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Molecular phylogeny of the cave beetle genus *Hadesia* (Coleoptera: Leiodidae: Cholevinae: Leptodirini), with a description of a new species from Montenegro

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Abstract

Hadesia is a charismatic and scientifically important genus of cave beetles. It is a classical representative of the subterranean fauna of the Dinaric Karst and the model upon which the ultra-specialized ecomorphological form of semi-aquatic, hygropetricolous filter feeders became known. We describe a new species, *Hadesia zetae* **sp.n.**, from southwestern Montenegro, and employ new morphological characters to build an identification key to all five species of the genus. Based on DNA sequence data from two nuclear and two mitochondrial genes and using multispecies coalescent methods, we corroborated their reciprocal monophyly and inferred a well-supported phylogenetic hypothesis. Molecular dating suggests that the most recent common ancestor of the extant *Hadesia* species lived about three million years ago, during the warm and wet mid-Pliocene, and diversified allopatrically through range fragmentation caused by habitat reduction during the Pleistocene. Because of their extreme rarity and observed negative impact of collection pressure, we discourage collecting of *Hadesia* at known sites.

Key words

Leptodirini, Anthroherponina, *Hadesia*, new species, molecular phylogeny, Dinaric Karst, troglobiont, subterranean, cave hygropetric.

1. Introduction

Since the beginning of the twentieth century, the Dinaric Karst has been widely recognized for its rich and highly diverse subterranean coleopteran fauna (Jeannel 1911, 1924, 1928; Müller 1917). A significant part of this richness can be attributed to the large tribe Leptodirini (Leiodidae: Cholevinae), known for its numerous highly specialized subterranean species. According to the presently accepted Leptodirini systematics (Guéorguiev 1976; Giachino et al. 1998; Newton 1998; Perreau 2000), the tribe is divided into six or seven subtribes restricted to the Western Palearctic (Fresneda et al. 2011). This contribution deals with the subtribe Anthroherponina, which consists of several morphologically highly evolved and ecologically ultra-specialized genera. Among them, *Hadesia*, *Croatodirus*, *Nauticiella*, *Velebitodromus* and *Kircheria* are specialist filter feeders, dwelling in films of water running down the cave walls. This unique habitat is known as the cave hygropetric (Sket 2004; Engel et al. 2013). The mouthparts of these genera have widened maxillae and mandibles, coupled with densely setose maxillipeds, forming filters for fine grained organic matter (Remy 1940; Casale et al. 2000a; Moldovan et al. 2004).

The first described and best known of all hygropetricolous beetles belong to the genus *Hadesia* (Figs. 1, 2).

Figs. 1, 2. *Hadesia* in its natural habitat: **1**: *Hadesia vasiceki* Müller in the cave Špilja za Gromačkom vlakom, Dubrovnik, Croatia (photo credit: B. Jalžić). **2**: *Hadesia zetae* **sp.n.** in its natural hygropetric habitat in the cave Lipska pećina, Cetinje, Montenegro.

The type species *H. vasiceki* (Fig. 1) was described by Müller (1911) from the cave Vjetrenica in southern Herzegovina. He noted *Hadesia*'s strangely looking mouthparts, bearing numerous long, gold-colored bristles on the clypeus. Jeannel (1924) presented the first detailed drawings of the special morphological adaptations in his monographic revision of the Bathysciinae (= Leptodirini). For over a decade, the species and genus had been known only from their type locality, when Zariquiey (1927) described a new subspecies, *H. vasiceki weiratheri*, based on the male specimen collected by Leo Weirather in Montenegro, in a cave referred to by Weirather as Höhle 9 and supposedly called Dobra Pećina at Droškorica, Lisac Gau. After 50 years this *nomen fictum* was resolved, and the type locality of. *H. vasiceki weiratheri* identified as the cave Vojvode Dakovića pećina at Grahovo, Grahovsko polje, Montenegro (Pretner 1974, 1977, 2011; Giachino & Lana 2006). While the type species *Hadesia vasiceki* is common in deeper parts of Vjetrenica Cave and is relatively easy to see, *H. weiratheri* is extremely difficult to find, and only a few specimens have been collected until now (Perreau & Pavićević 2008). Intensive cave sampling in Herzegovina, Montenegro and Croatia since the year 2000 yielded some additional sites for the genus *Hadesia*, and prompted a taxonomic revision of the genus (Perreau & Pavićević 2008). These authors redescribed the nominotypical *H. vasiceki vasiceki* with the inclusion of specimens from the cave Špilja pod Gromačkom vlakom near Dubrovnik, Croatia. Additionally, they described two new species: *Hadesia lakotai* from the cave Veliko Đatlo at Bileća, Herzegovina, and *Hadesia asamo* from the cave Jama Bravenik at Grab, Zubačko polje, Herzegovina (code-named pećina Vodenica, Zubei Gau, Orjen Gruppe – Höhle 6, Orjen, by Weirather; Pretner 2011). The same authors (Perreau & Pavićević 2008) raised the status of *H. vasiceki weiratheri* to species level.

Our recent cave explorations in western Montenegro resulted in the collection of individuals superficially resembling the *H. weiratheri*, but differing in morphological details and DNA sequences. In this paper we morphologically characterize and taxonomically validate the newly discovered species. We further apply a multi-locus molecular approach to infer phylogenetic relationships among all known *Hadesia* species, and test their status using several species delimitation procedures.

2. Material and methods

Specimen collections. Over a period of 10 years, we collected representatives of all known species of *Hadesia* from limestone caves in western Herzegovina and western Montenegro. Because of their special habitat requirements and feeding habits, adult *Hadesia* are rare and usually occur in remote and deep parts of caves in very small numbers. Several visits to a cave may be necessary in order to find a single individual specimen. Unlike most other cave beetles, *Hadesia* spp. are not attracted by baits, and therefore have to be searched for by visual inspection and collected by hand directly from the cave hygropetric. These limitations, along with conservation concerns and legal restrictions led us to take the absolute minimum number of specimens from nature that would still allow us to conduct the study with a sense of statistical confidence. A total of 15 specimens were collected and preserved in 96% ethanol for molecular analyses, or, alternatively 50% ethanol for morphological analyses. Information on sampling localities and specimen vouchers is accessible in Electronic Supplement 1 Table S1. Since *Hadesia* is traditionally perceived as a member of the Anthroherponina, three other representatives of this subtribe were added as outgroups (*Anthroherpon cylindricollis*, *Leptomeson dombrowskii* and *Croatodirus bozicevici*). The fieldwork was undertaken with permissions from the nature conservation authorities of Bosnia and Herzegovina (Republički zavod za zaštitu kulturno-istrorijskog i prirodnog nasleđa Republike Srpske; No. 07/1/625-248/16) and Montenegro (Agencija za zaštitu životne sredine; No. 02/UPI-341/6, No. 02/UPI-740/7).

DNA extraction and PCR amplification. Total Genomic DNA was extracted from the whole specimens using the Sigma Aldrich GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich Corporation, Saint Louis, Missouri, USA). The left-over chitin exoskeletons are deposited in Notranjska museum Postojna collection (NMPO), Slovenia. Six molecular markers – three mitochondrial (two parts of the Cytochrome Oxidase I gene and the 16S rRNA gene) and three nuclear DNA sequences (two parts of the 28S rRNA gene and the Histone H3 gene) – were amplified by polymerase chain reactions (PCR). The reactions were run using standardized primers and protocols given in Electronic Supplement 1 Table S3. PCR products were purified enzymatically using Exonuclease I and Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and sequenced bidirectionally with amplification primers by Macrogen Europe (Amsterdam, Netherlands).

Phylogenetic analyses. Sequence chromatograms were assembled, visually checked and edited in Geneious v8.0.4 (Biomatters Ltd., Auckland, New Zealand). The resulting sequences were aligned separately for each gene using MAFFT v7 (Katoh & Standley 2013). All sequences except 28S rRNA were indel-free and thus trivial to align. We used the E-INS-i algorithm to align 28S rDNA sequences with multiple conserved domains and long gaps, as implemented in MAFFT. The alignment revealed two additional stretches of 50 base pair and three of 4 base pairs in the *Croatodirus bozicevici* sequence. However, since these were unique to *C. bozicevici* and all other sequences were gap-free, downstream phylogenetic procedures were not affected. Optimal substitution models and partitioning schemes were assessed with the help of PartitionFinder v1.1.1 (Lanfear et al. 2012). The concatenated sequence alignment and the selected partitioning scheme with the corresponding models are provided in Electronic Supplements 1 and 2.

Phylogenetic relationships were inferred for each locus separately and on concatenated sequences in MrBayes 3.2 (Ronquist & Huelsenbeck 2003) using two parallel runs of four Markov chains for two million generations. Every 200th generation was sampled and the first 25% of the sampled trees were discarded as a burn-in. The remaining trees were summarized as 50% majority-rule consensus tree (Electronic Supplement 1 Fig. S1).

Additionally, temporal diversification of the genus was estimated in BEAST v1.8.2 (Drummond et al. 2012). For molecular dating, Leptodirine clock-rate estimates were taken from Cieslak et al. (2014) who used the tectonic separation of the Corso-Sardinian plate from the continent as calibration point. Mean lognormal clock priors were set to 0.015, 0.006, and 0.004 substitutions per nucleotide site per million years for the COI, 16S rRNA and 28S rRNA genes, respectively. The H3 gene substitution rate was estimated during the analysis relatively to the rates of the other markers. The Yule speciation model was used and the population size prior set to constant. Markov chains were run for 30 million generations and sampled every 1000th generation. Convergence of the runs and effective samples size were checked in Tracer v1.6. Four independent runs were combined in LogCombiner v1.8.2 after discarding the first 5000 of the resulting trees as a burn-in, while the remaining 25,001 trees were used to infer the maximum clade credibility tree using Tree Annotator v1.8.2 (Drummond et al. 2012).

Molecular species delimitation. Evolutionary independence of the morphologically recognized *Hadesia* species and their phylogenetic relationships were first assessed using a multi-locus and multispecies coalescent approach as implemented in Bayesian Phylogenetics & Phylogeography 3.1 (BPP) (Yang & Rannala 2014). No prior knowledge about species boundaries and relationships was assumed, therefore the A11 analysis option was chosen. Under this option, BPP performs joint species delimitation and species-tree inference via reversible-jump Markov chain Monte Carlo (rjMCMC) and nearest neighbor interchange on the multilocus molecular dataset. There were no missing data or ambiguities in the dataset. 100,000-generations rjMCMC run was sampled every fifth generation, and the first 20,000 generations were omitted as burn in before summarizing the output. The topology obtained from the concatenated phylogenetic analysis (Fig. 3) was used as starting tree. Priors for ancestral population size (θ) and root age (τ_0) were set according to Leaché & Fujita (2010) to i) 2, 2000 and 2, 2000; ii) 1, 10 and 1, 10; and iii) 1, 10 and 2, 2000; matching small ancestral population sizes and shallow divergences, large ancestral population sizes and deep divergences and large ancestral population sizes and shallow divergences, respectively. The heredity scalar was set to 1 for nuclear and 0.25 for mitochondrial loci, while fine tune parameters and locus rates were estimated during the run.

We further applied two single-locus approaches as part of the standard species delimitation toolkit: Bayesian Poisson Tree Process (bPTP; Zhang et al. 2013) and General Mixed Yule Coalescence (GMYC; Fujisawa & Barraclough 2013). For the bPTP analysis, the COI gene tree obtained in MrBayes (see previous section) was submitted to the web server at http://species.h-its.org/ptp/ for species delimitation. Bayesian posterior probabilities for putative species were acquired after running 500,000 generations, sampling every 100 generation and discarding the first 20% of the samples as a burnin. To perform the GMYC analysis, an ultrametric COI gene tree was inferred in BEAST with a clock prior set to 0.015 and all other settings as described in the previous section. The GMYC itself was run using the package splits (Ezard et al. 2014) in the R statistical environment.

Morphological analyses. Specimens of *H. zetae* **sp.n.** not used for the DNA extraction were dry card mounted, partly dissected and designated as type series. The studied specimens were dissected in 96% ethanol after maceration in 10% KOH at room temperature for 12 hours, washed in pure water and dehydrated by using ethanol at increasing concentrations (50–96%), studied and measured. Photo-

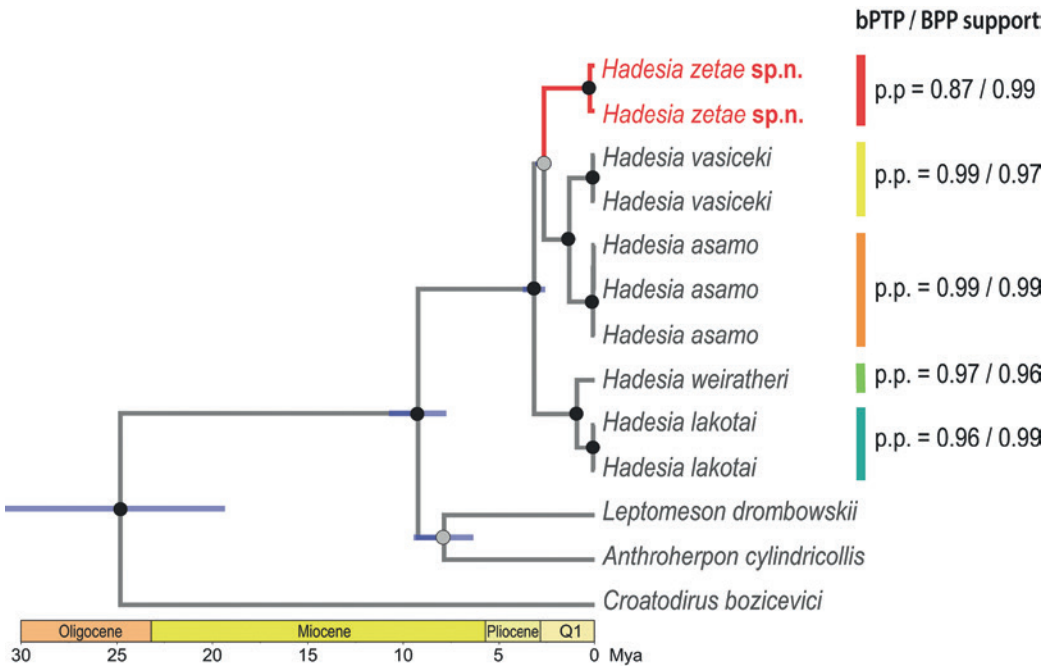


Fig. 3. Maximum clade credibility chronogram inferred in BEAST from concatenated COI, 16S, 28S and H3 gene sequences. Posterior probabilities for the nodes are indicated with circles, black circles p.p. = 1, grey circles p.p. = 0.95–0.99. Node bars indicate 95% confidence intervals for the estimated age of the nodes and correspond to the time scale at the bottom of the figure. Bayesian posterior probabilities for the morphologically identified species, acquired from BPP multilocus coalescent species delimitation, are indicated at the right side of the figure.

graphs were taken using a Leica MZ7.5 stereomicroscope (0.63 – 5.0 × 10 magnifications) (Leica Microsystems GmbH, Wetzler, Germany) and an Euromex microscope ME2665 (10 × 4, 10 × 10 and 10 × 40 magnifications) (Euromex Microscopen BV, Arnhem, the Netherlands). Taxonomically informative body parts (antennae, protarsi and genital parts) were separated and immersed in glycerine or Solakryl BMX. Photographs of the specimen habitus were made using a Nikon Coolpix 4500 digital camera and measured via the Image J software (National Health Institute, Bethesda, Maryland, USA). Digital microscope images were additionally edited in Adobe Photoshop. For scanning electron microscopy (SEM) two specimens were fixed in 0.5% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C overnight. Specimens were subsequently washed in 0.1 M phosphate buffer, dehydrated in an ascending ethanol series and transferred to pure acetone. After gradual substitution of acetone with hexamethyldisilazane (HMDS) the samples were air-dried and attached to metal holders with silver paint. Mounted specimens were coated with platinum and observed using a JEOL JSM-7500F field-emission scanning electron microscope.

Voucher specimens were deposited in the Entomological collection of Notranjska Museum Postojna (NMPO) and in the Zoological Collection of the Department of Biology (ZCDB), Biotechnical Faculty, University of Ljubljana; both in Slovenia. Two paratype specimens of *Hadesia zetae* **n.sp.** were deposited in the Natural History Museum of Montenegro (NHMM) in Podgorica, Montenegro.

Abbreviations. ***Institutes and collections***: **NMPO**: Notranjska Museum Postojna, Postojna (Slovenia); **ZCDB**: Zoological Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Slovenia); **NHMM**: Zoological Collection of Natural History Museum of Montenegro, Podgorica (Montenegro). — ***Measurements***: **BL**: Total body length; **AtL**: Antennae total length; **AmL**: Lengths of antennomeres; **PL**: Pronotum maximal length; **PW**: Pronotum maximal width; **EL**: Elytra maximal length; **EW**: Elytra maximal width; **TL**: Protarsomere length; **TsL**: Protarsomeres (summa) lengtH. — ***Type material***: **HT**: Holotype; **PT**: Paratype.

3. Results

3.1. Phylogeny and species delimitation

The genus was always strongly supported and clearly separated from its sister clade composed of *Anthroherpon* and *Leptomeson*, or *Anthroherpon* alone in the MrBayes analysis (Electronic Supplement 1 Fig. S1). Relationships within *Hadesia* obtained from the concatenated sequence matrix were fully resolved and concordant with single-locus topologies. On the other hand, the multilocus species tree topologies obtained by BPP without outgroup were sensitive to ancestral population size and root age priors, resulting in a strict consensus topology of (*H. zetae*, (*H. asamo*, *H. vasiceki*, (*H. lakotai*, *H. weiratheri*))).

All nominal species for which more than one individual was sequenced, including *H. zetae* **sp.n.**, were monophyletic with high posterior probabilities. Moreo-

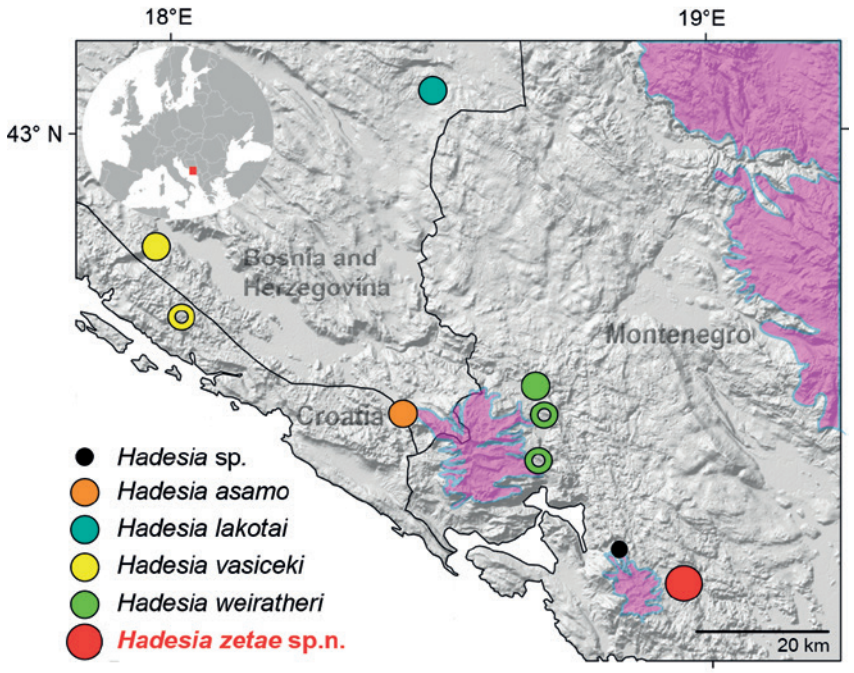


Fig. 4. Geographic distribution of *Hadesia* species with the maximum extent of the last glacial maximum glaciers (Hughes et al. 2010, 2011; Žebre & Stepišnik 2014) shown in purple. Animals from sites represented by empty circles were determined by morphology only, and not used in molecular analyses.

ver, the unilocus species delimitation procedures bPTP and GMYC supported the morphologically-based subdivision of the genus (Fig. 3). The BPP multilocus coalescent species delimitation analysis was largely insensitive to ancestral population size and root age priors and produced three alternative scenarios of which individuals might comprise potential species. The highest support was received by a combination of a large ancestral population size and shallow divergence, in which the current morphological subdivision of five species had a posterior probability (p.p.) of 0.96, while the support for a four species and three species scenario was negligibly low (p.p. = 0.037 and 0.005, respectively). Each of the five thus delimited species corresponded to one of the nominal *Hadesia* species including the new *H. zetae* **sp.n.**, their support ranging from p.p. = 0.96 to p.p. = 0.99 (Fig. 3).

The BEAST timetree analysis indicated that the basal split of the genus was of pre-Pleistocene age (2.7–3.6 million years), while further speciation events took place within the last two million years. The analysis suggested very recent coalescent times of individual lineages within species (0.05–0.24 million years), typical of small, weakly structured populations.

3.2. *Hadesia zetae* Delić, Polak & Trontelj sp.n.

Figs. 2, 5, 6–9, 10–21, 22E, 23E, 24E,F, 25E,F

Type locality. Cave Lipska pećina, situated near the village of Lipa, Cetinje (Montenegro) (Fig. 4).

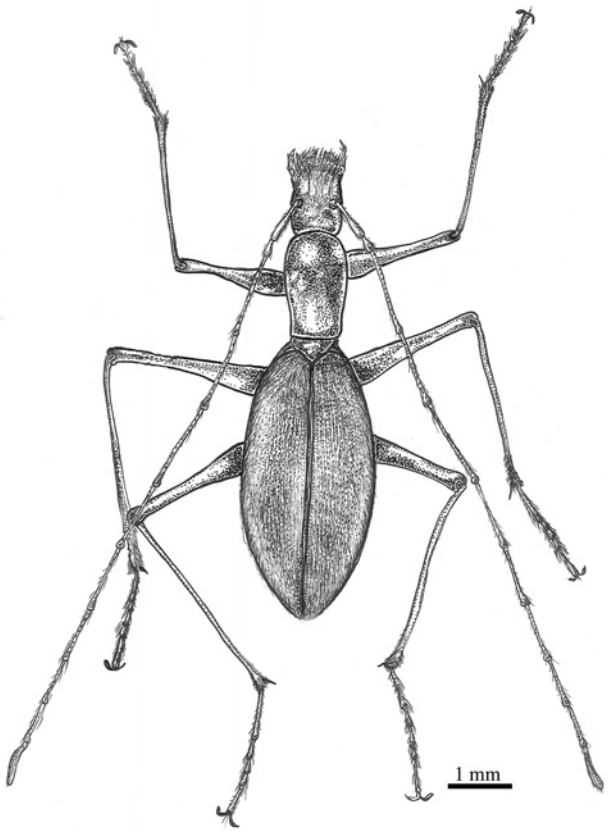
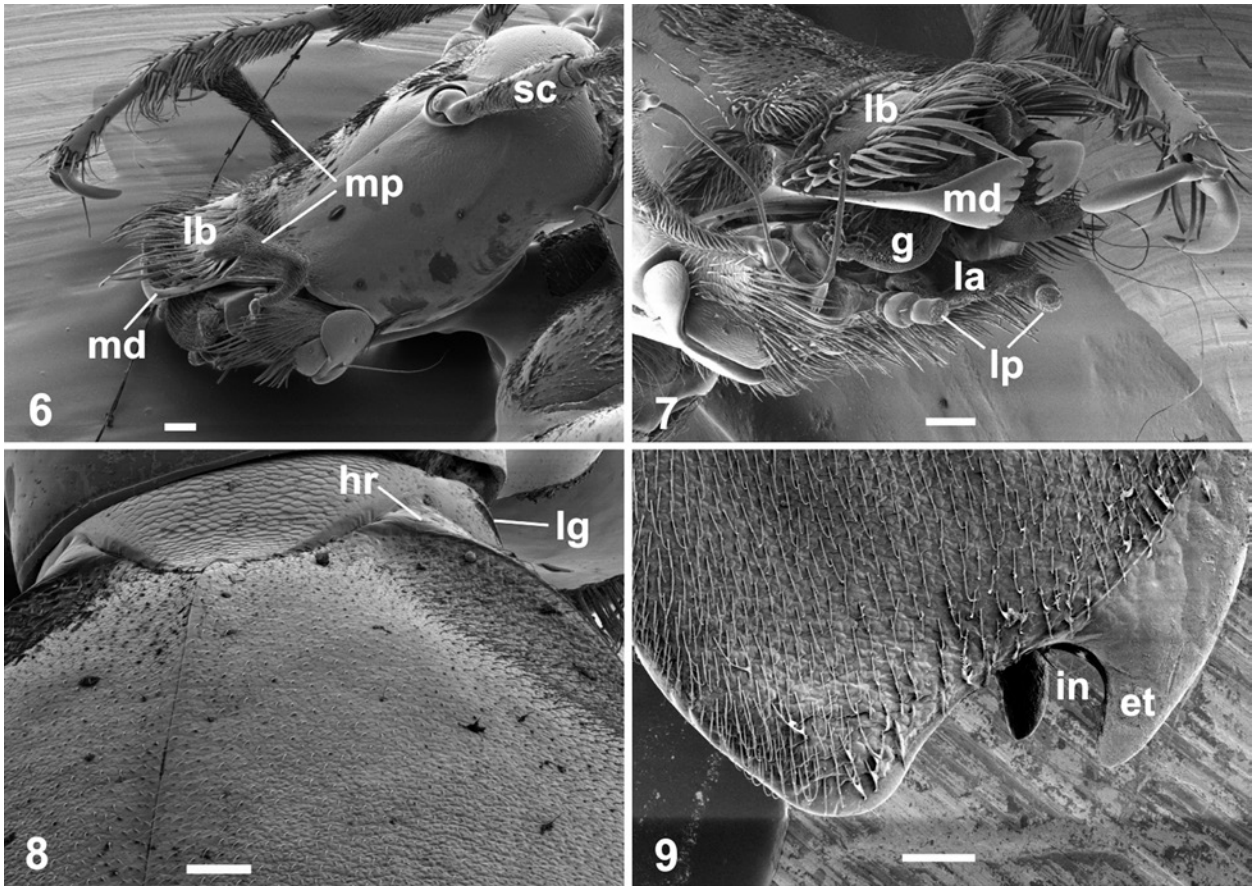


Fig. 5. *Hadesia zetae* **sp.n.** holotype male, habitus.

Description. Habitus as in Figs. 2, 5. Anophthalmous and depigmented. ***Size***: Total body length (BL) (measured with head in natural, hypognathous position) 5.92–6.08 mm in ♂♂ and 6.81–6.93 mm in ♀♀ (HT 6.08 mm). ***Colour***: Reddish-brown, antennae and legs slightly paler (Fig. 2).



Figs. 6–9. *Hadesia zetae* **sp.n.** female: **6**: Head lateral view. **7**: Mouthparts. **8**: Right shoulder. **9**: Preapical part of female epipleuron in lateral view. (Scale bars: 0.1 mm). — ***Abbreviations:*** et – epipleuron preapical tooth; g – galea; hr – thickened humeral region of epipleuron; in – deep indentation of epipleuron apex; la – labium; lb – labrum; lg – lateral grove of shoulder; lp – labial palp; md – mandible; mp – maxillary palp; sc – scape.

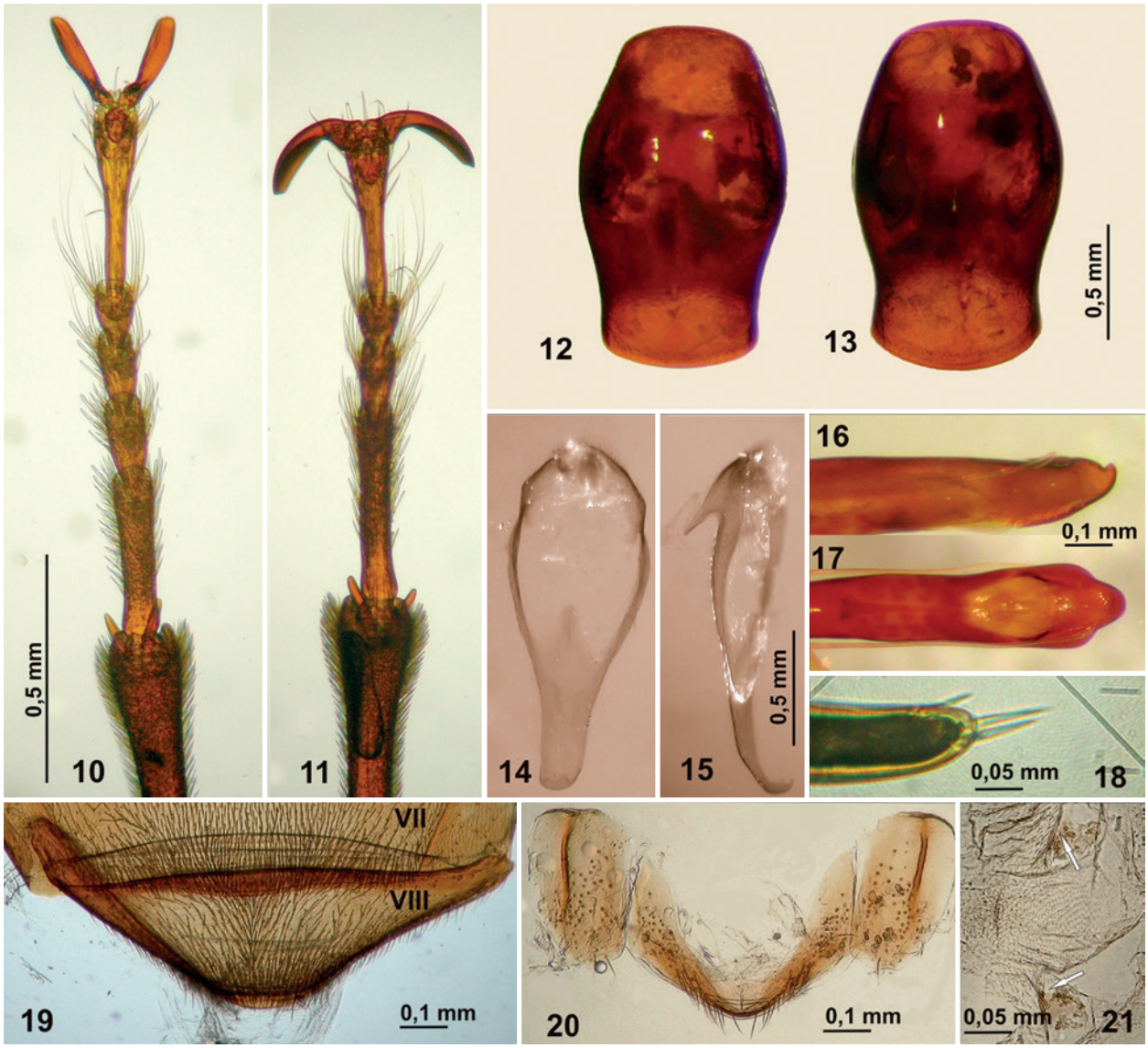
Head: Typical for genus with long bristles on the labrum and fine sparse decumbent setae on the central frontal part of the head (Figs. 6, 7). ***Antennae***: Inserted on the posterior-most quarter of the length of the head (Fig. 6), long and slender, longer than body. Antennae total length (AtL) 8.97–9.50 mm in ♂♂, 7.65–8.07 mm in ♀♀. Ratio Antennae length (AtL) / Body length (BL): 1.52–1.56 in ♂♂ and 1.12–1.16 in ♀♀. Lengths of antennomeres AmL (from scape to terminal antennomere, in mm) ♂ (N = 1): 0.57; 0.25; 1.25; 1.07; 1.32; 1.09; 0.88; 0.77; 0.64; 0.54; 0.59. ♀ (N = 1): 0.58; 0.21; 1.17; 0.96; 1.11; 0.79; 0.70; 0.68; 0.50; 0.44; 0.51. Antennomere ratio AtL/AmL (from scape to terminal antennomere, in %) ♂ (N = 1): 6.35; 2.79; 13.94; 11.93; 14.72; 12.15; 9.81; 8.58; 7.13; 6.02; 6.58. ♀ (N = 1): 7.57; 2.75; 15.29; 12.55; 14.51; 10.33; 9.15; 8.89; 6.54; 5.75; 6.67.

Thorax: Pronotum cylindrical, moderately elongate, the lateral edge in dorsal view rounded in the anterior half, slightly sinuate concave in the posterior half (♂ Fig. 12, ♀ Fig. 13), maximal width in the anterior third, dorsal face glabrous, macroscopic aspect shiny. Pronotum maximal length (PL) 1.52–1.56 mm in ♂♂, 1.57–1.65 mm in ♀♀; pronotum maximal width (PW) 0.93–1.01 mm in ♂♂ and 0.98–1.03 mm in ♀♀; ratio PL/PW approximately 1.6 in ♂♂ and ♀♀. Lateral groove on the shoulder present (Fig. 8). Scutellum wide and long, glabrous, with roughly scaled surface.

Elytra: Elongate-oval in ♂♂, elongate and slightly conical at the posterior (preapical) end in ♀♀, with maximum width approximately in the middle of elytra. Covered with pale, short, fine, dense and therefore hydrophobic pubescence. Elytra length (EL) 4.07–4.09 mm in ♂♂, 4.87–4.90 mm in ♀♀; elytra width (EW) 1.86–1.92 mm in ♂♂, 2.30–2.32 mm in ♀♀; ratio EL/EW 2.13–2.19 in ♂♂ and 2.11–2.12 in ♀♀. Preapical part of epipleuron in lateral view slightly indented near the apex in ♂♂, and with prominent and protruding tooth followed by deep indentation toward the apex in ♀♀ (Figs. 9, 24E,F). Humeral region of epipleuron thickened (Fig. 8).

Abdomen: Mesocoxal cavities strongly confluent. First visible ventrite (ventrite III) in ♀♀ with deep lateral concavity on each side. Ventrites on anterior margin with smooth area, posterior parts pilose. Edge between smooth part and pilose part distinctly carinate on ventrite III. Ventrite VIII in ♀♀ simple, without median expansion on anterior edge (Fig. 19).

Legs: Long and slender (Figs. 2, 5). Femora basally strongly widened, distally slightly thickened, mostly glabrous with short recumbent hairs basally and on thickened distal part. Tibiae slim and straight, distally slightly



Figs. 10–21. *Hadesia zetae* **sp.n.** males and females: **10**: Right male protarsus, ventral view. **11**: Right female protarsus, dorsal view. **12**: Male pronotum. **13**: Female pronotum. **14**: Male genital segment, dorsal view. **15**: Male genital segment, lateral view. **16**: Male aedeagus apex, lateral view. **17**: Male aedeagus apex, dorsal view. **18**: Male paramere apex. **19**: Female apical ventrites VII & VIII. **20**: Female urite IX with reduced epipleurites and mediotergite. **21**: Female reduced gonostyles, stylomeres marked with arrows.

curved inwards on the distal parts. Protibiae and mesotibiae without an apical ring of spines of equal size, but with a bunch of short and dense bristles on the distal end, and a row of short fine recumbent hairs on inner edge (Figs. 10, 11). Metatibiae are mostly glabrous with short spines distally. All tibiae without external row of spines and without an external apical spur but internally armed with two short and wide spurs (Figs. 10, 11). Spurs seem simple but are reduced trident under close microscopical examination. Male protarsi 5-segmented (Fig. 10), female protarsi 4-segmented (Fig. 11), not dilated. All tarsomeres strongly chaetose on the ventral side, laterally with long bristles. Tarsal empodium with two setae. Claws widely dilated and spoon-like (Figs. 10, 11). Protarsomere (mean, N = 2) lengths in mm (TL) ♂♂: 0.40, 0.22, 0.19, 0.15, 0.48; ♀♀: 0.50, 0.20, 0.14, 0.24; protarsomeres (summa) length (TsL) ♂♂ = 1.44 (1.23 mm measured in natural tarsomere overlapping position); ♀♀ = 1.08 (1.04 mm measured in natural tarsomere overlapping position); protarsomere ratio (TL/TsL in %) ♂♂: 27.78; 15.28, 13.19, 10.42, 33.33; ♀♀: 46.29, 18.52, 12.96, 22.22.

Male genitalia: Aedeagus in dorsal view (Fig. 22E) 1.82–1.96 mm long, straight and slender. Median lobe more or less parallel sided toward the slightly widened apex with terminal triangular beak (Figs. 17, 22E). Parameres filamentous, narrow and parallel with median lobe, shorter than median lobe, curved medially and dorsally at the apex (Figs. 16, 17), with two subapical and one apical long seta (Fig. 18). Aedeagus in lateral view (Fig. 23E) slender, moderately curved, distinctly thickened at terminal half. Median lobe apex in lateral view (Figs. 16, 23E) with slight preapical hump, and weak apical hook (Fig. 23). In dorsal view with small central depression

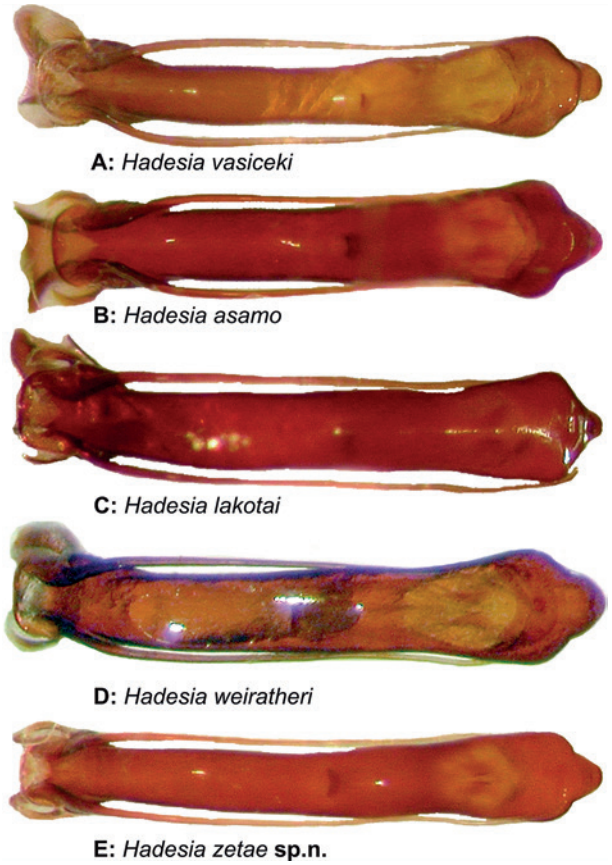


Fig. 22. *Hadesia* spp.: Aedeagus and parameres, dorsal view: **A**: *H. vasiceki* Müller. **B**: *H. asamo* Perreau & Pavićević. **C**: *H. lakotai* Perreau & Pavićević. **D**: *H. weiratheri* Zariquiey. **E**: *H. zetae* **sp.n.**, paratype.

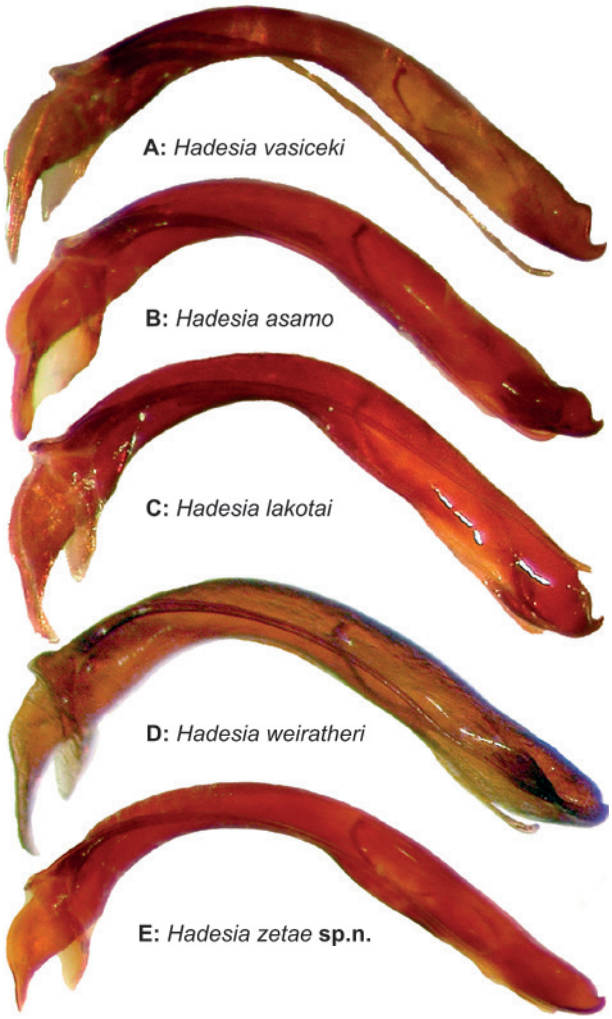


Fig. 23. *Hadesia* spp.: Aedeagus and parameres, lateral view: **A**: *H. vasiceki* Müller. **B**: *H. asamo* Perreau & Pavićević. **C**: *H. lakotai* Perreau & Pavićević. **D**: *H. weiratheri* Zariquiey. **E**: *H. zetae* **sp.n.**, paratype.

anterior to the apical hump, poorly visible in lateral view (Figs. 16, 17). Internal sac of aedeagus with long and strong stylus (Figs. 16, 17, 22, 23) visible in apical half of median lobe as in other *Hadesia* species. Male genital segment (Figs. 14, 15) reduced, weakly sclerotized, with strong non-hyaline lateral apophysis and long, bent ventral apophyses.

Female genitalia: Urite IX with reduced sclerotized parts, epipleurites reduced to circular lateral plates with some strong bristles apically, mediotergite reduced to semicircular arc strongly pilose apically (Fig. 20). Gonostyles reduced to simple plates with one apical stylomere (Fig. 21) attached to spermatheca via spermathecal duct. Spermatheca (Fig. 25E,F) sack-like (sacciform), curved, uniformly and weakly sclerotized, rounded distally. Clearly visible hyaline gland arising from the spermathecal duct at the proximal part of spermatheca.

Differential diagnosis. *Hadesia zetae* **sp.n.** differs from other *Hadesia* species by smaller body size (5.92–6.08 mm in ♂♂ and 6.8–6.93 mm in ♀♀), with exception of the even smaller *H. lakotai*. Females can be easily recognized by the shape of the preapical part of epipleuron in lateral view, bearing a distinct and prominent tooth (the biggest among the species) protruding almost to epipleuron apex followed by a deep and wide indentation toward the apex (Figs. 9, 24). Males are distinguished by the shape of aedeagus median lobe in lateral view, being narrower and more slender than in other species, moderately curved and thickened at the terminal half with slight low apical hump and weak short apical hook (Figs. 16, 23). Additionally, the aedeagus lobe in dorsal view is narrower, more slender than in other species, almost straight with weakly widened apex with distinct triangular beak (Figs. 17, 22).

Etymology. The species epithet is a patronym derived from the name of the medieval Montenegrin kingdom of Zeta.

Distribution. The new species is known only from its type locality.

Ecology. Adult specimens of *H. zetae* **sp.n.** were collected in the deep parts of the cave Lipska pećina, some 500

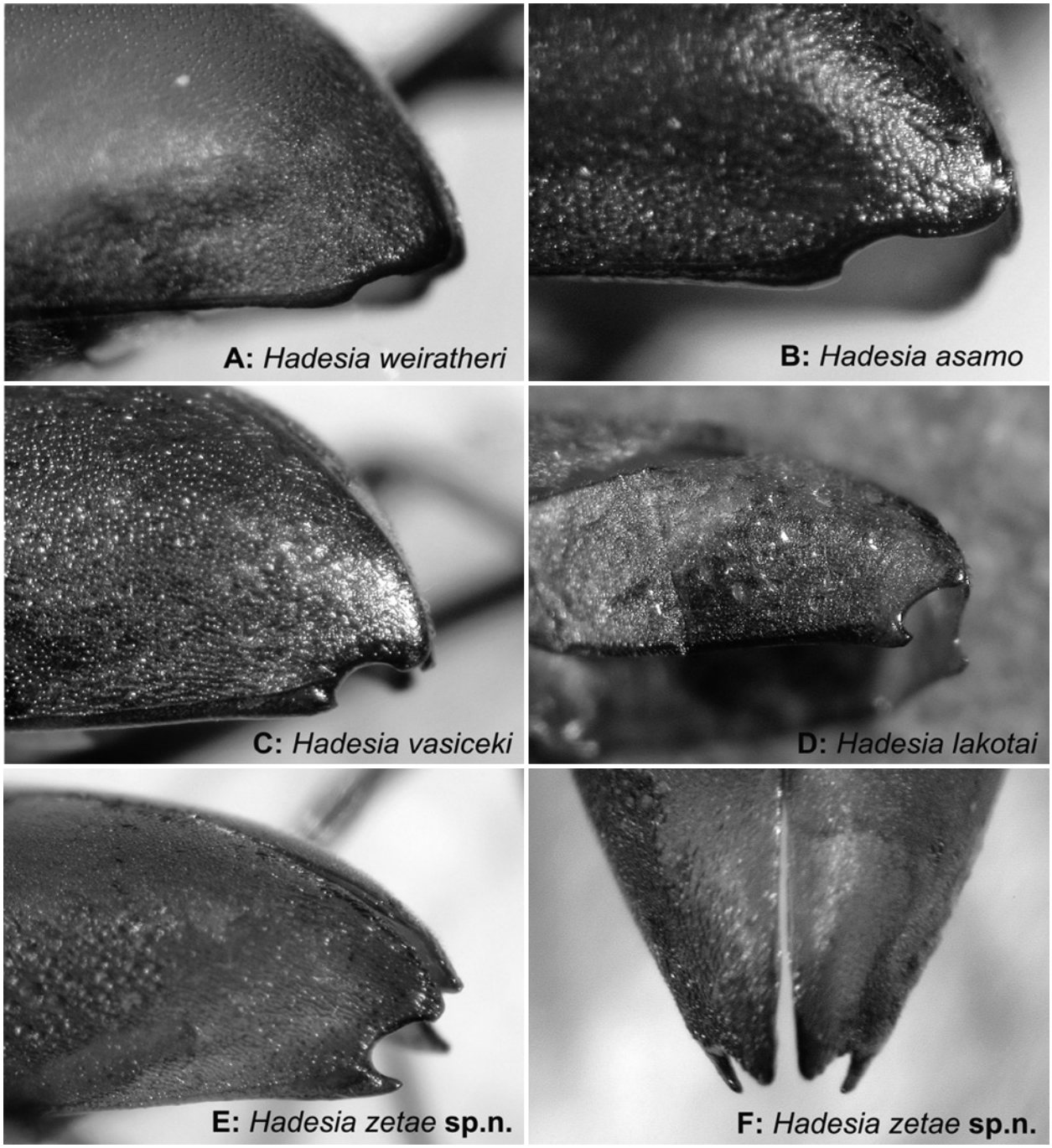


Fig. 24. *Hadesia* spp.: Preapical part of female elytron and its epipleuron in lateral (A–E) and dorsal (F) view: **A**: *H. weiratheri* Zariquiey. **B**: *H. asamo* Perreau & Pavićević. **C**: *H. vasiceki* Müller. **D**: *H. lakotai* Perreau & Pavićević. **E&F**: *H. zetae* **sp.n.**, paratype.

meters from the entrance, in the cave hygropetric with fast running water, together with the amphipod crustacean *Typhlogammarus mrazeki* Schaeferna, 1906.

Type series. Holotype ♂, glued to a white card, pinned dry, aedeagus dissected and glued to a transparent label below the specimen, labeled: MONTENEGRO | Cetinje, Lipa, Lipska pećina | 01.v.2015, Delić, T. leg. [rectangular white label, printed], HOLOTYPE | *Hadesia zetae* sp.n. ♂ | Delić, Polak & Trontelj det. [rectangular red label, printed] (NMPO) (Inv. No.: C-4541). — Paratypes: 1 ♀, specimen glued to white card, pinned dry, not dissected same locality and same date than the holotype [rectangular white label, printed] PARATYPE | *Hadesia zetae* sp.n. ♀ | Delić, Polak & Trontelj det. [rectangular yellow label, printed] (NMPO) (Inv. No.: C-4542); 1 ♂ same locality, 01.v.2013, 1 ♀ same locality, 01.v.2015, Delić, T., leg., DNA extracted, exoskeleton preserved in 96% ethanol and partly dissected, (aedeagus, genital segments, protarsi, antennae) preserved immersed in Solakryl BMX on separate microscope slides, both labeled [rectangular white label, printed] PARATYPE | *Hadesia zetae* sp.n. | Delić, Polak & Trontelj det. [rectangular yellow label, printed], (NMPO) (♂ Inv. no. C-4263,

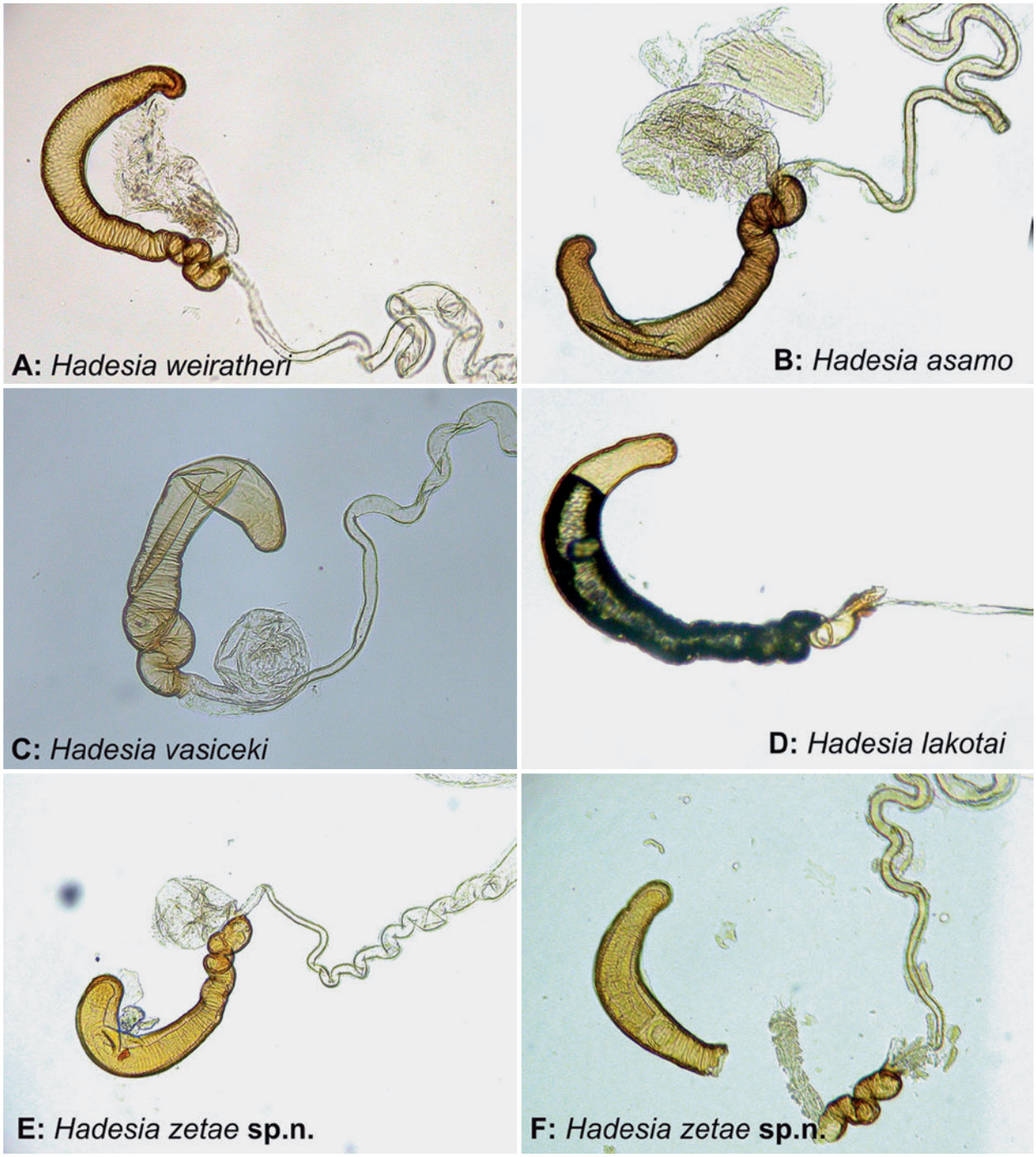


Fig. 25. *Hadesia* spp.: Female spermatheca: **A**: *H. weiratheri* Zariquiey. **B**: *H. asamo* Perreau & Pavićević. **C**: *H. vasiceki* Müller. **D**: *H. lakotai* Perreau & Pavićević. **E&F**: *H. zetae* **sp.n.**, paratype (spermatheca in F artificially broken).

♀ Inv. no. C-4502); 1 ♂, 1 ♀, same locality, 01.v.2015, Delić, T. leg., specimens glued to white card, pinned dry, not dissected, labeled [rectangular white label, printed] PARATYPE | *Hadesia zetae* sp.n. | Delić, Polak & Trontelj det. [rectangular yellow label, printed] (NHMM); 2 ♀, specimens prepared for scanning electron microscopy (SEM), coated with platinum, attached to metal holders with silver paint, same locality, 01.v.2015, Delić, T. leg., labeled [rectangular white label, printed] PARATYPE | *Hadesia zetae* sp.n. | Delić, Polak & Trontelj det. [rectangular yellow label, printed] (ZCDB).

3.3. Identification key to the species of the genus *Hadesia*

We propose a novel identification key for *Hadesia*, based on the indentation on the apex of elytra in females and the distinctive aedeagus shape in males. The key requires no morphometric measurements and differs from the one recently proposed by Perreau & Pavićević (2008), where spermatheca (Fig. 25) and the shape of the humeral region of epipleura were used as two essential characters for species identification. Both characters

show intraspecific variability and are therefore difficult to evaluate.

1 Protarsi 5-segmented (males) (Fig. 10) **2**
1' Protarsi 4-segmented (females) (Fig. 11) **6**
2 Aedeagus median lobe in lateral view with prominent dorsal protuberance and stronger apical hook (Fig. 23A,B,C) **3**
2' Aedeagus median lobe in lateral view with slight dorsal protuberance and weak apical hook (Figs. 16, 23D,E) **5**
3 Aedeagus median lobe dorsal protuberance in lateral view narrow angular, apical hook strong and long (Fig. 23A). Aedeagus median lobe in dorsal view narrowest on the basal third, slowly widened toward the elliptical apex with rounded apical beak (Fig. 22A) ..... ***vasiceki*** Müller
3' Aedeagus median lobe dorsal protuberance in lateral view distinctly rounded, apical hook strong but short (Fig. 23B,C). Aedeagus median lobe in dorsal view widened on the middle part and with distinct triangular beak (Fig. 22B,C) **4**
4 Aedeagus median lobe dorsal protuberance in lateral view distinctly rounded with strong depression before it (Fig. 23B) ..... ***asamo*** Perreau & Pavićević
4' Aedeagus median lobe dorsal protuberance in lateral view distinctly rounded with weak depression before it (Fig. 23C). Aedeagus median lobe in lateral view moderately constricted in the middle part and therefore making an acute angle (Fig. 23C) ..... ***lakotai*** Perreau & Pavićević
5 Aedeagus median lobe in dorsal view wide, widened on the basal part and on apical part with distinct triangular apex and wide rounded apical beak (Fig. 22D) ..... ***weiratheri*** Zariquiey
5' Aedeagus median lobe in dorsal view narrow, almost straight, slightly widened on apical part with triangular beak (Fig. 22E) ..... ***zetae*** **sp.n**.
6 Preapical part of female epipleuron in lateral view with a minute or without tooth (Fig. 24A,B) **7**
6' Preapical part of female epipleuron in lateral view with a distinct tooth (Fig. 24C,D,E,F) **8**
7 Preapical part of epipleuron in lateral view without tooth followed by week indentation toward the elytron apex (Fig. 24A) ..... ***weiratheri*** Zariquiey
7' Preapical part of epipleuron in lateral view with a minute tooth followed by weak indentation toward the elytron apex (Fig. 24B) ..... ***asamo*** Perreau & Pavićević
8 Preapical part of epipleuron in lateral view with a distinct tooth not protruding toward the elytron apex followed by deep indentation (Fig. 24C) ..... ***vasiceki*** Müller
8' Preapical part of epipleuron in lateral view with a prominent tooth protruding toward the elytron apex followed by deep indentation (Fig. 24D) ..... ***lakotai*** Perreau & Pavićević
8" Preapical part of epipleuron in lateral view with a prominent tooth protruding almost to elytron apex followed by deep and wide indentation (Fig. 24E,F) ..... ***zetae*** **sp.n.**

4. Discussion

4.1. The long way from a single-site endemic to a five-species anthroherponine genus

In the secretive world of subterranean life, *Hadesia* stands at a foremost position among arthropods, both as a charismatic taxon and in terms of scientific importance. It is the model upon which a new ecomorphological form of ultra-specialized cave beetles became known, the semi-aquatic, hygropetricolous filter feeders (Casale et al. 2000a, 2004; Sket 2004). Its uniqueness has been underscored and perhaps overstated by the fact that for most of the 20th century the world-famous Vjetrenica Cave was its only known site of occurrence. Leo Weirather's hide-and-seek play with the possible second *Hadesia* cave still intensified the mystery associated with the species (Pretner 1974, 1977, 2011). It was only during the last 15 years that, through the increased fieldwork effort by several independent biospeleological teams and by the employment of the rope technique, new *Hadesia* habitat became accessible and known. Further work using genital structures (Perraeu & Pavićević 2008) and, in this paper, multilocus coalescent species delimitation led to the current picture of the most diverse of all hygropetricolous genera. With five species from nine known sites, its range spans about 100 kilometers and covers an area of more than 2,000 km^2 split amongst Bosnia and Herzegovina, Croatia, and Montenegro (Fig. 4). The current taxonomy appears completely settled and straightforward. Morphological characters support the same subdivision as do several mutually agreeing molecular markers. Monophyly of the genus, using three putative anthroherponine outgroup genera, seems equally undisputable. However, and contrary to the phylogenetic hypothesis proposed by Perraeu & Pavićević (2008), the timetree analysis indicated that *Hadesia* might to be more closely related to *Antroherpon* and *Leptomeson* than to *Croatodirus*, with which it shares its hygropetricolous lifestyle (Casale et al. 2000b, 2004). This first discrepancy between expectations based on morphological similarities and molecular phylogenetics is just a foretaste of what we can expect in the future. Many more taxa need to be analyzed before we can propose more reliable hypotheses about relationships within Anthroherponina, and research towards this goal is already under way.

4.2. An evolutionary scenario for the current diversity of the genus

The increase of known diversity and range raises the question of how and when the genus evolved and diver-

sified. The ultra-specialized ecology of the genus allows us to suggest that already the last common ancestor of all known species possessed the same adaptations and life style. This further implies that its habitat was restricted to caves and limestone crevice systems, as well as to areas with sufficient rainfall to provide a constant flow of percolating water. As suggested by Giachino & Vailati (2006), hygropetricolus beetles require at least 2000 mm of precipitation per year. We may conjecture that the common ancestral *Hadesia* lineage had to be distributed over an area at least the size of the current range of the genus. The estimated age of the last common ancestor of about three million years coincides with the mid-Pliocene warm period that was not only warmer but also considerably wetter than the modern climate in the eastern Mediterranean area (Salzmann et al. 2011; Haywood et al. 2013). At that time cave formation in the Dinaric karst was already at its height (Mihevc 2007), so enough contiguous habitat was available for the *Hadesia* ancestor to disperse over the area of southeastern Herzegovina, the southernmost tip of Croatia, and southwestern Montenegro. The possibility that there existed a huge, at least temporarily interconnected cave and crevice system is supported by the fact that today this is one of the most highly karstified areas in the world (Milanović 2015).

The diversification leading to the current diversity of the genus started a bit later, in the Pleistocene. It seems plausible that during the cold and dry phases of this epoch caves with constantly flowing water became fewer and separated from each other. According to this scenario, speciation was strictly allopatric, induced by vicariant range fragmentation. Southern Dinaric Pleistocene glaciers might have formed a part of the barriers (Hughes et al. 2010, 2011; Žebre & Stepišnik 2014). Nevertheless, the only effect that can be inferred by superimposing the last glacial maximum (LGM) glacier on the known distribution of *Hadesia* is a retraction of range (Fig. 4). The LGM glaciers do not divide the ranges of any of the sister lineage pairs, nor does their age of about 21,000 years agree with the age of the splits. Vicariance of contiguous subterranean ranges with subsequent speciation has been predicted theoretically (Holsinger 2000), but probably never convincingly documented in nature. Similar patterns of allopatric speciation are known from other subterranean beetles (Faille et al. 2015), although the processes leading to range fragmentation were geological rather than climatic, and much older as in the case of *Hadesia*.

4.3. Future taxonomic practice and conservation issues

The recent discoveries of new species and new sites will doubtlessly kindle further intensive collection effort among professional researchers and amateurs. Generally, such a trend can be welcomed as increased knowledge of a taxon leads to better awareness and protection. Yet, in cave beetles and *Hadesia* spp. in particular, this trend is associated with a twofold problem. The first part is the extreme rarity of these animals. For example, *H. lakotai* was described based on chitin leftovers only. *Hadesia* spp. are bound to permanent flows of percolated water that are usually located in the deepest parts of the caves, if at all accessible. Perreau & Pavićević (2008) point out that, if and when the flow conditions cease, the beetles vanish leaving us without the slightest clue where they might have gone. Long term observations in Vjetrenica Cave have suggested that excessive collecting can lead to a severe population reduction (B. Lewarne, pers. comm.). As long as it is not clear whether the scarcity of finds is only apparent and periodical, or it reflects true population numbers, no specimens should be taken except those needed to determine the taxonomic identity of a population.

With this plea, we arrive at the second part of the problem, which is the practice of identification and future taxonomy. All known species can be positively identified using both, the here proposed identification key and sequencing of diagnostic DNA markers. Both methods are destructive and require the removal of individuals from caves. However, due to the strict allopatry and wide gaps separating their ranges, the geographical location is a reliable pointer to species identity. Therefore, for the purpose of monitoring and surveying no further specimens need and should be taken from known sites or caves in their immediate vicinity. This restriction can be met much easier than in other cave beetles, as *Hadesia* individuals cannot be baited and trapped but have to be searched for by eye. In our opinion, only discoveries at new sites well separated from the known caves, or at intermediate position between two ranges, still warrant collecting of a small number of specimens.

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Electronic Supplement Files

at http://www.senckenberg.de/arthropod-systematics ("Contents")

File 1: polak&al-leiodidaehadesia-asp2016-electronicsupplement1.docx. – **Table S1.** List of taxa and sequence data used in phylogenetic analysis. – **Table S2.** DNA partitions and their optimal substitution models as selected using the program PartitionFinder and used in subsequent phylogenetic analyses. – **Table S3.** List of primers and protocols used in the molecular analysis. – **Fig. S1.** Phylogenetic relationships among studied Anthroherponina taxa inferred by Bayesian analysis based on COI, 16S rRNA, 28S rRNA and H3 molecular markers. Values at nodes indicate posterior probabilities. Species names are followed by voucher codes in parentheses.

File 2: polak&al-leiodidaehadesia-asp2016-electronicsupplement2.nex. – Concatenated alignment as used in phylogenetic analyses.