction over the Tara River see Pešić *et al.* 2020b). At the Trebaljevo site located downstream, the negative impact was less pronounced - here, three individuals were collected.

Hygrobates limnocrenicus Pešić sp. nov.

Zoobank: F010A08A-BFD9-4E57-9E22-E9CE9CDA740A

Figs. 5C-F, 6, 7

Synonym – *Hygrobates setosus sensu* Pešić *et al.* 2018a: 170; Bańkowska *et al.* 2016: 1028; Pešić *et al.* 2019c: 471.

Material examined — Holotype ♂ [19. M19_20_5_E4], sequenced, dissected and slide mounted, Montenegro, Podgorica, Mareza, outflow of limnocrene spring, 42°28'44.01" N, 19°10'52.50" E, 24.6.2019, leg. Pešić & Zawal. Paratypes: 1♀ (dissected and slide mounted), Podgorica, Daljam, limnocrene spring "Kraljičino Oko", springbrook, 42°29'9.61" N, 19°10'25.28" E, 31.5.2018, leg. Pešić & Zawal; North Macedonia, Struga, Crni Drim River-outlet of Ohrid Lake, 41°11'3.39" N, 20°40'40.88" E, 12.9.2019, leg. Pešić, 1♀ [19. MECD2019_3.2_C2], sequenced, conserved in Koenike fluid.

Other material — Montenegro: Podgorica, Vitoja springs near Skadar Lake, 42°19'31.25" N, 19°21'45.93" E, 5.8.2017, leg. Pešić, 6♂♂, 8♀♀ (1♂ and 1♀ dissected and slide mounted); *ibid.*, 30.7.2014, leg. Pešić, 1♂, 1♀; *ibid.*, 31.5.2018, leg. Pešić & Zawal, 142 ex. (115 ex. in ethanol; 5♂♂, 4♀♀ in Koenike fluid; 2♂♂, 2♀♀ dissected and slide mounted).

Compared material – *Hygrobates setosus*, Germany, Farver Au, 54°16'0.5556" N, 10°48'9.0396" E, small stream, 5.9.1992, leg. P. Martin, 2♂♂, 3♀♀ (2♂♂, 1♀ dissected and slide mounted).

Diagnosis — Large in size (mL of Cx-I + gnathosoma > 350, L genital plate > 200, P-4 > 170 µm); Cx-I+II apodemes moderately protruding, mediocaudal margins of Cx-IV in females without prominent apodemes; anterior margin of male genital field with a knob-shaped medial projection; L of IV-L-6 proximoventral seta ♂ > 40, ♀ > 35; running waters.

Description — General features – Colour yellowish to brown (Fig. 8, inset). Integument finely striated. Posteromedial margin of Cx-I rounded, caudal apodemes of Cx-I+II well-developed (Figs. 6A, 7A); Cx-IV subtriangular. Genital field: Ac in a triangular position. 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 (Figs. 6B-C, 7C-D); P-4 ventral setae on the same level.

Male – Anterior margin of genital field convex, with a small knob-shaped medial projection, posterior margin indented, with a rounded central projection not extending beyond posterior genital plate margin (Figs. 5E).

Female – Genital plates distinctly longer than gonopore (Fig. 5C-D, F).

Measurements — **Female** (Holotype; in parentheses measurements of paratype from Crni Drim River, Macedonia; in square brackets measurements of specimens from Vitoja, given as range and mean, n = 3)

Idiosoma – L (1050), W (853) [1080-1210, χ = 1136]; coxal field: L 475 (525) [489-536, χ = 514]; Cx-II W 453 (484) [406-513, χ = 472]; Cx-III W 634 (627) [689-753, χ = 721]; mL of Cx-I + gnathosoma L 375 (403) [339-403, χ = 370]; distance between lateralmost ends of Cx-II apodemes, 203 (221) [197-213, χ = 202].

Genital field – L/W 241 (256) [259-281, χ = 271.7], W 353 (334) [356-386, χ = 371.3]; genital plate L 233-238 (236-241) [247-268, χ = 258]; gonopore L 194 (206) [209-238, χ = 224.3]; L gonopore/genital plate ratio 0.82-0.83 (0.85-0.87) [0.85-0.89, χ = 0.87]. L Ac 1-3: 109-116 (103) [109-116, χ = 112.7]; 108 (109-113) [112-117, χ = 114]; 84-86 (94) [106-124, χ = 112].

Palp – total L 537 [568-625, χ = 598]; dL: P-1, 41 [48-45, χ = 47]; P-2, 147 [157-172, χ = 165]; P-3, 94 [109-123, χ = 115]; P-4, 183 [194-209, χ = 203]; P-5, 72 [63-73, χ = 68.3]; H: P-1, 55 [53-57, χ = 55]; P-2, 97 [100-108, χ = 104.3]; P-3, 80 [82-86, χ = 84]; P-4, 52 [50-53, χ = 51]; P-5, 27 [23-25, χ = 23.7]; dL/H ratio: P-1, 0.74 [0.84-0.87, χ = 0.86]; P-2, 1.52 [1.54-1.64, χ = 1.59]; P-3, 1.18 [1.32-1.42, χ = 1.36]; P-4, 3.54 [3.88-4.15, χ = 3.99]; P-5, 2.7 [2.67-2.94, χ = 2.85]; P-2/P-4 ratio 0.8 [0.81-0.82, χ = 0.81]. Chelicera total L 434 [403-442, χ = 423.3],

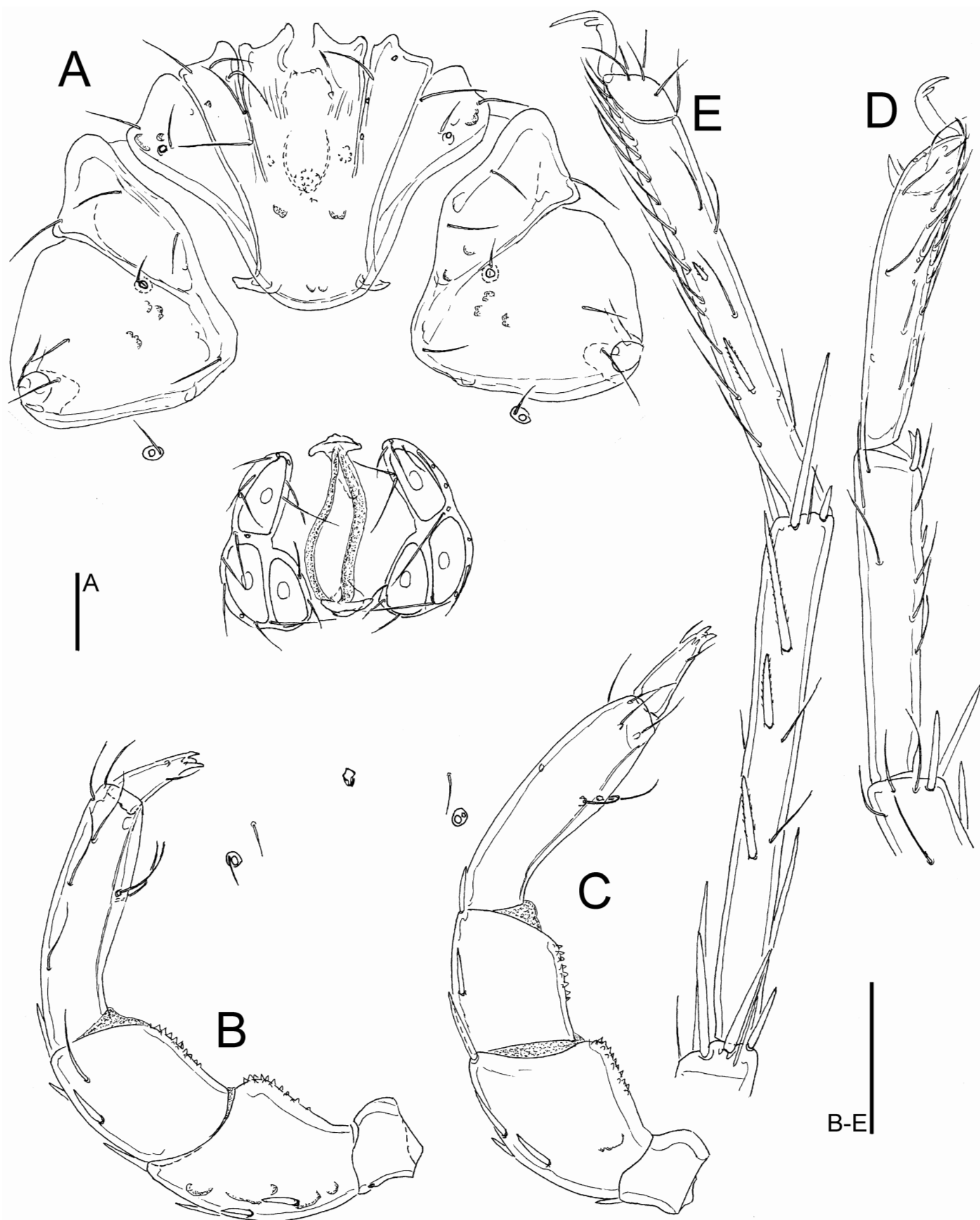


Figure 6 *Hygrobatella limnocrenicus* sp. nov., holotype ♀, Mareza, Montenegro: A – coxal and genital field; B – palp, lateral view; C – palp, medial view; D – I-L-5 and -6; E – IV-L-5 and -6. Scale bars = 100 μm.

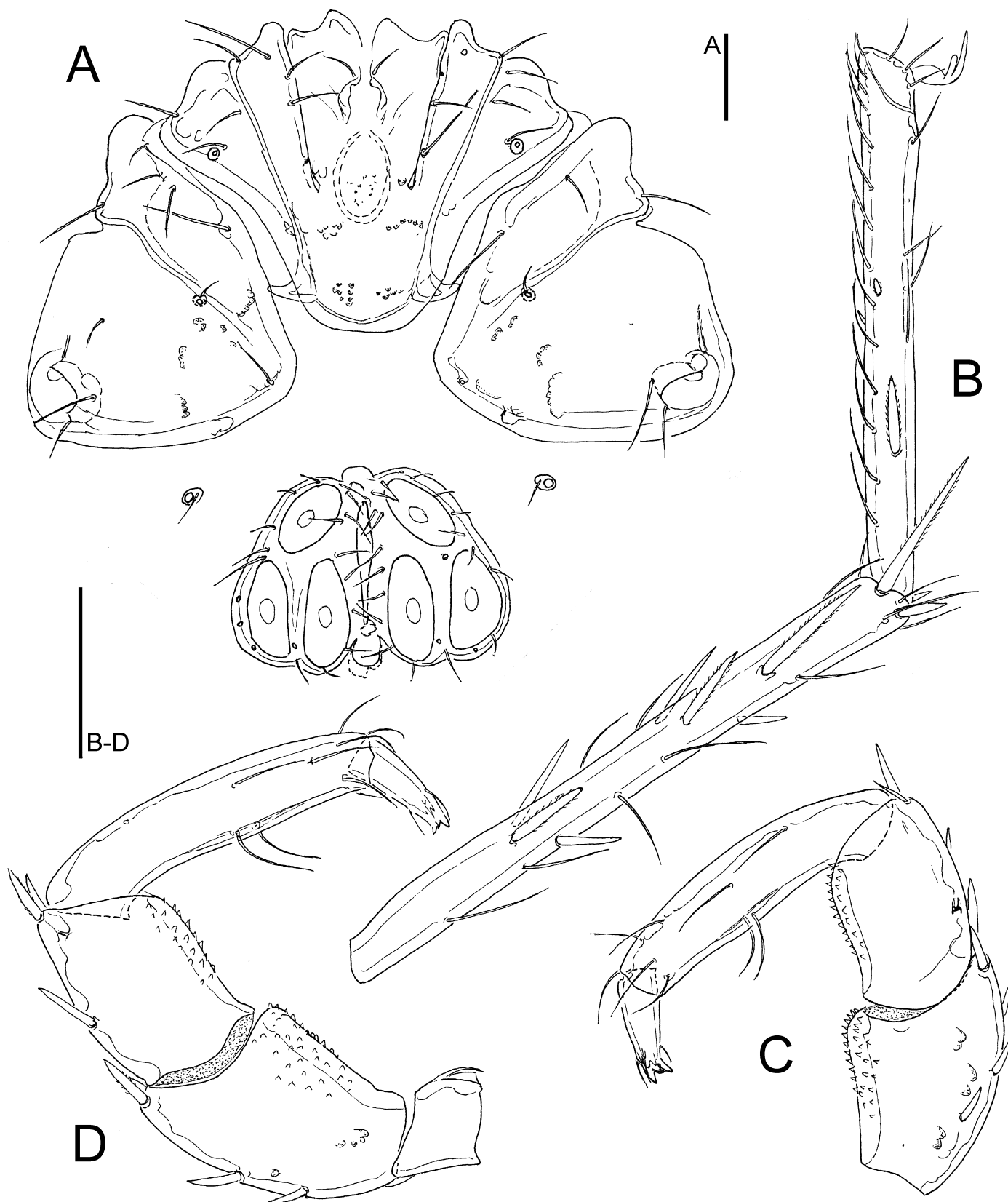


Figure 7 *Hygrobates limnocrenicus* sp. nov. (A-C, male; D, female), Vitoja, Montenegro: A – coxal and genital field; B – IV-L-5 and -6; C – palp, lateral view (P-1 lacking); D – palp, lateral view. Scale bars = 100 μ m.

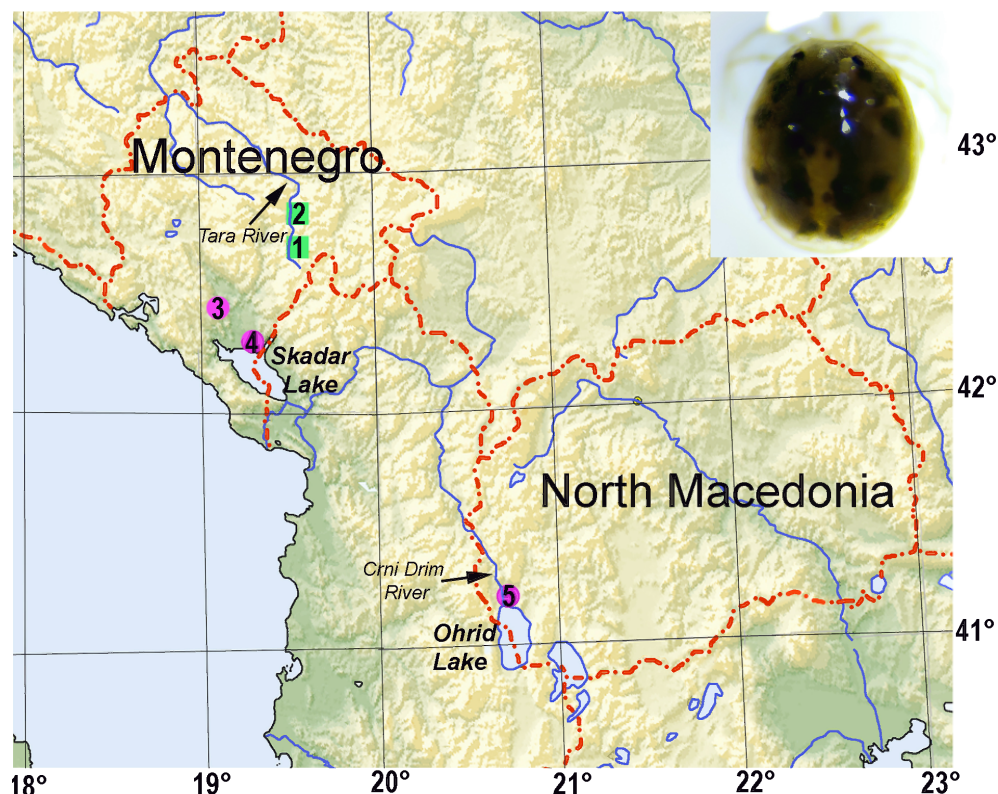


Figure 8 Distribution of *H. lacrima* sp. nov. (green square) and *H. limnocrenicus* sp. nov. (purple circle). Sampling site numbers: 1, River Tara near Mateševo; 2, River Tara near Trebaljevo; 3, Mareza and Kraljičino Oko (Queen's Eye) springs; 4, Vitoja springs; 5, Crni Drim River-outlet of Lake Ohrid. Inset: Photograph of *H. limnocrenicus* sp. nov.

L basal segment 278 [269-291, $\chi = 281$], claw 163 [153-172, $\chi = 160$], L basal segment/claw ratio 1.7 [1.65-1.87, $\chi = 1.76$].

Legs – dL of I-L-1-6: 84 (86) [84-89, $\chi = 86$]; 103 (116) [116-136, $\chi = 126.7$]; 144 (169) [160-172, $\chi = 168$]; 203 (244) [225-256, $\chi = 242.7$]; 222 (255) [245-272, $\chi = 260$]; 216 (240) [238-256, $\chi = 248$]. dL of IV-L-1-6: 163 [172-192, $\chi = 185$], 167 [188-205, $\chi = 198$], 266 [284-325, $\chi = 304$], 353 (411) [413-466, $\chi = 440$], 356 (414) [416-445, $\chi = 432$], 309 (325) [338-366, $\chi = 356$]. L of IV-L-6 proximoventral seta 41 [37-55, $\chi = 44$].

Male (specimens from Vitoja springs, Montenegro, measurements given as range and mean, $n = 3$)

Idiosoma – L 1040-1140, $\chi = 1086$; coxal field: L 487-511, $\chi = 500$; Cx-II W 459-500, $\chi = 474$; Cx-III W 631-700, $\chi = 656$; mL of Cx-I + gnathosoma L 372-386, $\chi = 381$; distance between very lateral ends of Cx-II apodemes, 190-216, $\chi = 206$. Genital field L 227-258, $\chi = 243$, W 300-322, $\chi = 311.7$. L Ac 1-3: 100-122, $\chi = 110$; 113-122, $\chi = 116$; 116-134, $\chi = 126$.

Palp – total L 546-585, $\chi = 561$; dL: P-1, 46-50, $\chi = 48$; P-2, 144-158, $\chi = 151.3$; P-3, 105-113, $\chi = 108$; P-4, 185-198, $\chi = 190.3$; P-5, 61-66, $\chi = 63.3$; H: P-1, 52-54, $\chi = 53$; P-2, 94-98, $\chi = 95.3$; P-3, 75-78, $\chi = 76$; P-4, 42-50, 46.7; P-5, 23, $\chi = 23$; dL/H ratio: P-1, 0.98-0.93, $\chi = 0.91$; P-2, 1.53-1.68, $\chi = 1.58$; P-3, 1.4-1.44, $\chi = 1.42$; P-4, 3.96-4.38, $\chi = 4.1$; P-5, 2.68-2.8, $\chi = 2.74$; P-2/P-4 ratio 0.77-0.82, $\chi = 0.8$. Chelicera total L 391-413, $\chi = 400.3$, L basal segment 254-273, $\chi = 266$, claw 147-153, $\chi = 150$, L basal segment/claw ratio 1.66-1.86, $\chi = 1.77$.

Legs– dL of I-L-1-6: 75-89, $\chi = 81.7$; 113-121, $\chi = 117$; 148-154, $\chi = 152$; 213-228, $\chi = 220$; 228-248, $\chi = 237$; 225-239, $\chi = 232$. dL of IV-L-1-6: 181-203, $\chi = 190$; 181-188, $\chi = 183$; 269-288, $\chi = 276$; 396-411, $\chi = 404$; 398-397, $\chi = 398$; 331-341, $\chi = 335.3$. L of IV-L-6 proximoventral seta 45-48, $\chi = 46.7$.

Etymology — The species is named after its dominant occurrence in limnocrane springs.

Remarks — Due to its larger dimensions (and also regarding habitat preference), the new species is similar to *H. lacrima* **sp. nov.** Females of the latter species differ from *H. limnocrenicus* **sp. nov.** in having well-developed apodemes at mediocaudal margins of Cx-IV (compare Fig. 3A with Fig. 6A), males in the medial projection of the genital field bluntly-pointed (knob-shaped in *H. limnocrenicus* **sp. nov.**; compare Fig. 5A and 5E), both sexes bear a shorter proximoventral seta on IV-L-6 (L *H. lacrima* vs. *H. limnocrenicus*, ♂♂: < 20 vs. > 40, ♀♀: < 35 vs. > 35 µm).

Analysis of COI sequences suggests that *H. limnocrenicus* **sp. nov.** is most closely related to *H. setosus* Besseling, 1942, which has also a preference for running water habitats. However, the latter species, as well as *H. lacrima* **sp. nov.**, was mostly found in pools of running waters, while *H. limnocrenicus* **sp. nov.** prefers deeper, fast flowing water, typically being found in the outflow of limnocrane springs or lake outlets (Figs. 9D-F). The COI divergence between *H. setosus* and *H. limnocrenicus* **sp. nov.** was 12.43% (SD = 1.47). K2P indicating a long independent history of these two species. The studied specimens of *H. setosus* (in parentheses) differ in both sexes by a shorter proximoventral seta on IV-L-6 (L *H. setosus* vs. *H. limnocrenicus*, ♂♂: < 30 vs. > 40, ♀♀: < 20 vs. > 35 µm) and in males by the comparatively less wide genital field (L/W ratio > 0.8), a smaller dimensions of acetabula (Ac-3 < 105 µm), and the medial projection of the genital field less pronounced, typically with irregular margin of a secondary sclerotization (see Figs. 5G-H).

Biology — Morphological and genetical analysis of populations from the Vitoja springs situated at the northeastern shore of the Lake Skadar indicates that the oviposition data published by Bańkowska *et al.* (2016) for *H. setosus* in fact refers to *H. limnocrenicus* **sp. nov.** The highest number of females laying eggs was found in springs, with an average number of eggs per female of 58.2±30.6. A lower number of females laying eggs was noted in rivers, but here the average number of eggs per female (80±24.5) was higher (Bańkowska *et al.* 2016). A statistically significant difference in number of laid eggs between May and October was found. The average time of hatching was 14.1 ± 5.29 days.

Distribution — Montenegro and North Macedonia (Fig. 8). Details of distribution are unknown due to the previous confusion with *H. setosus*, but the species is obviously widespread in the Balkans. It is likely that the records of the latter species from the limnocrane springs of the Mediterranean region of the Balkan refers to *H. limnocrenicus* **sp. nov.** For example, Pozojević *et al.* (2019) reported occurrence of *H. setosus* in the limnocrane spring Modro Oko in Southern Dalmatia, in a low abundance not exceeding 4 individuals per square meter.

Key to the European species of the *Hygrobates nigromaculatus*-complex

1. Median length of Cx-I + gnathosoma < 340 µm. Males: P-4 L < 130, genital plate L < 160. Females: P-4 L < 165, genital plate L < 175 µm. *Hygrobates nigromaculatus* Lebert, 1879; Central Europe (Preferably in standing waters)
— Median length of Cx-I + gnathosoma > 340 µm. Males: P-4 L > 140, genital plate L > 170. Females: P-4 L > 165, genital plate L > 175 µm. (preferably in running waters) 2
2. Male genital field with a narrow, bluntly-pointed anteromedial projection (Fig. 5A)..... *Hygrobates lacrima* **sp. nov.**; Montenegro (in pools of running waters)
— Male genital field with a more broad-based, knob-shaped anteromedial projection (Figs. 5E, G)..... 3
3. Male: genital field comparatively wider (L/W ratio < 0.8), acetabula larger in dimension, Ac-3 > 110 µm, anteromedial projection of genital field well pronounced (Fig. 5E); IV-L-6 proximoventral seta L: males, > 40, female, > 35 µm. *Hygrobates limnocrenicus* **sp. nov.**; Montenegro, Macedonia (Preferably in deeper, fast flowing water)

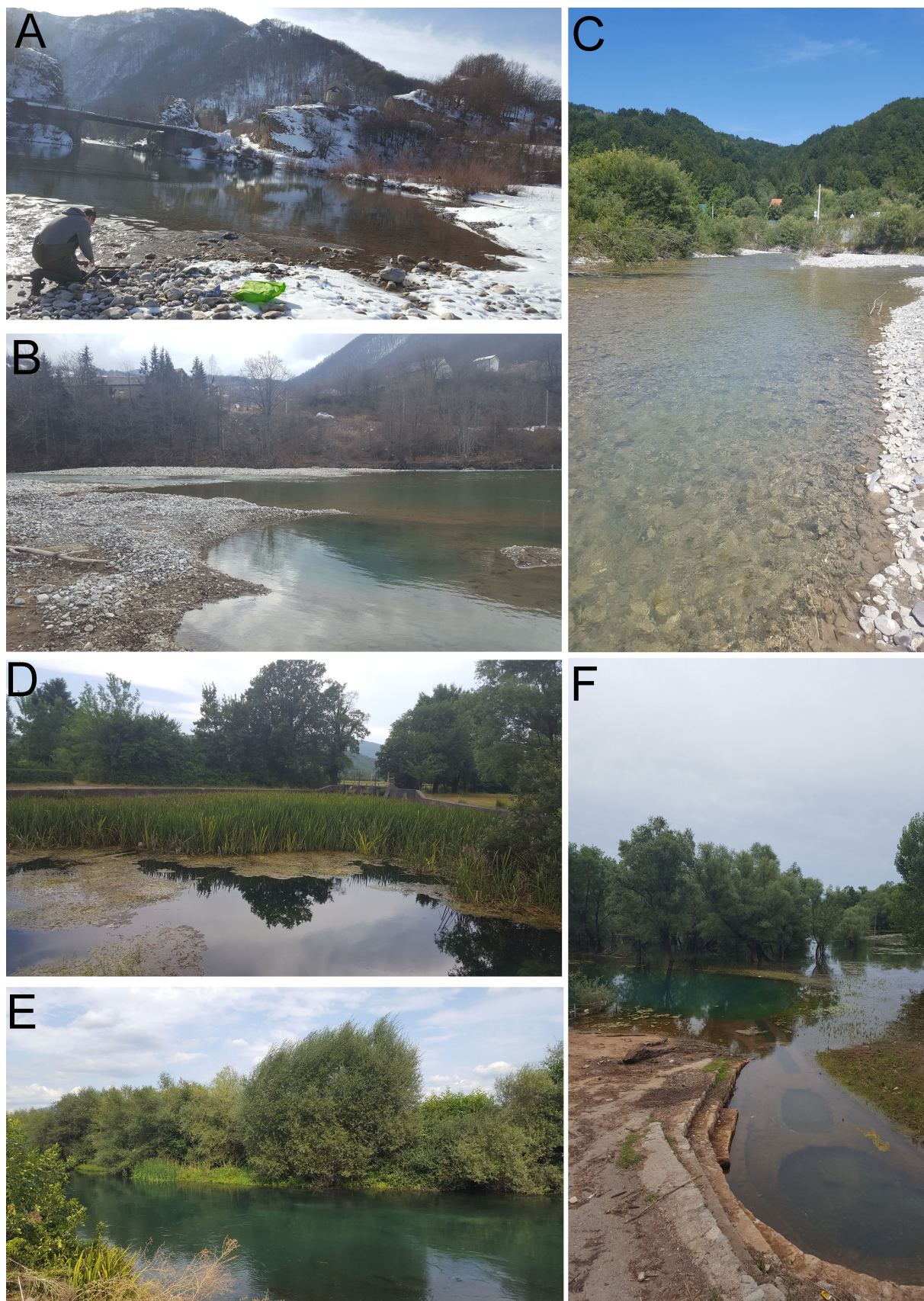


Figure 9 Sampling sites: A-B – River Tara near Trebaljevo; C – River Tara near Mateševo; D – the "Kraljičino Oko" (the Queen's Eye) spring near Podgorica; E – River Crni Drim near Struga; F – the Vitoja spring located on the north-eastern shore of Lake Skadar flooded in the winter and spring months.

— Male: genital field comparatively less wide (L/W ratio > 0.8), acetabula smaller in dimension, Ac-3 < 110 µm, anteromedial projection of genital field less pronounced, often with irregular border of secondary sclerotization (Figs. 5G-H); IV-L-6 proximoventral seta L: males, < 30, female, < 20 µm.
 .. *Hygrobatos setosus* Besseling, 1942; Central Europe (preferably in pools of running waters)

Discussion

The use of an integrative approach based on molecular data and on morphology allowed us to define a new species-complex in the genus *Hygrobatos*, the *H. nigromaculatus*-complex, containing at least four species, *H. nigromaculatus*, *H. setosus*, *H. lacrima* **sp. nov.**, and *H. limnocrenicus* **sp. nov.**

The study conducted by Martin *et al.* (2010) on *H. nigromaculatus* and *H. setosus* and our study on two new species from the Balkans reveal that these species substantially overlap in morphology. Without the results of molecular analyses, it would have been very difficult to circumscribe in this complex species boundaries, and to determine their similarities. For example, molecular data suggest that *H. lacrima* **sp. nov.** is closer to *H. nigromaculatus* than to *H. setosus* with which it shares a larger size and a preference for running waters.

From *H. setosus* and two new species described from the Balkans, *H. nigromaculatus* differs in smaller size and its habitat preference for standing waters, often preferring surge shores of the littoral zone of lakes (Martin 1996, Martin *et al.* 2010). *Hygrobatos setosus* is exclusively found in pools of streams with slow current (Martin *et al.* 2010). Similar to *H. setosus*, *H. lacrima* **sp. nov.** inhabits pools and shallow eddies along faster flowing waters such as the Tara River. Finally, *H. limnocrenicus* **sp. nov.** prefers deeper, fast flowing water, typically found in the outflow of a limnocrenic springs or a lake outlets.

During our survey, an abundant population of *H. limnocrenicus* **sp. nov.** was found in the Vitoja springs, located at the shore of Skadar Lake, and falling below the lake water level during high water periods in winter and early spring (Pešić *et al.* 2018b; see Fig. 9F). Probably, the proximity of the lake provides optimal conditions to sustain a large population of *H. limnocrenicus* **sp. nov.** compared with isolated water bodies where it was found in a low abundance only (regardless of the effort made to sample more material, only a few specimens were collected in the outflows of the Mareza and Kraljičino Oko (Queen's Eye) springs). It is worth mentioning that *H. limnocrenicus* **sp. nov.** was found also in the Crni Drim (Black Drin) River near the exit from the Ohrid Lake which shares the same watershed as the Skadar Lake. The latter finding supports the congruence of the distribution of this species in the Adriatic Sea watershed.

As demonstrated by Martin and Davids (2002), differences in life-cycle strategies may contribute to species discrimination within the *H. nigromaculatus*-complex. The stream-dwelling populations of *H. setosus* have a parasitic larva, whereas the lake-living populations of *H. nigromaculatus* have reduced larval parasitism (Martin and Davids 2002). The question if parasitism eventually went lost in a common ancestor of *H. lacrima* **sp. nov.** and the *H. nigromaculatus* merits particular attention. Martin *et al.* (2010) hypothesized that *H. nigromaculatus* secondarily lost parasitic larvae and probably originated from a stream-living *Hygrobatos* species with a larval stage able to disperse. Both new species have a relatively large body size which may be a first hint to a parasitic life style. Often, non-parasitic lineages are able to afford to be smaller by producing less abundant, but larger eggs (Smith 1998).

Although our study is based on a relatively small number of individuals that could be sequenced, it resulted in the discovery of two new species and the definition of a *H. nigromaculatus* species complex. Our results suggest that efforts to investigate mites of this complex should be intensified all over Europe.

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