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Marko Lukić

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UNIVERSITY OF LJUBLJANA  
BIOTECHNICAL FACULTY

Marko LUKIĆ

**SYSTEMATICS AND BIOGEOGRAPHY OF THE  
SUBGENUS *Heteromurus* (*Verhoeffiella*)  
(COLLEMBOLA: ENTOMOBRYIDAE) IN THE  
DINARIC KARST**

DOCTORAL DISSERTATION

Ljubljana, 2020



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*Heteromurus (Verhoeffiella)* (COLLEMBOLA: ENTOMOBRYIDAE) IN  
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DOCTORAL DISSERTATION

**SISTEMATIKA IN BIOGEOGRAFIJA PODRODU *Heteromurus*  
(*Verhoeffiella*) (COLLEMBOLA: ENTOMOBRYIDAE) NA  
DINARSKEM KRASU**

DOKTORSKA DISERTACIJA

Ljubljana, 2020

On the basis of the Statute of the University of Ljubljana and the decision made by the Senate of Biotechnical Faculty at the 11<sup>th</sup> session of the Commission for the Doctoral Studies of University of Ljubljana, held on November 11, 2014, it was confirmed that the candidate qualifies to perform doctorate of science at the Interdisciplinary doctoral study program of Biosciences, scientific field of Biology.

Doc. dr. Maja Zagmajster was approved as the supervisor and dr. sc. Louis Deharveng as the co-supervisor of the doctoral dissertation.

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AB We explored evolutionary pathways and diversification in subterranean environments using the collembolan cave genus *Verhoeffiella* (formerly a subgenus of *Heteromurus*) as a study model. We conducted thorough morphological analyses of *Verhoeffiella*, tested the monophyly of the genus, explored its relationship with putative surface relative *Heteromurus nitidus*, and its temporal patterns of molecular diversification. The basis of the study were 411 individuals collected from 105 sites, throughout the *Verhoeffiella* distributional range (Iberian Peninsula, the Alps, Dinarides). We have set up a morphological framework for describing species of *Verhoeffiella* and found new taxonomically relevant diagnostic characters. Based on our work, the number of described species rose from 9 to 14 species. Molecular phylogeny showed that the genera *Verhoeffiella* and *Heteromurus* form a clade nested within Heteromurinae. Within the clade, both *Verhoeffiella* and *Heteromurus* were not monophyletic, implicating the need for taxonomic re-arrangements at the generic level of Heteromurinae. Molecular species delimitation methods disclosed remarkable diversification of *Verhoeffiella* within Dinarides, where a complex geographical pattern of five *Verhoeffiella* lineages resulted in a high number of co-occurring molecular operational taxonomic units. Divergence time estimation showed two boosts of diversification, one linked to the Messinian salinity crisis and the other to Pleistocene climatic oscillations. The discovery of extensive subterranean diversification of *Verhoeffiella* and its closely related epigean relative *Heteromurus nitidus* makes *Verhoeffiella* a model of utmost interest within the subterranean fauna of temperate areas, where the phylogenetic origin of most troglobiotic taxa remain poorly known.

## KLJUČNA DOKUMENTACIJSKA INFORMACIJA

- ŠD Dd  
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IN SISTEMATIKA IN BIOGEOGRAFIJA PODRODU *Heteromurus* (*Verhoeffiella*) (COLLEMBOLA: ENTOMOBRYIDAE) NA DINARSKEM KRASU  
TD Doktorska disertacija  
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IJ en  
JI sl/en  
AI Raziskovali smo evolucijske poti in diverzifikacijo v podzemnih habitatih, pri čemer smo za študijski model uporabili podzemni rod skakačev *Verhoeffiella* (prej podrod rodu *Heteromurus*). Naredili smo podrobno morfološko študijo rodu *Verhoeffiella*, testirali monofilijo rodu, raziskali njegov odnos z domnevnim površinskim sorodnikom *Heteromurus nitidus*, in časovne vzorce molekulske diverzifikacije. Osnova študije je bil nabor 411 osebkov, ki so bili nabrani na 105 lokalitetah po območju razširjenosti rodu *Verhoeffiella* (Iberski polotok, Alpe, Dinaridi). Pripravili smo morfološki okvir za opisovanje vrst *Verhoeffiella* in našli nove razločevalne znake. Na podlagi našega dela je število opisanih vrst naraslo z 9 na 14 vrst. Molekulska filogenija je pokazala, da tvorita rodova *Verhoeffiella* in *Heteromurus* klad znotraj Heteromurinae. Znotraj klada pa noben od teh dveh rodov ni monofiletski, ki naazujejo da bodo potrebne taksonomske spremembe na rodovnem nivoju znotraj Heteromurinae. Molekulska delimitacija vrst je razkrila izjemno diverzifikacijo rodu *Verhoeffiella* znotraj Dinaridov, kjer se je pokazal kompleksen geografski vzorec petih linij z velikim številom sopojavljajočih se molekulskih operacijskih taksonomskih enot. Ocena časovne divergence je pokazala dva vrhova diverzifikacije, enega povezanega z Mesinijsko slanostno krizo, drugega pa s pleistocenskimi klimatskimi nihanji. Odkritje obsežne podzemne diverzifikacije rodu *Verhoeffiella* in bližnjega površinskega sorodnika *Heteromurus nitidus* postavlja rod *Verhoeffiella* na posebno zanimivo mesto znotraj podzemne favne zmernih klimatov, kjer je filogenetski izvor številnih troglobiotskih taksonov slabo poznan.

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- Lukić M., Porco D., Bedos A., Deharveng L. 2015. The puzzling distribution of *Heteromurus* (*Verhoeffiella*) *absoloni* Kseneman, 1938 (Collembola: Entomobryidae: Heteromurinae) resolved: detailed redescription of the nominal species and description of a new species from Catalonia (Spain). *Zootaxa*, 4039, 2: 249-275 8
- Lukić M., Delić T., Zagamajster M., Deharveng L. 2018. Setting a morphological framework for the genus *Verhoeffiella* (Collembola, Entomobryidae) for describing new troglomorphic species from the Dinaric karst (Western Balkans). *Invertebrate Systematics*, 32, 5: 1118-1170 36
- Lukić M., Delić T., Pavlek M., Deharveng L., Zagamajster M. 2020. Distribution pattern and radiation of the European subterranean genus *Verhoeffiella* (Collembola, Entomobryidae). *Zoologica Scripta*, 49: 86-100 90

## LIST OF ANNEXES

ANNEX A: Permission from publisher for the publication of the article Lukić et al. (2015)  
in the printed and electronic versions of the doctoral dissertation

ANNEX B: Permission from publisher for the publication of the article Lukić et al. (2018)  
in the printed and electronic versions of the doctoral dissertation

ANNEX C: Permission from publisher for the publication of the article Lukić et al. (2020)  
in the printed and electronic versions of the doctoral dissertation



## 1 INTRODUCTION

### 1.1 ORIGIN AND DISTRIBUTION PATTERNS OF SUBTERRANEAN SPECIES

Subterranean habitats differ from the surface ones in several features: constant darkness, paucity of food, stability of abiotic parameters e.g. temperature and humidity (Culver and Pipan, 2019). Many species have adapted to these conditions and can live exclusively in the subterranean realm. Subterranean habitats also strongly differ from surface ones in composition of higher taxonomic groups (Gibert and Deharveng, 2002). Only some of them are well represented below ground, while many others are completely absent (Juberthie and Decu, 1994, 1998).

Pre-adaptation or exaptation of some of the groups to subterranean lifestyle can sometimes explain their colonization and speciation success, e.g. Diplopoda (Golovatch and Kime, 2009; Golovatch, 2015) and Collembola (Christiansen, 1961, 1965). However, many that are ideal candidates for subterranean life have no or very few troglobiotic species, e.g. Symphyla (Juberthie-Jupeau, 1994). What hampers our understanding of colonization is the fact that the phylogenetic origin of most troglobiotic taxa remains poorly known. This might be due to strong morphological differences of troglobiotic species and their epigeal relatives that conceals their true relationship, or to the extinction of these epigeal relatives. Whether the closest relatives of subterranean taxa are known or not, a common view is that most subterranean taxa originated from independent colonization events. Evolutionary transition from surface ancestor to troglobiotic species is hypothesized to encompass four phases (Trontelj, 2018; Culver and Pipan, 2019). Active or passive colonization is followed by a “trial phase” during which many factors contribute to its success or failure. After successful colonization, taxa undergo speciation (Peck and Finston, 1993; Danielopol and Rouch, 2012; Howarth, 2019) and subterranean dispersal. Only in rare cases, there is evidence of surface dispersal of troglobiotic taxa (Rizzo et al., 2013).

Whatever colonization and speciation scenarios, they generally produce a similar distributional pattern of troglobionts in all taxonomic groups: small and fragmented distribution ranges (Trontelj et al., 2009; Niemiller and Zigler, 2013; Zagamajster et al., 2014). In extreme cases, the proportion of species known from single or only a few localities can represent more than half of the species pool in a region (Niemiller and Zigler, 2013; Bregović et al., 2019). Range sizes of subterranean taxa have so far been explained by the fragmentation of karstic landscapes, complex paleogeographic events and/or historic climate variability (Deharveng and Gers, 1993; Trontelj et al., 2009; Rizzo et al., 2013; Eme et al., 2014; Zagamajster et al., 2014). Aside many narrowly distributed troglobiotic species and genera, some taxa have large distribution ranges (Gibert and Deharveng, 2002; Zakšek et al., 2007; Trontelj et al., 2009; Baratti et al., 2010; Faille et al., 2010; Isaia et al., 2017), especially among Collembola (Christiansen et al., 1983; Lukić, 2019). Most intriguing are widespread troglobiotic genera occurring in geographic regions which can be hundreds or thousands of kilometers apart. For taxa with limited dispersal abilities, such

patterns are difficult to interpret and strongly contrast small distribution ranges of most troglobiotic taxa. Within Europe, the distribution of only a few terrestrial troglobionts illustrates this case. Interestingly, several troglobionts share similar disjunct distribution pattern, being known from the Iberian Peninsula, the Alps and the Dinarides (Lukić et al., 2010; Weigand et al., 2013; Ribera et al., 2014; Pavlek and Ribera, 2017; Taiti et al., 2018). These regions, which represent subterranean biodiversity hotspots, strongly differ in troglobiotic fauna composition. The disjunct distribution patterns of such taxa challenge the validity of their monophylies as well as the currently accepted scenarios of their origin and colonization.

## 1.2 THE DINARIDES – A SUBTERRANEAN BIODIVERSITY HOTSPOT

The Dinarides are a 650 km long and 150 km wide continuous karst region of more than 60.000 km<sup>2</sup>, that stretches from Italy (Trieste) to Albania (Prokletije Mt.) (Zupan Hajna, 2019). This area has a complex paleogeographic history and orogeny (Tari, 2002; de Leeuw et al., 2011) and is one of the most highly karstified areas in the world (Milanović, 2015). It is a world hotspot of subterranean biodiversity (Culver et al., 2006; Eme et al., 2017; Zagamajster et al., 2018) with a long tradition of research and nearly 1000 aquatic and terrestrial troglobiotic species (Sket et al., 2004; Sket, 2012). This high regional diversity offers many opportunities to approach general evolutionary questions. Aquatic crustaceans have been widely used in this respect, e.g. the cave shrimps of the genus *Troglocaris* (Zakšek et al., 2007; 2009), the amphipod genus *Niphargus* (Fišer et al., 2012; Trontelj et al., 2012; Delić et al., 2016; Zakšek et al., 2019), the aquatic isopods *Monolistra* (Prevorčnik et al., 2010) and *Asellus* (Konec et al., 2015). On the contrary, similar studies on terrestrial troglobionts are very few, mostly limited to Leiodidae beetles of the genera *Anthroherpon* and *Hadesia* (Polak et al., 2016; Njunjić et al., 2018). Other studies with molecular phylogenetic methods focused on mostly taxonomic issues, e.g. on the gastropod *Zospeum* (Weigand et al., 2013; Inäbnit et al., 2019), the terrestrial isopod *Alpioniscus* (Bedeck et al., 2019) and the harvestmen *Cyphophthalmus* (Boyer et al., 2005; Murienne et al., 2010). Most recent studies on terrestrial troglobionts deal however with taxonomy (e.g. Karaman, 2009; Giachino et al., 2011; Antić et al., 2014; Stoev et al., 2015; Papač et al., 2016; Pavlek and Ribera, 2017; Hlaváč et al., 2019) and mapping subterranean biodiversity (Zagamajster and Kováč, 2006; Bedeck et al., 2011; Polak et al., 2012).

## 1.3 COLLEMBOLA IN SUBTERRANEAN HABITATS

With 8128 species in four orders and 30 families (Janssens and Christiansen, 2011) Collembola are moderately diverse compared to many other Hexapoda groups. However, with regular and significant accumulation of new taxa, Collembola diversity grows at a fast rate (Deharveng, 2004). Many recent molecular studies have revealed high genetic diversity within different species, pointing to underestimated diversity within the group

(Porco et al., 2012, 2014; von Saltzwedel et al., 2017; Mateos et al., 2018; Zhang et al., 2019).

Collembola are numerically dominant in most terrestrial ecosystems (Deharveng, 2004) and are found from polar regions to tropical rainforests and deserts, in a variety of habitats from tree canopy to soil and caves, even in intertidal marine habitats, and on water surface (Hopkin, 1997). Many can tolerate freezing conditions, and some soil species are assumed to have persisted in situ through severe climatic changes like Pleistocene glacial cycles (von Saltzwedel et al., 2016; Faria et al., 2019). In subterranean habitats they are present from mesovoid shallow substratum (MSS) and oligotrophic cave habitats to eutrophic guano piles (Baquero et al., 2017; Zhang et al., 2018; Lukić, 2019). In tropics many species are linked to guano, of which some are cave restricted and can be considered troglotrophic guanobites (Gnaspini and Trajano, 2000). Eutroglophilic Collembola species (surface species that can establish exclusively subterranean populations) are well represented in caves, but troglotrophic species usually dominate in abundance (Lukić and Deharveng, 2008).

Troglotrophic Collembola have served as model arthropod organisms to coin the term that illustrates convergent morphological evolution occurring in caves (Howarth and Moldovan, 2018). Species that have such morphology were called ‘troglomorphes’ by Christiansen (1962), while associated characters are classically qualified as ‘troglomorphic’ (Christiansen, 2012). This striking convergent morphological evolution occurs in many unrelated living groups (Deharveng and Bedos, 2018; Howarth and Moldovan, 2018). Troglotrophic Collembola exhibit various troglomorphic traits known to occur also in many other taxonomic groups: larger body size, elongation of appendages, reduction of eyes and pigmentation, compared to their putative surface relatives. Collembola-specific troglomorphic traits are also well documented: basal shift and regression of inner and lateral teeth on claws, elongation of claw, regression of tibiotarsal tenent hair (being pointed instead of capitate), elongation of mucro and particularly its distal tooth, higher number and hypertrophy of specialized chaetae (sensilla) on antennae (Christiansen, 2012). In addition to such an exceptional variety of troglomorphic traits, some of them are particularly exaggerated, as appendage and claw elongation in some species (Lukić et al., 2018; Jantarit et al., 2019; Vargovitsh, 2019). All this generates a very high diversity in the expression of troglomorphy within Collembola.

Troglotrophic Collembola are highly diversified in subterranean habitats across the whole world (Thibaud and Deharveng, 1994; Deharveng and Bedos, 2018). Recent synthesis yielded a total number of 583 troglotrophic and cave restricted guanobiotic species from 14 families and 61 genera (Lukić, 2019). This diversity is unevenly distributed on the globe and several areas stand out with high species richness: southern Europe, central and eastern United States, Mexico, Japan, Thailand, and southwest China. Among them the Euro-Mediterranean region is the most species-rich with 298 troglotrophic species, with a diversity at the family and generic level that outmatches that of any other region in the

world (Lukić, 2019). Still, extensive knowledge on diversity and distribution of cave Collembola is lacking throughout many European karst areas, one of them being the Dinarides.

Collembola in Dinarides are one of the least studied groups in caves of the region. So far, occasional and unsystematic collection of Collembola in caves resulted in 30 described species (Bogojević, 1968; Lukić, 2019), but without good biogeographical data. However, recent preliminary barcode screening and morphological examination revealed a strong underestimation of Collembolan species diversity in the region (Lukić et al., 2012). Among them, the genus *Verhoeffiella* Absolon stands out with the highest number of described species and spectacular morphological adaptations to subterranean life.

#### 1.4 GENUS *VERHOEFFIELLA* AS A STUDY MODEL

Collembola have been traditionally used as evolutionary models for morphological adaptations within subterranean habitats (Christiansen, 1962, 1965), but their potential for testing evolutionary hypotheses using molecular approaches has rarely been exploited (Katz et al., 2018). In this perspective, the genus *Verhoeffiella* represents a promising model as it embodies several features of interest: (i) all species are found in caves and exhibit numerous troglomorphic traits; (ii) the genus has an intriguing disjunct distribution, occurring in four karst regions of Europe; (iii) its center of diversity is in the Dinarides; (iv) species of the genus are very similar to the non-troglobiotic and non-troglomorphic species *Heteromurus nitidus* (Templeton, 1835) regarding characters assumed to be non-adaptive (Templeton, 1835). These features are elaborated below.

The genus encompassed eight troglobiotic species, found exclusively in caves (Lukić et al., 2015). All species are troglomorphic, exhibiting at different degrees almost all troglomorphic traits of cave Collembola. Particularly impressive is the elongation of antennae. The most extreme case is the species *Verhoeffiella longicornis* (Absolon, 1900), which has antennae more than twice the length of the body, one of the longest among troglobiotic Collembola at a world scale (Absolon, 1900; Jantarit et al., 2019). This species and *V. anagastumensis* Lučić, 2008 (in Lučić et al. 2008) also exhibit gigantism with the body + head length of more than 4 mm, in contrast to the smallest species of the genus, *V. dallaii* Nosek & Paoletti, 1985 of only 2.5 mm (Nosek and Paoletti, 1985; Lučić et al., 2008). Further, varying degrees of elongation of unguis, reduction of teeth on unguis and regression of tenent hair are present in all species of the genus. This diversity of troglomorphic traits is unique within the subfamily Heteromurinae (Mari Mutt, 1980a; Cipola et al., 2016).

The genus has intriguing disjunct distribution, occurring in four karst regions of Europe – the Dinarides (Bogojević, 1968; Lučić et al., 2007, 2008), South-eastern Calcareous Alps (Nosek and Paoletti, 1985), southern Catalonia (da Gama, 1984) and Cordillera Cantabrica (Bonet, 1931). This disjunct pattern of troglobiotic *Verhoeffiella* populations could hardly

be a result of sampling bias, as no *Verhoeffiella* species has ever been recorded in thoroughly sampled regions in between, namely in the Western Alps, French southern Massif Central and the Pyrenees (Dallai and Malatesta, 1982; Jordana et al., 1990; Dallai et al., 1995; Thibaud, 2017). Moreover, two of the three species reported from Catalonia and Cordillera Cantabrica by da Gama (1984) have been considered conspecific with *V. absoloni* Kseneman 1938 and *V. longicornis* from the Dinarides. Such a wide disjunction in geographical distribution is an unusual case among troglobiotic arthropods, raising the question of the true identity of the Iberian populations.

Six species have been described from the Dinarides, while single species has been described from South-eastern Calcareous Alps and Cordillera Cantabrica. Within Dinarides the genus inhabits caves in south-eastern part, where its species sometimes present the dominant cave invertebrates in subterranean communities (Lukić and Deharveng, 2008). On the contrary, in the Iberian Peninsula and the Alps that are both well sampled for cave Collembola, *Verhoeffiella* species are only known from few caves (Nosek and Paoletti, 1985; Jordana et al., 1990).

A species that is similar to *Verhoeffiella*, and can often be found in caves, is *Heteromurus nitidus*. It is widely distributed in epigeal habitats across almost the entire Palearctic and Nearctic. Populations of this species also often colonize subterranean habitats, and many trogliphilic populations have been recorded in Europe and the United States (Christiansen and Bellinger, 1980; Jordana et al., 1990; Thibaud, 2017). The species is very common in caves of Dinarides where it is sometimes found in syntopy with *Verhoeffiella* species (Lukić and Deharveng, 2008). Species of *Verhoeffiella* are very similar to *H. nitidus* in important morphological characters, e.g. in dorsal chaetotaxic pattern (Loksa and Bogojević, 1967; Szeptycki, 1979; da Gama, 1984) that is widely used to separate species within the family Entomobryidae.

## 1.5 TAXONOMIC POSITION OF *VERHOEFFIELLA*

The genus *Verhoeffiella* is placed in the subfamily Heteromurinae of the family Entomobryidae. It had a ‘floating’ taxonomic status throughout the history of its study. Type species of the genus, *V. cavicola* Absolon, 1900, was described originally as *Heteromurus* (*Verhoeffiella*) *cavicola* for which Absolon erected a new subgenus. In the same paper Absolon described another new genus and species, *Typhlopodura longicornis*, but based on specimens with accidentally fused antennal segments. This mistake was later realized by Kseneman (1938) who transferred the species to the genus *Verhoeffiella*. The status of *Verhoeffiella* as a subgenus of *Heteromurus* was widely accepted by many authors (Bonet, 1931; Gisin, 1960; Mari Mutt, 1980a; Lučić et al., 2007, 2008), but it was also treated as a separate genus by others (Kseneman, 1938; Thibaud and Deharveng, 1994; Soto-Adames, 2008; Bedos, 2014; Cipola et al., 2016; Guru Pada Mandal, 2018). It was even considered as *Heteromurus sensu stricto* by da Gama (1984). Status change was

a direct consequence of the poor original descriptions of almost all the species of the genus and reflected subjective appreciation of the value of the third antennal segment annulation, the only character used to separate *Verhoeffiella* from *Heteromurus* s.str. (Mari Mutt, 1980a, 1980b; Cipola et al., 2016). All *Verhoeffiella* species were found to have the third and fourth antennal segment annulated, while species of *Heteromurus* s.str. have only the fourth antennal segment annulated. From 2018, we have considered *Verhoeffiella* as a genus in our publications, following the conception of Kseneman (1938). References before that date, including the time when the title of this dissertation was formally approved (in 2012), refer to *Verhoeffiella* as subgenus of the genus *Heteromurus*.

#### 1.6 DIAGNOSTIC CHARACTERS BETWEEN SPECIES OF THE GENUS *VERHOEFFIELLA*

Most of the species of the genus were described during the last century and original species description are all very brief, some even lacking a single illustration of diagnostic characters (e.g. Kseneman, 1938). Even most recently described species lack illustration and description of most characters used in modern taxonomy of Entomobryidae (Lučić et al., 2007, 2008). Body size, position and number of teeth on unguis and unguiculus, and differences in elongation of antennae were the sole characters used to discriminate the species. Antennae elongation were expressed as the ratio antennae: head diagonal, and antennae: body length, often loosely expressed only as ‘shorter’ or ‘longer’ than body. Antennal segments ratio has been described in some species, but not in a uniform way due to subdivision of first antennal segments in two subsegments. Therefore some authors considered antennae to be four-(Bonet, 1931; Lučić et al., 2008) and others five-segmented. Furthermore, third and fourth antennal segments are frequently fused and ratio of these fused antennal segments have been used in original species descriptions (Absolon, 1900), bringing additional difficulties for comparing antennal segments length among species of the genus.

Chaetotaxy of *Verhoeffiella* species have been rarely described and not used as a diagnostic character. Diversity of antennal chaetotaxy have been recognized by Bonet (1931), who gave preliminary description of several antennal chaetae types and their distribution, but nobody followed his approach in subsequent species descriptions. Dorsal body chaetotaxy that is widely used in species discrimination within Entomobryidae, has been illustrated only by da Gama (1984) and Loksa and Bogojević (1967), while illustration of Lučić et al. (2007) certainly represent inaccurate observations.

Trogomorphic traits are the most striking features of *Verhoeffiella*, and they have been often described in more details. Their morphological diversity led authors to almost exclusive use some of them as diagnostic characters in the taxonomy of the genus. A need for new diagnostic characters and the re-assessment of *Verhoeffiella* position within Heteromurinae has been stressed several times (Mari Mutt, 1980a; Cipola et al., 2016), but

due to lack of specimens in the collections, species of the genus were never included in recent morphological studies (Cipola et al., 2016) or molecular phylogenetic studies of the subfamily (Zhang et al., 2014, 2015).

## 1.7 THESIS AIMS AND HYPOTHESES

The overall goal of this dissertation is to explore diversification and distribution patterns in subterranean environments using the collembolan cave genus *Verhoeffiella* as a study model. The close relationship of the *Verhoeffiella* and the epigeal populations of species *Heteromurus nitidus* may offer an insight into the origin of disjunct distribution of genus *Verhoeffiella*, and contribute to a better understanding of colonization and diversification dynamics within the subterranean habitats in general. Thesis specific aims the work addresses are: (i) morphological and taxonomical study of *Verhoeffiella*; (ii) test of monophyly of *Verhoeffiella* and its phylogenetic position within Entomobryidae; (iii) relationships of *Verhoeffiella* and *Heteromurus nitidus*; (iv) diversification of *Verhoeffiella* in the Dinarides.

(i) To do a detailed morphological study and to explore diversity and variability of morphological characters of *Verhoeffiella* species, as well as to search for new diagnostic ones among the species. Special emphasis was given to chaetotaxy, diversity of chaetae and troglomorphic characters. These results are important as a basis for a new descriptive framework for further taxonomic advances in study of the genus.

(ii) To determine the position of the genus within the Entomobryidae family and its relationships to other genera of the subfamily Heteromurinae, in particular to the genus *Heteromurus*, using molecular phylogeny. Further, to test the monophyly of *Verhoeffiella* populations from remote European regions and to reconstruct phylogenetic relationships among the species.

(iii) To investigate whether the species *Heteromurus nitidus* is closely related to genus *Verhoeffiella*, as suggested by many morphological similarities. To explore relationships of *Verhoeffiella* species from remote European regions and populations of *H. nitidus* from corresponding regions. We assumed that the epigeal ancestors of *Verhoeffiella* resembled or were related to *H. nitidus*, and that molecular phylogeny will reveal *Verhoeffiella*–*H. nitidus* sister relationship. To investigate, whether remote European karst regions were independently colonized by these epigeal ancestors, while multiple independent colonization events were important on a smaller geographic scale.

(iv) To give a special emphasize to *Verhoeffiella* populations from Dinaric region, as a center of diversity of the genus. To compile new extensive dataset which enables exploration of cryptic diversity of the genus within the region. Finally, to explore the relationship between different populations of the genus, in order to reconstruct the evolutionary scenarios of diversification in relation to paleogeographic events.

## 2 SCIENTIFIC PAPERS

### 2.1 THE PUZZLING DISTRIBUTION OF *HETEROMURUS (VERHOEFFIELLA) ABSOLONI* KSENEMAN, 1938 (COLLEMBOLA: ENTOMOBRYIDAE: HETEROMURINAE) RESOLVED: DETAILED REDESCRIPTION OF THE NOMINAL SPECIES AND DESCRIPTION OF A NEW SPECIES FROM CATALONIA (SPAIN)

Lukić M., Porco D., Bedos A., Deharveng L. 2015. The puzzling distribution of *Heteromurus (Verhoeffiella) absoloni* Kseneman, 1938 (Collembola: Entomobryidae: Heteromurinae) resolved: detailed redescription of the nominal species and description of a new species from Catalonia (Spain). *Zootaxa*, 4039, 2: 249-275

V prispevku smo podali podrobno redeskripcijo vrste *Heteromurus (Verhoeffiella) absoloni* Kseneman, 1938, kot tudi identifikacijsko molekulsko črtno kodo na osnovi osebkov s tipske lokalitete v Črni gori. Določili smo neotip. Prvič za rod *Verhoeffiella* smo opisali dorzalno S-hetotaksijo. Podrobno smo analizirali morfologijo het in njihovo razporeditev, še posebno na antenah, kjer smo prepoznali kar 12 tipov het. Številne morfološke značilnosti smo opisali na novo tako za rod kot tudi za poddružino Heteromurinae. Široko razširjeno disjunktno razširjenost vrste smo obravnavali z molekulsko in morfološko primerjavo osebkov s tipske lokalitete v Črni gori in iz populacij iz Katalonije. Ugotovili smo, da v Kataloniji živi druga, doslej neznana vrsta, ki smo jo opisali kot *Heteromurus (Verhoeffiella) gamae* sp. nov. Predlagamo tudi novo kombinacijo za *Heteromurus (Verhoeffiella) constantinellus* (Ćurčić & Lučić in Lučić, Ćurčić & Mitić 2007) comb. nov. Podali smo pregled vseh vrst podrodo, predstavili problematiko razlikovanja vrst znotraj podrodo in posebej obravnavali taksonomski status podrodo *Verhoeffiella*.



**The puzzling distribution of *Heteromurus* (*Verhoeffiella*) *absoloni* Kseneman, 1938 (Collembola: Entomobryidae: Heteromurinae) resolved: detailed redescription of the nominal species and description of a new species from Catalonia (Spain)**

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**Abstract**

The species *Heteromurus* (*Verhoeffiella*) *absoloni* Kseneman, 1938 is redescribed in detail and characterized by its barcode, based on specimens from its type locality in Montenegro. A neotype is designated. Dorsal S-chaetotaxy is given for the first time in the subgenus *Verhoeffiella*. Chaeta morphology and distribution are thoroughly analyzed, in particular on antennae where 12 chaetal types are recognized. Several morphological features are newly described for the genus and for Heteromurinae. The widely disjunct distribution of the species is approached through morphological and molecular comparison of specimens from the type locality in Montenegro and from the Catalan population. We established that this last record is a new species described here as *Heteromurus* (*Verhoeffiella*) *gamae* sp. nov. New combination is proposed *Heteromurus* (*Verhoeffiella*) *constantinellus* (Čurčić & Lučić in Lučić, Čurčić & Mitić 2007) comb. nov. A table of all species of the subgenus is provided. The taxonomic status of *Verhoeffiella* and the problems of species discriminations in the subgenus are discussed.

**Key words:** taxonomy, chaetotaxy, troglomorphy, cave fauna, Dinarids, disjunct distribution, DNA barcodes

**Introduction**

*Verhoeffiella* Absolon, 1900 is a subgenus of *Heteromurus* Wankel, 1860 (considered as a full genus by Soto-Adames *et al.* 2008) which includes 9 troglomorphic species. They are present in several European karst areas: Balkans (Dinarids, Macedonia) (7 species), southeastern Italian Alps (one species), southern Catalonia (one species) and Cordillera Cantabrica (two species) in Spain, and Northeastern France (one species). In some areas (e.g. Dinarids) these species count among the dominant cave invertebrates in subterranean communities (Lukić & Deharveng, 2008) while in the Iberian Peninsula they are only known from a few caves (Jordana *et al.* 1990). No species has ever been recorded from intermediate regions (particularly Western Alps, southern Massif Central and the Pyrenees), a disjunct pattern extremely intriguing from a biogeographical and evolutionary point of view. Moreover, the species reported from Catalonia and Cordillera Cantabrica in Spain by Gama (1984) and from Northeastern France by Thibaud (1970) have been considered by these authors conspecific with three Dinaric species, *H. (V.) absoloni* Kseneman, 1938, *H. (V.) longicornis* (Absolon, 1900) and *H. (V.) cavicola* Absolon, 1900. Such a situation would probably represent the most extreme case of disjunct distribution among terrestrial troglomorphic species in Europe.

In spite of spectacular morphological modifications of several species in relation to cave life and the puzzling distribution pattern of the subgenus summarized above, *Verhoeffiella* has never been the subject of detailed morphological study, and the available descriptions remain basic. This may be due to the fact that chaetotaxi-

patterns widely used to separate species in Entomobryidae are of little help in *Verhoeffiella*. Having collected a large number of specimens and species from several Dinaric caves, we initiate in the present paper a review of the subgenus with two objectives. First, to re-describe in detail *H. (V.) absoloni* and to set a reference descriptive framework for species of the subgenus, that are all very poorly described. Second, to compare specimens from the type locality in Montenegro with specimens of the Catalan locality from where it was recorded in 1984 by Gama.

*Heteromurus (Verhoeffiella) absoloni* was described in 1938 by Kseneman from a cave of Montenegro (pećina Golodražnica), and is only known from this locality within the Dinarids. The original description of *H. (V.) absoloni* was extremely succinct, lacking most characters used in modern taxonomy of Entomobryidae. The extensive re-description of the species given here is based on fresh material collected in the type locality, using modern chaetotaxic and mouthpart characters. A special emphasis is put on the diversity of chaetae, especially on antenna that has been only partially studied so far in the literature (Bonet, 1931), whereas it might be a rich mine of new character for the taxonomy of the Heteromurinae *sensu* Zhang & Deharveng (2015) where chaetotaxic patterns seem to be very uniform within the genus *Heteromurus*.

Forat del Rastre, a cave in southern Catalonia 1500 km from the Dinarid locality, is the second locality from where *H. (V.) absoloni* has been recorded (Gama 1984). Such a wide disjunction in geographical distribution is an unusual case among troglobiotic arthropods, raising the question of identification accuracy of the Catalan species in a group where interspecific discriminant characters are all considered as adaptive. We compare here specimens from this Catalan cave with topotypical specimens, using morphological and barcoding approaches.

#### Material and methods

**Sampling.** The deposition of the type specimens of *Heteromurus (Verhoeffiella) absoloni* is not mentioned in the original description, and it has been impossible to locate them in regional Museum. We relied upon fresh specimens collected by hand in the type locality, among which a neotype was designated. For the Spanish record of the species, we used specimens collected in Forat del Rastre in the Mola de Cati range (Tarragona, Catalonia), the only locality where the species has been recorded in Spain.

**Abbreviations.** Abd.—abdominal segment, AIIO—apical organite of Ant. II, AIII—apical organite of Ant. III, Ant.—antennal segment, Th.—thoracic segment, Tita—tibiotarsus, mac—macrochaetae, mes—mesochaetae, mic—microchaeta, ms—smooth, very short, thick, pointed S-chaetae, sens—smooth, short and hyaline, subcylindrical, thin, blunt S-chaetae, N—number of measured specimens.

**Formulae.** Formulae are given per half tergite from Th. II to Abd. IV for dorsal macrochaetae and pseudopores, number of mac in formula correspond to: Th. II anterior + posterior, Th. III lateral + posterior, Abd. IV medial + posteromedial + anterolateral + posterolateral (“≥” or “?” are used in formula for anterior or lateral mac usually not given in old descriptions); from Th. II to Abd. V for S-chaetae; from head to Abd. IV for trichobothria.

**Chaetotaxy systems.** The dorsal macrochaetotaxy pattern observed in our specimens is almost identical to that of *Heteromurus (H.) nitidus* (Templeton, 1835) described by Szeptycki (1972, 1979). We give on Fig. 23 the chaetal notation proposed by this author for tergites, as well as another notation by groups of chaetae for head (after Mari-Mutt 1979) and for tergites, that we used rather than individual chaetal notation, in order to avoid uncertainties of chaeta-to-chaeta homologies due to the reduced macrochaetotaxy of the studied species.

**Vocabulary.** Antenna 1 and 2 of authors (Mari-Mutt 1980a, 1980b) are here named Ia and Ib to preserve homologies with other Entomobryidae, as they correspond to the splitting of Ant. I of Collembola; Ant. 3, 4 and 5 are here Ant. II, III and IV.

**Chaetal nomenclature.** The basic distinction between ordinary chaetae (“chaetae”) and S-chaetae (“sensilla” of authors) follow the rationale of Deharveng (1983). S-like chaetae correspond to the “setulae” of Szeptycki (1979: 10) when they cannot be assigned unambiguously to S-chaetae. On the tergite, ms designate short pointed S-chaetae, and sens larger ones, following the convention used in Potapov (2001) for Isotomidae and Zhang & Deharveng (2015) for Entomobryidae. On the antennae, where chaetotaxy is extremely diversified in Entomobryoidea, we described all kinds of chaetae that were able to discriminate morphologically, but did not try to match the notation systems previously published in the literature which are less complete. This important task will require extensive comparative investigations beyond the scope of this paper.

**Deposition of studied material.** Collection of Croatian Biospeleological Society (CBSS), Croatia; Muséum National d'Histoire Naturelle de Paris (MNHN), France.

**TABLE 1.** Specimens of *H. (V.) absoloni*, *H. (V.) gamae* sp. nov. and *H. (H.) nitidus* used in molecular analyses.

Taxa	Locality (sample code)	Date	Legator	Number of specimens	BOLD sample ID
<i>H. (V.) absoloni</i>	Montenegro, Risan, Golodražnica (CLL2278)	24.04.2010.	Jana Bedek	6	4028C01_MNECLL2278
					4028C02_MNECLL2278
					4028C03_MNECLL2278
					4028C04_MNECLL2278
					4028C05_MNECLL2278
					4028C06_MNECLL2278
<i>H. (V.) gamae</i> sp. nov.	Spain, Tarragona, Mola de Cati, Forat del Rastre (ETA-052)	13.05.2003	Arnaud Faille	1	4027C08_ETA052
<i>H. (V.) gamae</i> sp. nov.	Spain, Tarragona, Mola de Cati, Forat del Rastre (ETA-050)	04.12.2002	Arnaud Faille	2	10785A11_ETA050
					10785A10_ETA050
<i>H. (H.) nitidus</i>	Bosnia and Herzegovina, Rogatica, Konjokrad, Golubina pećina (CLL2325)	14.08.2010	Marko Lukić	6	4028E07_BHCLL2325
					4028E08_BHCLL2325
					4028E09_BHCLL2325
					4028E10_BHCLL2325
					4028E11_BHCLL2325
					4028E12_BHCLL2325

**Molecular analyses.** DNA barcodes were obtained from 9 specimens of *Heteromurus* (*Verhoeffiella*) and 6 specimens of *Heteromurus* (*Heteromurus*) *nitidus* for genetic examination of the divergences (Tab. 1). DNA was extracted from entire specimens in 30µl of lysis buffer ([http://www.ccdb.ca/docs/CCDB\\_DNA\\_Extraction.pdf](http://www.ccdb.ca/docs/CCDB_DNA_Extraction.pdf)) and proteinase K incubated at 56°C overnight. DNA extraction followed a standard automated protocol using 96-well glass fibre plates (Ivanova *et al.* 2006). Specimens were recovered after DNA extraction using a specially designed work flow allowing their morphological examination (Porco *et al.* 2010). The 5' region of COI used as a standard DNA barcode was amplified using M13 tailed primers LCO1490 and HCO2198 (Folmer *et al.* 1994). Samples that failed to generate an amplicon were subsequently amplified with a pair of internal primers combined with full length ones (C\_LepFolF/C\_LepFolR) (Ivanova—published on [www.boldsystems.org](http://www.boldsystems.org)). The standard PCR reaction protocol of the Canadian Center for DNA Barcoding was used for amplifications (<http://www.dnabarcodes2011.org/conference/preconference/CCDB-Amplification-animals.pdf>), and products were checked on a 2% E-gel 96Agarose (Invitrogen). Unpurified PCR amplicons were sequenced in both directions using M13 tailed primers, with products subsequently purified using Agencourt CleanSEQ protocol and processed using BigDye version 3.1 on an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were assembled and edited with Sequencher 4.5 (GeneCode Corporation, Ann Arbor, MI, USA). The alignment was obtained using BIOEDIT version 7.0.5.3 (Hall 1999). Sequences are publicly available on BOLD via the following DOI ([dx.doi.org/10.5883/DS-CAHETABS](http://dx.doi.org/10.5883/DS-CAHETABS)) and can also be retrieved on Genbank (JN275258, JN275273-JN275278, JN275288-JN275292, KT381926-KT381928).

**Data analyses.** Distance analyses were performed with MEGA6 (Tamura *et al.* 2013), utilizing a Neighbor-Joining algorithm (Saitou & Nei 1987) with the Kimura-2 parameter model to estimate genetic distances (Kimura 1980). The robustness of nodes was evaluated through bootstrap re-analysis of 1000 pseudoreplicates.

## Taxonomy

### Genus *Heteromurus* Wankel, 1860

#### Subgenus *Verhoeffiella* Absolon, 1900

Type species: *Heteromurus* (*Verhoeffiella*) *cavicola* Absolon, 1900: p. 429

**Diagnosis of *Heteromurus* (*Verhoeffiella*).** The diagnosis of the subgenus provided below is provisional, as diagnostic characters are unknown in several species.

Medium to large size (1.6–5.3 mm). Eyes absent. Body white, yellowish or pale orange, usually with pigment as orange dots. Scales present, rounded, their surface covered with numerous short spicules arranged longitudinally. Mac present dorsally on head and body, in small number, with typical arrangement (not known in several species). Ant. I divided in two subsegments, Ant. II entire, Ant. III and IV annulated. Antennae of various length, slightly shorter to longer than body. Abd. IV 1.5 to 2 times as long as Abd. III (contradictory statements in the original description of *H. (V.) dallai*, where the ratio is given as “nearly two times” and “2.8-3 times” elsewhere in the text. This last ratio would be unusual, rather pointing to a *Pseudosinella* species). Unguis thin and elongate, usually with one inner pair of small basal teeth, and one inner small unpaired tooth. Tita with thin, acuminate tenent hair. Dens devoid of spines. Mucro bidentate with basal spine.

**Remark.** The subgenus *Verhoeffiella* only differs from *Heteromurus* by the annulation of Ant. III.

#### Checklist of the species of *Heteromurus* (*Verhoeffiella*)

The nine species that can be assigned to *Heteromurus* (*Verhoeffiella*) are listed below with their detailed geographical distribution.

**1. *Heteromurus* (*Verhoeffiella*) *absoloni* Kseneman, 1938**

*Verhoeffiella absoloni* Kseneman, 1938

*Heteromurus absoloni* Kseneman, 1938 (in Gama 1984)

Type locality: Montenegro, Boka kotorska, Risan, Golodražnica (cave); "Jugoslawien, Risan in Boka Kotorska, Höhle "Golodražnica" legít J. Kratochvil am 3.VII.1935."

Other record: Spain, "Forat del Rastre (Mola de Catí), Tarragona, 20-III-1976, 15 exemplaires, leg. Comas" (Gama 1984, dubious identification)

**2. *Heteromurus* (*Verhoeffiella*) *anagastumensis* Lučić, 2008 (in Lučić, Tomić, Brajković & Čurčić 2008)**

Type locality: Montenegro, Nikšić, Budoš, Velja peč (cave); "Velja Peč Cave near the city of Nikšić, Montenegro; collected on 7 September 2000 by B. P. M. Čurčić and S. B. Čurčić. The type specimen is deposited in the collections of the Institute of Zoology, Faculty of Biology, University of Belgrade, 11000 Belgrade, Serbia (IZB 2004)."

Only known from the type specimens.

**3. *Heteromurus* (*Verhoeffiella*) *cavicola* Absolon, 1900, type species of the subgenus**

*Verhoeffiella cavicola* Absolon, 1900

*Verhoeffiella cavatica* Absolon (in Lang 1935, *lapsus calami*)

*Heteromurus cavicola* Absolon, 1900 (in Thibaud, 1970)

Type locality: Bosnia and Herzegovina, Sarajevo, Bjelašnica, Laništa, Megara (cave); "Insurgentenhöhle im Ivangebirge der Herzegowina; belebt sehr häufig die tief in der Höhle gelegenen Stalaktiten. Wurde im Oktober 1897 daselbst von Herrn Dr. C. Verhoeff entdeckt und in vielen Exemplaren gesammelt."

Other records: Bosnia and Herzegovina, "jeskiny Megara...*Verhoeffiella cavatica* Absln. (loc. typ.)" (Absolon 1912). Bosnia and Herzegovina, "Loc. Nr. 148. Unbenannte Höhle in der Šemeč planina im Drinagebiet bei Višegrad im früheren Ost-bosnien, ca. 1180 m hoch...2.IX.1913. (C. Weirather)" (Lang 1935). France, "Grotte des Excentriques (Pierre-la-Treiche, Meurthe-et-Moselle) située a 230 m d'altitude" (Thibaud 1970, dubious record).

**4. *Heteromurus* (*Verhoeffiella*) *constantinellus* (Čurčić & Lučić in Lučić, Čurčić & Mitić 2007) **comb. nov.****

*Heteromurus constantinellus* Čurčić & Lučić (in Lučić, Čurčić & Mitić 2007)

Type locality: Macedonia, Holotype male, from the Peštera Momiček Cave, village of Belica, Mt. Dautica, FYROM; 21 June 2002, collected by S. B. Čurčić, S. E. Makarov, V. T. Tomić, and B. M. Mitić. The type specimen is deposited in the collection of the Center of Biospeleology, Institute of Zoology, Faculty of Biology, University of Belgrade, 11000 Belgrade, Serbia."

Only known from the type specimen. This species is assigned here to the subgenus *Verhoeffiella* on account of two troglomorphic characters (elongated claw and long fourth antennal segment).

**5. *Heteromurus (Verhoeffiella) constantius*** Ćurčić & Lučić (in Lučić, Ćurčić & Tomić 2007)

Type locality: Bosnia and Herzegovina, Gacko, Avtovac, Mulje (cave); "five males (holotype and four paratypes), allotype female and a paratype female, all from a cave near the Mušnica Gorge (Avtovac Mulje), near Trebinje, Herzegovina; collected on 10 September 2006 by I.M. Karaman."

Only known from the type specimens.

**6. *Heteromurus (Verhoeffiella) dallaii*** Nosek & Paoletti, 1985

Type locality: Italy, "Holotype male mounted in Swan's medium. Venetian Prealps, Grotta della Fous, (229 FR) 1979 Camatta leg. In coll. Museum d'Histoire naturelle of Geneve. Allotype with some paratypes from the same locality kept in coll. Museo Civico di Storia Naturale of Venice and 4 paratypes in the Museum d'Histoire naturelle of Geneve."

Only known from the type specimens.

**7. *Heteromurus (Verhoeffiella) hispanica*** Bonet, 1931

*Heteromurus hispanicus* Bonet, 1931 (in Gama 1984, 1985)

Type locality: Spain, "Un ejemplar de la cueva de la Loja. Museo de Madrid."

Other records: Spain, "Prov. de Oviedo: Cueva del Espinoso, La Franca" (Bonet 1931). Spain, "Sima 0-3, Ocoño (Peñamellera Alta), Oviedo, 16-IV-1981, 3 exemplaires, leg. Escolà. 2 exemplaires en alcool, sont déposés au Museu de Zoologia de Barcelona et 1 exemplaire, en préparation, au Museu Zoològic da Universidade de Coimbra; Cueva del Agua, Picos de Europa, Santander, 3-VIII-1979, 2 exemplaires, leg. Chapman. 1 exemplaire, en alcool, se trouve au Museu de Zoologia de Barcelona et 1 exemplaire, en préparation, au Museu Zoològic da Universidade de Coimbra." (Gama 1984). Spain, "Cueva Pruneda, Bobia de Abajo, Asturias, 2-VIII-1976, 3 exemplaires, leg. Comas; Cueva Merín, Villar, Asturias, 7-VIII-1976, 6 exemplaires, leg. Comas." (Gama 1985).

**8. *Heteromurus (Verhoeffiella) longicornis*** (Absolon, 1900)

*Typhlodura longicornis* Absolon, 1900

*Heteromurus Typhlodura longicornis* Absln. (in Absolon, 1912)

*Verhoeffiella longicornis* Absolon, 1900 (in Kseneman 1938)

*Heteromurus cf longicornis* (Absolon, 1900) (in Gama 1984)

Type locality: Bosnia and Herzegovina, Trebinje, Kličanj, Ilina pećina (cave); "Sie wurde durch Herrn Dr. C. Verhoeff in der Eliashöhle bei Trebinje (Herzegowina) (April 1900) in wenigen (3) Exemplaren gesammelt, wo sie tief in der Höhle an Stalaktiten herumläuft."

Other records: Montenegro, "Höhle "Vilina pećina" bei Crkvice Bezirk, Kotor stammen (legit. J. Kratochvil am 23.VII.1935)" (Kseneman 1938).

Spain, "Cueva Coventosa (Arredondo), Santander, 2 -VI-1974, 6 exemplaires, leg. Comas. 4 exemplaires, en alcool, sont déposés au Museu de Zoologia de Barcelona et 2 exemplaires, en préparation, au Museu Zoològic da Universidade de Coimbra; Cueva del Agua, Picos de Europa, Santander, 3-VIII-1979, 2 exemplaires, leg. Chapman, 1 exemplaire, en alcool, se trouve au Museu de Zoologia de Barcelona et 1 exemplaire, en préparation, au Museu Zoològic da Universidade de Coimbra." (Gama 1984, dubious identification).

**9. *Heteromurus (Verhoeffiella) medius*** Loksa & Bogojević, 1967

*Heteromurus medius* Loksa et Bogojević, 1967 (in Bogojević 1975)

*Verhoeffiella media* (Loksa & Bogojević, 1967) (in Cukrov & Ozimec 2014)

Type locality: Croatia, Dubrovnik, Osojnik, Somino brdo, Močiljska špilja (cave); "Die neue Art wurde in der Mociljska-Höhle gesammelt. Die Aufbewahrung der Holotype sowie der Paralypen erfolgte wie bei den oben beschriebenen neuen Arten...Er wird in der Sammlung des Zoosystematischen Institutes der Eötvös-Loránd Universität, Budapest, aufbewahrt. Der Paratypus – vom gleichen Fundort stammend – wird in der Sammlung des Zoologischen Institutes der Landwirtschaftlichen Fakultät, Belgrade aufbewahrt."

Other records: Croatia, Dubrovnik, Komolac, Vilina špilja—Ombla (cave system) (Cukrov & Ozimec 2014).

Croatia, Dubrovnik, island Lokrum " *Orno-Quercetum ilicis* facies *Laurus nobilis* communities" (Bogojević 1975, dubious identification). Montenegro, "Rožaje, Donje Biševo; Pečina u Dubokom potoku cave" (Lohaj *et al.* 2013).

#### Redescription of *Heteromurus* (*Verhoeffiella*) *absoloni* Kseneman, 1938

**Designation of neotype.** The type material of *Heteromurus* (*Verhoeffiella*) *absoloni* has not been retrieved in spite of thorough investigations in regional Museums and in Czech republic, including: Natural History Museum of Montenegro, Podgorica, National Museum of Bosnia and Herzegovina, Sarajevo, Croatian Natural History Museum, Zagreb, The Moravian Museum, Brno, Mendel University in Brno - Department of Zoology, Fisheries, Hydrobiology and Apiculture (formally Zoologický ústav vysoké školy zemědělské v Brně where Kseneman was working, pers. com. Jan Bezdek). It was also not possible to locate Ksenemans collection (Rusek and Weiner, pers. com.). Type material can be therefore considered as lost. We propose here as neotype a specimen from the type locality.

**Material examined.** Montenegro, Boka kotorska, Risan, Golodražnica (cave) (coordinates WGS84 X=18.69962°, Y=42.50282°, alt.110 m.). Neotype adult female on slide, 04.xii.2014, M. Lukić leg.(CLL 4557); 5 ex. on slides and 19 ex. in 96% alcohol, same data (CLL 4555). Three ex. directly mounted on slides and one ex. mounted on slide after DNA extraction (BOLD Sample ID:4028C01\_MNECLL2278), 24 ex. in 96% alcohol, 24.iv.2010, J. Bedek leg.(CLL 2278); 3 ex. on slides, 8 ex. in 96% alcohol, 24.iv.2010, A. Kirin leg.(CLL 2279); 2 ex. on slides, 4 ex. in 96% alcohol, 24.iv.2010, A. Komerički leg.(CLL 2280); 3 ex. on slides, 34 ex. in 96% alcohol, 31.iii.2012, J. Bedek leg.(CLL 4000); 2 ex. on slides, 77 ex. in 96% alcohol, 31.iii.2012, M. Lukić leg. (CLL 4001); 17 ex. in 96% alcohol, 31.iii.2012, A. Komerički leg. (CLL 4185).

**Material deposition.** All material in 96% alcohol and following ex. on slides, including neotype, deposited in collection of the Croatian Biospeleological Society, Zagreb, Croatia: 2 ex. (CLL 2278), 3 ex. (CLL 2279), 1 ex. (CLL 2280), 1 ex. (CLL 4000), 1 ex. (CLL 4001), 6 ex. (CLL 4555). Following ex. on slides deposited in the collection of the Muséum National d'Histoire Naturelle de Paris: 1 ex. (CLL 2278), 1 ex. (CLL 2280), 2 ex. (CLL 4000), 1 ex. (CLL 4001).



FIGURE 1. *Heteromurus* (*Verhoeffiella*) *absoloni*: habitus (scale 500  $\mu$ m).

**Diagnosis.** Color pale orange, pigment as orange dots. Scales present on dorsal and ventral side of head and body, on Ant. Ib and on Ant. II, on legs, ventrally and dorsally on manubrium, ventrally on dens. Antennae 0.5 to 0.7 as long as (head + body) length, Ant. III and IV annulated, often fused. Ant. segment ratio as Ia:Ib:II:III:IV = 0.15:0.61:1:1.5:2.0. Tita of leg III longer than those of legs I and II; length 0.40, 0.49, 0.58 mm for Tita I, II, III. Labial triangle with 9–12 smooth chaetae, and no scales. Dorsal macrochaetotaxy as (3+4),(3+3)/0,2,3,(3+0+4+4).



S-chaetotaxy: ms formula 1,?1/1,0,1,0,0; sens formula, 2,2/1,3,3,3,3, with S-like chaetae at least 10+10 on Abd. IV. Unguis thin and elongated, with one pair of inner basal teeth at 25% from its base and one unpaired inner tooth at 50% from its base; unguiculus pointed, 75% as long as inner edge of unguis; tenent hair acuminate, smooth. Manubrium with about 15 smooth pointed mac dorsally, unpaired ventro-basal smooth mac and 2+2 ventro-apical serrated mes; dens with one short internal basal spine, mucro bidentate with one basal spine.

**Redescription.** Head and body length combined without antennae: 2.7 to 3.2 mm (N=8). Habitus as in Fig. 1. Abd. IV 1.75 – 2 times as long as Abd. III. Color pale orange, pigment scattered as orange dots ventrally and dorsally on body, manubrium, coxa, paler on trochanter and femur. Intersegment area, mouth parts, antennae, other leg segments, and dens unpigmented. Body dorsally and laterally with white regular patches on an orange background (Fig. 1). Eyes absent, no pigment patch at their place. Body, mouthparts and appendages with a large diversity of phaneres. Most can be assigned to the traditional four types of chaetae: ordinary chaetae, S-chaetae, trichobothria and scales; several other kinds of specialized chaetae occur on mouth and antennae. Pseudopores of same size as mac socket, observed on antennae, tergites, legs and manubrium.

**Antennae** (Figs 2–14). Antennae 0.54 to 0.68 as long as (head + body) length (N=8). Antennae 3 times longer than head diagonal. Ant. segment ratio as Ia:Ib:II:III:IV = 0.15:0.61:1:1.5:2.0 (mean values, N=9). Ant. III and IV annulated, except the proximal part of Ant. III and the distal part of Ant. IV. Antennae often asymmetrical, because of the fusion of Ant. III and IV. Pseudopores present ventro-apically on Ant. Ib (2–5), Ant. II (8–14) and Ant. III (5), located in the intersegment just above sensorial areas.

Antennal phaneres very diversified (ordinary chaetae, S-chaetae, scales and papillae). The following categories have been recognized (Fig. 2):

- Type a—serrated mac, dorsally on Ant. Ib and II, shorter than those of head and body.
  - Type b—thick serrated mes, on all antennal segments (Fig. 9).
  - Type c—thin serrated mes, less ciliated than type b, on Ant. III and IV (Fig. 9).
  - Type d—pointed smooth mac, ventrally on Ant. Ib and II.
  - Type e—pointed smooth mes, ventrally on Ant. Ia, Ib and II.
  - Type f—acuminate smooth mes, thin, basally thinner than e-type mes, straight and inserted perpendicular to integument, on all antennal segments but only ventrally on Ant. Ia, Ib (Fig. 9).
  - Type g—pointed smooth mic similar in shape to e-type mes but shorter, basally on Ant. Ia and II (Fig. 10).
  - Type h—swollen S-chaetae (Fig. 11), dorso-externo-apically on Ant. Ib (2), II (4–6) and III (2) (Figs 4, 5, 6).
  - Type i—thick subcylindrical S-chaetae, on all antennal segments (Fig. 12): 3–4 ventrally on Ant. Ia (Fig. 3), several grouped in ventro-externo-distal sensorial fields on Ant. Ib, II and III (Fig. 4), and 2–7 distally on Ant. IV (Fig. 7).
  - Type j—short, pointed, bent S-chaetae (Fig. 13), 2–8 distally on Ant. II and 3–4 on Ant. III, located near sensorial area; not seen on Ant. Ib.
  - Type k—thin, short, hyaline S-chaetae (Fig. 14), on the whole length of Ant. III and IV and a few ventrally on Ant. Ib and II, some larger and intermediate with type i, especially on Ant. IV.
  - Type l—thin, long S-chaetae (Fig. 10), similar in shape and size to f-type mes, but blunt at the tip, thinner proximally and not straight, present ventrally on Ant. Ia and Ib.
- Scales rounded, of medium size, their surface covered with numerous short spicules arranged longitudinally; numerous dorsally on Ant. Ib and II, few ventrally on Ant. Ib; absent on Ant. Ia, III and IV.

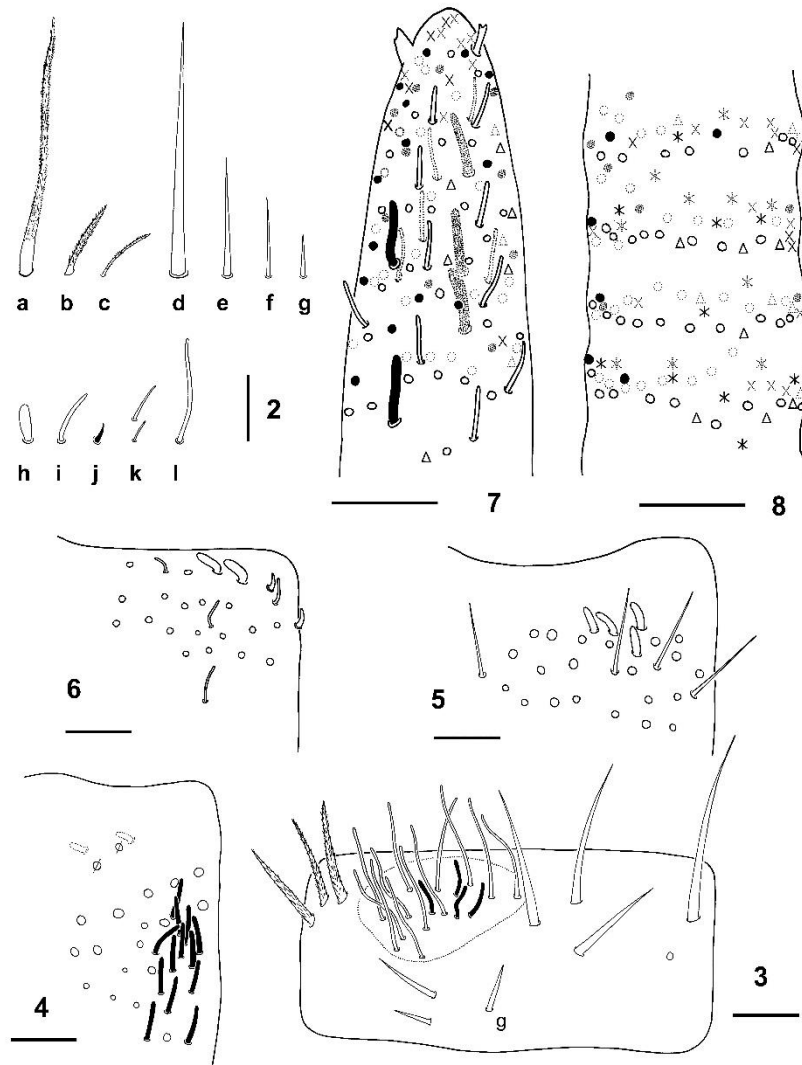
Subapical organite of Ant. IV long, thick and slightly bifurcate (Fig. 7).

Subapical papilla of Ant. IV, devoid of "pin seta" (Fig. 7).

The different categories of phaneres are distributed as follows on each antennal segment:

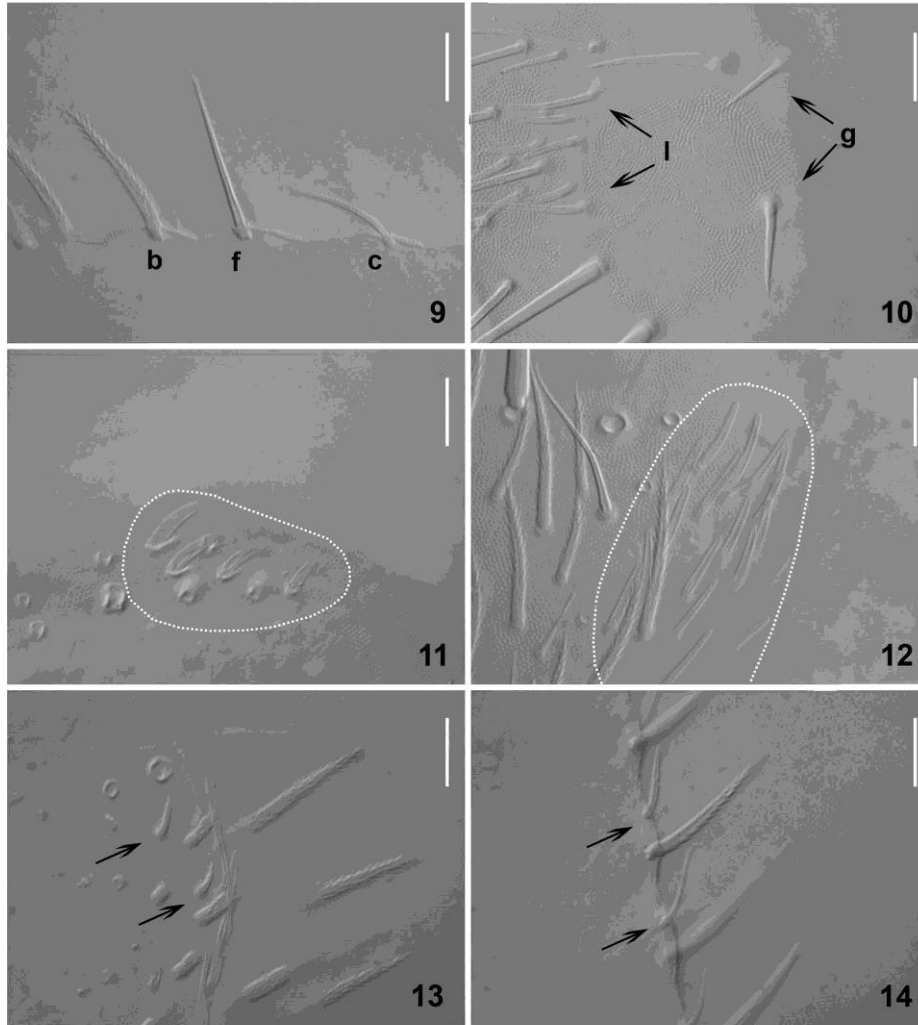
Ant. Ia dorsally with 2 basal mic (type g) and thick serrated mes (type b); ventrally with 3 basal mic (type g), pointed smooth mes (type e), acuminate smooth mes (type f) and sensorial area constituted of 10–12 S-chaetae of type l and 3–4 S-chaetae of type i (Fig. 3). No scales.

Ant. Ib dorsally with dense cover of scales, without chaetae except apical row of serrated mac (type a), thick serrated mes (type b), and two externo-apical small swollen S-chaetae (type h); ventrally with very few scales, large number of pointed smooth mes (type e), thick serrated mes (type b), few acuminate smooth mes (type f), distal row of pointed smooth mac (type d) or large pointed smooth mes (type e), ventro-external sensorial field constituted of several S-chaetae of type i, few short S-chaetae of type k externo-apically to sensorial field, and several S-chaetae of types i, k and l proximally to field. No basal mic.



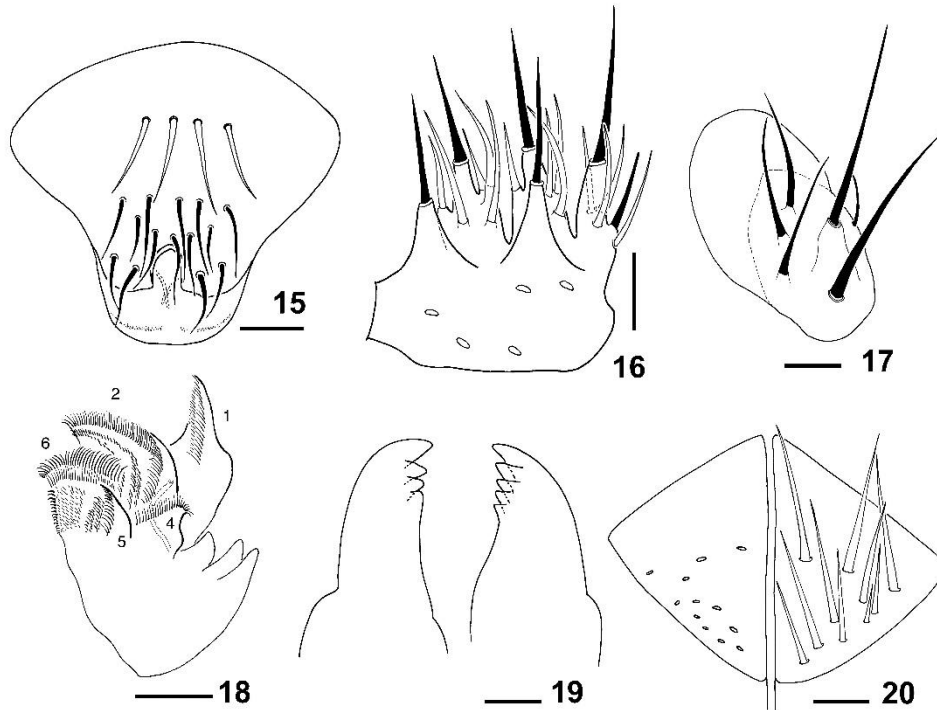
**FIGURES 2–8.** *Heteromurus* (*Verhoeffiella*) *absoloni*, antennal chaetae: 2, types of antennal chaetae: a—serrated mac, b—thick serrated mes, c—thin serrated mes, d—pointed smooth mac, e—pointed smooth mes, f—acuminate smooth mes, g—pointed smooth mic, h—swollen S-chaeta, i—thick subcylindrical S-chaeta, j—short pointed bent S-chaeta, k—thin short S-chaeta, l—thin, long S-chaetae; 3, Ant. Ia, ventral side with g-type of chaetae, and sensorial field (circled by dotted line) composed of i- and l-types of chaetae (i in black); 4, Ant. Ib, ventro-externo-distal sensorial field (i-type chaetae in black), pseudopores (cross-circles) and the 2 h-type chaetae of the dorsal side in dotted line; 5, Ant. II, dorso-externo-apical organite (AIIO) with acuminate thin mes (f) and swollen S-chaetae (h); 6, Ant. III, dorso-externo-apical organite (AIII O), with sockets of f-type and b-type chaetae; 7, Ant. IV, distal part: hollow circles—thick serrated mes (b), triangles—thin serrated mes (c), black circles—acuminate smooth mes (f), x—fallen chaetae, hollow lines—thin short S-chaetae (k), filled lines—thick subcylindrical S-chaetae (i); 8, whorls of Ant. IV, middle part; symbols same as in Fig. 7, in addition asterisk—thin short S-chaetae (k). All scales 20  $\mu$ m.



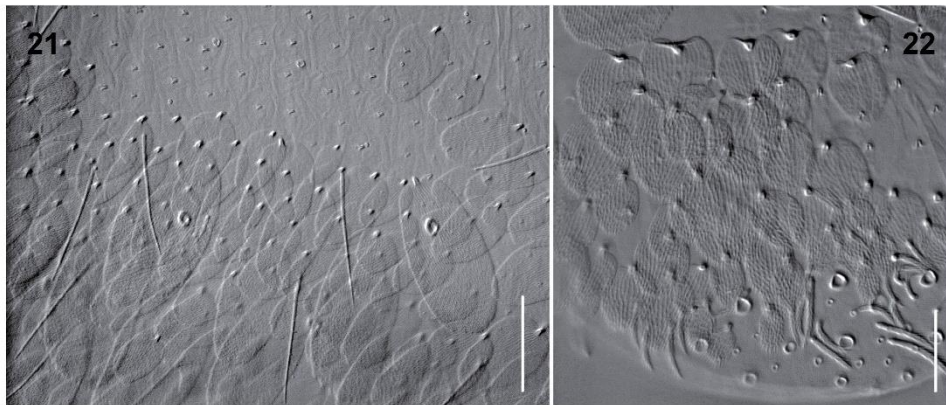


**FIGURES 9–14.** *Heteromurus* (*Verhoeffiella*) *absoloni*, optical microscope photos of antennal chaetae (chaetae types as in Fig. 2): 9, Ant. IV, with chaetae of types b, c and f; 10, Ant. Ia ventro-proximally, with chaetae of types g and I; 11, Ant. II, with its dorso-distal group of h-type chaetae (AIIO); 12, Ant. II, with its ventro-externo-distal sensorial field of i-type chaetae; 13, Ant. II ventro-externo-distally, with chaetae of type j; 14, Ant. IV laterally, with chaetae of type k. All scales 10  $\mu$ m.

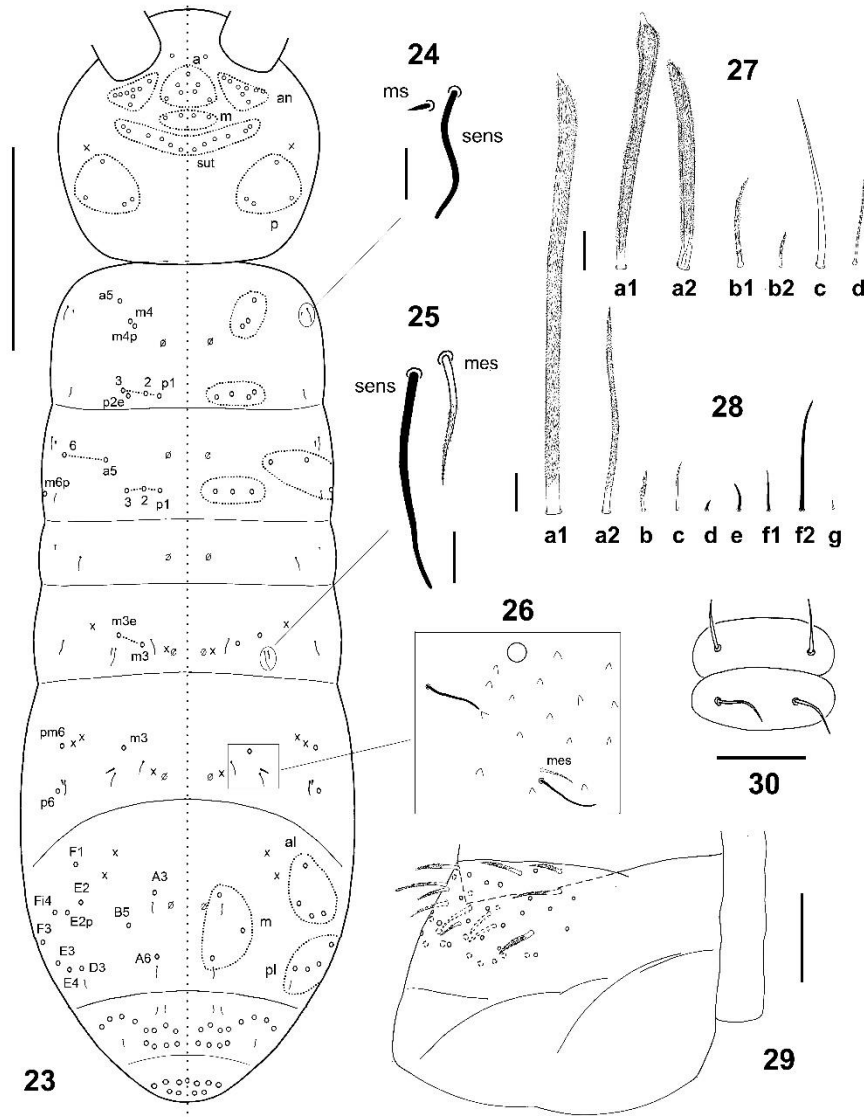
Ant. II dorsally with dense cover of scales mixed with sparse serrated mac and mes (types a and b), distally a few acuminate smooth mes (type f), apical row of small mac (type d) or large straight mes (type e) and externo-apical group of 4–7 swollen S-chaetae of type h (AIIO, Fig. 5); ventrally without scales, with many short thick serrated mes (type b), pointed smooth mes (type e, less numerous), acuminate smooth mes (type f), distal row of small mac (type d) or large smooth mes (type e) and externo-apical sensorial field constituted of 25–30 S-chaetae of type i, similar to that of Ant. Ib, and 2–8 chaetae of type j; 2–3 short S-chaetae of type k present externo-distally between sensorial field and AIIO; longer S-chaetae of type k present laterally to and mostly proximally to sensorial field. Basal mic present (1 dorsal, 2 ventral, 1 latero-external, 1 latero-internal).



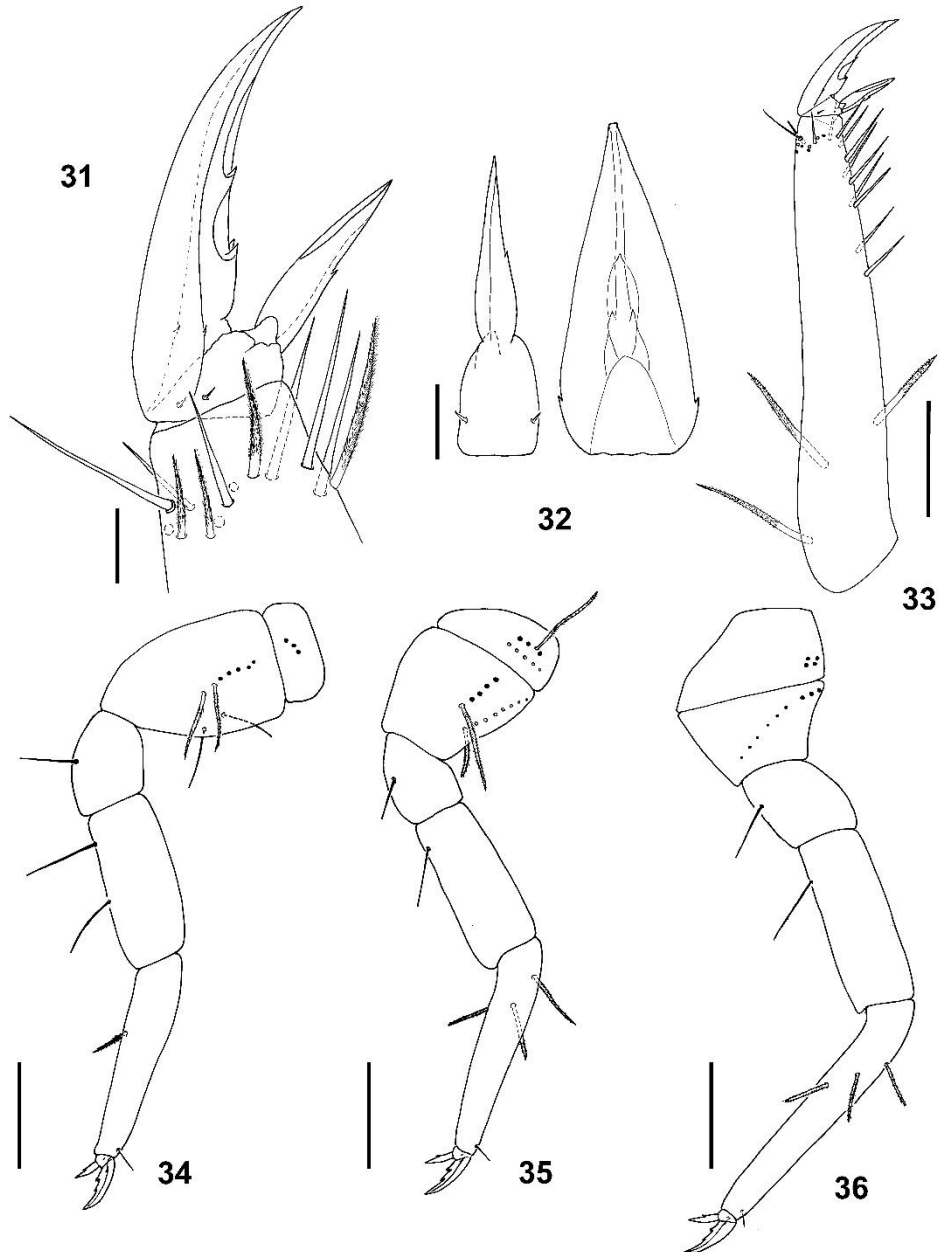
FIGURES 15–20. *Heteromurus* (*Verhoeffiella*) *absoloni*, mouthparts: 15, labrum; 16, labial palp; 17, maxillary outer lobe; 18, maxilla; 19, mandible; 20, labial triangle. Scales: 40  $\mu\text{m}$  (15), 20  $\mu\text{m}$  (16–20).



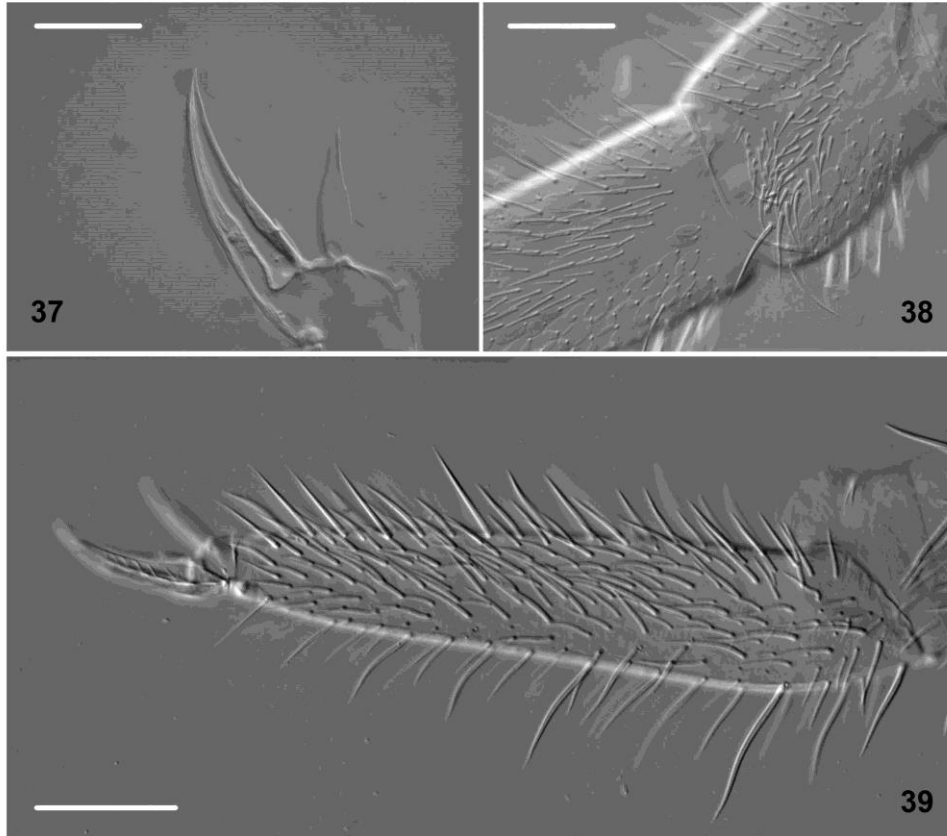
FIGURES 21–22. *Heteromurus* (*Verhoeffiella*) *absoloni*, optical microscope photos of Abd. IV and VI: 21, scales and long S-like chaetae on Abd. IV; 22, scales and serrated mes on Abd. VI. Scales 50  $\mu\text{m}$ .



**FIGURES 23–30.** *Heteromurus* (*Verhoeffiella*) *absoloni*: 23, body chaetotaxy; chaeta notation after Szeptycki (1979); chaeta groups circled with dotted lines; circles—mac, x—trichobothria, crossed circles—pseudopores, lines—S-chaetae, a—anterior mac, an—antennal mac, m—medial mac, sut—sutural mac, p—posterior mac, al—antero-lateral mac, pl—postero-lateral mac; S-like chaetae not given on Abd. IV; 24, sensillar complex of Th. II; 25, sensillar complex of Abd. II; 26, part of sensillar complex of Abd. III; 27, head chaetae: a1, clubbed mac, a2, subcylindrical mac, b1, normal serrated mes, b2, small serrated mes, c, smooth mes, d, trichobothria; 28, tergite chaetae: a1, big serrated mac, a2, lateral pointed mac, b, serrated mes, c, thin serrated mes, d, short pointed S-chaetae, e, thin subcylindrical S-chaetae, f1, short S-like chaetae on Th. III, f2, long S-like chaetae on Abd. IV, g, mic; 29, latero-distal flap of ventral tube, anterior side; sockets—smooth mes; 30, female genital plate. Scales: 500  $\mu$ m (23); 40  $\mu$ m (29); 20  $\mu$ m (27, 28, 30); 10  $\mu$ m (24, 25).



**FIGURES 31–36.** *Heteromurus* (*Verhoeffiella*) *absoloni*: 31, unguis and unguiculus of leg III, posterior view (scale 20  $\mu$ m); 32, unguis and unguiculus of leg II (scale 20  $\mu$ m); 33, Tita II, posterior view chaetae, hollow circles—anterior chaetae; 34, leg I, posterior view; 35, Leg II, posterior view, hollow circles—anterior chaetae; 36, leg III, anterior view. Scales: 200  $\mu$ m (34–36); 100  $\mu$ m (33); 20  $\mu$ m (31, 32).



**FIGURES 37–39.** *Heteromurus* (*Verhoeffiella*) *absoloni*, optical microscope photos of legs: 37, internal structure of unguis; 38 trochanteral organ; 39, Tita I, posterior view; the oblique ridges visible on the the claw are an artefact. Scales: 50  $\mu$ m (37, 38); 100  $\mu$ m (39).

Ant. III–IV chaetotaxy with tendency to form whorls of chaetae, sometimes spiraling, without clear serial pattern across successive whorls (Fig. 8) that are composed of 4 types of chaetae variously interspersed (b, c, f and k, Fig. 9); dorsally with less c-type chaetae and all chaetae smaller in size compared to the ventral ones. Ant. IV with several thick subcylindrical S-chaetae (type i) in distal part (Fig. 7).

AHIO located dorso-externo-apically, with 2 swollen S-chaetae side by side (type h), surrounded internally by one thin short S-chaeta (type k) and externally by three short external S-chaetae, of which 2 pointed and bent (type j), and 1 thin (type k); few additional S-chaetae of type k present below (Fig. 6). Ant. III ventro-externo-distally with sensorial field constituted of about 20–30 i-type chaetae, and 1–3 small pointed j-type chaetae. Basal mic absent or undifferentiated on Ant. III and absent on Ant. IV. Scales absent on Ant. III–IV. Ant. IV with large slightly bifurcate subapical organite and a subapical papilla (Fig. 7).

When Ant. III and IV are fused, the described pattern of Ant. III distal chaetae is strongly modified.

**Mouthparts** (Figs 15–20). Labral formula 4/5,5,4. No labral papillae. Prelabral and labral chaetae smooth, acuminate, of similar size. Labrum margin with inverted medial U-form intrusion devoid of primary granules, distal smooth area large (Fig. 15). Ventro-distally on labrum two unequal combs with one apical row of about 10 and 15 teeth respectively, and a pair of sinuous rods (Fig. 15). Labial triangle with 9–12 chaetae, all smooth (N=9) and no scales (Fig. 20); lateral area (submentum) with 5 smooth chaetae. Labial palp with 5 proximal chaetae and 5

papillae (A,B,C,D,E); guard chaeta of papilla A integrated in papilla B complex which thus appears to have 5 guard chaetae (Fjellberg 1999: 325); papilla C without guard chaeta; papilla D with 4 guard chaetae; papilla E with 4 guard chaetae and well developed labial process (Fig. 16). Maxillary outer lobe with one basal chaeta, simple maxillary palp, three sublobal hairs and one smaller chaeta (Fig. 17); basal fold with one chaeta. Maxilla with strong tridentate claw, 5 lamellae and a thin rod, long and bent (Fig. 18, after Fjellberg 2007: 160). Mandible asymmetrical with 5 teeth on right and 4 on left on all examined specimens (Fig. 19). Clypeus with many pointed smooth and serrated mesochaetae. About 10+10 smooth post-labial chaetae (PLQ of Mari-Mutt 1985 *pro parte*) interspersed with scales.

**Head and body** (Figs 21–30). Chaetae are rather diverse on head (Fig. 27) and body (Fig. 28), with the following categories usually relatively easy to recognize:

- serrated mac, either clubbed (*sensu* Stach 1960: plate I) or subcylindrical-pointed (Figs 27a1–a2 and 28a1–a2);
- serrated mes on head and body (Figs 27b1–b2 and 28b);
- thin mes slightly serrated on its distal half and associated with thin S-chaetae (Figs 25, 26 and 28c);
- smooth mes, only on head (Fig. 27c);
- trichobothria on head (Fig. 27d) and tergites, often fallen in our specimens; those of head (1+1) located laterally and rather short (50 µm), those of tergites longer;
- S-chaetae, limited to tergites, smooth, very short, thick, pointed (ms, Figs 24, 28 d);
- thin S-chaetae, limited to tergites, smooth, short and hyaline, subcylindrical, blunt (sens, Figs 24–26, 28e);
- smooth, thin, blunt, short to long S-like chaetae, longer, less transparent and slightly thicker than thin S-chaetae, only on Abd. IV (Fig. 28 f1 and f2);
- mic, 2 minute on each anal valve, and 2+2 on female genital plate (Fig. 28 g);

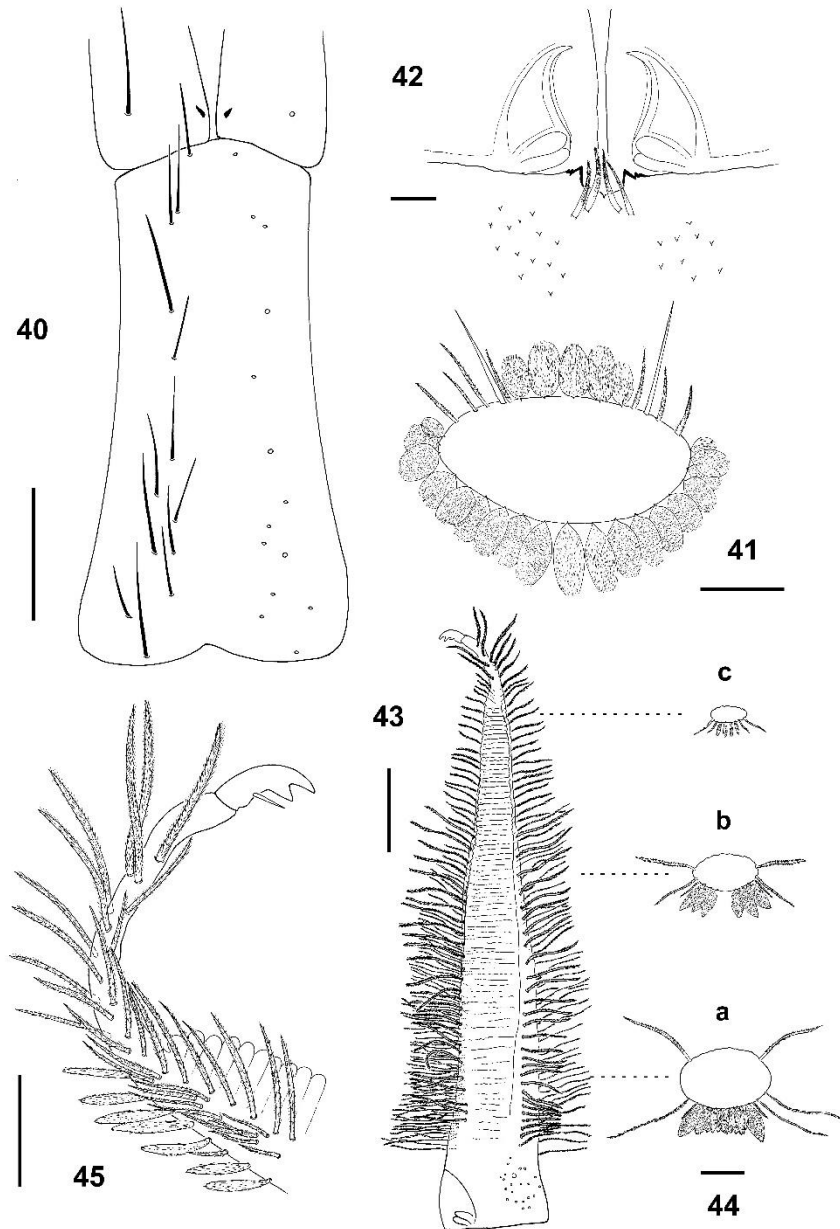
Scales on dorsal and ventral side of head and body, rounded to oval-elongated, sometimes truncated, hyaline, overlapping, of various size (from 15 to 100 µm long), with a dense cover of short spicules arranged in more or less regular lines (Figs 21, 22).

Chaetae, scales and trichobothria of dorsal side of head and body usually detached in microscopic preparations or on skins retrieved after DNA extraction. Chaetae have circular or oval sockets, those of mac and large mes easily recognizable by their size, but those of tergite S-chaetae not distinguishable from those of small mes. Scale sockets are characterized by cupuliform morphology of their socket. Trichobothria have small round socket often with minute point in the middle, but often indistinguishable from mes sockets.

Head dorsally with macrochaetae arranged as in Fig. 23, and dense cover of small to large scales and short serrated mes; laterally with scales mixed with medium size serrated mes; between antennae with few smooth mes; posteriorly with row of serrated mes regularly interspersed with large scales; ventrally with smooth and serrated mes and small to medium-size scales; 1+1 trichobothria posterior to ocular area.

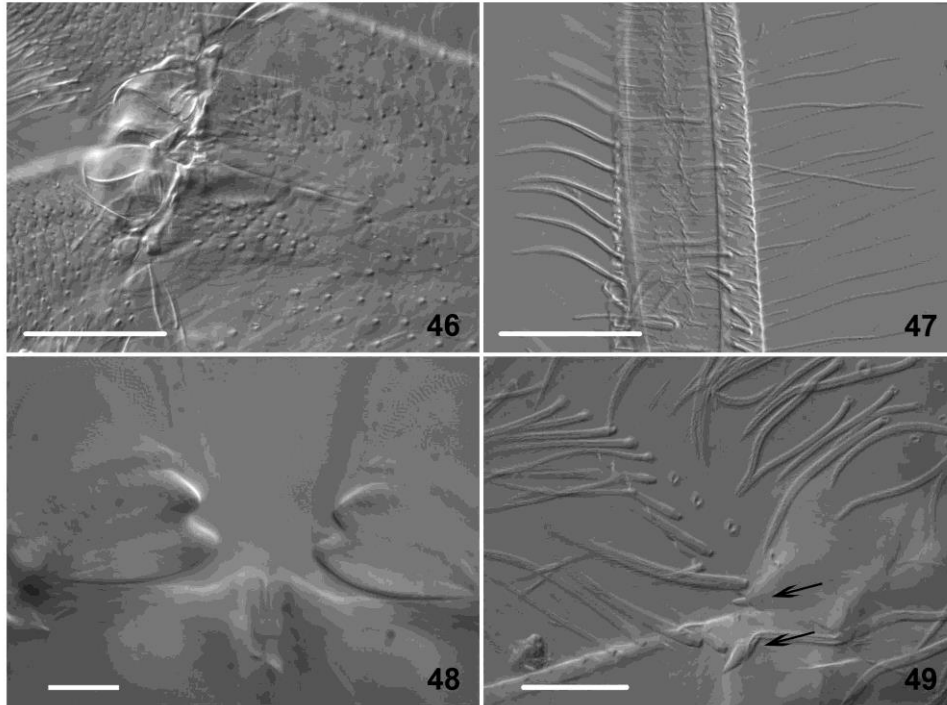
Dorsal chaetotaxy illustrated in Fig. 23. Pseudopore formula 1,1/1,1,1,1. Trichobothrial formula 1/0,0/0,2,3,2. Macrochaeta formula (3+4),(3+3)/0,2,3,(3+0+4+4). Macrochaeta arrangement stable; multiplets *sensu* Szeptycki 1979 only present anteriorly on Th. II; Th. II with 3 anterior and line of 4 posterior mac; Th. III with 3 posterior and 3 lateral mac; Abd. IV with 3 medial, 4 antero-lateral and 4 postero-lateral mac; Abd. V with 12 to 16 mac; Abd. VI with 11 mac in two rows, including an uneven one in the anterior row (Fig. 23). Serrated mes present on anterior part on Th. II, dorsally on Abd. V and VI (Fig. 22) and very few laterally on all tergites. Many rather long S-like chaetae on Abd. IV, 50% longer than mes of other tergites, arranged symmetrically (Fig. 21). Sens difficult to observe, often detached or hidden by scales, 3–4 times longer than mac socket; ms very short, about the length of mac socket. S-chaetotaxy given in Tab. 2 and Figs 23–26. Sens associated with thin serrated mes in tandem on Abd. II–III (also possibly in other locations). S-chaetotaxy: ms formula 1,?1/1,0,1,0,0 (ms on Th. III observed in two specimens, possibly an aberration as it would be unique in Entomobryoidea); sens formula, 2,2/1,3,3,3,3, with S-like chaetae at least 10+10 on Abd. IV.

Scales rounded, of various sizes: medium-size on all tergites except Abd. VI where they are smaller (Fig. 22); larger posteriorly (at least twice as large as the anterior ones) on all tergites except Abd. VI. No special arrangement or morphological modification of scales and chaetae around mac and trichobothria.



**FIGURES 40–45.** *Heteromurus* (*Verhoeffiella*) *absoloni*: 40, distribution of smooth chaetae on manubrium and proximal part of dens, dorsal view; 41, schematic cross section of manubrium; 42, manubrium and basal part of the dens, ventro-distal sclerification, chaetae and scale insertions; 43, dens, dorsal side; 44, schematic cross sections of dens: a, proximal part, b, medial part, c, distal part; 45, distal part of the dens and mucro. Scales: 200  $\mu\text{m}$  (40), 100  $\mu\text{m}$  (41, 43); 50  $\mu\text{m}$  (44); 40  $\mu\text{m}$  (45); 20  $\mu\text{m}$  (42).





**FIGURES 46–49.** *Heteromurus* (*Verhoeffiella*) *absoloni*, optical microscope photos of the dens: 46, dens, ventro-distal area; 47, dens; 48, dens thickening; 49, dens internal spines. Scales: 100  $\mu$ m (46–47); 50  $\mu$ m (49); 10  $\mu$ m (48).

**TABLE 2.** Tergite S chaetotaxy of *H. (V.) absoloni* from the type locality. Mesochaetae are mentioned when they are in tandem with sens, with (sens+mesochaeta) given between brackets.

Area on tergite	S chaetae and mesochaetae
ThII antero-lateral	(1 ms+1 sens)
ThII postero-lateral	1 sens
ThIII antero-lateral	(1 ms+1 sens)
ThIII postero-lateral	1 sens
Abd. I lateral	1 ms, 1 sens
Abd. II median	1 sens
Abd. II medio-lateral	(1 sens+1 mesochaeta)
Abd. II lateral	1 sens
Abd. III median	1 sens
Abd. III medio-lateral	(1 sens+1 mesochaeta)
Abd. III lateral	(1 ms+1 sens)
Abd. IV median	2 sens, >10 long S-like chaetae
Abd. IV postero-lateral	1 sens
Abd. V median	2 sens
Abd. V lateral	1 sens



Ventral tube with smooth pointed and serrated mes and mac. Posteriorly with about 125 sockets (serrated and smooth mes in unknown proportion, impossible to recognize from their socket once they have fallen) and 6–7 larger sockets (possibly mac); no scale observed except a single scale in place in one specimen. Anteriorly with 46–49 + 46–49 sockets (serrated or smooth mes). Each latero-distal flap with 45 mes, about 12 of them serrated and others smooth (Fig. 29). Genital plate of female with 2+2 mic (Fig. 30), that of adult male not observed.

**Legs** (Figs 31–39). Unguis relatively thin and elongated, with one inner pair of equal basal teeth at 25% from its base and one inner unpaired tooth at 50% from its base (Figs 31, 32). Large hyaline lamella covers claw, and bears 1+1 small latero-basal teeth (Figs 31, 32). Internal structure of unguis illustrated in Fig. 37. Unguis (internal edge): unguiculus = 1.6:1. Unguiculus robust, pointed, about 75% as long as inner edge of unguis, with two external ridges, without basal lamella (Figs 31, 32). Small outer tooth, sometimes difficult to see at 50% from its base on anterior ridge of unguiculus I, II and posterior ridge of unguiculus III (Figs 31, 32).

Legs with ordinary chaetae and scales, often detached, including the following categories:

- serrated mac, cylindrical-pointed, on subcoxae 2, coxae and tibiotarsi (similar to Figs 27 a2 and 28 a2);
- serrated mes, thick, on all leg segments (Fig. 31)
- smooth mac, pointed, on femur, trochanter, coxa
- smooth mes, pointed, on Tita, femur, trochanter, coxa (Fig. 31)
- tenent hairs, smooth, acuminate, of similar length but thinner than smooth mes, located dorso-apically on Tita (Fig. 31)
- smooth, pointed mes, inserted perpendicular to integument, forming the trochanteral organ (Fig. 38)
- mic of pretarsus (Fig. 31)
- scales small, elongated, subequal, present on all leg segments.

Detailed distribution of the different types of chaetae on each leg segment as follows: pretarsus with 1+1 minute mic (Fig. 31). Tibiotarsus with a large number of serrated mes, few serrated mac, smooth mes, thin tenent hair and scales (Fig. 39). Distal whorl of Tita with 11–14 (usually 13) chaetae including 5, 5, 4 smooth mes and the tenent hair on leg I, II, III. Serrated mac as 1, 3, 3 on Tita I, II, III (Fig. 33–36). Beside the apical ones, smooth mes present ventrally in two longitudinal rows on Tita I, II, III, anterior one with 3, 3–6, 5–7 chaetae, posterior one with 4–5, 3–4, 6–7 chaetae (Fig. 33). Few scales present in the proximal third of Tita I, more numerous in the proximal half of Tita II, and many distributed on the whole length of Tita III. Tita of leg III longer than those of legs I and II; length 0.40, 0.49, 0.58 mm for Tita I, II, III.

Femur with many serrated mes (some almost as large as serrated mac), smooth mac, smooth mes, and scales. Ventrally about 1–2, 1–2, 1 smooth macrochaetae on femora I, II, III and few additional smooth mes on femora I (Figs 34–36). Biggest smooth mac as long as 0.35–0.45 length of femur. Group of ventro-proximal serrated mes without interspersed scales (Fig. 38).

Trochanter with many chaetae ranging in size from mes to mac including many serrated mes, 1–2 smooth mac, and smooth mes. Smooth chaetae about 9–10 on trochanter I, 3–6 on trochanter II, many on trochanter III in adult, of which 55 to 100 forming the trochanteral organ of leg III (Fig. 38). Scales in small number on trochanter I, II and in large number on trochanter III.

Coxa with pseudopores, thick serrated mes, smooth mes, smooth mac, multiplets of serrated chaetae and scales. Pseudopores as 1–2, 3–6, 6–10 on coxae I, II, III. Smooth mac as 2, 0, 0 on dorso-anterior side of coxa I, II, III; multiplet of 5–7 dorso-posterior serrated chaetae on coxa I, two multiplets (dorso-anterior one and dorso-posterior one) of 5–8 serrated chaetae on coxa II; chaetae in multiplets of coxae I and II decreasing in size from mac (distally) to mes (proximally) (Figs 34–35). Dorsally on coxa III, a transversal line of 3 serrated mac and 5–8 serrated mes (Fig. 36).

Separation between subcoxae 1 and 2 indistinct. Subcoxae with scales. Subcoxa I with 3 dorso-posterior serrated mac (Fig. 34). Subcoxa II with 4–6 dorso-anterior serrated chaetae ranging in size from mac to mes and 3–5 dorso-posterior serrated mac (Fig. 35). Subcoxa III, with group of 4–5 dorso-posterior serrated mac and some serrated mes (Fig. 36).

**Furca** (Figs 40–49). Furca with pseudopores, smooth mac, different kinds of serrated mes and scales of various morphologies. Pseudopores as 6–8 dorsally on each side of manubrium, among distal group of chaetae. Manubrium dorsally with two longitudinal stripes of serrated mes, each one interspersed with  $14 \pm 2$  smooth

pointed mac (Fig. 40); between these stripes, loose cover of scales except in basal 1/3 where few serrated mes are present; externally, each longitudinal stripe of mes is bordered by a very narrow stripe devoid of chaetae and scales. Cross-section of manubrium as shown in Fig. 41. Manubrium covered ventrally by medium size scales, without chaetae, except an unpaired ventro-basal serrated and thick mac and 2+2 apical serrated mes near the axis (Fig. 42); scales evenly distributed, except on two ventro-distal areas where scale cover is more dense with larger and more elongated scales (Fig. 46). Distal manubrial thickening with 2 to 5 small teeth on each side (Fig. 42).

Dens not articulated, but constituted of a short basal part hardly annulated, a long medial part annulated dorsally and a short, thinner, non annulated distal part about 4 times as long as mucro (Figs 43, 45); covered by various chaetae and scales arranged in a complex pattern as shown in schematic cross-sections (Fig. 44). All dental chaetae serrated except one smooth mac basally on dorsal part of each dens (Fig. 40). Two types of serrated chaetae can be recognized (Fig. 47); the thickest ones arranged dorso-laterally in two longitudinal rows on basal and medial part of dens; the thinner ones, ventro-laterally, very long on basal part and shorter on medial part, arranged in two longitudinal rows, with two irregular lines of serrated chaetae internally on basal part (Fig. 44). Small serrated mes present between rows and ventral scales. Ventrally between two rows of thin serrated chaeta, dense cover of medium size elongated scales, small and rounded on basal part of dens, increasing in size laterally to large, fusiform, pointed apically (Fig. 44); scales absent in distal non-annulated part (Fig. 45). Dens thickening with two faint transversal structures (Figs 42, 48). One short and thick internal spine basally on each dens (Figs 40, 49). Ratio manubrium: dens: mucro = 10: 14: 0.5. Mucro bidentate with one basal spine, sometimes not observed (probably detached) (Fig. 45). Tenaculum with 1 serrated chaeta and 4 teeth on each ramus.

**Discussion.** The extensive re-description of *H. (V.) absoloni* given here is the first detailed description of a species of the subgenus, all existing ones being very succinct and lacking most characters used in modern taxonomy of Entomobryidae. Several morphological features, described for the first time for the subgenus, revealed interesting characters, e.g. the presence of a chaeta ms on Th. III. It was observed in two specimens only, but S-chaetae are often very difficult to detect; if it is not an aberration, it would be unique among Entomobryodea. The detailed description of antennal chaetotaxy shows a huge diversity in chaetae morphological types. Their distribution on antennae follows recognizable but loose patterns, as chaetae-to-chaetae homologies are not possible, with a few exceptions, between adults of a same species or between whorls of Ant. III–IV. The descriptive framework set up in this paper will be used as a reference in the ongoing subgenus revision.

#### ***Heteromurus* (*Verhoeffiella*) *gamae* sp. nov.**

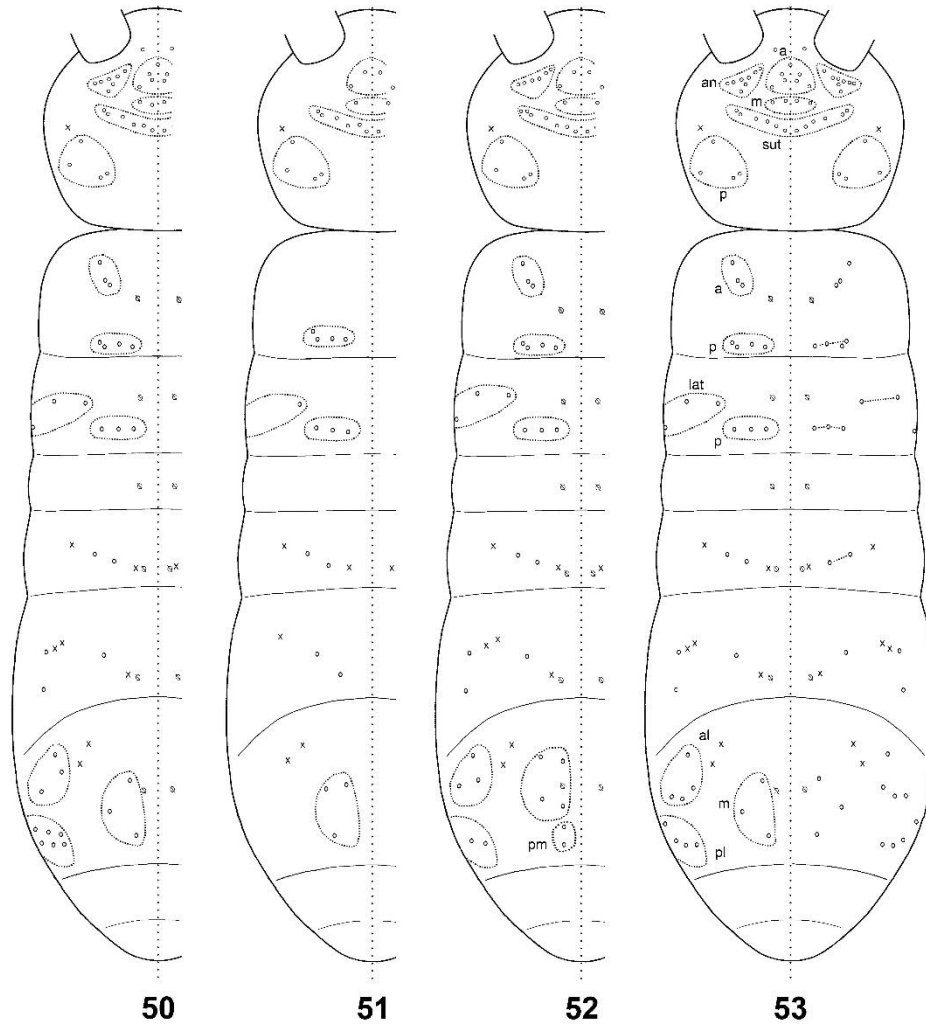
Figs 52, 55, 58, 60

**Type material.** Holotype: adult male on slide, Spain, Tarragona, Mola de Cati, Forat del Rastre (cave) (coordinates WGS84 X=0.283331°, Y=40.799838°, alt.1240 m.), 08.ii.2014, Carles Fontgüell Dalmau leg. (CLL 4559); paratypes: 1 subadult female on slide, 1 juvenile ex. in 96% alcohol, 08.ii.2014, Carles Fontgüell Dalmau leg. (CLL 4279); skins of 2 barcoded ex., 04.xii.2002., Arnaud Faille leg. (ETA-050); skin of 1 barcoded ex., 13.v.2003, Arnaud Faille leg. (ETA-052).

**Type material deposition.** Holotype and three paratypes (ETA-050, ETA-052) deposited in MNHN, Paris. Two paratypes (CLL 4279) deposited in CBSS, Zagreb, Croatia.

**Etymology.** The new species is named in honor of Maria Manuela da Gama, for her essential contribution to the knowledge of cave Collembola of the Iberian Peninsula.

**Diagnosis.** Color whitish or very pale orange. Scales present on dorsal and ventral side of body and dorsal side of head, on Ant. Ib and Ant. II, on legs, ventrally on manubrium and ventrally on dens. Antennae 0.85 as long as (head + body) length, Ant. III and IV annulated. Ant. segment ratio as Ia:Ib:II:III:IV = 0.12:0.5:1:1.25:3.1. Tita of leg III longer than those of legs I and II; length 0.42, 0.48, 0.60 mm for Tita I, II, III. Tita with thin acuminate, smooth tenent hair, large number of serrated mes, few serrated mac and the ventro-distal smooth mes of Tita III. Labial triangle with 9–12 chaetae, at least one serrated and others smooth and no scales. Dorsal macrochaetotaxy as (3+4),(3+3)/0,2,3,(4+2+3+2). Unguis very slender and elongated with external edge basally sinuous, with one inner pair of equal basal teeth at 15 % from its base and one inner unpaired tooth at 40% from its base; unguiculus slender and pointed with small outer tooth at 30% from its base. Manubrium dorsally only with serrated mes, 2+2 ventro-apical serrated mes; dens with one short internal basal spine, mucro bidentate with one basal spine.



FIGURES 50–53. Semi-diagrammatic macrochaetotaxy: 50, *Heteromurus* (*Heteromurus*) *nitidus* from Golubina pečina; 51, *Heteromurus* (*Verhoeffiella*) “*absoloni*” from Forat del Rastre as given by Gama (1984); 52, *Heteromurus* (*Verhoeffiella*) *gamae* sp. nov., our observation, pm-posteromedial mac; 53, *Heteromurus* (*Verhoeffiella*) *absoloni*, type population.

**Description.** Head and body length combined without antennae: 2.2mm. Abd. IV about 2 times as long as Abd. III. Color white, eyes absent, no pigment patch at their place.

**Antennae.** Antennae 0.85 as long as (head + body) length. Antennae 4,5 times longer than head diagonal. Ant. segment ratio as Ia:Ib:II:III:IV = 0.12:0.5:1:1.25:3.1. Ant. III and IV annulated, except the proximal part of Ant. III and the distal part of Ant. IV. Scales rounded, of medium size, their surface covered with numerous short spicules arranged longitudinally; numerous dorsally on Ant. Ib and II, absent on Ant. Ia, III and IV.

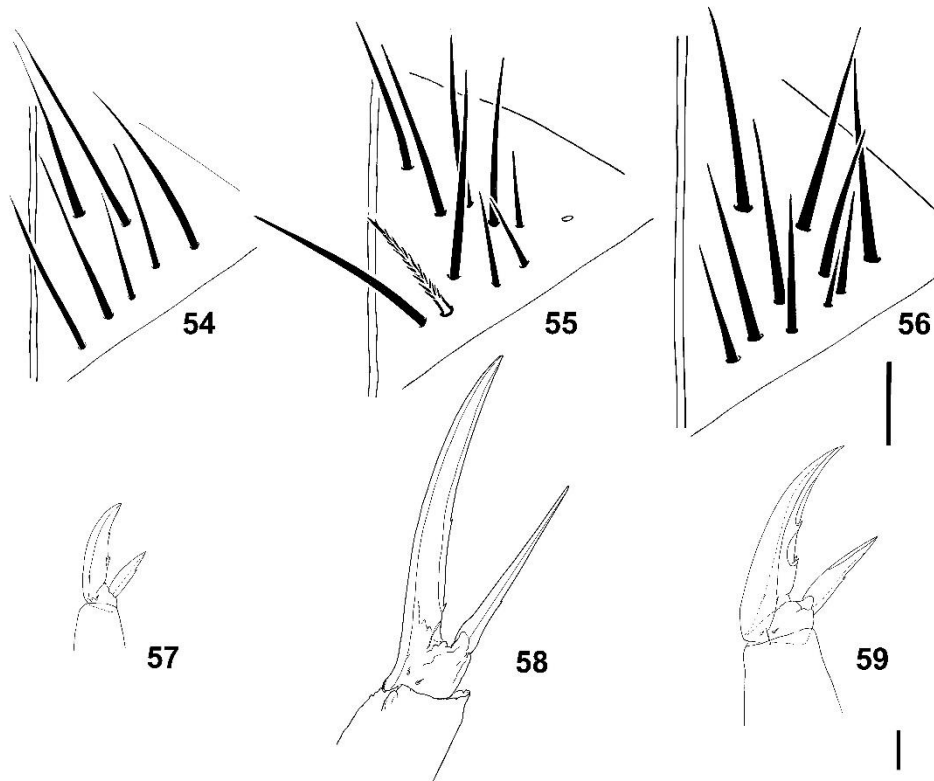
Ant. III–IV chaetotaxy with tendency to form whorls of chaetae, sometimes spiraling, without clear serial pattern across successive whorls. AIII located dorso-externo-apically, with 2 swollen S-chaetae side by side,

surrounded internally by 1 thin short S-chaeta and externally by 1 pointed and bent, and 1 thin S-chaetae. Ant. IV with several thick subcylindrical S-chaetae in distal part, long, thick bifurcate subapical organite and subapical papilla devoid of "pin seta".

**Mouthparts.** Labral formula 4/5,5,4. No labral papillae. Prelabral and labral chaetae smooth, acuminate, of similar size. Labrum margin with inverted medial U-form intrusion devoid of primary granules, the distal smooth area large. Ventro-distally on labrum two unequal combs with one apical row of about 10 and 15 teeth respectively, and a pair of sinuous rods. Labial triangle with 9-12 chaetae, at least one serrated and others smooth, and no scales (Fig. 55); lateral area (submentum) with 5 smooth chaetae. Maxillary outer lobe with one basal chaeta, a simple maxillary palp, 3 sublobal hairs and one smaller chaeta. Mandible asymmetrical with 5 teeth on the right and 4 on the left.

**Head and body.** Scales on head and body rounded to oval-elongated, hyaline, overlapping, of various size with a regular and dense cover of short spicules arranged in more or less regular lines. Chaetae, scales and trichobothria of the dorsal side of head and body are usually detached in microscopic preparations or on skins retrieved after DNA extraction.

Head dorsally with macrochaetae arranged as in Fig. 52, and a dense cover of small scales and short serrated mes; laterally with scales mixed with medium size serrated mes; ventrally with smooth and serrated mes and no scales; 1+1 trichobothria posterior to ocular area.



**FIGURES 54–59.** *Heteromurus* (*Heteromurus*) *nitidus* from Golubina pečina (54, 57), *Heteromurus* (*Verhoeffiella*) *gamae* sp. nov. (55, 58), *Heteromurus* (*Verhoeffiella*) *absoloni* from type locality (56, 59): 54–56, labial triangle chaetotaxy; 57–59, claw of leg III. Scales: 20  $\mu$ m.

Dorsal chaetotaxy illustrated in Fig. 52. Pseudopore formula 1,1/1,1,1,1. Trichobothrial formula 1/0,0/0,2,3,2. Macrochaeta formula (3+4),(3+3)/0,2,3,(4+2+3-2). Th. II with 3 anterior and a line of 4 posterior mac; Th. III with 3 posterior and 3 lateral mac; Abd. IV with 4 medial, 1 or 2 posteromedial, 3 antero-lateral and 2 postero-lateral mac (Fig. 52). S-chaetae arrangement not observable.

Ventral tube with smooth and serrated, pointed mes and mac, scales not observed. Overall chaetae number posteriorly and anteriorly not possible to observe. Each latero-distal flap with about 30 chaetae, most smooth pointed mes and several serrated mes. Genital plate of adult male consisting of numerous smooth mes, overall number not observable. Genital plate of subadult female with 2+2 mic.

**Legs.** Unguis very slender and elongated with external edge basally sinuous, with one inner pair of equal basal teeth at 15 % from its base and one inner unpaired tooth at 40% from its base (Fig. 58). Small hyaline lamella covers claw, and bears 1+1 small latero-basal teeth (Fig. 58). Unguis (internal edge): unguiculus = 1.2:1. Unguiculus slender and pointed with small outer tooth at 30% from its base (Fig. 58).

Pretarsus with 1+1 minute mic.

Tibiotarsus with large number of serrated mes, thin tenent hair, large sockets probably of serrated mac (one observed in place on Tita II) and ventro-distal smooth mes of Tita III. Distal whorl of Tita with 10–12 serrated chaetae including thin, smooth acuminate tenent hair. Tita of leg III longer than those of legs I and II; length 0.42, 0.48, 0.60 mm for Tita I, II, III.

Femur with many serrated mes (some almost as large as serrated mac), smooth mac and scales. Ventrally at least 1, 1, 1 smooth macrochaetae on femora I, II, III. Biggest smooth mac as long as 0.32–0.4 the length of femur.

Trochanter with many chaetae ranging in size from mes to mac including many serrated mes, 1–2 smooth mac, and few smooth mes. Trochanteral organ of leg III with around 60 smooth mes. Scales not observed on trochanter I, present in small number on trochanter II and more numerous on trochanter III.

Chaetotaxy of coxa and subcoxa not observable.

**Furca.** Furca with pseudopores, different kinds of serrated mes and scales of various morphologies. Pseudopores present dorsally on each side of manubrium among distal group of chaetae, their overall number difficult to estimate. Manubrium dorsally with serrated mes and no scales; ventrally with medium size scales, without chaetae, except 2+2 apical serrated mes near axis. Ventro-basal part of manubrium not observable. Distal manubrial thickening with 5 to 7 small teeth on each side.

Dens not articulated, but constituted of short basal part hardly annulated, long medial part annulated dorsally and short, thinner, non annulated distal part about 4 times as long as mucro; covered by serrated chaetae and scales. Ventrally dense cover of medium size elongated scales, small and rounded on basal part of dens, increasing in size laterally to large, fusiform, pointed apically; scales absent in distal non-annulated part. One short and thick internal spine basally on each dens. Ratio manubrium: dens: mucro = 11,2: 20: 0.5. Mucro bidentate with one basal spine (Fig. 60). Tenaculum with 4 teeth on each ramus and one chaeta socket (chaeta fallen on all specimens).

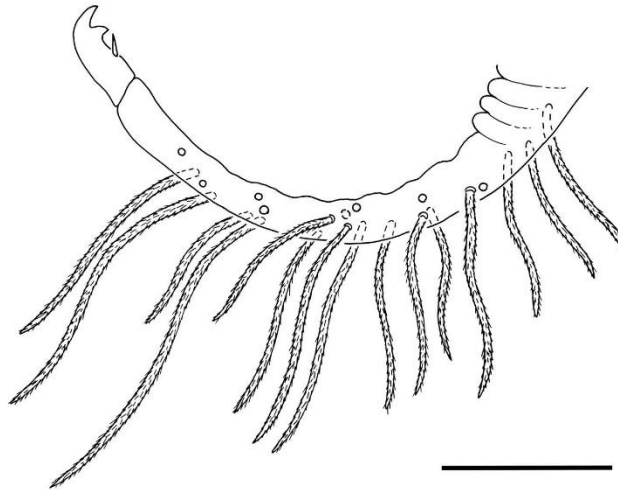


FIGURE 60. *Heteromurus* (*Verhoeffiella*) *gamae* sp. nov.: mucro. Scale 40µm.

**Discussion.** The characters on which the *Verhoeffiella* specimens of Forat del Rastre have been identified as *H. (V.) absoloni* by Gama (1984) are not specified by the author. However, given that Gama did not examine the type material of the species, she must have relied on antenna length and inner tooth position on claw, which are the only features mentioned, but not illustrated, in the original and sole description of the species by Kseneman (1938). Gama (1984) characterized the Forat del Rastre specimens of *H. (V.) absoloni*, by four features: 1) antennae long but shorter than body; 2) claw elongated, with a pair of inner proximal teeth and an uneven unpaired inner tooth at 50% of its inner edge; 3) unguiculus long with a small outer tooth; 4) mucro curved, with apical tooth larger than the ante-apical one, with a thin basal chaeta. In addition, the author figured the pattern of dorsal macrochaetotaxy and the claw. Based on our own observation and Gama characterization of specimens from Forat del Rastre together with molecular comparison of specimens from the type locality in Montenegro and from the Catalan population we established that the population from Forat del Rastre is a new species described here as *Heteromurus (Verhoeffiella) gamae* sp. nov.

The discriminant characters between the different species of the subgenus *Verhoeffiella*, summarized in Tab. 3, rely on a few characters usually considered as adaptive, and most published descriptions, except those of Gama (1984), lack information on modern taxonomic characters. Labrum, labial palp and mouthparts have not been described by authors. Dorsal macrochaetotaxy is available for 5 species. Most characters used in the redescription of *H. (Verhoeffiella) absoloni* and in the description of *H. (V.) gamae* sp. nov. given here are unknown in other species of the subgenus and therefore not included in the Tab. 3. Considering the very basic available descriptions and the reference descriptive framework given here, all species of the subgenus need to be redescribed.

**TABLE 3.** Differential characters between species of the subgenus *Verhoeffiella* after the literature, with our own observations for *H. (V.) absoloni*. Most new characters used in the redescription of *H. (V.) absoloni* are not given in the table as they are unknown in other species. Antennal segment ratio: values between brackets when given together for (Ant Ia + Ib) or for fused segments. Asterisk (\*) links the data in cells to source reference.

	Source	Body length (mm)	Antenna: body ratio	Antennal segment ratio	Antenna: head diagonal ratio	Abd III-IV ratio	Dorsal macrochaeta formula
<i>absoloni</i> (original description)	Kseneman 1938		shorter than body				
<i>absoloni</i> (redescription)	our observation	2.7-3.2	Ant body+head = 0.54-0.68:1	0.15:0.61:1:1.5:2.0	3	1 : 1.5	(3+4),(3+3)/ 0,2,3,(3+0+4+4)
<i>anagastumensis</i>	Lučić <i>et al.</i> 2008	4.35	Ant. body = 1.34:1	(0.7):1.2.64:3.67	5		
<i>cavicola</i>	Absolon 1900*, Nosek & Paoletti 1985**	3.5-4.32		0.2:0.7:1:1.55:2.65		1 : 2	
<i>constantius</i>	Lučić <i>et al.</i> 2007	3.98	equal to body	0.13:0.53:1.2:13:2.47	4.7		
<i>constantinellus</i>	Lučić <i>et al.</i> 2007	5.06	shorter than body	(0.21):1:(3.62)	2.4		
<i>dallai</i>	Nosek & Paoletti 1985	2.5-3.0	shorter than body	0.23:0.67:1:1.27:2.77	2.5	1 : 2.8-3.0	
<i>gamae</i> sp. nov.	our observation	2.2	Ant. body+head = 0.85:1	0.12:0.5:1:1.25:3.1	4.5	1 : 2	(3+4),(3+3)/ 0,2,3,(4+2+3+2)
<i>hispanica</i>	Bonet 1931***, Gama 1984****, Nosek & Paoletti 1985	4	shorter than body	(0.8):1.2.3.2.4	4.3	1 : 1.5	(7+4),( >1+3)/ 0,3,3,(3+0+7+7)
<i>longicornis</i>	Absolon 1900*, Kseneman 1938, Nosek & Paoletti 1985**	5.3	Ant III-IV longer than body	0.12:0.67:1:(7.76)		1 : 1.5	
cf. <i>longicornis</i> (Spanish records)	Gama 1984		Ant III-IV not longer than body				(7+3),( >1+2)/ 0,1,0,(1+0+7+7)
<i>medius</i>	Loksa & Bogojević 1967	1.6-2.1	shorter than body	0.13:0.5:1:1.75:2.63			(3+4),( >1+3)/ 0,2,>1,(3+0+>2+>2)

... ..continued on the next page

TABLE 3. (Continued)

Character	Pair of basal teeth on unguis	Inner unpaired tooth on unguis	Outer tooth on unguiculus	Unguis : unguiculus ratio	Manubrium : (macro+dens) ratio
<i>absoloni</i> (original description)	big basal teeth	big, at 50% from its base	present		
<i>absoloni</i> (redescription)	present	at 50% from its base	present, at 50% from its base	1.6 : 1	1 : 1.45
<i>anagastumenstis</i>	single tooth		absent		1 : 1.3
<i>cavicola</i>	one tooth in the first half*; small proximal teeth**			1.7 : 1	1 : 2
<i>constantius</i>	single tooth		absent	1.4 : 1	1 : 1.25
<i>constantinellus</i>	single tooth		absent		1 : 1.35
<i>dallati</i>	present	at 50% from its base	present	1.4 : 1	1 : 1.4
<i>gamae</i> sp. nov.	present	at 40% from its base	present	1.2 : 1	1 : 1.8
<i>hispanica</i>	present	absent	present strong***; small, not always visible****	1.3 : 1	1 : 1.2
<i>longicornis</i>	absent	absent		1.6 : 1	1 : 1.3*; 1 : 1.4-1.6**
cf. <i>longicornis</i> (Spanish records)	absent	absent			
<i>medus</i>	small basal teeth	absent	present, at 50% from its base		1 : 1.65

**Comparison of *H. (V.) absoloni*, *H. (V.) gamae* sp. nov. and *H. (H.) nitidus***

**Additional material examined.** *H. (V.) nitidus*, Bosnia and Herzegovina, Romanija, Rogatica, Golubovići, Golubina pećina (cave) (coordinates WGS84 X=18.91642°, Y=43.78418°, alt. 800 m.), 4 ex. on slide, 3 ex. in 96% alcohol, 14.xiii.2010, M. Lukić leg. (CLL 2325).

**Dorsal macrochaetotaxy** (Figs 50–53). Dorsal macrochaetotaxy provides the main diagnostic characters to separate species and genera across Entomobryoidea when there is no color pattern, as it is the case for troglomorphic species. Macrochaetotaxic pattern of *H. (V.) absoloni* and *H. (V.) gamae* sp. nov. is identical on most tergites but exhibits important differences on head and Abd. IV (Figs 52, 53). Differences on head concern the number of mac in anterior (3+3 with one unpaired vs 2+2 without unpaired), median (2+2 vs 1+1), antennal (8 vs 6) and sutural (6+6 vs 6–7+6–7) groups on head. The 1+1 chaetae between antennae are absent in *H. (V.) gamae* sp. nov. Differences on body concern the chaetotaxy of Abd. IV (Figs 52, 53): number of mac in medial (3 vs. 4), anterolateral (4 vs 3) and posterolateral (4 vs 2) groups in *H. (V.) absoloni* versus *H. (V.) gamae* sp. nov. Additionally, specimens of *H. (V.) gamae* sp. nov. have 1 or 2 mac in posteromedial group.

Described macrochaetotaxic pattern of *H. (V.) gamae* also differs from the standard macrochaetotaxic pattern of Heteromurinae recognized in *H. (H.) nitidus* (Fig. 50, see also Szeptycki 1972, Szeptycki 1979) and *H. (H.) major* (see Hüther 1970, Mari-Mutt 1980a) in several features such as: absence of unpaired mac in anterior group, difference in the number of mac in anterior and antennal group, absence of 1+1 chaetae between antennae and macrochaetotaxic pattern on Abd. IV. The most troglomorphic species of the subgenus *Verhoeffiella* regarding claw elongation, collected in a cave of Cantabria and identified as *V. cf. longicornis* by Gama (1984), departs even more from the standard pattern, while *H. (V.) hispanica* from province Oviedo has, according to Gama (1984), the standard pattern of Heteromurinae. The evolutionary stasis of the dorsal macrochaetotaxy pattern, suggested by its remarkable similarity between several species very different otherwise, is therefore not retrieved in 2 of the 3 iberic species of the genus.

**Labial triangle** (Figs 54–56). Number of chaetae of the labial triangle is variable in the genus *Heteromurus* according to Mari-Mutt (1980b), who did not consider it as a good taxonomic character. However, the author did not analyze this variability in populations, and used the presence/absence of ciliated chaetae (but not their number) as one of the character for separating *Heteromurus* (*H.*) in two groups (group *major* and group *nitidus*). Actually, from our observations, the variation in number of chaetae is relatively limited within populations. The number of chaetae in the labial triangle, expressed as ranges, may be therefore a valuable taxonomic character

within Heteromurinae. In both *H. (V.) absoloni* and *H. (V.) gamae* **sp. nov.** this number fluctuates within a narrow range of 9–12–9–12 chaetae, compared to 6–8+6–8 in *H. (H.) nitidus* examined from various localities. All chaetae of examined populations of *H. (H.) nitidus* and those of *H. (V.) absoloni* are smooth like in *H. (H.) nitidus* group of Mari Mutt (1980), while in *H. (V.) gamae* **sp. nov.** there is at least 1+1 serrated chaetae among the smooth ones (Fig. 55).

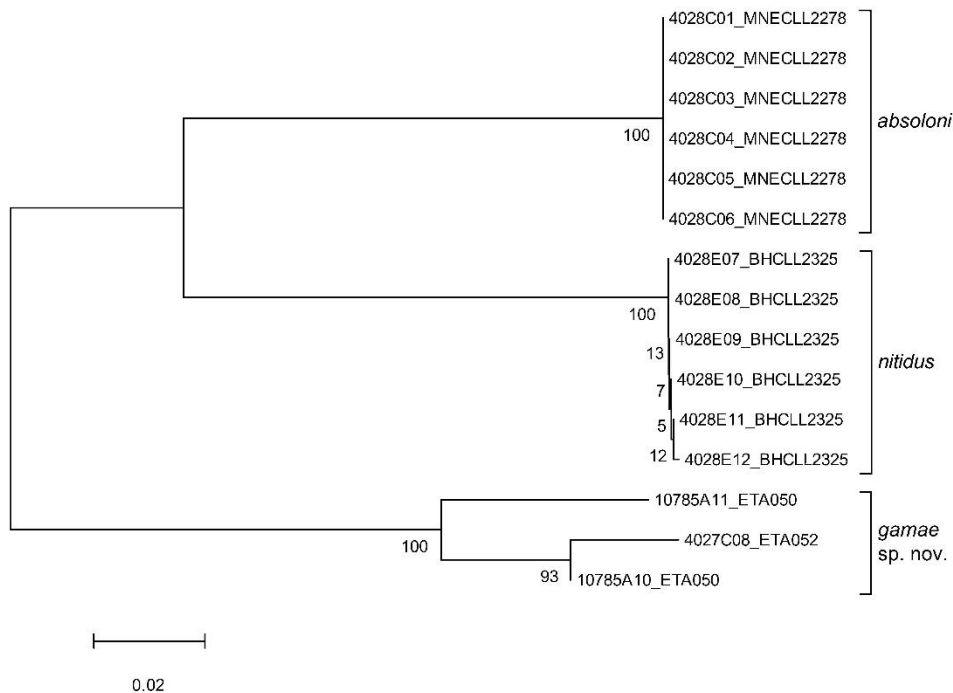
**Tibiotarsus.** *H. (V.) gamae* **sp. nov.** has no smooth mes on tibiotarsus beside the dorsal tenent hair and the ventro-distal smooth mes of Tita III, contrary to *H. (V.) absoloni* where there are several smooth mes arranged in two rows in the 1/3 ventro-distal part of Tita, and to *H. (H.) nitidus*, where these two rows run all along Tita length.

**Claw** (Figs 57–59). Claw is very different in the three forms of interest in its degree of elongation, curvature and position of inner tooth. Unguis of *H. (V.) gamae* **sp. nov.** is very slender and elongated, with external edge basally sinuous, bearing smaller pair of basal teeth and of inner unpaired tooth. Unguiculus is slender and pointed with small outer tooth at 30% from its base. Unguis and unguiculus of *H. (V.) absoloni* are less elongated than those of the Catalan population, being intermediate with those of *H. (H.) nitidus*. The ratio body+head:inner edge of claw clearly illustrates these differences: about 45 for *H. (V.) nitidus*, about 37 for *H. (V.) absoloni*, and about 20 for *H. (V.) gamae* **sp. nov.**

**Furca.** The manubrium of *H. (V.) gamae* **sp. nov.** is dorsally beset only with serrated mes, without interspersed smooth pointed mac as in the population of *H. (V.) absoloni*.

#### Genetic divergence

Fig. 61



**FIGURE 61.** Neighbor joining tree (K2P) of *Heteromurus* (*Verhoeffiella*) *absoloni*, *H. (V.) gamae* **sp. nov.**, and *Heteromurus* (*H.*) *nitidus* from Golubina pečina based on the COI 5' 'barcoding fragment'. Bootstrap support values showed on the branches.



The mean genetic divergence observed among *H. (H.) nitidus*, *H. (V.) absoloni* and *H. (V.) gamae sp. nov.* was 20.63% with the highest divergence found between *H. (V.) absoloni* and *H. (V.) gamae sp. nov.* (22.41%). The divergence measured between *H. (H.) nitidus* and the topotypical population of *H. (V.) absoloni* was 17.38% while its divergence from *H. (V.) gamae sp. nov.* was 22.13%. These divergences between *H. (V.) absoloni* and *H. (V.) gamae sp. nov.* and the morphologically well-defined species *H. (H.) nitidus* strongly support the independent specific status of the Catalan population. Moreover, in a previous study, the mean divergence observed in *Heteromurus* species ranged up to 23% (Porco *et al.* 2012) supporting further this hypothesis. Although it could be sampling bias related, no intraspecific variability was found in the Dinaric population of *H. (V.) absoloni* and minor variation in *H. (H.) nitidus* whereas *H. (V.) gamae sp. nov.* exhibited a 4.03% among 3 specimens only.

#### Taxonomical and biogeographical implications

Morphological and genetic evidence compiled in this paper unambiguously establish that *Heteromurus* (*Verhoeffiella*) of Forat del Rastre is different from *H. (V.) absoloni* from Montenegro and that it represents a new species, *H. (V.) gamae sp. nov.*, well characterized morphologically and genetically. It differs from Dinaric *H. (V.) absoloni* in several important characters: dorsal macrochaetotaxy on head and body, distribution of scales on head, labial triangle, tibiotarsus, femur and manubrium chaetotaxy, and claw morphology. To evaluate whether these characters, beyond their value in discriminating the species of interest, represent differences at supra-specific level between Iberic and Dinaric *Verhoeffiella*, further morphological study of other species from both areas are needed. As a result of this paper, the remarkable disjunct distribution of *H. (V.) absoloni* is no more a biogeographical problem and is proven here to originate from the poor original description of the Dinaric species. But the disjunct distribution of the subgenus *Verhoeffiella*, that comprises species in several largely remote karst areas, remains to be explained. On-going phylogenetic analyses based on molecular data are expected to solve this puzzling biogeographical riddle.

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## 2.2 SETTING A MORPHOLOGICAL FRAMEWORK FOR THE GENUS *VERHOEFFIELLA* (COLLEMBOLA, ENTOMOBRYIDAE) FOR DESCRIBING NEW TROGLOMORPHIC SPECIES FROM THE DINARIC KARST (WESTERN BALKANS)

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Zadnje raziskave jamskih skakačev (Collembola) so razkrile visoko raznolikost rodu *Verhoeffiella* Absolon, 1900 na Dinarskem krasu. Najbolj izjemna lastnost vrst rodu *Verhoeffiella* je visoko število troglomorfnih znakov, zaradi česar je rod zanimiv model za študije morfološke diverzifikacije in adaptacije na podzemna okolja. Raziskali smo različne morfološke modifikacije, za katere smo domnevali, da so povezane s podzemnim življenjem. Podrobno smo opisali štiri nove vrste in dodali redeskripciji dveh vrst, vključno s tipsko vrsto rodu. Identiteto vrst smo potrdili z eno-lokusnimi (citokrom c oksidaza I) delimitacijami vrst, ki temeljijo na Poissonovih procesih (PTP) in distančnih pristopih (metoda ABGD). Oba pristopa sta dala enake rezultate. Obravnavali smo morfološke spremembe, ki veljajo kot adaptivne za življenje v jamah, kot tudi nove potencialno troglomorfne znake skakačev. Pri teh znakih smo opazili veliko raznolikost med vrstami. Ugotovili smo, da morfološke spremembe različnih znakov med seboj niso povezane, tako da se pojavljajo različne kombinacije sprememb znotraj različnih vrst. Te razlike smo izpostavili na primeru dveh parov vrst rodu *Verhoeffiella*, ki žive v sintopiji - vrste imajo znake različno troglomorfno spremenjene. Nadalje podajamo več novih znakov, ki so uporabni za vrstno in morda rodovno ali nad-rodovno razlikovanje. Poleg tega smo prvič pri skupini Collembola opisali "distalni organ" na anteni IV.

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## Setting a morphological framework for the genus *Verhoeffiella* (Collembola, Entomobryidae) for describing new troglomorphic species from the Dinaric karst (Western Balkans)

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**Abstract.** Recent research of cave Collembola in Dinaric karst resulted in discovery of high regional diversification of the genus *Verhoeffiella* Absolon, 1900. The most striking feature of *Verhoeffiella* species is the high number of troglomorphic traits, which makes this genus a good model for studying morphological diversification and adaptation in subterranean environments. We explore the expression of various morphological modifications assumed to be linked to subterranean life, through detailed descriptions of four new species and redescription of two species including the type species of the genus. Species delimitations are confirmed by single locus (cytochrome *c* oxidase I) tree-based (Poisson tree processes) and distance-based (automatic barcode gap discovery) species delimitation approaches, which gave identical results. Morphological changes classically considered as adaptive for cave life and new, potentially troglomorphic characters for Collembola are discussed. For several of these characters, high morphological diversity between species and large decoupling in the development of different traits within species are recorded. Such a decoupling is also illustrated in the finding of two cases of *Verhoeffiella* species pairs at different levels of troglomorphy living in syntopy. We further provide several new differential characters of specific and possibly generic or supra-generic importance and describe for the first time among Collembola an original 'distal organ' on Ant. IV.

**Additional keywords:** *COI* species delimitation, Heteromurinae, new species, sensory fields, subterranean, syntopy, troglobiont, troglomorphy.

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### Introduction

Even though Collembola are a common part of subterranean fauna in caves and other subterranean habitats, they are often understudied compared with other groups. Even in the Dinaric karst of the Western Balkans, which is considered a world hotspot of subterranean biodiversity (Culver *et al.* 2006; Sket 2012; Zagmajster *et al.* 2014), extensive knowledge on distribution and diversity of cave Collembola is lacking. Yet, this region harbours one of the most promising models for studying diversification and testing evolutionary hypotheses in subterranean habitats, that is, the troglobiotic genus *Verhoeffiella* Absolon, 1900 of the family Entomobryidae. Its potential usefulness is based on the combination of the following features: (i) all species are found in caves and exhibit a large range of troglomorphic traits; (ii) species of the genus are very similar to the non-troglobiotic and non-troglomorphic species

*Heteromurus nitidus* (Templeton, 1835) in several important morphological characters such as dorsal body macrochaetotaxy (Bonet 1931; Mari Mutt 1980b; Lukić *et al.* 2015); (iii) the genus is highly diversified along the Dinaric karst; (iv) the genus in its current conception has an intriguing disjunct distribution, occurring in five karst regions of Europe – the Dinaric karst, Jakupica (Macedonia), south-eastern calcareous Alps, southern Catalonia and Cordillera Cantabrica (excluding a dubious record from north-eastern France) (Thibaud 1970; Lukić *et al.* 2015). The diversity of troglomorphic traits and characters observed in *Verhoeffiella* are striking features within Heteromurinae (Lukić *et al.* 2015). It includes, for example, *V. longicornis* (Absolon, 1900), which exhibits one of the most modified habitus among cave Collembola regarding elongation of antennae and body size. The large range of troglomorphic traits documented within *Verhoeffiella* and its high regional



diversification are unmatched by any other cave Collembola genus in the Dinaric karst.

The taxonomic status of genus *Verhoeffiella* has been treated differently by authors and the need for re-assessment of its position within Heteromurinae has been stressed several times (Mari Mutt 1980a; Lukić *et al.* 2015; Cipola *et al.* 2016). Originally, it was described as a subgenus of *Heteromurus* Wankel, 1860, a status widely accepted by many authors (Bonet 1931; Gisin 1960; Mari Mutt 1980a; Lučić *et al.* 2007a, 2008, Lukić *et al.* 2015). It was subsequently treated as a separate genus by other authors (Denis 1935; Kseneman 1938; Thibaud and Deharveng 1994; Soto-Adames *et al.* 2008; Bedos 2014; Cipola *et al.* 2016), and even considered as *Heteromurus sensu stricto* by da Gama (1984). Its 'floating' status was a direct consequence of the poor and sketchy original descriptions of almost all the species in the genus, which lack most of the characters used in modern taxonomy of Entomobryidae. Status change reflected merely a difference of appreciation of the value of Ant III annulation, the single discriminant character used so far between *Verhoeffiella* and *Heteromurus* s.str. (Mari Mutt 1980a, 1980b; Cipola *et al.* 2016). In the present study, *Verhoeffiella* is given generic rather than subgeneric status, following the recent publication of Cipola *et al.* (2016) and because of the discovery of several new differential characters presented herein that might be exclusive to *Verhoeffiella* within Heteromurinae.

So far, studies of this genus have been impeded by a lack of specimens in museum collections and its difficult taxonomy, exacerbated by poor original species descriptions (e.g. Absolon 1900; Kseneman 1938; Lučić *et al.* 2007a, 2007b, 2008). A recent increase in available material coming from intensive fieldwork and advances in morphological descriptive tools have enabled significant progress in the study of this group. A descriptive framework was recently proposed in the redescription of *V. absoloni* Kseneman, 1938 from the Dinaric karst (Lukić *et al.* 2015), allowing the recognition of its disjunct 'population' from Catalonia (Spain) as an independent species. This confirmed the need to apply and evaluate new morphological characters for redescription of the existing species, as well as description of the new *Verhoeffiella* species.

In this study, we focus on the populations from the Dinaric karst region, from which the largest number of *Verhoeffiella* species have been described (six species, including the type species of the genus, *V. cavicola* Absolon, 1900). We describe four new species, redescribe and designate neotypes for two species (*V. cavicola* and *V. longicornis*) and complement the description of *V. absoloni* (Lukić *et al.* 2015). The existence of separate species is independently confirmed by molecular species delimitation for all ten species described from Dinaric karst. With these descriptions of new species, the distributional range of genus *Verhoeffiella* in the Dinaric karst is significantly enlarged.

The descriptive framework set up in Lukić *et al.* (2015) is used in the present study, completed with the introduction of new morphological characters and the evaluation of all taxonomic characters. A wide range of known collembolan troglomorphic traits and potentially new ones are documented. Finally, we show that a thorough exploration of species morphology offers an efficient way to discover characters

of specific, generic or even supra-generic importance, which have been lacking at various taxonomic levels within the Entomobryidae family (Deharveng 2004).

## Materials and methods

### Studied populations and sampling

The study was conducted at six different caves in the Dinaric karst of the Western Balkans. These caves range from 40 m to 2500 m in total length, with entrances at elevations between 220 m and 1320 m above sea level (ASL). All specimens used for morphological study, molecular analyses and designation of neotypes were collected at type localities by hand and preserved in 96% alcohol. Sampling was not designed to systematically explore microhabitat preferences of species; however, some field observations (by first author) were undertaken to complement species descriptions.

To retrieve the type material for species redescrptions, we searched for Absolon's collection and type specimens of *V. cavicola* and *V. longicornis*. The collections were not retrieved despite searches in the Moravian museum in Brno (P. Baňář, pers. comm.) and the National Museum in Prague (J. Hájek, pers. comm.), the institutions where the collection might be deposited. Therefore, we consider Absolon's collection of Collembola, including type specimens of both aforementioned species, to be lost.

### Species morphological descriptions

Specimens were preserved in 96% alcohol, cleared in lactic acid and 10% KOH solution and then mounted on slides in Marc-André II. The slides were studied with a Leica MZ16 stereoscope, Zeiss Primo Star and Leica DMLB2 microscope with differential interference contrast. Photographs were taken with a Jenoptik ProgRes C10+ camera. Scanning electron micrographs were taken with a Hitachi SU 3500 scanning electron microscope. For image stacking Helicon Focus software was used. Photographs *in situ* were taken with Canon 400D, macro lens Canon MP-65 and Canon ring flash.

Redescription of the type species of the genus, *V. cavicola*, was undertaken following the descriptive framework set up in Lukić *et al.* (2015). In description of other species, only characters that differ and are variable among *Verhoeffiella* species are given to avoid repetition and shorten descriptions. To ease comparison, figures are compiled by character rather than species, and are presented on a single scale. All measurements were taken from adult and subadult type specimens, while given ratios represent mean values of several measured specimens. Measurement of claw in posterior view was carried out as follows: unguis length (length of inner edge) – from tip to internal basis; unguiculus length (length of inner edge) – from tip to internal basis; width of unguis – from internal basis to external basis; width of unguiculus – from internal basis to external basis; position of teeth on unguis – from internal basis to tooth; position of outer tooth on unguiculus – from external basis to tooth; width of empodium – from external basis of unguis to external basis of unguiculus. Subdivisions of Ant. I are named Ia and Ib to preserve homologies with antennae of other Entomobryidae. Structure of the maxilla follows Fjellberg

(2007), of the labial palp Fjellberg (1999) and of unguiculus Hühner (1986).

Abbreviations used in descriptions include: Abd., abdominal segment; AIO, apical organite of Ant. II; AIII, apical organite of Ant. III; Ant., antennal segment; Th., thoracic segment; Tita, tibiotarsus; mac, macrochaetae; mes, mesochaetae; mic, microchaeta; ms, smooth, very short, thick, pointed S-chaetae; psp, pseudopores; sens, smooth, short and hyaline, subcylindrical, thin, blunt S-chaetae; N, number of measured specimens; CBSS, Croatian Biospeleological Society; MNHN, Muséum National d'Histoire Naturelle de Paris; CLL, specimen/sample code; CA, voucher for specimens used in molecular analysis.

Formulae are given per half tergite from Th. II to Abd. IV for dorsal macrochaetae and pseudopores; from Th. II to Abd. V for S-chaetae; from head to Abd. IV for trichobothria. Number of mac in formula correspond to: Th. II anterior + medial + posterior (anterior fringe mac excluded), Th. III lateral + posterior, Abd. IV medial + postero-medial + antero-lateral + postero-lateral ('>' or '?' are used in formula for anterior or lateral mac usually not given in old descriptions).

Distinction between ordinary chaetae and S-chaetae follows the rationale of Deharveng (1979). S-like chaetae ('setulae' in Szeptycki 1979) are chaetae that cannot be assigned unambiguously to S-chaetae. On the tergite, ms designates short pointed S-chaetae, and sens larger ones, following Potapov (2001) for Isotomidae and Zhang and Deharveng (2015) for Entomobryidae. Chaetal notation for tergites follows Szeptycki (1972, 1979), for notation by groups of chaetae on head Mari Mutt (1979). We followed Lukić *et al.* (2015) for notation by groups of chaetae for tergites, that we used rather than individual chaetal notation, to avoid uncertainties of chaeta-to-chaeta homologies because of the reduced macrochaetotaxy of studied species. On figures illustrating dorsal body chaetotaxy, mesochaetae are not illustrated. Chaetae of uncertain homology are followed by a question mark (?). The subcoxae chaetotaxy follows Yosii (1959). For the types of chaetae and their general distribution on the antennae, legs, head and body we follow Lukić *et al.* (2015). We did not intend to match the notation systems for antennal chaetae published for Cyphoderidae and Paronellidae (Jantarit *et al.* 2013, 2014), as such a task, though important, would require extensive comparative investigations beyond the scope of this study.

#### DNA extraction and sequencing

Barcodes were obtained in BOLD (Canada), as well as in the laboratories of SubBioLab, Biotechnical Faculty, University of Ljubljana (Slovenia) and Ruder Bošković Institute (Croatia). Two different protocols were used for DNA extraction.

In the first protocol, DNA extraction from whole individuals followed the Canadian Center for DNA Barcoding (CCDB) DNA extraction protocol (Ivanova *et al.* 2006). Specimens were recovered after DNA extraction using a specially designed work flow allowing morphological examination (Porco *et al.* 2010). Standard DNA barcode region mitochondrial (mtDNA) cytochrome *c* oxidase I (*COI*) was amplified using primers listed in Table A1 of Appendix 1. The standard PCR reaction protocol of the CCDB was used for amplifications ([\[www.dnabarcodes2011.org/conference/preconference/CCDB-Amplification-animals.pdf\]\(http://www.dnabarcodes2011.org/conference/preconference/CCDB-Amplification-animals.pdf\)\), and products were checked on a 2% E-gel 96Agarose \(Invitrogen, Carlsbad, California, USA\). Unpurified PCR amplicons were sequenced in both directions with products subsequently purified using Agencourt CleanSEQ protocol and processed using BigDye \(ver. 3.1\) on an ABI 3730 DNA Analyzer \(Applied Biosystems, Foster City, California, USA\).](http://</a></p></div><div data-bbox=)

In the second protocol, DNA was extracted from whole individuals using the GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, USA) or with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The cuticle was deposited in the CBSS collection. We amplified the mitochondrial *COI* gene, run under standard amplification settings. A list of primers used is available in Table A1. Exonuclease I and Fast AP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) were used to purify PCR products.

The resulting PCR products were sequenced using amplification primers by MacroGen Europe (Amsterdam, Netherlands). Sequences were assembled and chromatograms were visually inspected in Geneious (ver. 8.0.4. and ver. 9.1.7, Biomatters, Auckland, New Zealand). The edited sequences were aligned in MAFFT (ver. 7, Katoh and Standley 2013) under the E-INS-i algorithm.

#### Phylogenetic analyses, species delimitation and nucleotide divergence

We compiled a dataset containing 82 sequences of all *Verhoeffiella* species (10) described from Dinaric karst, and closely and distantly related Entomobryidae (Table A2). Phylogenetic relationships were reconstructed using Bayesian inference in MrBayes (ver. 3.2.6, Ronquist *et al.* 2012). The best-fitted evolutionary model for each gene partition as well as the optimal partitioning scheme were chosen by PartitionFinder (ver. 2.1.1, Lanfear *et al.* 2017). As *COI* is a protein coding gene, codon specific models were selected (Table A3 of Appendix 1). Phylogenetic relationships were inferred by running two parallel Markov chain Monte Carlo searches with four cold chains each for 5 million generations in MrBayes. Every 500th generation was sampled, and the first 25% of the sampled trees were discarded as burn-in. The remaining trees were assembled into a majority-rule consensus tree with the node confidence assessed by posterior probabilities.

We used two alternative species delimitation methods, one tree-based and one distance-based. In the first step we performed single locus delimitation using a Poisson tree processes model (PTP) (Zhang *et al.* 2013). The analysis was run on a reduced dataset, comprising 33 unique haplotype sequences (accessible in Table A2), accessed by the R package SIDIER (Muñoz-Pajares 2013). PTP is a phylogeny-based species delimitation method based on the assumption that intra and inter-specific nucleotide substitution levels differ notably and can be modelled as two independent Poisson processes. The resulting consensus tree was used to run the Bayesian implementation of Poisson tree processes (bPTP) analysis on the species delimitation server <http://species.h-its.org/>, accessed 5 March 2018). Bayesian posterior probabilities for tentative species were acquired after



running 500 000 generations, sampling every 500 generation, and discarding the first 25% of the samples as burn-in.

In the second step we employed a distance-based delimitation method, empirically determining the hypothetical species boundaries by the pairwise differences between nucleotide sequences. Given the premise that the divergence among organisms belonging to the same species is smaller than divergence among organisms from different species, the automatic barcode gap discovery (ABGD) (Puillandre *et al.* 2012) method infers the barcode gap from the given dataset and partitions the sequences into putative species based on this distance. The ABGD analysis was conducted on the complete *COI* sequence dataset, and under the default settings recommended by the authors of the software. Minimum and maximum allowed intraspecific distances set to 0.001 and 0.1 respectively. The eventual effect of different evolutionary models on the results of species delimitation were tested by running the ABGD analyses under the two evolutionary models, K2P and JC69, and a simple distance analysis available on the ABGD web portal (available at <http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>, accessed 5 May 2018).

Additionally, insights into nucleotide divergence on intraspecific and interspecific levels were accessed by uncorrected p-distances using MEGA software (ver. 7, Kumar *et al.* 2016).

#### Systematics

##### Genus *Verhoeffiella* Absolon

(Figs 1–21)

Type species: *Verhoeffiella cavicola* Absolon, 1900, p. 429.

#### Diagnosis

Medium to large size (1.6–5.3 mm). Eyes absent. Body white, yellowish or pale orange, usually with pigment as orange dots. Scales hyaline, rounded to oval-elongated, sometimes truncated, with a dense cover of short spicules arranged in more or less regular longitudinal lines, present dorsally on: Ant. Ib and II, dorsal and sometimes ventral side of head, dorsal and ventral side of body, ventral side of furca, trochanter III, femora I, II, III, Tita III. Pseudopores present on Ant. Ib, II, III, Th. II–Abd. IV, laterally on coxa and dorso-distal part of the manubrium (Fig. 5–7, 11, 15). Ant. I divided in two subsegments (Ia, Ib), Ant. II entire, Ant. III and IV annulated with whorls of chaetae, sometimes spiraling. Antennae of various length, slightly shorter to two times longer than body. Sensory fields present on Ant. Ia, Ib, II and III (Fig. 3, 5, 6, 11). Externo-apical group of swollen S-chaetae present on Ant. II (AIIIO) and Ant. III (AIIIO) (Fig. 6, 10). One or two large subapical S-chaetae on Ant. IV (Fig. 12). Subapical organite of Ant. IV thick and slightly bifurcate (Fig. 12). Ant. IV without apical bulb. Prelabral and labral formula 4/5,5,4, chaetae smooth (Fig. 15). Labral papillae absent. Labrum margin with inverted medial U-form intrusion devoid of primary granules. Maxillary outer lobe with one basal chaeta, simple maxillary palp and four sublobal hairs (Fig. 15). Mandible asymmetrical with five teeth on right and four on left (dorsal view). Pseudopore formula 1,1/

1,1,1,1. Trichobothrial formula 1/0,0/0,2,3,2. Multiplets *sensu* Szeptycki (1979) present anteriorly on Th. II. Sens associated in tandem with thin serrated mes present on Abd. II and III (except in *V. cavicola*) (Fig. 17, 18). Sens formula 2,2/1,3,3,3–4,2–3. Abd IV 1.5–2× as long as Abd. III. Trochanteral organ with 20 to 100 spine-like mes. Smooth mac present on femora I, II and III. Smooth mes present ventrally on Tita I, II and III (Fig. 19). Distal whorl of Tita with smooth and serrated chaetae. Tenent hair thin, acuminate (Fig. 20). One small mic on anterior and posterior pretarsus. Unguis relatively thin and elongated with 1 + 1 small latero-basal teeth, with one inner pair of equal basal teeth, sometimes with one inner small unpaired tooth (Fig. 20). Ventral tube with serrated and smooth chaetae, scales present or absent (Fig. 15). Manubrium with unpaired ventro-basal mac and usually more than 4 + 4 dorso-distal pseudopores (Fig. 15). Distal manubrial thickening ventrally with 2–5 small teeth on each side (Fig. 15). Dens dorso-internally devoid of dental lobe and spines, except one short and thick internal spine basally (Fig. 15). Mucro usually bidentate, sometimes tridentate, with a basal spine (Fig. 21).

#### Remarks

*Verhoeffiella* is given generic rather than subgeneric status here as in Lukić *et al.* (2015), following the recent publication of Cipola *et al.* (2016). However, *Verhoeffiella* resembles *Heteromurus* in numerous characters used at generic level within Heteromurinae (see table 1 in Cipola *et al.* 2016). So far, its generic or subgeneric status reflected merely a difference of appreciation of the value of Ant III annulation, no other character having been searched for nor found to separate it from *Heteromurus*. In this study we introduce characters that are, at the moment, exclusive for *Verhoeffiella* within Heteromurinae: well-developed sensory fields on Ant. Ia, Ib, II and III, distal manubrial thickening with small teeth and usually a large number of dorso-distal pseudopores on the manubrium. However, these characters are rarely documented in other Heteromurinae genera and should be further studied (for more details see discussion on characters of generic value).

Many of the diagnostic characters are unknown in several species of *Verhoeffiella*, for which original descriptions are very brief. For example, dorsal head and body macrochaetotaxy are not given in diagnoses, not only because they are not known for several species but also because some observations in original descriptions are doubtful (see Lučić *et al.* 2007a).

##### *Verhoeffiella cavicola* Absolon

(Fig. 1–17, 19–21)

#### Material examined

*Neotype.* Type material is considered lost (for details see 'Materials and methods'). We propose here as neotype a specimen from the type locality. Adult ♀ on slide: Bosnia and Herzegovina: Sarajevo: Laništa: Bjelašnica Mt., Megara (cave) (WGS84 43°42'52.7"N; 18°05'04.1"E; 1320 m ASL), M. Lukić and A. Kirin leg., 05.xi.2015 (CLL4953).

*Other material examined.* The same data as neotype, 1 adult ♀ on slide, 12 ex. in 96% alcohol (CLL4791); 1 adult ♀ and 3 subadult ♀ on slides, 2 ex. mounted for SEM, 49 ex. in 96% alcohol, J. Jakupić leg. (CLL4787); 2 ex. mounted for SEM, 2 ex. for DNA (CA007, CA008), 19 ex. in 96% alcohol,



M. Lukić and U. Tulić leg., 20.xi.2011 (CLL3235); 1 ex. for DNA (CA256), M. Lukić, A. Kirin and J. Jakupić leg., 05.xi.2015 (CLL4762).

*Material deposition.* 1 adult ♀ on slide (CLL4787), 4 ex. mounted for SEM (CLL4787, CLL3235), 4 ex. in 96% alcohol (CLL4791) deposited in MNHN. All other material including neotype deposited in CBSS.

#### Diagnosis

Head and body length without antennae: 3.2–3.6 mm. Colour pale orange. Scales present on dorsal and ventral side of body, on dorsal side of head and Ant. II, on legs, and ventrally on the manubrium and dens. Antennae 0.9–1.0 × as long as (head and body) length; ratio Ant. II : (III+IV) = 1 : 4.5. Pseudopores as 4–6, 6–10, 4–6 distally on Ant. I, II, III. Ventral sensory field of Ant. Ia with 10–17 S-chaetae of type l and 4–7 S-chaetae of type k; S-chaetae (type h) absent on Ant. Ib, 5–7 on Ant. II; subapical papilla of Ant. IV present, distal organ of Ant. IV absent. Labral papillae absent. Labial triangle with 9–11 smooth chaetae. Proximal serrated post-labial chaetae along linea ventralis as (3–4)+(3–4). Dorsal macrochaetotaxy as (3+4), (3+3)0,2,3,(2+0+3+3). Trochanteral organ with 50–60 chaetae. Smooth mac as 3, 2, 1 on femora I, II and III. Serrated mac as 0, 1, 1 on Tita I, II and III; smooth mes as 33–43, 40–53, 55–71 on Tita I, II and III, not in rows. Tenent hair acuminate, smooth; ratio tenent hair : width of empodium for leg I 1.5 : 1. Unguis thin and elongated, with one pair of inner basal teeth; ratio unguis I length : unguis I width at basis 3.5 : 1; unguiculus pointed, ratio unguiculus I length : unguiculus I width at basis 7.2 : 1. Manubrium with 10–14 dorso-distal psp, dorsally without scales and 9–12 smooth pointed mac, 1+1 ventro-apical serrated mes; 2–5 basal smooth mac dorsally on dens; mucro bidentate with one basal spine.

#### Redescription

Head and body length combined without antennae: 3.2 to 3.6 mm ( $n=4$ ), 3.5 mm in neotype. Habitus as in Fig. 1D, 2D. Abd. IV 1.6–1.9 × as long as Abd. III ( $n=3$ ), 1.6 × in neotype. Antennae slightly shorter than or equal to (head and body) length. Colour pale orange, pigment scattered as orange dots ventrally and dorsally on body, Ant. I, manubrium, coxa paler on trochanter and femur. Intersegment area, mouth parts, other Ant. and leg segments, and dens unpigmented. Body dorsally and laterally with white regular patches on an orange background (Fig. 1D, 2D). Eyes absent, no pigment patch in their place. Body, mouthparts and appendages with a large diversity of phaneres. Most can be assigned to the traditional four types of chaetae: ordinary chaetae, S-chaetae, trichobothria and scales; the other kinds of specialised chaetae that occur on mouth and antennae of Entomobryidae are present. Pseudopores of same size as mac socket, observed on antennae, tergites, legs and manubrium.

*Antennae* (Fig. 3–13). Antennae 0.9 to 1.0 × as long as (head and body) length ( $n=4$ ), 0.9 × in neotype. Antennae 4.7 × longer than head diagonal in neotype. Ant. segment ratio as: Ia : Ib : II : III : IV = 0.1 : 0.61 : 1 : 1.78 : 2.71 ( $n=7$ ), for neotype 0.1 : 0.6 : 1 : 1.84 : 2.56; Ant. II : (III+IV) = 1 : 4.49 ( $n=7$ ), for neotype 1 : 4.4. Ant. III and IV annulated, except the 1/4 proximal part of Ant. III and the tip of Ant. IV. Antennae often asymmetrical, because of the fusion of Ant. III and IV. Pseudopores present ventro-apically on Ant. Ib (4–6), Ant. II (6–10) and Ant. III (4–6), located in the intersegment just

above sensory fields. Antennal phaneres very diversified (ordinary chaetae, S-chaetae, scales and papillae).

Scales rounded, of medium-sized, with a dense cover of short spicules arranged in more or less regular longitudinal lines; numerous dorsally on Ant. Ib and proximally on Ant. II (absent in distal half), sometimes a few ventrally on Ant. Ib; absent on Ant. Ia, III and IV.

The different categories of phaneres are distributed as follows on each antennal segment.

Ant. Ia dorsally with two basal mic (type g) and thick serrated mes (type b); ventrally with three basal mic (type g) (Fig. 3A), pointed smooth mes (type e), acuminate smooth mes (type f) and sensory field consisting of 10–17 S-chaetae of type l and 4–7 S-chaetae of type k (Fig. 3A, 4A).

Ant. Ib dorsally without chaetae except apical row of serrated mac (type a), thick serrated mes (type b), one small basal thick serrated mes (type b) and four dorso-latero-internal serrated mac (type a). Externo-apical small swollen S-chaetae (type h) absent. Ant. Ib ventrally with large number of pointed smooth mes (type e), several smooth mac (type d), thick serrated mes (type b), few acuminate smooth mes (type f), distal row of pointed smooth mac (type d) or large pointed smooth mes (type e); ventro-external sensory field consisting of S-chaetae of type k and 15–30 S-chaetae of type i. A few S-chaetae of type k always present externo-apically to sensory field (Fig. 4B, 5A). S-chaetae of type l proximally to sensory field absent. Basal mic absent.

Ant. II dorsally with sparse serrated mac and mes (types a and b), distally with a few acuminate smooth mes (type f) and thin serrated mes (type c), apical row of small serrated mac (type a) and several small smooth mac (type d), with an externo-apical group of 5–7 swollen S-chaetae of type h (AIIIO) (Fig. 4C, D, 6A). Ant. II ventrally without scales, with many short thick serrated mes (type b), pointed smooth mes (type e, less numerous), small smooth mac (type d), acuminate smooth mes (type f), distal row of small mac (type d) or large smooth mes (type e) and externo-apical sensory field consisting of 15–25 S-chaetae of type i, 2–7 chaetae of type j and of S-chaetae of type k (2–3 short S-chaetae of type k always present externo-distally between sensory field and AIIIO) (Fig. 4C, 6A). Basal mic present (one dorsal, three ventral, one latero-external, one latero-internal).

Ant. III–IV chaetotaxy with tendency to form whorls of chaetae, sometimes spiraling, without clear serial pattern across successive whorls that are composed of four types of chaetae variously interspersed (b, c, f and k) (Fig. 8A, 9A).

AIIIO located dorso-externo-apically, with two swollen h-type S-chaetae side by side (Fig. 7B), surrounded internally by one thin short k-type S-chaeta and externally by two short external S-chaetae, of which one pointed and bent j-type and one thin k-type (Fig. 7A, 10A); a few additional S-chaetae of k-type present below (Fig. 7C, 10A). Ant. III ventro-externo-distally with sensory field consisting of 13–20 S-chaetae of i-type, two small pointed j-type chaetae and of k-type chaetae (Fig. 7D, 8B, 11A). In proximal part of sensory field, S-chaetae of i-type and k-type smaller in size (Fig. 7E, F). Two ventro-basal mic present on Ant. III, absent or undifferentiated on Ant. IV. Ant. IV with at least four thick subcylindrical i-type S-chaetae in distal part and one large subapical j-type S-chaeta

(Fig. 9B, 12A). On Ant. IV except in distal part some S-chaetae of k-type are larger and intermediate with i-type. Scales absent on Ant. III–IV. Ant. IV with large slightly bifurcate subapical organite and a subapical papilla devoid of 'pin seta' (Fig. 12A, 13A). Distal organ of Ant. IV absent.

When Ant. III and IV are fused, the described pattern of Ant. III distal chaetae is strongly modified.

**Mouthparts** (Fig. 14–16). Clypeus with 50–60 mes, of which 2–3 serrated and others smooth (Fig. 14A). Prelabral and labral formula 4/5,5,4, chaetae smooth acuminate, of similar size. Labrum margin with inverted medial U-form intrusion devoid of primary granules, distal smooth area large (Fig. 15A). No labral papillae. Ventro-distally on labrum two unequal combs, each with one apical row of ~15 and 19 teeth respectively, and a pair of sinuous rods (Fig. 15A). Labial triangle with 9–11 smooth chaetae and no scales (Fig. 16A); basolateral field (mentum) with five smooth chaetae (A3–5, L1–2). Labial palp with five proximal chaetae and five papillae (A, B, C, D, E); guard chaeta of papilla A integrated in papilla B complex, which thus appears to have five guard chaetae (Fjellberg 1999, p. 325); papilla C without guard chaeta; papilla D with four guard chaetae; papilla E with five guard chaetae and well-developed labial process (Fig. 15B). Main hypostomal chaeta (H) with two accessory chaetae (h1 and h2) present. Maxillary outer lobe with one basal chaeta, simple maxillary palp, four sublobal hairs (Fig. 15C). Maxilla with strong tridentate claw, five lamellae and a thin rod, long and bent. Mandible asymmetrical with five teeth on right and four on left on all examined specimens. Post-labial chaetae (9–11) + (9–11), of which (3–4) + (3–4) most proximal ones are small and serrated and others are smooth without interspersed scales.

**Head and body** (Fig. 8, 9, 16–18). Scales present on dorsal and ventral side of body and only dorsal side of head, rounded to oval-elongated, sometimes truncated, hyaline, overlapping, with a dense cover of short spicules arranged in more or less regular lines (Fig. 8C, 9C, D), of various sizes: medium-size on all tergites except Abd. VI where they are smaller; larger posteriorly (at least twice as large as the anterior ones) on all tergites except Abd. VI. No special arrangement or morphological modification of scales and chaetae around mac and trichobothria.

Head dorsally with stable macrochaetae arrangement as in Fig. 17A; antennal mac (5–6)+2; 1+1 trichobothria posterior to ocular area; dense cover of small to large scales and short serrated mes; very small and thin serrated mes present (Lukić *et al.* 2015, type b in fig. 28), their exact pattern not possible to reconstruct; laterally with scales mixed with medium-sized serrated and smooth mes; the posterior row of chaetae consisting of serrated mes regularly interspersed with large scales. Head ventrally with smooth and serrated mes.

Dorsal chaetotaxy of the tergites and head illustrated in Fig. 17A. Pseudopore formula 1,1/1,1,1,1; trichobothrial formula 1/0,0,2,3,2; ms formula 1,0/1,0,1,0,0 (Fig. 8D, 17A); sens formula 2,2/1,3,3,3,3 (Fig. 8D, 9D, 17A); macrochaeta formula (3+0+4),(3+3)/0,2,3,(2+0+3+3). Macrochaeta arrangement stable; multiplets only present anteriorly on Th. II; Th. II with three anterior and four posterior mac; Th. III with three posterior and three lateral mac; Abd. IV with two medial, three antero-lateral

and three postero-lateral mac (A6, F2, E3 are mes); Abd. V with (10–11)+(10–11) mac; Abd. VI with 7–8 mac. On each anal valve serrated mac and mes, two smooth mac and two mic present. Medium-sized serrated mes present on anterior part on Th. II, dorsally on Abd. V and VI and very few laterally on all tergites. Dorsally on all tergites very small and thin serrated mes present (Lukić *et al.* 2015, type b2 in fig. 27), their exact pattern not possible to reconstruct. On Abd. IV 20–25 + 20–25 rather long S-like chaetae, 50% longer than mes of other tergites (Fig. 8C, 9C). Tandem of thin serrated mes associated with sens absent on all tergites on all observed specimens.

**Legs** (Fig. 19, 20). Legs with ordinary chaetae and scales. Detailed distribution of the different types of chaetae on each leg segment as follows.

Separation between subcoxae 1 and 2 indistinct. Subcoxae with scales. Subcoxa I with 3–4 dorso-posterior serrated mac. Subcoxa II with 5–8 dorso-anterior serrated chaetae ranging in size from mac to mes and 4–5 dorso-posterior serrated mac. Subcoxa III with group of 4–7 dorso-posterior serrated mac and some serrated mes.

Coxae with pseudopores, thick serrated mes, smooth mes, smooth mac, multiplets of serrated chaetae and scales. Pseudopores as 1, 4–7, 5–8 on coxae I, II and III. Smooth mac as 1, 1, 0 on dorso-anterior side of coxa I, II and III; multiplet of 5–6 dorso-posterior serrated chaetae on coxa I, two multiplets (dorso-anterior one and dorso-posterior one) of 6–8 serrated chaetae on coxa II; chaetae in multiplets of coxae I and II decreasing in size from mac (distally) to mes (proximally). Dorsally on coxa III, a transversal line of three mac sockets and six mes sockets.

Trochanter with many chaetae ranging in size from mes to mac including many serrated mes, smooth mac and mes. Trochanter I with 17–22 smooth chaetae, trochanter II with 18–20, trochanter III, with 6–7 and the trochanteral organ consisting of 50–60 shorter, straight, spine-like mes. Scales in small number on trochanter I, II and in large number on trochanter III.

Femur with serrated mes, serrated mac, smooth mes and smooth mac. Scales present in proximal half on femur I, numerous on whole length of femora II and III. Ventrally, 3, 2, 1 smooth mac on femora I, II and III and a few additional smooth mes on femora I, II and III. Largest smooth mac 0.35–0.55 × length of femur. Group of ventro-proximal smooth mes without interspersed scales on femora III.

Tibiotarsus with a large number of serrated mes, few serrated mac, smooth mes, thin, pointed and smooth tenent hair, and scales. Distal whorl of Tita with 14–16 (usually 15) chaetae including 4–5, 5, 5–7 smooth mes and one tenent hair on legs I, II and III (Fig. 20A). Serrated mac as 0, 1, 1 on Tita I, II and III (Fig. 19A1, A2, A3). Besides the apical ones, smooth mes present ventrally and dorsally on Tita I, II and III. On Tita I and II at least four rows of smooth mes ventrally, smooth mes not arranged in clear rows dorsally (Fig. 19A1, A2). On Tita III smooth mes present dorsally and ventrally, not arranged in rows (Fig. 19A3). In total, 33–43, 40–53, 55–71 smooth mes on Tita I, II and III (Fig. 19A1, A2, A3). Scales absent on Tita I and II, and a few scales in the proximal third of Tita III. Tita of leg III longer than those of legs I and II (0.55, 0.62, 0.82 mm for Tita I, II, III) ( $n = 3$ ).





**Fig. 1.** Habitus *in situ*. (A) *Verhoeffiella longicornis* (Absolon, 1900); (B) *V. polychaetosa*, sp. nov.; (C) *V. verdemontana*, sp. nov.; (D) *V. cavicola* Absolon, 1900; (E) *V. milosi*, sp. nov.; (F) *V. margusi*, sp. nov. Photo: M. Lukić. Scale bars: 1 mm.

Pretarsus with one anterior and one posterior mic (Fig. 20A). Unguis relatively thin and elongated, with one inner pair of equal basal teeth at 21, 24, 26% from its base for unguis I, II and III ( $n=3$ ), 1 + 1 small latero-basal teeth (Fig. 20A), inner unpaired tooth absent. Unguis (internal edge) 1.5, 1.5, 1.3 $\times$  longer than unguiculus for claws I, II and III ( $n=3$ ). Unguis (internal edge) 3.5 $\times$  longer than width of unguis at basis for claw I ( $n=3$ ), 3.6 $\times$  in neotype. Unguiculus robust, pointed, 0.65–0.67 $\times$  as long as inner edge of unguis for claw I and II, 0.75 $\times$  for claw III, with four lamellae (antero-extern, antero-intern, postero-extern,

postero-intern), without basal lamella (Fig. 20A). Small outer tooth, sometimes difficult to see at 35% from its base ( $n=3$ ) on antero-external lamella of unguiculus I, II and postero-external lamella of unguiculus III (Fig. 20A). Unguiculus 7.2 $\times$  longer than its width at basis for claw I ( $n=3$ ). Tenent hair 1.5 $\times$  longer than width of empodium and 0.5 $\times$  the length of unguis (internal edge) for claw I ( $n=3$ ).

*Ventral tube* (Fig. 15G, II). Ventral tube with smooth and serrated mes and mac, all pointed. Posteriorly 150–160

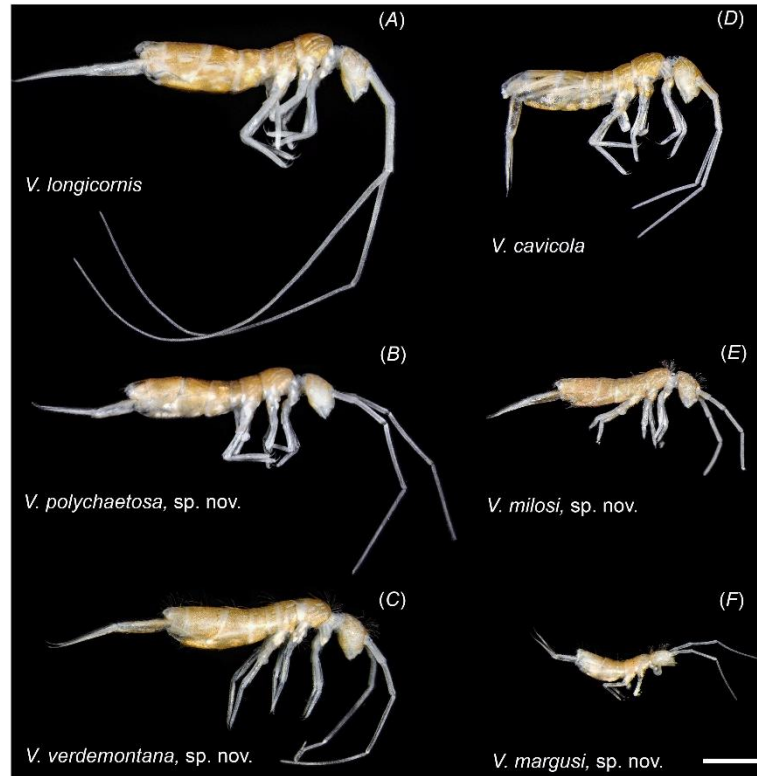


Fig. 2. Habitus, all at same scale. (A) *Verhoeffiella longicornis* (Absolon, 1900); (B) *V. polychaetosa*, sp. nov.; (C) *V. verdemontana*, sp. nov.; (D) *V. cavicola* Absolon, 1900; (E) *V. milosi*, sp. nov.; (F) *V. margusi*, sp. nov. Photo: M. Lukić. Scale bar: 1 mm.

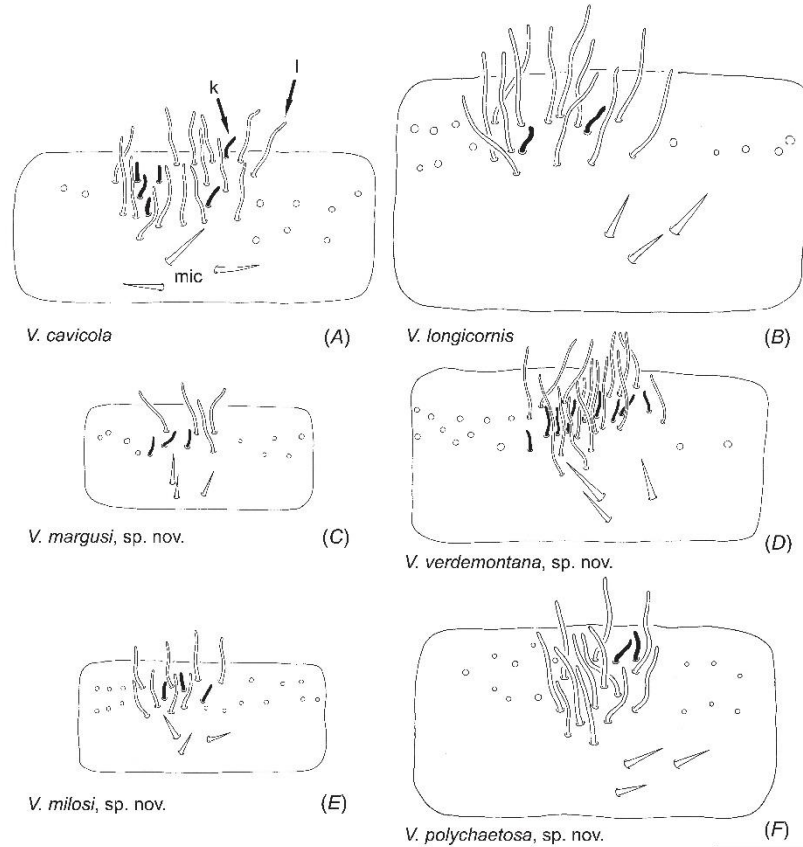
smooth mes, and no scales. Anteriorly with 45–75+45–75 smooth mes (sometimes some weakly serrated present), 2+2 smooth mac in distal part, and several large smooth mes. Each latero-distal flap with 55–60 mes, of which 5–9 are serrated and others smooth.

Genital plate of female with 2+2 mic (Fig. 15D), that of adult male not observed. Tenaculum with one serrated chaeta and four teeth on each ramus.

*Furca* (Fig. 15, 21). Furca with pseudopores, smooth mac, different kinds of serrated mes and scales of various morphologies. Pseudopores as 10–14 dorso-distally on each side of the manubrium, among distal group of chaetae (Fig. 15E). Manubrium dorsally with two longitudinal stripes of serrated mes, each one interspersed with 9–12 smooth pointed mac (Fig. 21A); scales absent between these stripes; externally, each longitudinal stripe of mes is bordered by a very narrow stripe devoid of chaetae and scales. Manubrium covered ventrally by medium-sized scales, without chaetae,

except an unpaired ventro-basal smooth and thick mac, and 1+1 apical serrated mes near the axis. Distal manubrial thickening with two teeth on each side.

Dens curved and tapering, consisting of a short basal part hardly annulated, a long medial part annulated dorsally and a short, thinner, non-annulated distal part ~4× as long as mucro (Fig. 21A). All dental chaetae serrated except 2–5 smooth mac dorsally on basal part of each dens (Fig. 21A). Two types of serrated chaetae can be recognised: dorso-laterally, two longitudinal rows of thicker chaetae on basal and medial part of dens; ventro-laterally, two longitudinal rows of thinner chaetae, very long on basal part and shorter on medial part. Small serrated mes present between rows and ventral scales. Ventrally between the two rows of thin serrated chaetae, dense cover of medium-sized elongated scales, small and rounded on basal part of dens, increasing in size laterally to large, fusiform, pointed apically; scales absent in distal non-annulated part. Dens thickening with two faint transversal



**Fig. 3.** Ant. Ia, ventral side with basal mic and sensory field composed of S-chaetae of type I and type k (in black). (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. (A, C, E) Left antenna, (B, D, F) right antenna. Scale bar: 50 µm.

structures. One short and thick internal spine basally on each dens. Ratio manubrium : dens : mucro = 25 : 39 : 1. Mucro + dens 8.3× longer than unguis III (internal edge) and 1.4× longer than Tita III. Mucro bidentate with one basal spine reaching the apex of the proximal tooth, distal tooth elongated (Fig. 21A1).

**Ecology.** Type locality cave Megara: entrance 1320 m ASL; cave length: 243 m; air temperature at the end of the cave 4.6–5.8°C. Specimens were very abundant in the last chamber, usually on decaying wood or around it on wet clay.

#### Discussion

The original and only existing description of *V. cavicola* is very brief and lacks most characters used in modern taxonomy

of Entomobryidae. We give here a detailed redescription of reference for this species, which is the type species of the genus. *Verhoeffiella cavicola* differs from *V. constantius* Čurčić and Lukić (in Lukić *et al.* 2007a) and *V. margusi*, sp. nov. in antennal segment ratio (Table 1) and from *V. verdemontana*, sp. nov. and *V. polychaetosa*, sp. nov. in the number of serrated mac on Tita I, II, III, of smooth mac dorsally on the manubrium and of smooth mac dorso-basally on dens (Table 2). *Verhoeffiella cavicola* differs from all species of the genus that are described in detail in the following characters: absence of sens in tandem with serrated mes on all tergites; 0, 1, 1 serrated mac on Tita I, II and III; presence of numerous smooth mes dorsally on Tita. For other diagnostic characters see Tables 1–3.



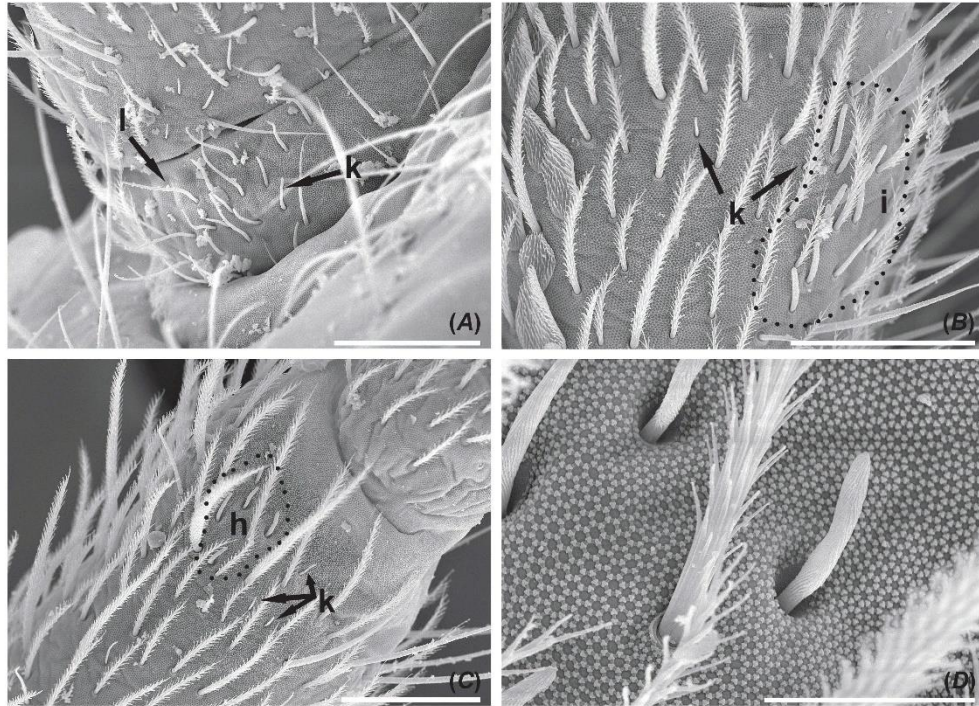


Fig. 4. *Verhoeffiella cavicola* Absolon, 1900, SEM images of Ant. Ia, Ib and II. (A) Ant. Ia, ventral sensory field with S-chactae of type l and type k; (B) Ant. Ib, ventro-external sensory field with S-chactae of type i and k; (C) Ant II, AIIO with S-chactae of type h and k; (D) Ant II, swollen S-chactae of type h of AIIO. Scale bars: 40 µm (A, B, C); 10 µm (D).

***Verhoeffiella longicornis* Absolon**

(Fig. 1–3, 5–6, 10–17, 19–21)

*Material examined*

*Neotype.* Type material is considered lost (for details see 'Materials and methods'). We propose here as neotype a specimen from the type locality. Adult ♂ on slide: Bosnia and Herzegovina: Trebinje: Bihovo: Pavlova pećina (cave) (WGS84 42°39'59.0"N; 18°18'28.0"E; 420 m ASL), J. Bedek leg., 25.x.2015 (CLL4954).

*Other material examined.* The same data as neotype, 1 adult and 1 subadult ♂ on slides, 14 ex. in 96% alcohol (CLL4779); 1 adult and 2 subadult ♀ on slides, 9 ex. in 96% alcohol, M. Lukić leg., 30.iii.2012 (CLL4229); 2 adult ♀ on slides, 28 ex. in 96% alcohol, V. Sudar leg., 25.x.2015 (CLL4777); adult ♀ and subadult ♀? on slides, 7 ex. in 96% alcohol, J. Jakupić leg., 06.xi.2015 (CLL4782); 2 subadult ♂ on slides, 6 ex. for DNA (4028F01, 4028F02, 4028F03, 4028F04, 4028F05, 4028F06), 3 ex. in 96% alcohol, M. Lukić leg., 19.iv.2010 (CLL2283).

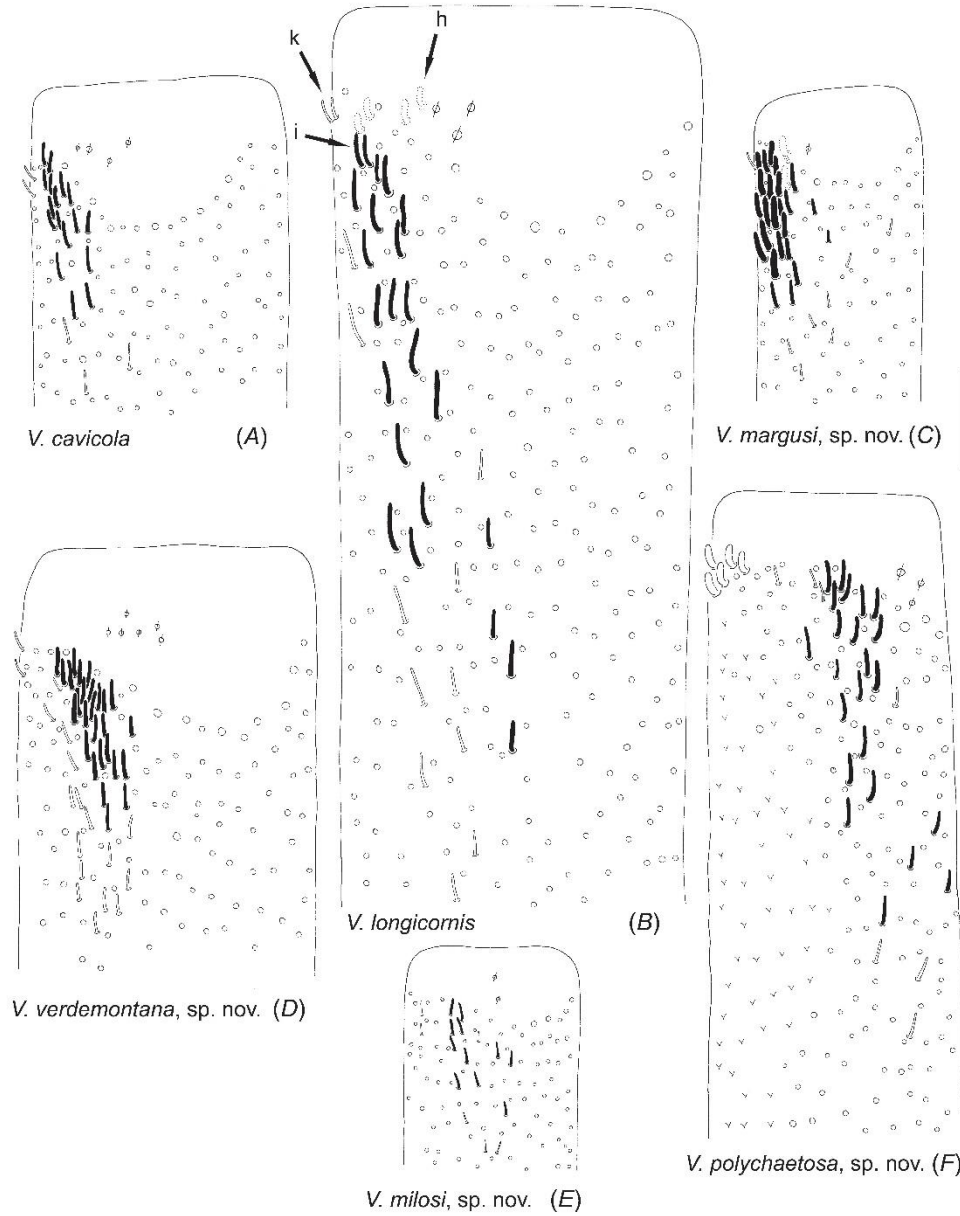
*Material deposition.* 1 adult and 1 subadult ♂ on slides (CLL4779, CLL4229) deposited in MNHN. All other material, including neotype deposited in CBSS.

*Type locality*

In original text from Absolon (1900) type locality is given as: 'Eliashöhle bei Trebinje (Herzegowina)'. Literal translation of 'Eliashöhle' to 'Ilijina pećina' led to logical conclusion that a cave today called Ilijina pećina located ~2 km north-west of Trebinje is the type locality, and it is given like this in Lukić *et al.* (2015). However, in Willmann (1941, p. 16) a photo of the cave entrance cited as 'Eingang zu der Ilijina Pećina ... original photograph taken by Absolon 20.VII.1912.' shows the entrance to a cave today called Pavlova pećina located ~6 km south-west of Trebinje. Moreover, in the text near the photograph this cave is cited as 'Eliashöhle'. Therefore, type locality of *V. longicornis* is: Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave).

*Diagnosis*

Head and body length without antennae: 3.8–4.2 mm. Colour pale orange. Scales present on dorsal and ventral side of head and body, dorsal side of Ant. II, on legs, ventrally on the manubrium and dens. Antennae 2–2.2 as long as (head and



**Fig. 5.** Ant. lb, ventro-externo-distal sensory field composed of S-chaeatae of type i (in black), S-chaeatae of type k (hollow line), pseudopores (cross-circles). Swollen S-chaeatae of type h of the dorsal side in dotted line (except in *F* where in full line). (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bar: 50  $\mu$ m.

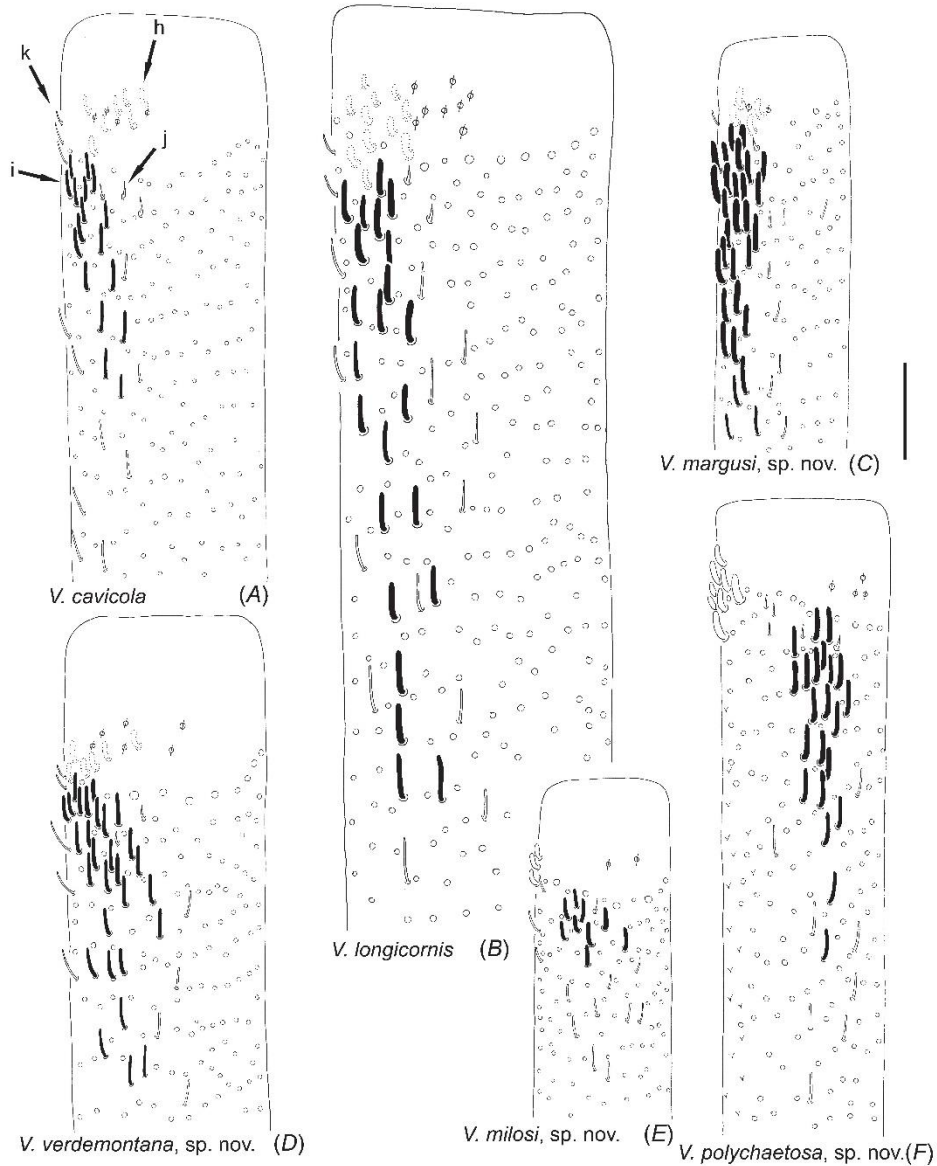
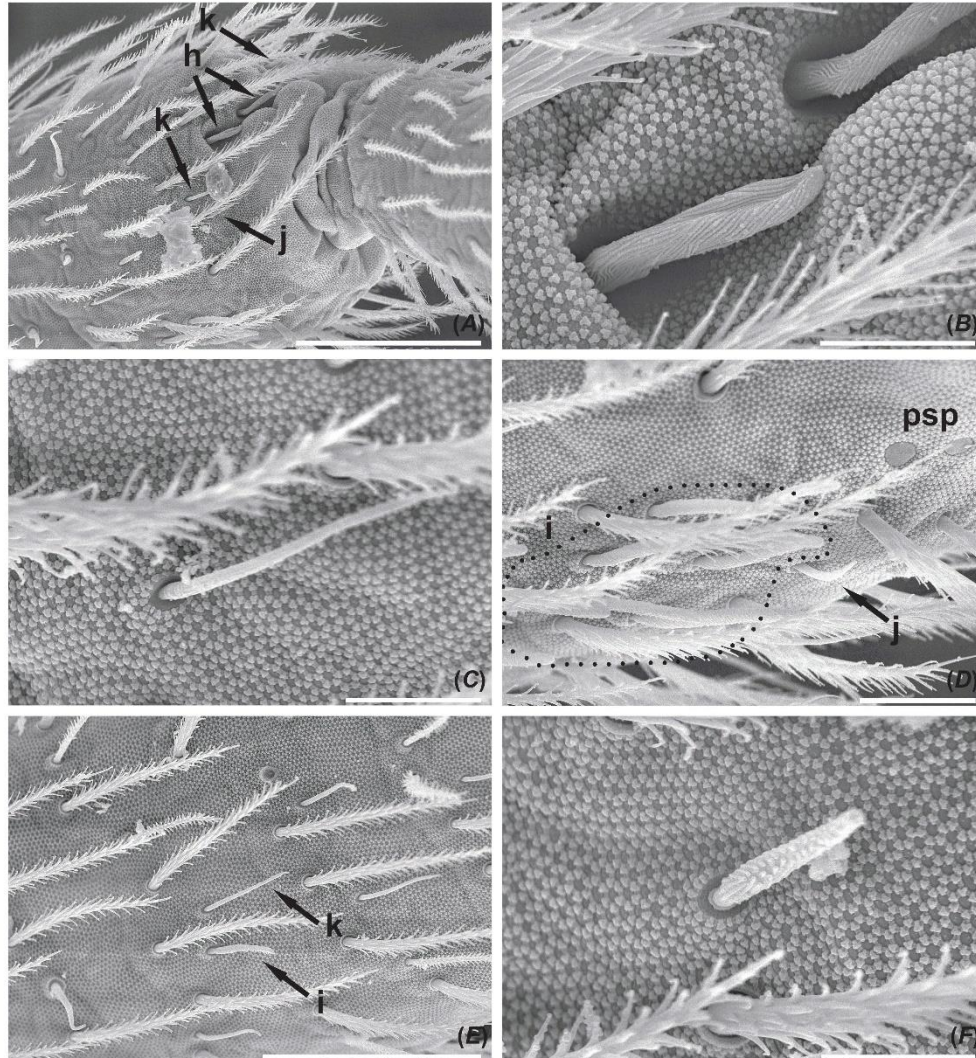
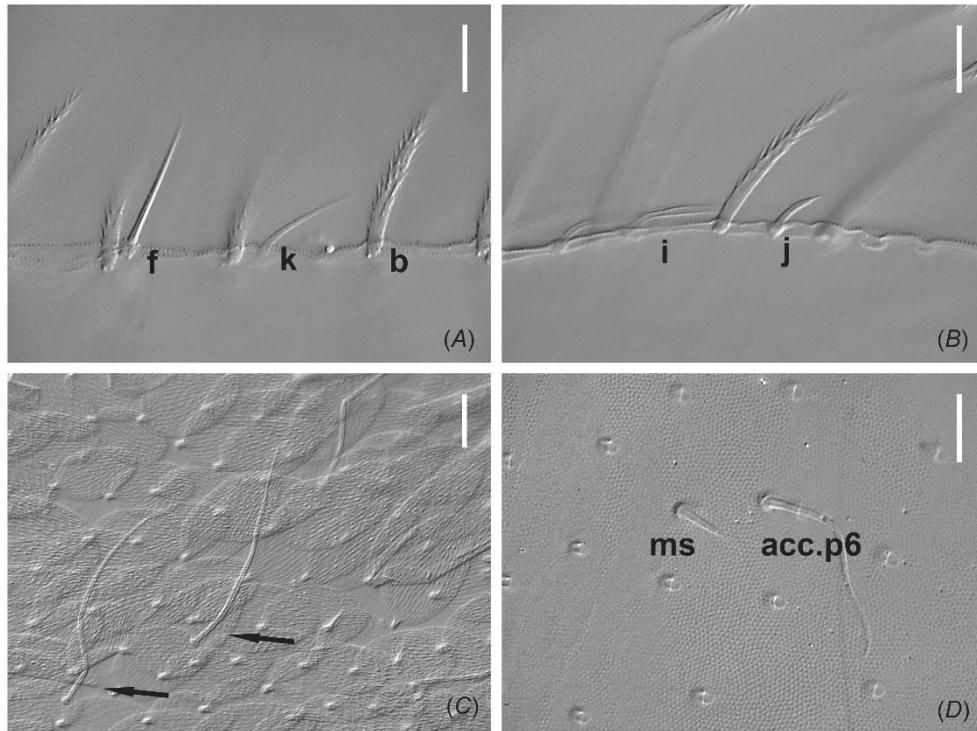


Fig. 6. Ant. II, ventro-externo-distal sensory field composed of S-chaetae of type i (in black) and type j (short, pointed), S-chaetae of type k (hollow line), pseudopores (cross-circles), swollen S-chaetae of type h of the dorsal side (AIIIO) in dotted line (except in *E* and *F* where in full line). (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bar: 50  $\mu$ m.





**Fig. 7.** *Verhoeffiella cavicola* Absolon, 1900, SEM images of Ant. III. (A) AIII with S-chaetae of type h, j and k and part of sensory field; (B) AIII, swollen S-chaetae of type h; (C) S-chaetae of type k below AIII; (D) ventro-external sensory field with S-chaetae of type i and j and pseudopores (psp); (E) proximal part of sensory field with small S-chaetae of type i and type k; (F) small S-chaetae of type i in the lateral part of the sensory field. Scale bars: 40  $\mu$ m (A); 5  $\mu$ m (B, C, D); 10  $\mu$ m (E); 30  $\mu$ m (F).



**Fig. 8.** *Verhoeffiella cavicola* Absolon, 1900, optical microscope photos of antenatal and tergal chaetae. (A) Ant. IV, with ordinary chaetae of types b, f and S-chaetae of type k; (B) Ant. III, with S-chaetae of types i and j; (C) Abd. IV with scales and S-like chaetae; (D) Abd. III, with S-chaetae (ms and sens acc.p6). Scale bars: 10 µm (A, B, D); 20 µm (C).

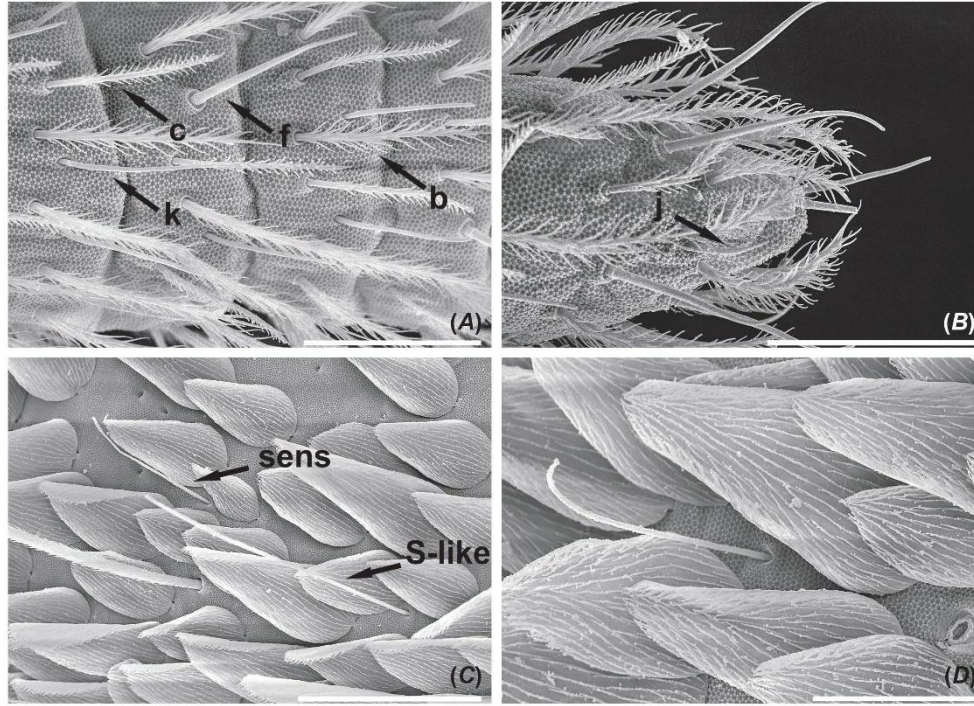
body) length; ratio Ant. II : (III+IV) = 1 : 8.51. Pseudopores as 2–3, 6–7, 4–5 distally on Ant. I, II and III. Ventral sensory field of Ant. Ia with 12–14 S-chaetae of type l and two S-chaetae of type k; four S-chaetae (type h) on Ant. Ib, 7–11 on Ant. II; subapical papilla of Ant. IV present, distal organ of Ant. IV present. Labial triangle with 8–10 smooth chaetae. Proximal serrated post-labial chaetae along linea ventralis as (1–3) + (1–3) with one scale present proximally. Dorsal macrochaetotaxy as (3+1+4), (3+4)/0,2,3, (3+0+2–4+2–4). Trochanteral organ with 55–65 chaetae. Smooth mac as 3, 3–4, 1–2 on femora I, II and III. Serrated mac as 3, 3, 3 on Tita I, II and III; smooth mes as 20–23, 23–27, 23–28 on Tita I, II and III, arranged in four rows. Tenent hair acuminate, smooth; ratio tenent hair : width of empodium for leg I 1 : 1. Unguis thin and elongated, with one pair of inner basal teeth; ratio unguis I length : unguis I width at basis 3.4 : 1. Manubrium with 6–8 dorso-distal psp, dorsally without scales and 20–25 smooth pointed mac, 1 + 1 ventro-apical serrated mes; 4–6 basal smooth mac dorsally on dens; mucro usually bidentate, sometimes tridentate with one basal spine.

#### Redescription

Head and body length combined without antennae: 3.8 to 4.2 mm ( $n=7$ ), 4.1 mm in neotype. Habitus as in Fig. 1A, 2A. Abd. IV 1.3× as long as Abd. III ( $n=3$ ), 1.3× in neotype. Antennae two times longer than (head and body) length. Colour pale orange, pigment scattered as orange dots ventrally and dorsally on body, manubrium, coxa, paler on trochanter and femur. Intersgment area, mouth parts, antennae, other leg segments and dens unpigmented. Body dorsally and laterally with white regular patches on an orange background (Fig. 1A, 2A). Eyes absent, no pigment patch in their place.

**Antennae** (Fig. 3, 5, 6, 10–13). Antennae 2 to 2.2× as long as (head and body) length ( $n=4$ ), 2× in neotype. Antennae 13.8× longer than head diagonal. Ant. segment ratio as: Ia : Ib : II : III : IV = 0.08 : 0.62 : 1 : 2.66 : 5.85 ( $n=5$ ), for neotype 0.08 : 0.6 : 1 : 2.72 : 6.07; Ant. II : (III+IV) = 1 : 8.51 ( $n=5$ ), for neotype 1 : 8.79. Ant. III and IV annulated, except the 1/10 proximal part of Ant. III and the tip of Ant. IV. Pseudopores present ventro-apically on Ant. Ib (2–3), Ant. II (6–7) and Ant. III (4–5).





**Fig. 9.** *Verhoeffiella cavicola* Absolon, 1900, SEM images of Ant. IV and Abd. IV and V. (A) Ant. IV, distal part with ordinary chaetae of type b, c and f and S-chaetae of type k; (B) Ant. IV tip with S-chaetae of type j; (C) Abd. IV with thin subcylindrical S-chaetae (sens) and long S-like chaetae (S-like); (D) Abd. V with thin subcylindrical S-chaetae (sens). Scale bars: 20 µm (A, B, D); 50 µm (C).

Scales numerous dorsally on Ant. Ib and the whole length of Ant. II (except in most distal part where absent), absent on Ant. Ia, III, IV and ventrally on Ant. Ib.

Ant. Ia dorsally with a group of centro-dorsal chaetae, two of which probably mic (type g) and other slightly serrated; ventrally acuminate smooth mes (type f) absent and sensory field consisting of 12–14 S-chaetae of type l and two S-chaetae of type k (Fig. 3B).

On Ant. Ib dorsally with one small basal thick slightly serrated mes (type b) and 3–5 dorso-latero-internal serrated mac (type a). Externo-apical four small swollen S-chaetae (type h). Ant. Ib with very few pointed smooth mes (type e); ventro-external sensory field consisting of S-chaetae of type k and 17–25 S-chaetae of type i. A few S-chaetae of type k always present externo-apically to sensory field (Fig. 5B). S-chaetae of type l proximally to sensory field present, located just above Ant. Ia sensory field.

Ant. II dorsally with thin serrated mes (type c) and apical row of small serrated mac (type a) and serrated mes (type b), with an externo-apical group of 7–11 swollen S-chaetae of type h (AIIO) (Fig. 6B). Ant. II ventrally with pointed smooth mes (type e) absent and apical row of small smooth mac (type d)

or large smooth mes (type e) and externo-apical sensory field consisting of 20–30 S-chaetae of type i, 2–4 chaetae of type j and of S-chaetae of type k (2–3 short S-chaetae of type k always present externo-distally between sensory field and AIIO) (Fig. 6B). Basal mic present one dorsal, ventrally six small smooth chaetae, impossible to determine the number of mic, one latero-external, one latero-internal).

AIII located dorso-externo-apically, with two swollen h-type S-chaetae side by side, surrounded internally by one thin short k-type S-chaeta and externally by two short external S-chaetae, of which one pointed and bent j-type, and 1–2 thin k-type ( $n=4$ ) (Fig. 10B); a few additional S-chaetae of k-type present below (Fig. 10B). Ant. III ventro-externo-distally with sensory field consisting of 20–25 S-chaetae of i-type and 1–2 small pointed j-type chaetae and of k-type chaetae (Fig. 11B). Ant. IV with at least two thick subcylindrical i-type S-chaetae in medio-distal part and two large subapical j-type S-chaeta (Fig. 12B). On Ant. IV except in distal part some S-chaetae of k-type are larger and intermediate with i-type. Ant. IV tip with very few thin serrated c-type mes and no thin short k-type S-chaetae, with a subapical papilla devoid of 'pin seta' (Fig. 12B). Distal organ of Ant. IV well developed

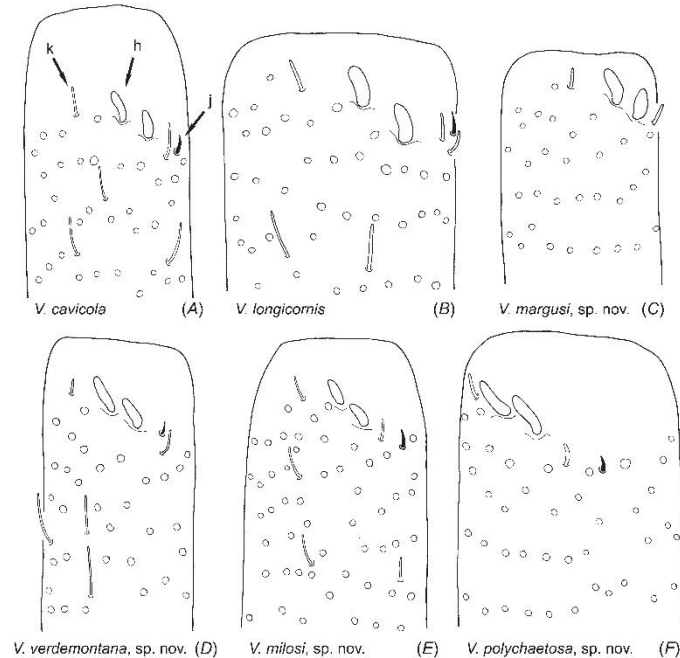


Fig. 10. Ant. III dorso-externo-apical organite (APIO) with S-chactae of type h, type k and type j (in black). (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bar: 20  $\mu$ m.

with several cuticular channels opening to the surface cuticle just behind papilla (Fig. 13B).

**Mouthparts** (Fig. 14B, 16B). Clypeus with 40–45 mes, of which 15–20 serrated and other smooth (Fig. 14B). Ventro-distally on labrum two unequal combs, each with one apical row of ~15 and 19 teeth respectively, and a pair of sinuous rods. Labial triangle with 8–10 chaetae, all smooth and no scales (Fig. 16B). Post-labial chaetae (10–11)+(10–11), of which (1–3)+(1–3) most proximal ones small, serrated and others smooth, with only one scale present in proximal part.

**Head and body** (Fig. 17B). Head dorsally with stable macrochaetae arrangement as in Fig. 17B; antennal mac 5+(2–3); laterally with scales mixed with medium-sized serrated and smooth mes; ventrally with small to medium-sized scales, smooth and serrated mes.

Dorsal chaetotaxy of the tergites and head illustrated in Fig. 17B. Ms formula 1,0/1,0,1,0,0; sens formula 2,2/1,3,3,3,3; macrochaeta formula (3+1+4),(3+4)/0,2,3,(3+0+2 4+2 4). Macrochaeta arrangement stable, except variation in lateral mac on Abd. IV; Th. II with three anterior, one medial (m2?) and four posterior mac; Th. III with four posterior and three lateral mac; Abd. IV with three medial, 2–4 antero-lateral and 2–4 postero-lateral mac (sometimes 1–2 antero-lateral and postero-lateral are mes), chaetae E2 is slightly displaced

compared with other species; Abd. V with (10–11)+(10–11) mac; Abd. VI with 14–16 mac. On Abd. IV 40–45+40–45 S-like chaetae. Thin serrated mes associated with sens in tandem on Th. III, Abd. II and Abd. III.

**Legs** (Fig. 19, 20). Subcoxa I with three dorso-posterior serrated mac. Subcoxa II with 3–4 dorso-anterior serrated chaetae ranging in size from mac to mes and three dorso-posterior serrated mac. Subcoxa III with group of three dorso-posterior serrated mac and some serrated mes.

Coxa with pseudopores as 1, 7 12, 7 13 on coxa I, II and III. Smooth mac as 2, 1, 0 on dorso-anterior side of coxa I, II and III; multiplet of five dorso-posterior serrated chaetae on coxa I, two multiplets (dorso-anterior one and dorso-posterior one) of 6–7 serrated chaetae on coxa II. Dorsally on coxa III, a transversal line of four mac sockets and seven mes sockets (sometimes all mac sockets).

Trochanter I with 20–25 smooth chaetae, trochanter II with 15–18, trochanter III with 25–30, and trochanteral organ consisting of 55–65 shorter, straight, spine-like mes. Scales in large number on trochanter I, II and III.

Femur with many serrated mes, serrated mac, smooth mes and smooth mac. Scales numerous on femora I, II and III. Ventrally, 3, 3–4, 1–2 smooth mac on femora I, II and III and few additional smooth mes on femora I, II and

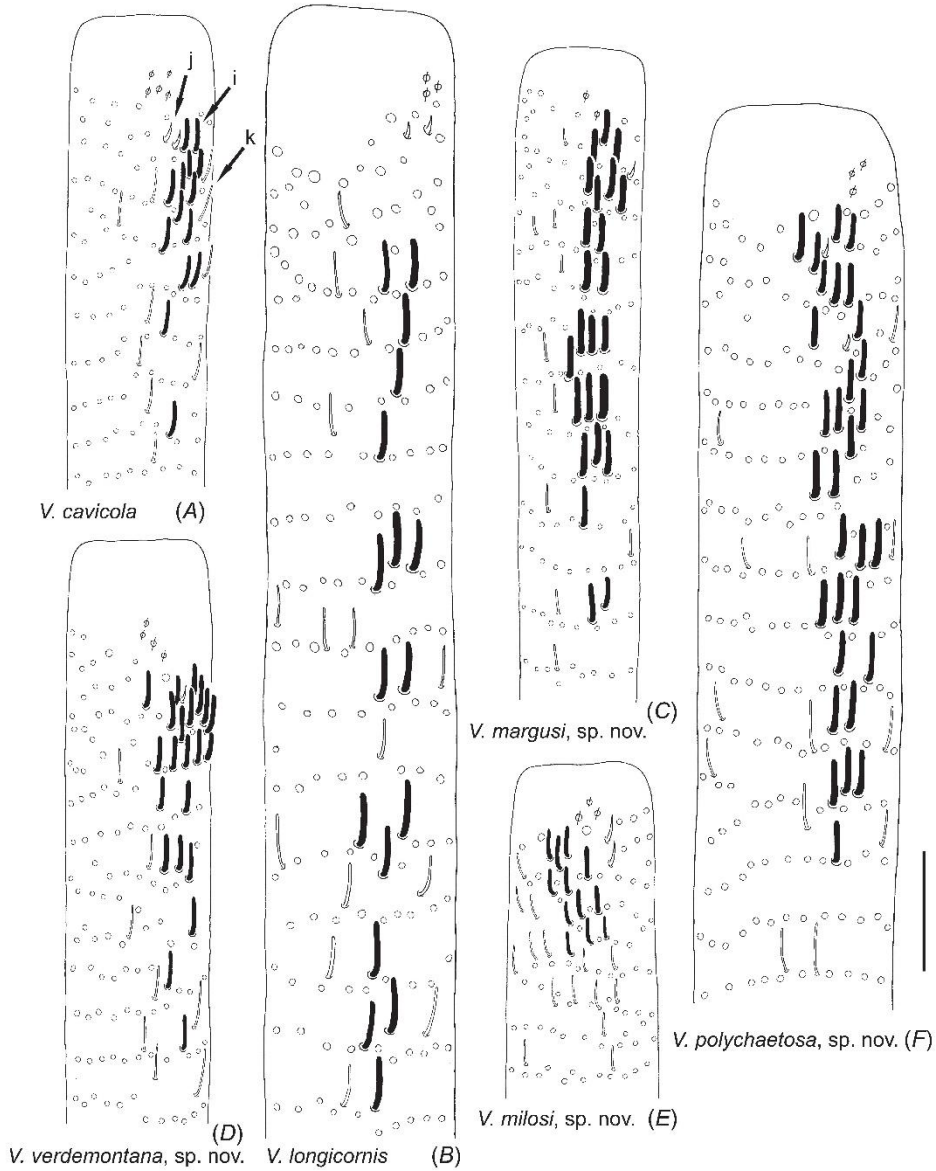
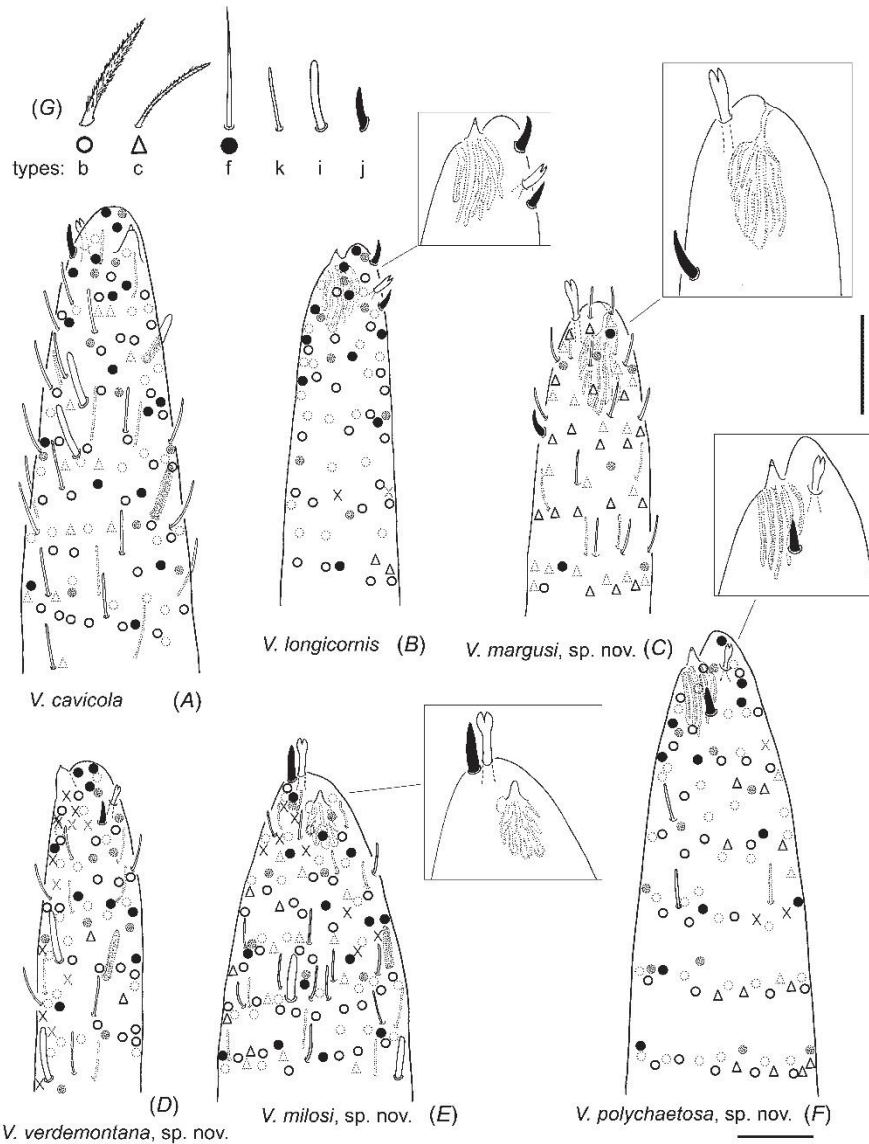
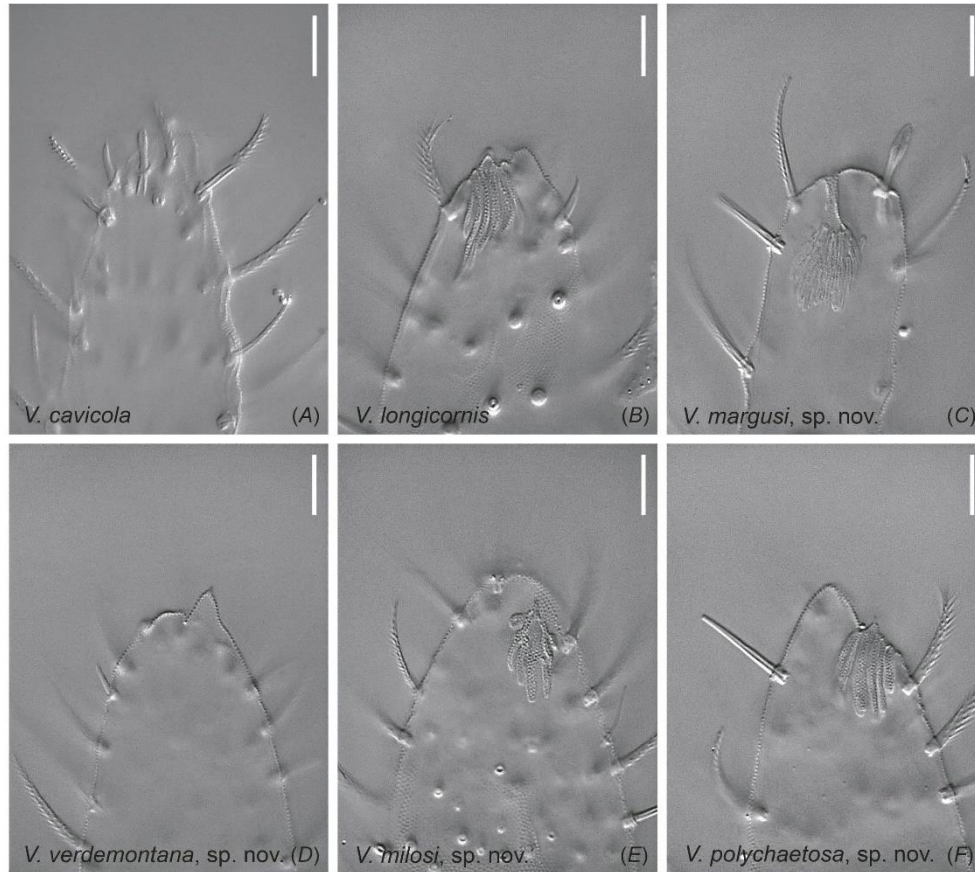


Fig. 11. Ant. III, ventro-externo-distal sensory field composed of S-chaeata of type i (in black) and type j (short, pointed), S-chaeata of type k (hollow line), pseudopores (cross-circles). (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bar: 50 μm.





**Fig. 12.** Ant. IV, distal part, with enlarged tip with distal organ, bifurcate subapical organite, subapical papilla and S-chaetae of type j; hollow circles – thick serrated mes (b); triangles – thin serrated mes (c); black circles – acuminate smooth mes (f); X – fallen chaetae; hollow lines – thin short S-chaetae (k); thick hollow lines – thick subcylindrical S-chaetae (i); in black – large subapical S-chaetae (j). (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov.; (G) types of chaetae and symbols used. All scale bars: 20  $\mu\text{m}$  (vertical bar for enlarged Ant. tips and G; horizontal bar for Ant. IV distal parts).



**Fig. 13.** Optical microscope photos of Ant. IV tip. (A) *Verhoeffiella cavicola* Absolon, 1900, with subapical organite and large subapical S-chaetae of type j; (B) *V. longicornis* (Absolon, 1900) with distal organ and large subapical S-chaetae of type j; (C) *V. margusi*, sp. nov. with distal organ and subapical organite; (D) *V. verdemontana*, sp. nov., with subapical papilla; (E) *V. milosi*, sp. nov., with distal organ; (F) *V. polychaetosa*, sp. nov. with distal organ. Scale bars: 10  $\mu$ m.

III. Largest smooth mac 0.27–0.36 $\times$  length of femur. Group of ventro-proximal smooth mes without interspersed scales on femora III.

Distal whorl of Tita with 13–15 (usually 15) chaetae including 6, 5, 5 smooth mes and one tenent hair on legs I, II and III (Fig. 20B). Serrated mac as 3, 3, 3 on Tita I, II and III (Fig. 19B1, B2, B3). Besides the apical ones, smooth mes present ventrally on Tita I, II and III arranged in four rows. On Tita II several smooth mes interspersed between rows. In total, 20–23, 23–27, 23–28 smooth mes in four rows on Tita I, II and III (Fig. 19B1, B2, B3). Scales absent on Tita I, a few scales present in proximal part of Tita II and III. Tita of

leg III longer than those of legs I and II; length 0.76, 0.80, 0.96 mm for Tita I, II and III ( $n=2$ ).

Unguis with one inner pair of equal basal teeth at 19, 23, 25% from its base for unguis I, II and III ( $n=3$ ) (Fig. 20B), inner unpaired tooth absent. Unguis (internal edge) 1.7, 1.6, 1.4 $\times$  longer than unguiculus for claws I, II and III ( $n=3$ ). Unguis (internal edge) 3.4 $\times$  longer than width of unguis at basis for claw I ( $n=3$ ), 3.0 $\times$  in neotype. Unguiculus robust, pointed, 0.60–0.64 $\times$  as long as inner edge of unguis for claw I and II, 0.72 $\times$  for claw III (Fig. 20B). Small outer tooth, sometimes difficult to see 33% from its base ( $n=3$ ) on antero-external lamella of unguiculus I, II and postero-external lamella of unguiculus III

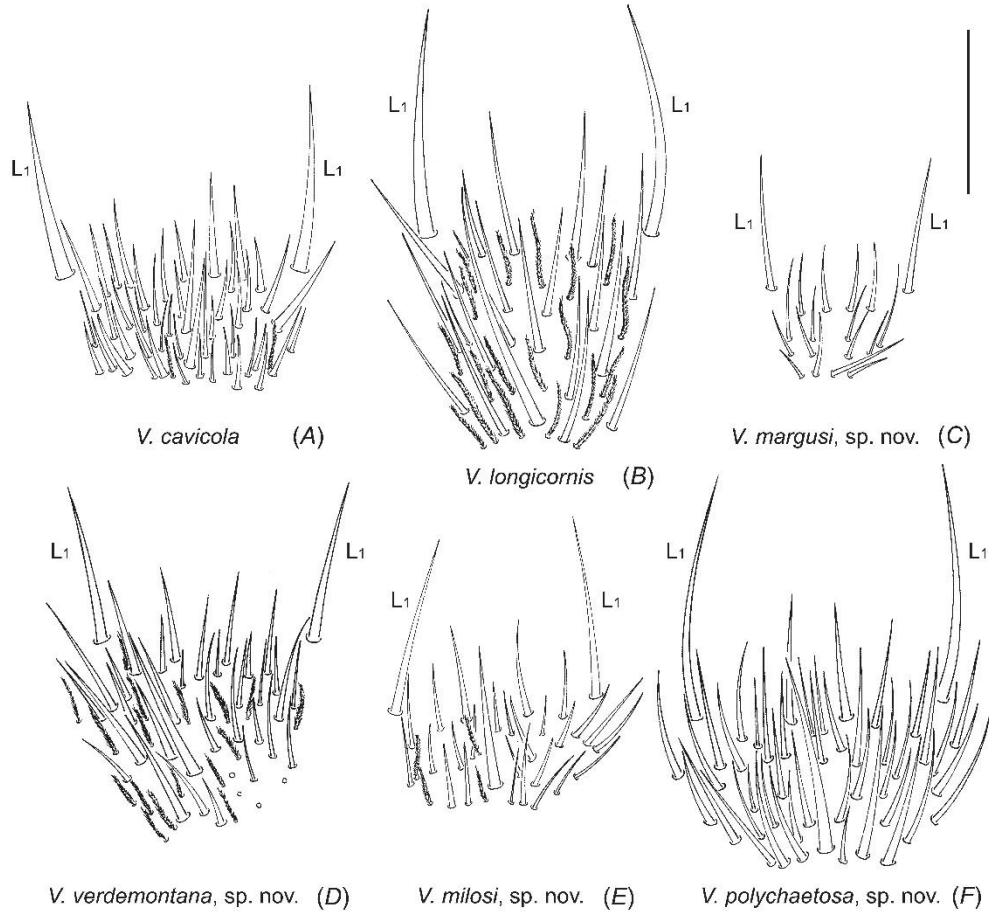


Fig. 14. Clypeus, L<sub>1</sub>, lateral chaetac. (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bar: 100 μm.

(Fig. 20B). Unguiculus 6× longer than its width at basis for claw I ( $n=3$ ). Tenent hair as long as width of empodium and 0.4× the length of unguis (internal edge) for claw I ( $n=2$ ).

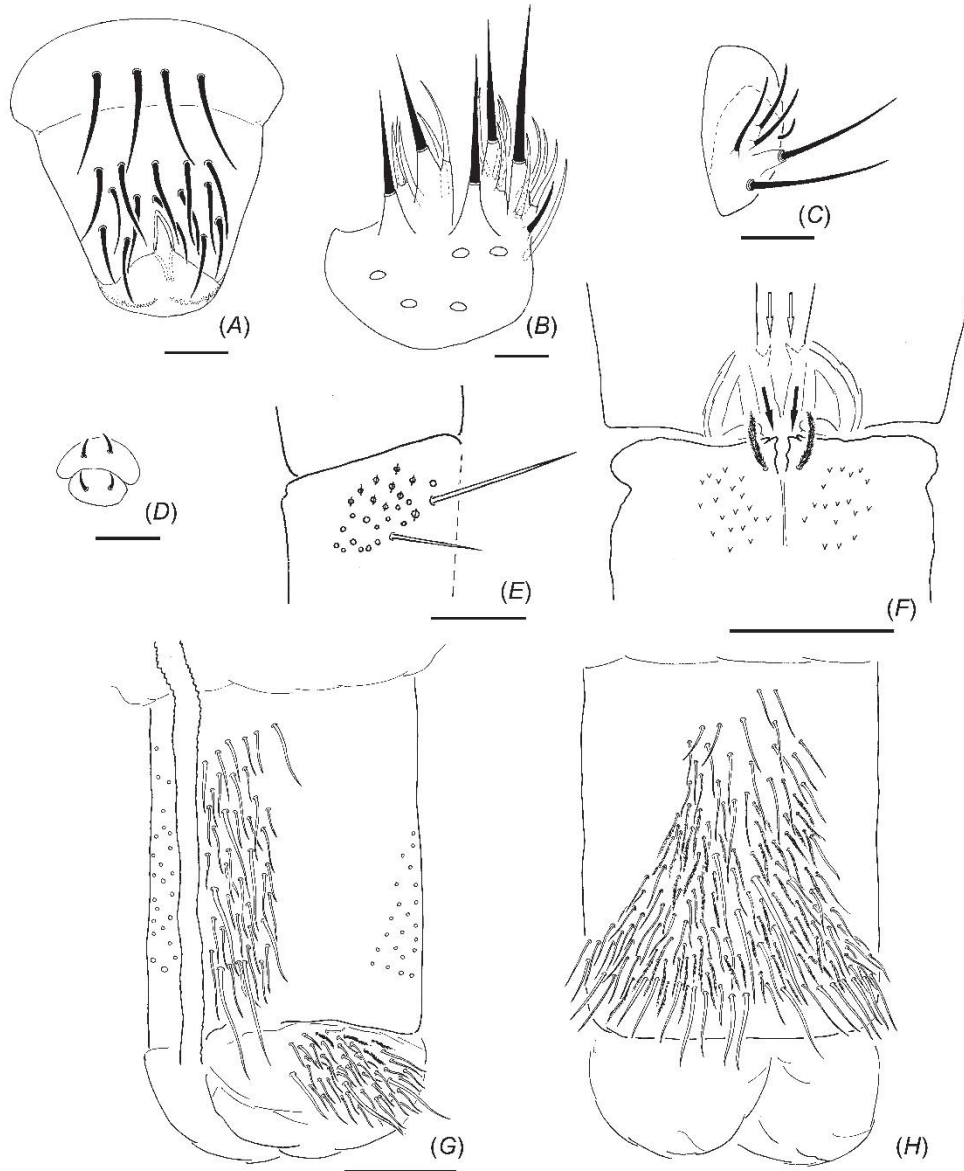
Ventral tube posteriorly with 50–60 serrated and smooth mes, several mac and large smooth mes, with 15–20 scales. Anteriorly with 33–40+33–40 smooth mes (sometimes some weakly serrated), 2+2 smooth mac in distal part, and several large smooth mes. Each latero-distal flap with 45–50 mes, all smooth or with up to two serrated mes.

*Furca* (Fig. 21B, B1, B2). Pseudopores as 6–8 dorso-distally on each side of the manubrium, among distal group of chaetac. Manubrium dorsally with two longitudinal stripes of serrated

mes, each one interspersed with 20–25 smooth pointed mac (Fig. 21B); scales absent between these stripes. Manubrium ventrally without chaetac, except an unpaired ventro-basal serrated and thick mac, and 1+1 apical serrated mes near the axis. Distal manubrial thickening with 2–3 teeth on each side.

Dens non-annulated distal part ~4× as long as mucro (Fig. 21B1). All dental chaetac serrated except 4–6 smooth mac dorsally on basal part of each dens (Fig. 21B). Ventrally dense cover of medium-sized elongated scales, small and rounded on basal part of dens, increasing in size laterally to large, fusiform, pointed apically. Dens thickening with two faint transversal structures. Ratio manubrium:dens:mucro =





**Fig. 15.** *Verhoeffiella cavicola* Absolon, 1900. (A) Labrum; (B) labial palp; (C) maxillary outer lobe; (D) female genital plate; (E) dorso-distal part of the manubrium (crossed circles – pseudopores, circles – serrated mes); (F) ventro-distal part of the manubrium and basal part of dens (v – scales, black arrows – distal manubrial thickening with 2–2 small teeth, hollow arrows – internal spines); (G) ventral tube, anterior view (circles – smooth chaetae); (H) ventral tube, posterior view. Scale bars: 50  $\mu\text{m}$  (A, C, D, E); 20  $\mu\text{m}$  (B); 100  $\mu\text{m}$  (F, G, H).

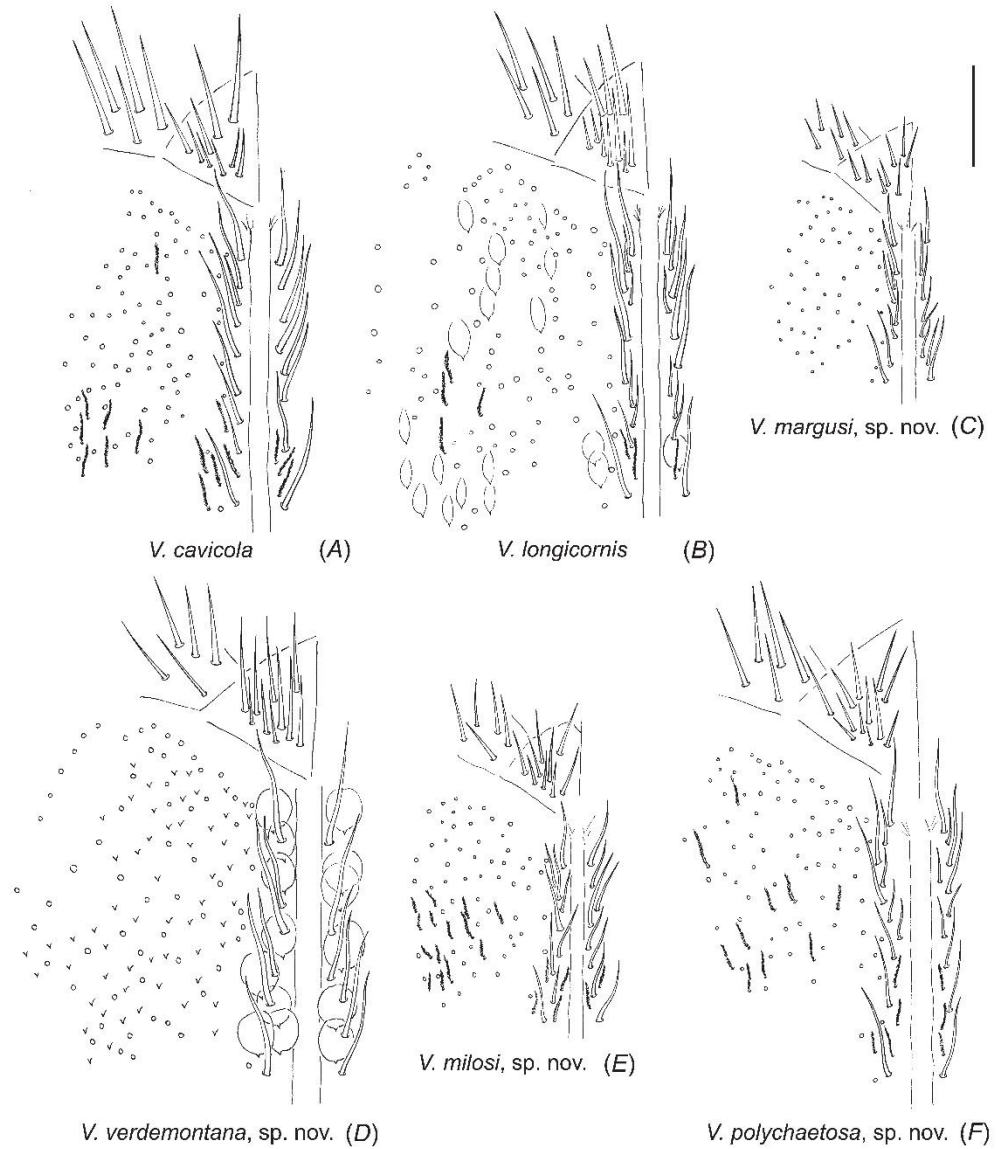


Fig. 16. Head, ventral view, labial triangle, basolateral field, post-labial chaetae, hollow circles – smooth chaetae, v – scales. (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bar: 100  $\mu$ m.

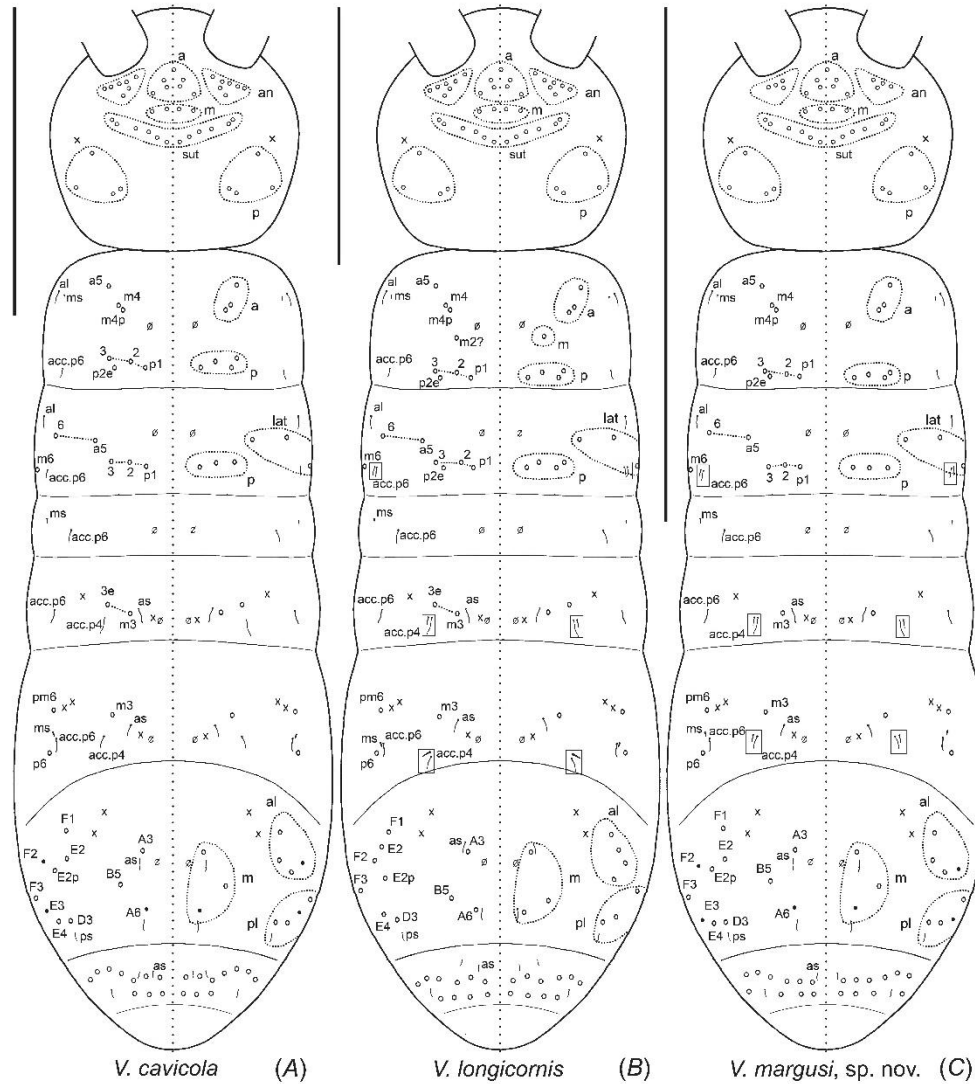
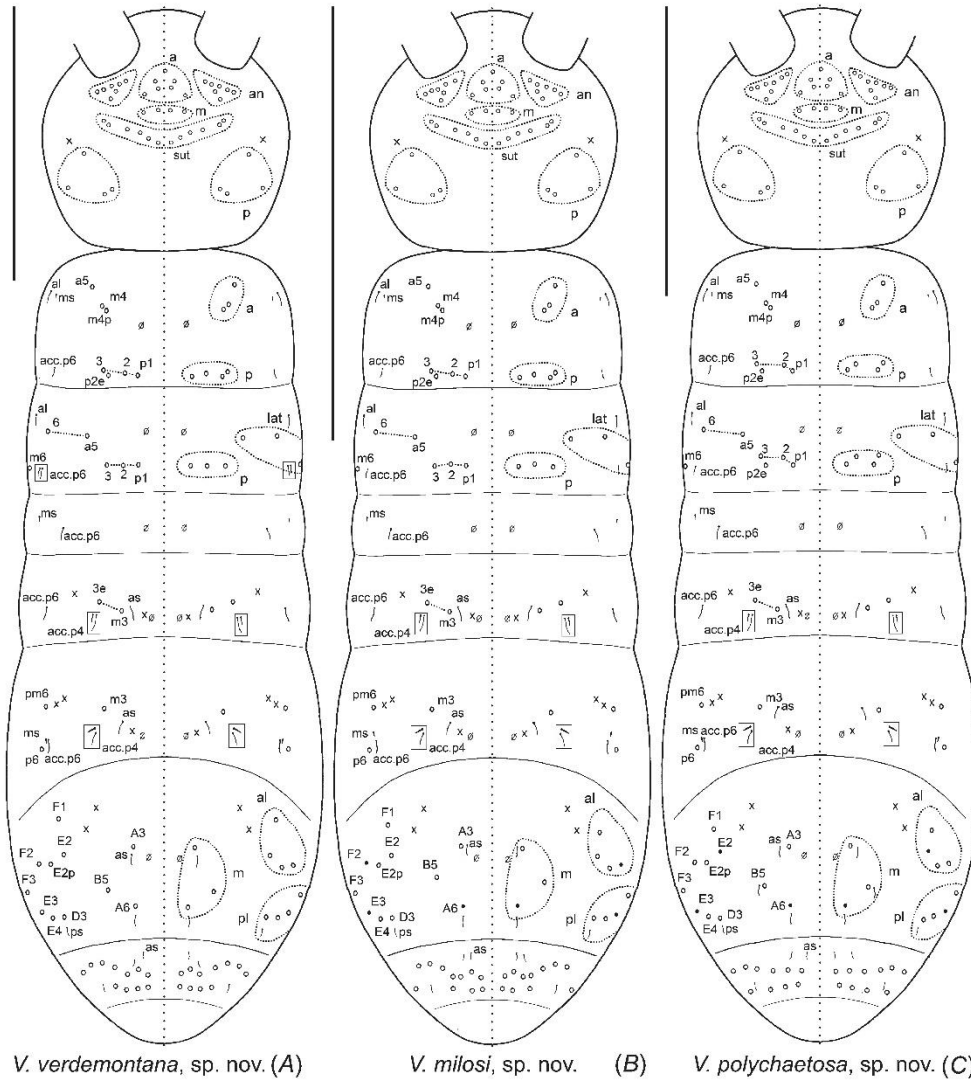
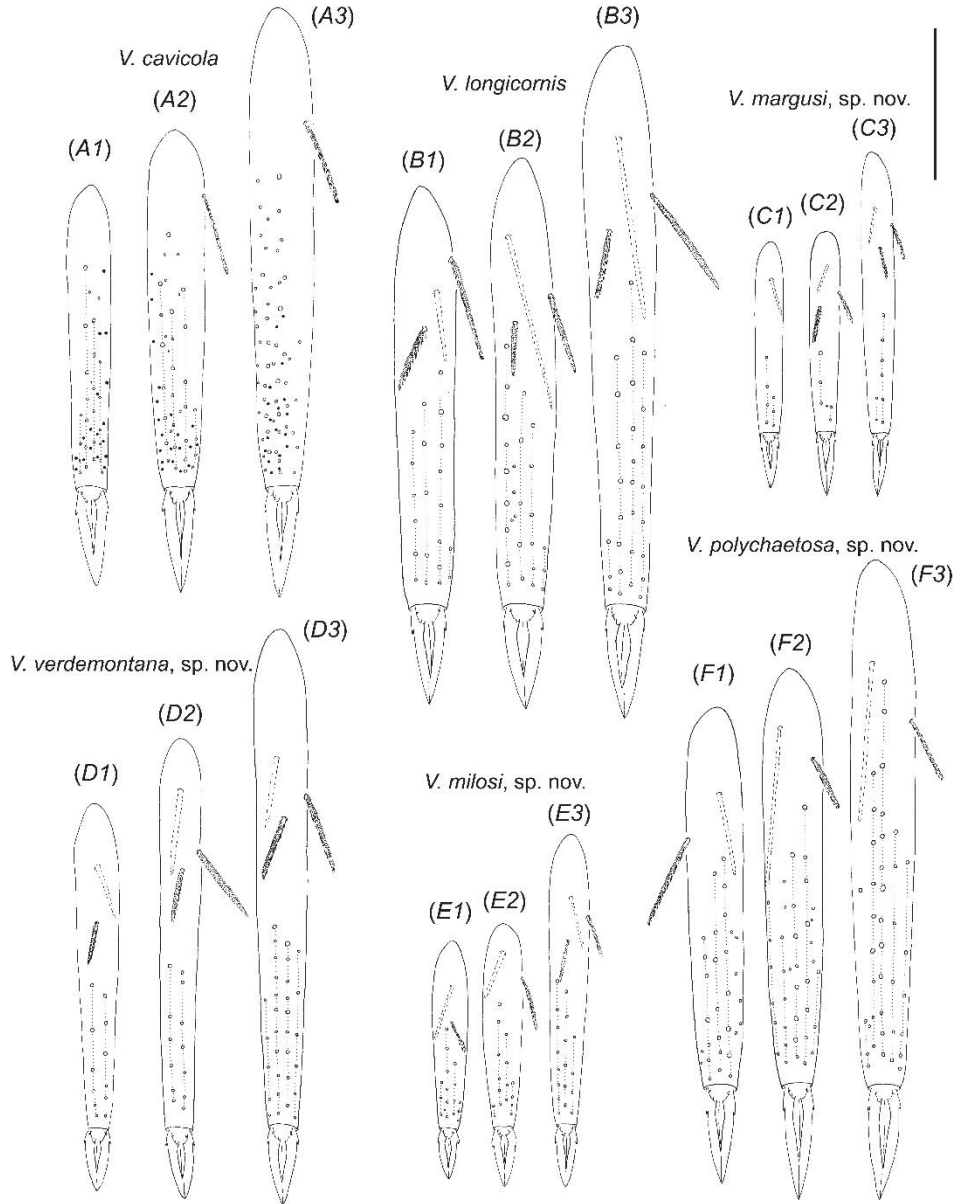


Fig. 17. Dorsal body chaetotaxy. Chaetal groups circled with dotted lines; rectangles – thin serrated mes associated with sens in tandem; circles – mac, black circles – mes, x – trichobothria, crossed circles – pseudopores, lines – S-chaetae and thin serrated mes in tandem; a, anterior mac; an, antennal mac; m, medial mac; lat, lateral mac; sut, sutural mac; p, posterior mac; al, antero-lateral mac; pl, postero-lateral mac; mesochaetae on all tergites and head, S-like chaetae on Abd. IV, anterior fringe on Th. II and mac on Abd. VI not figured: (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov. Scale bars: 1 mm.

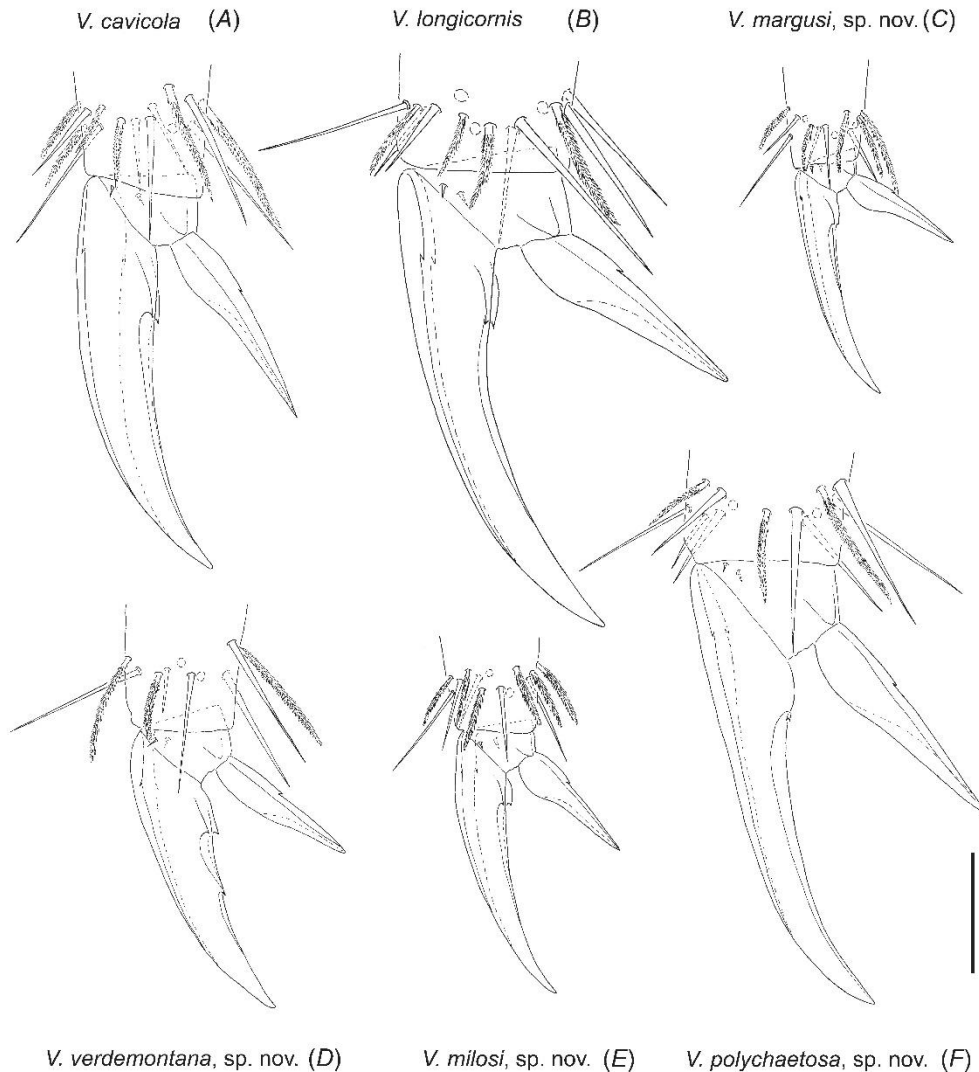


**Fig. 18.** Dorsal body chaetotaxy. Chaetal groups circled with dotted lines; rectangles – thin serrated mes associated with sens in tandem; circles – mac, black circles – mes, x – trichobothria, crossed circles – pseudopores, lines – S-chaetae and thin serrated mes in tandem; a, anterior mac; an, antennal mac; m, medial mac; sut, sutural mac; p, posterior mac; al, antero-lateral mac; pl, postero-lateral mac; msc, mesochaetae on all tergites and head, S-like chaetae on Abd. IV, anterior fringe on Th. II and mac on Abd. VI not figured; (A) *Verhoeffiella verdemontana*, sp. nov.; (B) *V. milosi*, sp. nov.; (C) *V. polychaetosa*, sp. nov. All scale bars: 1 mm.



**Fig. 19.** Tita I, II and III, ventral view, hollow circles – anterior smooth meses, black circles – posterior smooth meses. (A1–A3) *Verhoeffiella cavicola* Absolon, 1900; (B1–B3) *V. longicornis* (Absolon, 1900); (C1–C3) *V. margusi*, sp. nov.; (D1–D3) *V. verdemontana*, sp. nov.; (E1–E3) *V. milosi*, sp. nov.; (F1–F3) *V. polychaetosa*, sp. nov. Scale bar: 250 µm.

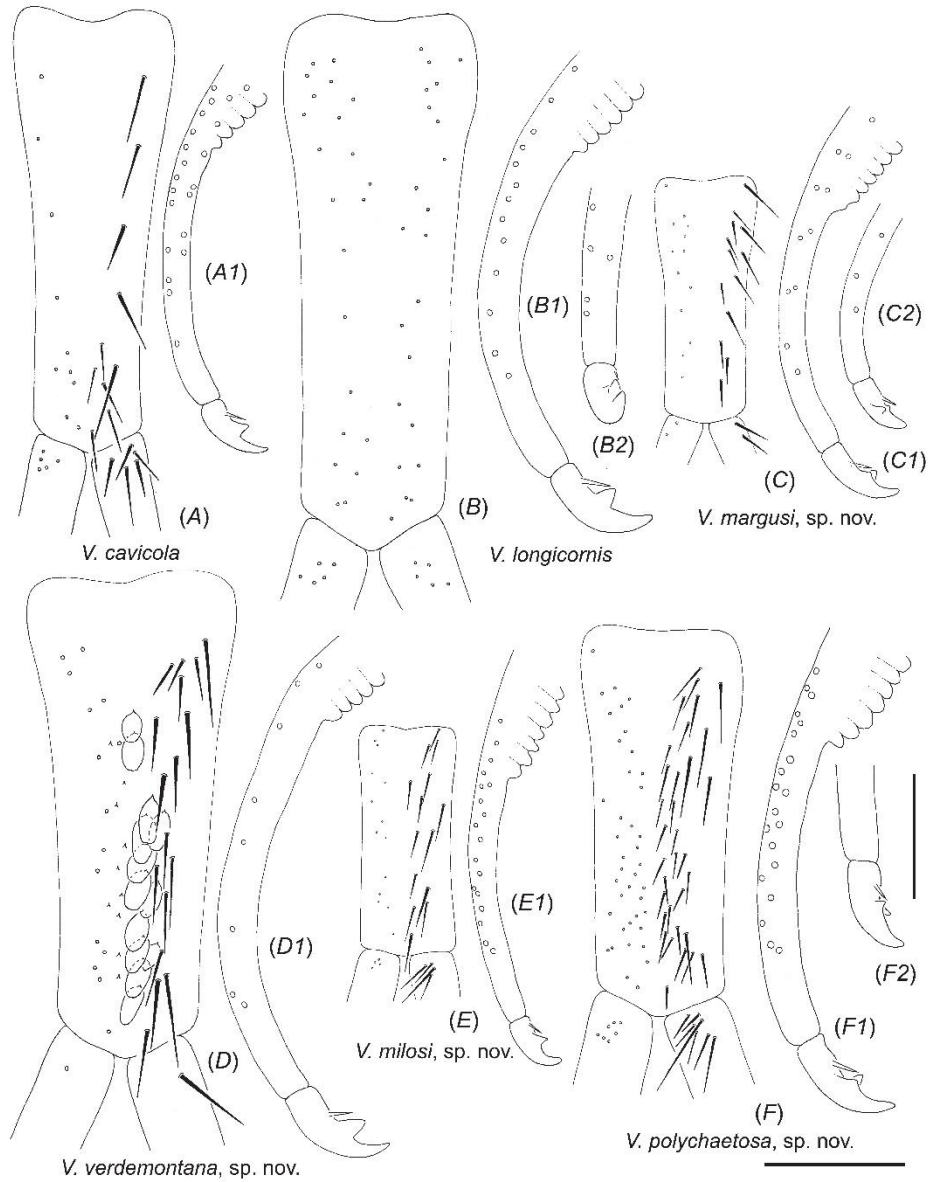




**Fig. 20.** Claw I, posterior view, hollow dotted circles – posterior serrated meses. (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bar: 50  $\mu$ m.

32:42:1. Mucro + dens 8.3 $\times$  longer than unguis III (internal edge) and 1.4 $\times$  longer than Tita III. Mucro usually bidentate, sometimes tridentate (Fig. 21B1, B2). Two of the 10 observed specimens have small third tooth on mucro. Mucro with one basal spine reaching the apex of the proximal tooth, distal tooth elongated (Fig. 21B1).

*Ecology.* The type locality, Pavlova pečina, is a cave with a bat colony and therefore bat guano: entrance 420 m  $\Lambda$ SL; cave length 168 m; air temperature in the deeper parts of the cave 12.7–13.8 $^{\circ}$ C. Specimens were relatively rare and found usually on oligotrophic habitats on wet clay or spelcothems in the deeper part of the cave, but not on guano.



**Fig. 21.** Furca, (A–F) dorsal view on distribution of smooth chaetae and scales on the manubrium and proximal part of dens, hollow circles – anterior smooth chaetae, (A1–F2) – distal part of the dens and mucro, hollow circles – anterior serrated chaetae. (A) *Verhoeffiella cavicola* Absolon, 1900; (B) *V. longicornis* (Absolon, 1900); (C) *V. margusi*, sp. nov.; (D) *V. verdemontana*, sp. nov.; (E) *V. milosi*, sp. nov.; (F) *V. polychaetosa*, sp. nov. Scale bars: vertical bar 50 µm (A1, B1, B2, C1, C2, D1, E1, F1, F2); horizontal bar 250 µm (A, B, C, D, E, F).

#### Remarks

In the species description, only characters that differed and were variable among *Verhoeffiella* species have been given. All other characters are the same as in the type species of the genus, *V. cavicola* (see 'Materials and methods' for more details).

#### Discussion

The original description of *V. longicornis* is very brief, with an illustration of the habitus showing a long-terminal segment resulting from the fusion of antennae III and IV (Absolon 1900, p. 429, fig. 1). The redescription given here confirms that the antennae of the species are extremely long when normal, a character unmatched in other species of the genus. With large head and body length of 3.8–4.2 mm and antennae 2–2.2× longer than head and body length, this species is easily distinguishable from all other species of the genus. *Verhoeffiella longicornis* is similar to *V. verdemontana* and *V. absoloni* in having scales ventrally on head. *Verhoeffiella longicornis* differs from all species of the genus that are described in detail in the following characters: two subapical S-chaetae (type j) on Ant. IV; Ant. IV tip with very few thin serrated mes (type c) and no thin short S-chaetae (type k); one medial mac (m2?) on Th. II, 3, 3, 3 serrated mac on Tita I, II and III and 15–20 scales posteriorly on ventral tube. For other diagnostic characters see Tables 1–3.

#### *Verhoeffiella margusi* Lukić & Deharveng, sp. nov.

(Fig. 1–3, 5–6, 10–17, 19–21)

*Verhoeffiella*, sp. nov. Marguš *et al.*, 2012, pp. 60, 122, 190, 222.

*Verhoeffiella*, sp. nov. Marguš *et al.*, 2013, pp. 107, 122.

ZooBank lsid:9054EA90-39BB-422D-90A4-CAF69F4F4F92

#### Material examined

**Holotype.** Adult ♀ on slide: Croatia: Driš: Oklaj: Punjane, Jazinka (cave) (WGS84 43°58'33.2"N; 16°01'47.0"E; 220 m ASL), M. Lukić leg., 20.ii.2016 (CLL4955).

**Paratypes.** The same data as holotype, 2 adult and 3 subadult ♀, 2 juveniles on slides, 26 ex. in 96% alcohol (CLL4759); 1 ex. for DNA (CA067), 1 ex. in 96% alcohol, M. Lukić leg., 10.x.2010 (CLL2850); 2 ex. for DNA (CA365, CA366), 2 ex. in 96% alcohol, M. Lukić leg., 6.ix.2016 (CLL4731).

**Material deposition.** 1 adult ♀ and 1 subadult ♀ on slide, 3 ex. in 96% alcohol (CLL4759) deposited in MNHN. All other material, including holotype deposited in CBSS.

#### Diagnosis

Head and body length without antennae: 1.9–2.1 mm. Colour pale orange. Scales present on dorsal and ventral side of body, dorsal side of head and Ant. II, on legs, ventrally on manubrium and dens. Antennae 0.8–0.9× as long as (head and body) length; ratio Ant. II: (III+IV)=1:4.09. Pseudopores as 1–2, 2, 2–3 distally on Ant. I, II and III. Ventral sensory field of Ant. Ia with 3–5 S-chaetae of type l and 1–3 S-chaetae of type k; 2–5 S-chaetae (type h) on Ant. Ib, 4–6 on Ant. II; subapical papilla of Ant. IV absent, distal organ of Ant. IV present. Labial triangle with 7–8 smooth chaetae. All post-labial chaetae smooth without interspersed scales. Dorsal macrochaetotaxy as (3+4),(3+3)/0,1,3,(2+0+3+3). Trochanteral organ with 20–25

chaetae. Smooth mac as 3, 1, 1 on femora I, II and III. Serrated mac as 1, 3, 3 on Tita I, II, III; smooth mes as 6–7, 5–6, 8–9 on Tita I, II and III, arranged in four rows. Tenent hair acuminate, smooth; ratio tenent hair: width of empodium for leg I 1.4:1. Unguis thin and elongated, with one pair of inner basal teeth; ratio unguis I length: unguis I width at basis 4.4:1. Manubrium with 2–4 dorso-distal psp, dorsally without scales and 10–13 smooth pointed mac, 1+1 ventro-apical serrated mes; 1–2 basal smooth mac dorsally on dens; mucro bidentate or tridentate with one basal spine.

#### Description

Head and body length combined without antennae: 1.9 to 2.1 mm ( $n=4$ ), 2.0 mm in holotype. Habitus as in Fig. 1F, 2F. Abd. IV 1.6× as long as Abd. III in holotype. Antennae slightly shorter than (head and body) length. Colour pale orange, pigment scattered as orange dots ventrally and dorsally on body, manubrium, coxa, paler on trochanter and femur. Intersegment area, mouth parts, antennae, other leg segments, and dens unpigmented. Body dorsally and laterally with white regular patches on an orange background (Fig. 1F, 2F). Eyes absent, no pigment patch in their place.

**Antennae** (Fig. 3, 5, 6, 10–13). Antennae 0.8 to 0.9× as long as (head and body) length ( $n=3$ ), 0.8× in holotype. Antennae 4.1–4.6× longer than head diagonal ( $n=3$ ). Ant. segment ratio as: Ia: Ib: II: III: IV = 0.13:0.55:1:1.76:2.7 ( $n=5$ ), for holotype 0.17:0.57:1:1.86:2.81; Ant. II: (III+IV) = 1:4.46 ( $n=5$ ), for holotype 1:4.67. Ant. III and IV annulated, except the 1/10 proximal part of Ant. III and the tip of Ant. IV. Pseudopores present ventro-apically on Ant. Ib (1–2), Ant. II (2) and Ant. III (2–3).

Scales numerous dorsally on Ant. Ib, in proximal half of Ant. II (absent in distal half), absent on Ant. Ia, III, IV and ventrally on Ant. Ib.

Ant. Ia dorsally with a group of centro-dorsal chaetae, two of which are probably mic (type g) and others slightly serrated; ventrally with acuminate smooth mes (type f) and sensory field consisting of 3–5 S-chaetae of type l and 1–3 S-chaetae of type k (Fig. 3C).

On Ant. Ib dorsally with one small basal thick slightly serrated mes (type b) and one dorso-latero-internal serrated mac (type a). Externo-apical 2–5 small swollen S-chaetae (type h). Ant. Ib with large number of pointed smooth mes (type e), externo-apical sensory field consisting of 22–27 S-chaetae of type i and of S-chaetae of type k (few short S-chaetae of type k present externo-distally between sensory field and AIO) (Fig. 5C). S-chaetae of type l proximally to sensory field absent.

Ant. II dorsally with thin serrated mes (type c), apical chaetae small serrated mac (type a) and serrated mes (type b) not in row, with an externo-apical group of 4–6 swollen S-chaetae of type h (AIO) (Fig. 6C). Ant. II ventrally with pointed smooth mes (type e), apical chaetae regular serrated mes (type b) not in row, and externo-apical sensory field consisting of 27–34 larger inflated S-chaetae of type i, one chaetae of type j and of S-chaetae of type k (1–2 short S-chaetae of type k always present externo-distally between sensory field and AIO) (Fig. 6C). Basal mic present (one dorsal, two ventral, one latero-external, one latero-internal).



**Table 1. Principal differential characters previously used between species of the genus *Verhoeffiella*: antennae and dorsal chaetotaxy**  
 Antennal segment ratio, values between brackets when given together for (Ant I+II) or for fused segments; '?', unknown

Species	Body+head length (mm)	Ant.: body ratio	Ant. segment ratio	Ant. (III+IV): II	Ant.: head diagonal	Abd. IV: III	Dorsal macrochaeta formula
<i>absoloni</i> <sup>G</sup>	2.7–3.2	0.54–0.68	0.15:0.61:1:1.5:2.0	3.5	3	1.5	(3+0+4)(3+3)/ 0.2.3.(3+0+4+4)
<i>anagastumenensis</i> <sup>F</sup>	4.35	Ant.: body = 1.34:1	(0.7):1:2.64:3.67	6.31	5	?	?
<i>cavicol</i> <sup>I</sup>	3.2–3.6	0.9–1.0	0.1:0.61:1:1.78:2.71	4.49	4.7	1.6–1.9	(3+0+4)(3+3)/ 0.2.3.(2+0+3+3)
<i>constantinellus</i> <sup>D</sup>	5.06	Shorter than body	(0.21):1:(3.62)	3.62	2.4	?	?
<i>constantinus</i> <sup>E</sup>	3.98	Equal to body	0.13:0.53:1:2.13:2.47	4.6	4.7	?	?
<i>dallai</i> <sup>H</sup>	2.5–3.0	Shorter than body	0.23:0.67:1:1.27:2.77	4.04	2.5	2.0; 2.8–3.0 <sup>I</sup>	?
<i>gomae</i> <sup>G</sup>	2.2	0.85	0.12:0.5:1:1.25:3.1	4.35	4.5	2	(3+0+4)(3+3)/ 0.2.3.(4+2+3+2)
<i>hispanica</i> <sup>A, B, H</sup>	4	Shorter than body	(0.8):1:2.3:2.4	4.7	4.3	1.5	(?+0+4)(≥1+3)/ 0.3.3.(3+0+?+?)
<i>longicornis</i> <sup>I</sup>	3.8–4.2	2–2.2	0.08:0.62:1:2.66:5.85	8.51	13.8	1.3	(3+1+4)(3+4)/ 0.2.3.(3+0+2–4?+2–4?)
cf. <i>longicornis</i> (Spanish records) <sup>B</sup>	?	Ant III–IV not Longer than body	?	?	?	?	(?+0+3)(≥1+2)/ 0.1.0.(1+0+?+?)
<i>margusi</i> , sp. nov. <sup>I</sup>	1.9–2.1	0.8–0.9	0.13:0.55:1:1.76:2.7	4.46	4.1–4.6	1.6	(3+0+4)(3+3)/ 0.1.3.(2+0+3+3)
<i>medius</i> <sup>C</sup>	1.6–2.1	Shorter than body	0.13:0.5:1:1.75:2.63	4.38	?	?	(3+0+4)(≥1+3)/ 0.2. ≥1.(3+0+ ≥2+ ≥2)
<i>mitosi</i> , sp. nov. <sup>I</sup>	2.2–2.5	0.6	0.15:0.59:1:1.57:2.3	3.87	3.4	1.6	(3+0+4)(3+3) 0.2.3.(2+0+3+3)
<i>polycchaetosa</i> , sp. nov. <sup>I</sup>	3.5–3.6	1.1–1.2	0.09:0.63:1:2.12:2.96	5.08	5.5–5.7	1.4–1.7	(3+0+4)(3+4)/ 0.2.3.(2+0+3+3)
<i>verdemontana</i> , sp. nov. <sup>I</sup>	3.4–4.0	0.9–1.2	0.09:0.65:1:1.96:2.59	4.55	6.4	1.5–1.8	(3+0+4)(3+3)/ 0.2.3.(3+0+4+3–4)

(continued next page)

**Table 1.** (continued): legs and furca

	Pair of basal teeth on unguis	Inner unpaired tooth on unguis	Outer tooth on unguis	Unguis: unguiculus ratio	(micro+dens): manubrium
<i>absoloni</i>	+	At 50% from its base	At 50% from its base	1.6	1.45
<i>anagastimensis</i>	Single tooth	?	–	?	1.3
<i>cavicola</i>	At 21, 24, 26% from its base for unguis I, II, III	–	At 35% from its base	1.5, 1.5, 1.3 for claws I, II, III	1.6
<i>constantinellus</i>	Single tooth	?	–	?	1.35
<i>constantius</i>	Single tooth	?	–	1.4	1.25
<i>dallaii</i>	+	At 50% from its base	+	1.4	1.4
<i>ganuae</i>	+	At 40% from its base	+	1.2	1.8
<i>hispanica</i>	+	–	Strong/small, not always visible	1.3	1.2
<i>longicornis</i>	At 19, 23, 25% from its base for unguis I, II, III	–	At 33% from its base	1.7, 1.6, 1.4 for claws I, II, III	1.34
cf. <i>longicornis</i> (Spanish records)	–	–	?	?	?
<i>margasi</i> , sp. nov.	–	–	–	–	–
<i>medius</i>	At 14, 15, 17% from its base for unguis I, II, III	–	At 35% from its base	1.6, 1.5, 1.4 for claws I, II, III	1.52
<i>mitosi</i> , sp. nov.	Small basal teeth	–	At 50% from its base	?	1.65
	At 14, 16, 19% from its base for unguis I, II, III	–	At 32% from its base	1.5, 1.5, 1.4 for claws I, II, III	1.45
<i>polychaetosus</i> , sp. nov.	At 18, 20, 24% from its base for unguis I, II, III	–	At 35% from its base	1.5, 1.4, 1.3 for claws I, II, III	1.44
<i>verdemontana</i> , sp. nov.	At 21, 21, 25% from its base for unguis I, II, III	At 50% from its base	At 43% from its base	1.5, 1.5, 1.5 for claws I, II, III	1.47

<sup>A</sup>Bonet (1931).

<sup>B</sup>da Gama (1984).

<sup>C</sup>Loksa and Bogojević (1967).

<sup>D</sup>Lukić *et al.* (2007b).

<sup>E</sup>Lukić *et al.* (2007a).

<sup>F</sup>Lukić *et al.* (2008).

<sup>G</sup>Lukić *et al.* (2015).

<sup>H</sup>Nosek and Paoletti (1985).

<sup>I</sup>Our observation.

<sup>J</sup>Contradictory statements given for the ratio Abd. III and IV in the original description of *V. dallaii* as 'nearly two times' and '2.8–3 times' elsewhere in the text.

**Table 2. Differential characters between all *Verhoeffiella* species that are described and redescribed in detail (after Lukić *et al.* 2015; and our observation)**  
 Discriminant values (see 'Discussion'): I, high differential value; II, low interspecific and intraspecific variation with exceptions; III, larger overlapping ranges

Character	Discriminant value	<i>absoloni</i>	<i>cavicola</i>	<i>longicornis</i>	<i>margasi</i> , sp. nov.	<i>milosi</i> , sp. nov.	<i>polychaetosa</i> , sp. nov.	<i>verdantiana</i> , sp. nov.
psp on Ant. I, II, III	III	2-5, 8-14, 5	4-6, 6-10, 4-6	2-3, 6-7, 4-5	1-2, 2, 2-3	1-2, 2-4, 3-4	3-4, 3-5, 3-4	4-6, 10-15, 5
S-chaetae (type l) in sensory field of Ant. Ia	I	10-12	10-17	12-14	3-5	5-8	12-15	16-17
S-chaetae (type k) in sensory field of Ant. Ia	I	3-4	4-7	2	1-3	3-4	2-3	8
S-chaetae (type h) on Ant. Ib, II	I	2, 4-7	~5-7	4, 7-11	2-5, 4-6	~3-4	3-7, 6-10	~7-10
S-chaetae (type i) in sensory field of Ant. Ib, II, III	III	14-17, 25-30, 20-30	15-30, 15-25, 13-20	17-25, 20-30, 20-25	22-27, 27-34, 20-25	8-12, 10-12	20-25, 20-30, 20-30	20-40, 20-30, 20-25
S-chaetae (type j) of AIIIO	II	1	1	1	-	1	1	1
External S-chaetae (type k) of AIIIO	II	1	1	1-2	1	1	1	1
S-chaetae (type j) on Ant. IV	II	1	1	2	1	1	1	1
Subapical papilla of Ant. IV	II	+	+	+	-	+	+	+
Distal organ of Ant. IV	I	+	-	+	+	+	+	+
Mes on clypeus	II	45-50	50-60	40-45	16-17	30-35	40-50	50-55
Serrated mes on clypeus	II	10-15	2-3	15-20	-	3-4	-	15-20
Labial triangle chaetae	III	9-12	9-11	8-10	7-8	7-12	9-17	10-11
Post-labial chaetae	III	10+10	(9-11)+(9-11)	(10-11)+(10-11)	(8-10)+(8-10)	(8-11)+(8-11)	(11-13)+(11-13)	6+6
Proximal serrated post-labial chaetae	I	+	(3-4)+(3-4)	(1-3)+(1-3)	-	(1-2)+(1-2)	(2-4)+(2-4)	-
Serrated mes ventrally on head	II	+	+	+	-	+	+	+(few)
Sens formula	II	2,2/1,3,3,3,3	2,2/1,3,3,3,3	2,2/1,3,3,3,3	2,2/1,3,3,3,2-3	2,2/1,3,3,3,3	2,2/1,3,3,3,3-4,3	2,2/1,3,3,3,3,3
Tandem on Th. III, Abd. II, Abd. III	II	1,1,1	~,-,-	1,1,1	1,1,1	~,-,-	~,-,-	1,1,1
Unguis I length: unguis I width at basis	I	2.7	3.5	3.4	4.4	3.4	3.2	2.8
Unguis I length: unguis I width at basis	III	5.1	7.2	6.1	6.3	6	7	5.7
Tenent hair: width of empodium for leg I	I	1.2	1.5	1	1.4	1.2	1	1.8
Serrated mac on Tita I, II, III	I	1,3,3	~1,1	3,3,3	1,3,3	2,2-3,3	2,2,2	2,3,3
Smooth mes/rows on Tita I, II, III	I	7-82, 6-102, 11-142	33-43, >40-53, >45-71, not in rows	20-23/4, 23-27/4, 23-28/4	6-7/2, 5-6/2, 8-9/2	12-17/4, 10-163/5, 12-18/3	29-31/3-4, 33-38/5, 32-39/5	8-12/2, 9-13/2, 19-25/3-4
Smooth mac on femora I, II, III	I	1-2, 1-2, 1	3, 2, 1	3, 3, 4, 1-2	3, 1, 1	2, 2, 1	3, 2, 1	3, 1, 1
Smooth mes on femora I, II, III	III	1	1, II, III	1, II, III	-	1	1, II, III	-
Chaetae of trochanteral organ	I	55-100	50-60	55-65	20-25	35-40	40-45	70-80
Mes posteriorly on ventral tube	I	120-130	150-160	55-60	25-30	40-45	120-150	110-130
Mes anteriorly on ventral tube	I	46-49	45-75	33-40	12-15	20-26	28-30	30-37
Mes on latero-distal flap	I	40-45	55-60	43-50	14-16	23-30	55-60	35-43
Serrated mes on latero-distal flap	I	12	5-9	0-2	-	0-1	6-8	8-10
psp on manubrium (one side)	I	6-8	10-14	6-8	2-4	4-8	10-12	10-13
Smooth mac dorsally on manubrium (one side)	I	12-16	9-12	20-25	10-13	14-15	33-46	13-17
Apical serrated mes ventrally on manubrium	II	2+2	1+1	1+1	1+1	1+1	2+2	2+2
Smooth mac dorsally on dens	I	1	2-5	4-6	1-2	3-4	7-9	1
Mucro	II	bidentate	bidentate	bidentate (n=8)/tridentate (n=2)	bidentate (n=3)/tridentate (n=3)	bidentate	bidentate (n=2)/tridentate (n=4)	bidentate

**Table 3. Distribution of scales between all species of the genus *Verhoeffiella* that are described and redescribed in details (after Lukić *et al.* 2015; and our observation)**

Character	<i>absoloni</i>	<i>cavicola</i>	<i>longicornis</i>	<i>margusi</i> , sp. nov.	<i>milosi</i> , sp. nov.	<i>polychaetosa</i> , sp. nov.	<i>verdemontana</i> , sp. nov.
Dorsally on Ant. II	Whole length, absent in most distal part	Many in proximal half, absent in distal half	Whole length, absent in most distal part	Whole length, absent in most distal part	Whole length, absent in most distal part	Whole length, absent in most distal part	Whole length, absent in most distal part
Post-labial area	Interspersed with mes	—	One scale present proximally	—	—	—	Interspersed with mes
Ventrally on head	+	—	+	—	—	—	+
Tita I, II, III	Few proximally on Tita I, numerous in proximal half on Tita II, numerous on whole length of Tita III	Absent on Tita I and II, few proximally on Tita III	Absent on Tita I, few proximally on Tita II, III	Absent on Tita I and II, few proximally on Tita III	Absent on Tita I and II, numerous in proximal half on Tita III	Absent on Tita I and II, numerous in proximal half on Tita III	Few proximally on Tita I and II, numerous on whole length of Tita III
Femora I, II, III	Numerous on whole length of femora I, II, III	Present in proximal half on femur I, numerous on whole length of femora II and III	Numerous on whole length of femora I, II, III	Few on whole length of femur I, numerous on whole length of femora II and III	Numerous on whole length of femora I, II, III	Numerous on whole length of femora I, II, III	Numerous on whole length of femora I, II, III
Trochanters I, II, III	Few on trochanters I, II, numerous on trochanter III	Few on trochanters I, II, numerous on trochanter III	Numerous on trochanters I, II, III	Absent on trochanters I, II, numerous on trochanter III	Absent on trochanters I, II, numerous on trochanter III	Absent on trochanter I, few on trochanter II, numerous on trochanter III	Numerous on trochanters I, II, III
Posteriorly on ventral tube	0-1	—	15-20	—	—	—	1-3
Dorsally on manubrium	+	—	—	—	—	—	+

AIII located dorso-externo-apically, with two swollen h-type S-chaetae side by side, surrounded internally by one thin short k-type S-chaeta and externally by one thin short k-type S-chaeta (Fig. 10C); a few additional S-chaetae of k-type below AIII absent (Fig. 10C). Ant. III ventro-externo-distally with sensory field consisting of 20–25 S-chaetae of i-type, one small pointed j-type chaetae and of k-type chaetae (Fig. 11C). Ant. IV with numerous thick subcylindrical i-type S-chaetae distributed along the whole length and one large subapical j-type S-chaeta (Fig. 12C). Ant. IV tip with very few thick serrated b-type meses and with no subapical papilla (Fig. 12C). Distal organ of Ant. IV well developed, inserted deep into antenna with one cuticular channel opening to the surface cuticle (Fig. 13C).

**Mouthparts** (Fig. 14C, 16C). Clypeus with 16–17 smooth meses arranged symmetrically (Fig. 14C). Ventro-distally on labrum two unequal combs, each with one apical row of ~13 and 17 teeth respectively, and a pair of sinuous rods. Labial triangle with 7–8 chaetae, all smooth and without scales (Fig. 16C). Post-labial chaetae (8–10) + (8–10), all smooth, without interspersed scales.

**Head and body** (Fig. 17C). Head dorsally with stable macrochaetae arrangement as in Fig. 17C; antennal mac (3–4) +1; laterally with scales mixed with medium-sized serrated and smooth meses; ventrally without scales, with only smooth meses.

Dorsal chaetotaxy of the tergites and head illustrated in Fig. 17C. Ms formula 1,0/1,0,1,0,0; sens formula 2,2/1,3,3,3,2–3. Number of sens on Abd. V usually two (one lateral + one medial;  $n=10$ ), sometimes three (one lateral + two medial;  $n=1$ ) or one (one lateral + none medial;  $n=1$ ). Macrochaeta formula (3+0+4), (3+3)/0,1,3,(2+0+3+3). Macrochaeta arrangement stable, except variation in lateral mac on Abd. IV; Th. II with three anterior and four posterior mac; Th. III with three posterior and three lateral mac; Abd. II with one medial mac; Abd. IV with two medial (A6 is mes), three antero-lateral and three postero-lateral mac (F2 and E3 are absent or mes); Abd. V with 9 + 9 mac; Abd. VI with 8–10 mac. On Abd. IV 23–27 + 23–27 S-like chaetae. Thin serrated meses associated with sens in tandem on Th. III, Abd. II and Abd. III.

**Legs** (Fig. 19, 20). Subcoxa I with 2–3 dorso-posterior serrated mac. Subcoxa II with 4–5 dorso-anterior serrated chaetae ranging in size from mac to mes and 3–4 dorso-posterior serrated mac. Subcoxa III with group of three dorso-posterior serrated mac and some serrated meses.

Coxae with pseudopores as 1, 3, 2 on coxae I, II and III. Smooth mac on dorso-anterior side of coxae I, II and III not observable; ~10 dorso-posterior serrated and smooth chaetae on coxa I not in multiplet, two multiplets (dorso-anterior one and dorso-posterior one) of 2–5 serrated chaetae on coxa II. Dorsally on coxa III, a transversal line of three mac sockets and 4–5 mes sockets.

Trochanter I with 15–19 smooth chaetae, trochanter II with 8–10, trochanter II with 4–5, and trochanteral organ consisting of 20–25 shorter, straight, spine-like meses. Many scales on trochanter III (not observable for trochanter I and II).

Femur with many serrated meses, serrated mac and smooth mac. A few scales present in proximal third on femur I, numerous on whole length of femora II and III. Ventrally, with 3, 1, 1 smooth macrochaetae on femora I, II and III, additional smooth meses on femora absent. Largest smooth mac 0.29–0.31 × length of femur.

Distal whorl of Tita with 11–14 chaetae including 4–5, 4, 4–5 smooth meses and one tenent hair on legs I, II and III (Fig. 20C). Serrated mac as 1, 3, 3 on Tita I, II and III (Fig. 19C1, C2, C3). Besides the apical ones, smooth meses present ventrally on Tita I, II and III arranged in two rows. In total, 6–7, 5–6, 8–9 smooth meses in two rows on Tita I, II and III (Fig. 19C1, C2, C3). Scales absent on Tita I and II, a few scales present in proximal part of Tita III. Tita of leg III longer than those of legs I and II; length 0.31, 0.32, 0.47 mm for Tita I, II and III ( $n=2$ ).

Unguis with one inner pair of equal basal teeth at 14, 15, 17% from its base for unguis I, II and III ( $n=4$ ) (Fig. 20C), inner unpaired tooth absent. Unguis (internal edge) 1.6, 1.5, 1.4 × longer than unguiculus for claws I, II and III ( $n=4$ ). Unguis (internal edge) 4.4 × longer than width of unguis at basis for claw I ( $n=4$ ), 4.4 in holotype. Unguiculus robust, pointed, 0.63–0.68 × as long as inner edge of unguis for claws I and II, 0.73 × for claw III (Fig. 20C). Small outer tooth, sometimes difficult to see at 35% from its base ( $n=3$ ) on antero-external lamella of unguiculus I, II and postero-external lamella of unguiculus III (Fig. 20C). Unguiculus 6.3 × longer than its width at basis for claw I ( $n=4$ ). Tenent hair 1.4 × longer than width of empodium and 0.5 × the length of unguis (internal edge) for claw I ( $n=3$ ).

Ventral tube posteriorly with 25–30 weakly serrated and smooth meses, with large smooth meses, and no scales. Anteriorly with 12–15 + 12–15 smooth meses (sometimes some weakly serrated present), and several large smooth meses. Each latero-distal flap with 14–16 smooth meses.

**Furca** (Fig. 21C, C1, C2). Pseudopores as 2–4 dorso-distally on each side of the manubrium, among distal group of chaetae. Manubrium dorsally with two longitudinal stripes of serrated meses, each one interspersed with 10–13 smooth pointed mac (Fig. 21C); scales absent between these stripes. Manubrium ventrally without chaetae, except an unpaired ventro-basal serrated and thick mac and 1 + 1 apical serrated meses near the axis. Distal manubrial thickening with one tooth on each side.

Dens non-annulated distal part ~4 × as long as mucro (Fig. 21C1). All dental chaetae serrated except 1–2 smooth mac dorsally on basal part of each dens (Fig. 21C). Ventrally dense cover of medium-sized elongated scales, small and rounded on basal part of dens, increasing in size laterally to large, fusiform, pointed apically. Dens thickening with two faint transversal structures. Ratio manubrium : dens : mucro = 17 : 25 : 1. Mucro + dens 8 × longer than unguis III (internal edge) and 1.4 × longer than Tita III. Mucro usually tridentate, sometimes bidentate (Fig. 21C1, C2). Three of the six observed specimens had a small third tooth on mucro. Mucro with one basal spine reaching the apex of the proximal tooth, distal tooth elongated (Fig. 21C1).

**Ecology.** The type locality is the small cave Jazinka, which has three chambers with tight passages between them: entrance 220 m ASL; cave length 40 m; air temperature in the last chamber 12.5 °C. Numerous collecting trips and a two-year monitoring protocol of cave fauna were conducted in this cave (in 2015 and 2016). During monitoring, the cave was visited every three months (once in each season) and the species was found every time. The species was present only at the end of the last chamber, always in oligotrophic habitat



on wet clay, rocks and near water puddles, but never on decaying wood. The species was always found in small numbers of up to 6–7 specimens, except once in February 2016 when 34 specimens were found.

#### Discussion

With head and body length of 1.9–2.1 mm, *Verhoeffiella margusi*, sp. nov. is one of the smallest species of the genus (*V. media* body length is 1.6–2.1 mm (Loksa and Bogojević 1967)). It is similar to *V. verdemontana* in serrated proximal post-labial chaetae and the absence of smooth meses on femora I, II and III. It is similar to *V. longicornis* and *V. polychaetosa* as it sometimes has tridentate mucro. *Verhoeffiella margusi* differs from all species of the genus that are described in detail in the following characters: external S-chaetae (type j) of AIII absent; S-chaetae (type i) in sensory field of Ant. Ib, II, III more inflated and relatively large compared with small body size; subapical papilla of Ant. IV absent; most developed distal organ of Ant. IV (Fig. 12C); Abd. II with one mac; reduced number of antennal mac ((3–4)+1, with some meses present); reduced number of smooth meses on Tita, arranged in two rows (6–7, 5–6, 8–9 smooth meses on Tita I, II, III). For other diagnostic characters see Tables 1–3.

#### Remarks

In the species description, only characters that differed and were variable among *Verhoeffiella* species have been given. All other characters are the same as in type species of the genus, *V. cavicola* (see 'Materials and methods' for more details).

#### Etymology

The species is named after Drago Marguš, expert leader of National Park Krka where the type locality is positioned, for his essential contribution and support to the research and protection of subterranean biodiversity of the wider area of National Park Krka.

#### *Verhoeffiella verdemontana* Lukić & Deharveng, sp. nov.

(Fig. 1–3, 5–6, 10–16, 18–21)

ZooBank lsid:3BC79DAF-E624-4FA4-979D-103D35CFF6B8

#### Material examined

**Holotype.** Adult ♂ on slide: Bosnia and Herzegovina: Trebinje: Bihovo, Pavlova pečina (cave) (WGS84 42°39'59.0"N; 18°18'28.0"E; 420 m ASL), M. Lukić leg., 30.iii.2012 (CLL4956).

**Paratypes.** The same data as holotype, 2 subadult (1 ♂ and 1 unknown sex), N. Kuharić leg., 25.x.2015 (CLL4780); 1 subadult ♂, 1 adult ♀ on slides, 5 ex. for DNA (4028D01, 4028D02, 4028D03, 4028D04, 4028D05), M. Lukić leg., 19.iv.2010 (CLL2282); 1 subadult ♀ on slide, 5 ex. in 96% alcohol, M. Lukić leg., 30.iii.2012 (CLL4228); 1 subadult ♀, 1 subadult ♂ on slides, M. Lukić and A. Kirin leg., 6.xi.2015 (CLL4796); 1 ex. for DNA (CA234), M. Lukić, A. Kirin and J. Jakupić leg., 6.xi.2015 (CLL4753).

**Material deposition.** 1 subadult ♀ on slide (CLL4228); 1 subadult ♀ on slide (CLL4796) deposited in MNHN. All other material, including holotype deposited in CBSS.

#### Diagnosis

Head and body length without antennae: 3.4–4.0 mm. Colour pale orange. Scales present on dorsal and ventral side of head and body, dorsal side of Ant. II, on legs, ventrally and dorsally on manubrium and ventrally on dens. Antennae 0.9–1.2× as long as (head and body) length; ratio Ant. II : (III+IV) 1 : 4.55. Pseudopores as 4–6, 10–15, 5 distally on Ant. I, II and III. Ventral sensory field of Ant. Ia with 16–17 S-chaetae of type l and eight S-chaetae of type k; S-chaetae (type h) absent on Ant. Ib, 7–10 on Ant. II; subapical papilla of Ant. IV present, distal organ of Ant. IV absent. Labial triangle with 10–11 smooth chaetae. All post-labial chaetae smooth and interspersed with scales. Dorsal macrochaetotaxy as (3+4),(3+3)/0,2,3,(3+0+4+3-4). Trochanteral organ with 70–80 chaetae. Smooth mac as 3, 1, 1 on femora I, II and III. Serrated mac as 2, 3, 3 on Tita I, II and III; smooth meses as 8–12, 9–13 on Tita I and II respectively, arranged in two rows, 19–25 smooth meses on Tita III arranged in 3–4 rows. Tenent hair acuminate, smooth; ratio tenent hair : width of empodium for leg I 1.8 : 1. Unguis thin and elongated, with one pair of inner basal teeth; ratio unguis I length : unguis I width at basis 2.8 : 1. Manubrium with 10–13 dorso-distal pseudopores, dorsally without scales and 13–17 smooth pointed mac, 2 + 2 ventro-apical serrated meses; one basal smooth mac dorsally on dens; mucro bidentate with one basal spine.

#### Description

Head and body length combined without antennae: 3.4 to 4.0 mm ( $n=4$ ), 3.6 mm in holotype. Habitus as in Fig. 1C, 2C. Abd. IV 1.5–1.8× as long as Abd. III ( $n=3$ ), 1.8 in holotype. Antennae equal to (head and body) length. Colour pale orange, pigment scattered as orange dots ventrally and dorsally on body, Ant. I and II, manubrium, coxa, anterior and posterior part of ventral tube paler on trochanter and femur. Intersegment area, mouth parts, antennae, other Ant. and leg segments, and dens unpigmented. Body dorsally and laterally with white regular patches on an orange background (Fig. 1C, 2C). Eyes absent, no pigment patch in their place.

**Antennae** (Fig. 3, 5, 6, 10–13). Antennae 0.9 to 1.2× as long as (head and body) length ( $n=3$ ), 1.0× in holotype. Antennae 6.4× longer than head diagonal in holotype. Ant. segment ratio as: Ia : Ib : II : III : IV = 0.09 : 0.65 : 1 : 1.96 : 2.59; Ant. II : (III+IV) = 1 : 4.55 ( $n=6$ ), Ant. III and IV fused in holotype. Ant. III and IV annulated, except the 1/4 proximal part of Ant. III and the tip of Ant. IV. Pseudopores present ventro-apically on Ant. Ib (4–6), Ant. II (10–15) and Ant. III (5).

Scales numerous dorsally on Ant. Ib, distributed on the whole length of Ant. II (except in most distal part where absent), sometimes very few scales present ventrally on Ant. Ib, absent on Ant. Ia, III and IV.

Ant. Ia dorsally with two basal mic (type g) and thick serrated meses (type b); ventrally with acuminate smooth meses (type f) and sensory field consisting of 16–17 S-chaetae of type l and eight S-chaetae of type k (Fig. 3D).

On Ant. Ib dorsally with one small, basal, thick, slightly serrated mes (type b) and two dorso-latero-internal serrated mac (type a). Externo-apical, small, swollen S-chaetae (type h) absent. Ant. Ib ventrally with large number of pointed smooth meses (type e), ventro-external sensory field consisting

of S-chaetae of type k and 20–40 S-chaetae of type i. A few S-chaetae of type k always present externo-apically to sensory field (Fig. 5D). S-chaetae of type l proximally to sensory field present, located just above Ant. Ia sensory field.

Ant. II dorsally with thin serrated mes (type c), apical row of small serrated mac (type a) and serrated mes (type b), with an externo-apical group of 7–10 swollen S-chaetae of type h (AIIO) (Fig. 6D). Ant. II ventrally with pointed smooth mes (type e), distal row of smooth mac (type d) and serrated mes (type e), and externo-apical sensory field consisting of 20–30 S-chaetae of type i, 2–4 chaetae of type j and of S-chaetae of type k (2–3 short S-chaetae of type k always present externo-distally between sensory field and AIIO) (Fig. 6D). Basal mic present (one dorsal, three ventral, one latero-external, one latero-internal).

AIIIO located dorso-externo-apically, with two swollen h-type S-chaetae side by side, surrounded internally by one thin short k-type S-chaeta and externally by two S-chaeta, of which one pointed and bent j-type and one thin k-type (Fig. 10D); a few additional S-chaetae of k-type present below AIIIO (Fig. 10D). Ant. III ventro-externo-distally with sensory field consisting of 20–25 S-chaetae of i-type, one small pointed j-type chaetae and of k-type chaetae (Fig. 11D). Ant. IV with at least four thick subcylindrical i-type S-chaetae in distal part one large subapical S-chaeta of j-type (Fig. 12D). On Ant. IV except in distal part some S-chaetae of k-type are larger and intermediate with i-type. Ant. IV tip with subapical papilla devoid of 'pin seta' (Fig. 12D, 13D). Distal organ of Ant. IV absent.

*Mouthparts* (Fig. 14D, 16D). Clypeus with 50–55 mes, of which 15–20 are serrated and others smooth (Fig. 14D). Ventro-distally on labrum two unequal combs, each with one apical row of ~15 and 19 teeth respectively, and a pair of sinuous rods. Labial triangle with 10–11 chaetae, all smooth and no scales (Fig. 16D). Post-labial chaetae 6+6, all smooth, interspersed with scales.

*Head and body* (Fig. 18A). Head dorsally with stable macrochaetae arrangement as in Fig. 18A; antennal mac 5+2; laterally with scales mixed with medium-sized serrated mes; ventrally with scales, smooth mes and few serrated mes.

Dorsal chaetotaxy of the tergites and head illustrated in Fig. 18A. Ms formula 1,0/1,0,1,0,0; sens formula 2,2/1,3,3,3,3; macrochaeta formula (3+0+4),(3+3)0,2,3,(3+0+4+3-4). Macrochaeta arrangement stable, except variation in post-lateral mac on Abd. IV; Th. II with three anterior and four posterior mac; Th. III with three posterior and three lateral mac; Abd. IV with three medial, four antero-lateral and 3–4 postero-lateral mac (D3 are sometimes mes); Abd. V with (9–12) + (9–12) mac; Abd. VI with 15–16 mac. On Abd. IV 25–30 + 25–30 S-like chaetae. Thin serrated mes associated with sens in tandem on Th. III, Abd. II and Abd. III.

*Legs* (Fig. 19, 20). Subcoxa I with three dorso-posterior serrated mac. Subcoxa II with six dorso-anterior serrated chaetae ranging in size from mac to mes and four dorso-posterior serrated mac. Subcoxa III with group of four dorso-posterior serrated mac and some serrated mes.

Coxae with pseudopores as 5–9, 8–9, 14–15 on coxae I, II and III. Two smooth mac on dorso-anterior side of coxa I, not observable for coxae II and III; multiplet of 6–7 dorso-posterior serrated chaetae on coxa I, two multiplets (dorso-anterior one

and dorso-posterior one) of 6–8 serrated chaetae on coxa II. Dorsally on coxa III, a transversal line of three mac sockets and 4–6 mes sockets.

Trochanter I with 8–11 smooth chaetae, trochanter II with 8–10, trochanter III with 5–8, and trochanteral organ, which has 70–80 shorter, straight, spine-like mes. Scales in large number on trochanters I, II and III.

Femur with many serrated mes, serrated mac and smooth mac. Scales numerous on the whole length of femora I, II and III. Ventrally, 3, 1, 1 smooth mac on femora I, II and III, additional smooth mes on femora absent. Largest smooth mac 0.35–0.39× length of femur. Group of ventro-proximal smooth mes without interspersed scales on femora III.

Distal whorl of Tita with 10–14 chaetae including 4, 4–5, 4–5 smooth mes and one tenent hair on legs I, II and III (Fig. 20D). Serrated mac as 2, 3, 3 on Tita I, II and III (Fig. 19D1, D2, D3). Besides the apical ones, smooth mes present ventrally on Tita I, II, III arranged in two rows on Tita I and II, in 3–4 rows on Tita III. In total, 8–12, 9–13 smooth mes in two rows on Tita I and II, 19–25 smooth mes in 3–4 rows on Tita III (Fig. 19D1, D2, D3). A few scales present proximally on Tita I and II, and many distributed on the whole length of Tita III. Tita of leg III longer than those of legs I and II; length 0.52, 0.62, 0.8 mm for Tita I, II and III ( $n=2$ ).

Unguis with one inner pair of equal basal teeth at 21, 21, 25% from its base for unguis I, II and III (Fig. 20D), inner unpaired tooth at 50% from its base ( $n=3$ ). Unguis (internal edge) 1.5, 1.5, 1.5× longer than unguiculus for claws I, II and III ( $n=3$ ). Unguis (internal edge) 2.8× longer than width of unguis at basis for claw I ( $n=3$ ), 2.9 in holotype. Unguiculus robust, pointed, 0.66× as long as inner edge of unguis for claws I and II, 0.68× for claw III (Fig. 20D). Small outer tooth, sometimes difficult to see at 43% from its base ( $n=3$ ) on antero-external lamella of unguiculus I, II and postero-external lamella of unguiculus III (Fig. 20D). Unguiculus 5.7× longer than its width at basis for claw I ( $n=3$ ). Tenent hair 1.8× longer than width of empodium and 0.7× the length of unguis (internal edge) for claw I ( $n=2$ ).

Ventral tube posteriorly with 110–130 serrated and smooth mes, with large smooth mes and 1–3 scales. Anteriorly with 30–37 + 30–37 serrated and smooth mes, 2 + 2 smooth mac distally and several large smooth mes. Each latero-distal flap with 35–43 mes, of which 8–10 are serrated and others smooth. Genital plate of female with 2 + 2 mic, of male with numerous circumgenital chaetae, genital chaetae not observable.

*Furca* (Fig. 21D, D1). Pseudopores as 10–13 dorso-distally on each side of manubrium, among distal group of chaetae. Manubrium dorsally with two longitudinal stripes of serrated mes, each one interspersed with 13–17 smooth pointed mac (Fig. 21D); between these stripes, loose cover of scales present. Manubrium ventrally without chaetae, except an unpaired ventro-basal serrated and thick mac, and 2 + 2 apical serrated mes near the axis. Distal manubrial thickening with 2–3 teeth on each side.

Dens non-annulated distal part ~4× as long as micro (Fig. 21D1). All dental chaetae serrated except one smooth mac dorsally on basal part of each dens (Fig. 21D). Ventrally dense cover of medium-sized elongated scales, small and rounded on basal part of dens, increasing in size laterally to



large, fusiform, pointed apically. Dens thickening with two faint transversal structures. Ratio manubrium : dens : mucro = 23 : 33 : 1. Mucro + dens 12.4× longer than unguis III (internal edge) and 1.6× longer than Tita III. Mucro bidentate with one basal spine reaching the apex of the proximal tooth, distal tooth elongated (Fig. 21D1).

#### Ecology

See description for *V. longicornis*. Microhabitat preference data are not available.

#### Discussion

The species is similar to *V. cavicola* and *V. absoloni* in absence of distal organ on Ant. IV; *V. margusi*, in absence of smooth mes on femur. Unguis of *V. verdemontana* is also similar to *V. absoloni* being less elongated and unguis bearing one inner unpaired tooth at 50% from its base. *Verhoeffiella verdemontana* differs from all species of the genus that are described in detail by the following combination of characters: well-developed sensory field of Ant. Ia with more numerous S-chaetae of type l (16–17) and type k (8); absent distal organ of Ant. IV; reduced number of post-labial chaetae (6+6) with interspersed scales; 8–12, 9–13 smooth mes on Tita I, II arranged in two rows, 19–25 smooth mes on Tita III arranged in four rows; absent smooth mes on femur; 2+2 apical serrated mes ventrally on manubrium; scales present dorsally on manubrium. For other diagnostic characters see Tables 1–3.

#### Remarks

In the species description, only characters that differed and were variable among *Verhoeffiella* species have been given. All other characters are the same as in the type species of the genus, *V. cavicola* (see 'Materials and methods' for more details).

#### Etymology

The species is named after Speleological club Zelena brda (English 'green mountains'; Latin *viridis*, *verdis* = green, *montana* = mountain) from Trebinje for the invaluable help of its members during many field trips and efforts to locate type localities of cave fauna in Herzegovina.

***Verhoeffiella milosi*** Lukić & Deharveng, sp. nov.

(Fig. 1–3, 5–6, 10–16, 18–21)

ZooBank lsid:715738D3-F0CD-4BCB-8A4F-445897FDEE08

#### Material examined

**Holotype.** Adult ♀ on slide: Montenegro: Nikšić: Budoš, Velja peč (cave) (WGS84 42° 43' 54.4"N; 18° 57' 07.5"E; 630 m ASL), M. Lukić leg., 11.xi.2015 (CLL4957).

**Paratypes.** The same data as holotype, 3 subadult ♀ on slides, M. Lukić leg., 11.xi.2015 (CLL4803); 2 subadult ♀ on slides, 2 ex. for DNA (4028B01, 4028B02), A. Kirin leg., 26.iv.2010 (CLL2270); 1 adult and 1 subadult ♀ on slide, M. Lukić leg., 11.xi.2015 (CLL4792); 2 ex. for DNA (CA076, CA077), M. Lukić leg., 1.iv.2012 (CLL4211); 4 ex. for DNA (CA237, CA238, CA239, CA240), M. Lukić, A. Kirin and J. Jakupić leg., 11.xi.2015 (CLL4755); 3 ex. for DNA (CA241, CA242, CA243), M. Lukić, A. Kirin and J. Jakupić leg., 11.xi.2015 (CLL4756).

**Material deposition.** 2 subadult ♀ on slide (CLL4803) deposited in MNHN. All other material, including holotype deposited in CBSS.

#### Diagnosis

Head and body length without antennae: 2.2–2.5 mm. Colour pale orange. Scales present on dorsal and ventral side of body, dorsal side of head and Ant. II, on legs, ventrally on manubrium and dens. Antennae 0.6 as long as (head and body) length; ratio Ant. II : (III+IV) = 1 : 3.87. Pseudopores as 1–2, 2–4, 3–5 distally on Ant. I, II and III. Ventral sensory field of Ant. Ia with 5–8 S-chaetae of type l and 3–4 S-chaetae of type k; S-chaetae (type h) absent on Ant. Ib, 3–4 on Ant. II; sensory field of Ant. Ib, II, III with 10–12, 8–12, 10–12 S-chaetae of type i; subapical papilla of Ant. IV present, distal organ of Ant. IV present. Labial triangle with 7–12 smooth chaetae. Post-labial chaetae without interspersed scales, (1–2)+(1–2) proximal serrated post-labial chaetae. Dorsal macrochaetotaxy as (3+4),(3+3)/0,2,3,(2+0+3+3). Trochanteral organ with 35–40 chaetae. Smooth mac on femora I, II and III as 2, 2, 1. Serrated mac on Tita I, II and III as 2, 2–3, 3; on Tita I 12–17 smooth mes in four rows, on Tita II 10–16 smooth mes in three or four rows, on Tita III 12–18 smooth mes in three rows. Tenent hair acuminate, smooth; ratio tenent hair : width of empodium for leg I 1.2 : 1. Unguis thin and elongated, with one pair of inner basal teeth; ratio unguis I length : unguis I width at basis 3.4 : 1. Manubrium with 4–8 dorso-distal psp, dorsally without scales and 14–15 smooth pointed mac, 1+1 ventro-apical serrated mes; 3–4 basal smooth mac dorsally on dens; mucro bidentate with one basal spine.

#### Description

Head and body length combined without antennae: 2.2 to 2.5 mm ( $n=4$ ), 2.3 mm in holotype. Habitus as in Fig. 1E, 2E. Abd. IV 1.6× as long as Abd. III in holotype. Antennae approximately half of the (head and body) length. Colour pale orange, pigment scattered as orange dots ventrally and dorsally on body, Ant. I, manubrium, coxa, paler on trochanter and femur. Intersegment area, mouth parts, antennae, other Ant. and leg segments, and dens unpigmented. Body dorsally and laterally with white regular patches on an orange background (Fig. 1E, 2E). Eyes absent, no pigment patch in their place.

**Antennae** (Fig. 3, 5, 6, 10–13). Antennae 0.6 as long as (head and body) length ( $n=4$ ), 0.6 in holotype. Antennae 3.4× longer than head diagonal in holotype. Ant. segment ratio as: Ia : Ib : II : III : IV = 0.15 : 0.59 : 1 : 1.57 : 2.3 ( $n=5$ ), for holotype 0.15 : 0.58 : 1 : 1.4 : 2.36; Ant. II : (III+IV) = 1 : 3.87 ( $n=5$ ), for holotype 1 : 3.76. Ant. III and IV annulated, except the 1/3 proximal part of Ant. III and the tip of Ant. IV. Pseudopores present ventro-apically on Ant. Ib (1–2), Ant. II (2–4) and Ant. III (3–5).

Scales numerous dorsally on Ant. Ib, along the whole length of Ant. II (except in most distal part where absent), absent on Ant. Ia, III, IV and ventrally on Ant. Ib.

Ant. Ia dorsally with two basal mic (type g) and thick serrated mes (type b); ventrally with acuminate smooth mes (type f) and sensory field consisting of 5–8 S-chaetae of type l and 3–4 S-chaetae of type k (Fig. 3E).



On Ant. Ib dorsally with one small, basal, thick, slightly serrated mes (type b), dorso-latero-internal serrated mac (type a) absent. Externo-apical, small, swollen S-chaetae (type h) absent. Ant. Ib ventrally with large number of pointed smooth mes (type e), ventro-external sensory field consisting of S-chaetae of type k and 10–12 S-chaetae of type i. A few S-chaetae of type k always present externo-apically to sensory field (Fig. 5E). S-chaetae of type l proximal to sensory field present, located just above Ant. Ia sensory field.

Ant. II dorsally with thin serrated mes (type c), apical row of serrated mac (type a) and smooth mac (type d), with an externo-apical group of 3–4 swollen S-chaetae of type h (AIIO) (Fig. 6E). Ant. II ventrally with pointed smooth mes (type e), distal row of small smooth mac (type d) or large smooth mes (type e) and large serrated mes (type e), and externo-apical sensory field consisting of 8–12 S-chaetae of type i, 1–2 chaetae of type j and of S-chaetae of type k (one short S-chaetae of type k always present externo-distally between sensory field and AIIO) (Fig. 6E). Basal mic present (one dorsal, three ventral, one latero-external, one latero-internal).

AIIIO located dorso-externo-apically, with two swollen h-type S-chaetae side by side, surrounded internally by one thin short k-type S-chaeta and externally by two S-chaeta, of which one is pointed and bent j-type and one is thin k-type (Fig. 10E); a few additional S-chaetae of k-type present below AIIIO (Fig. 10E). Ant. III ventro-externo-distally with sensory field consisting of 10–12 S-chaetae of i-type, 1–2 small pointed j-type chaetae and of k-type chaetae (Fig. 11E). Ant. IV with at least three thick subcylindrical i-type S-chaetae in distal part and one large subapical S-chaeta of j-type (Fig. 12E). On Ant. IV except in distal part some S-chaetae of k-type are larger and intermediate with i-type. Ant. IV tip with subapical papilla devoid of 'pin seta' (Fig. 12E, 13E). Distal organ of Ant. IV developed with several cuticular channels opening to the surface cuticle just behind papilla (Fig. 13E).

**Mouthparts** (Fig. 14E, 16E). Clypeus with 30–35 mes, of which 3–4 are serrated and others are smooth (Fig. 14E). Ventro-distally on labrum two unequal combs, each with one apical row of ~13 and 15 teeth respectively, and a pair of sinuous rods. Labial triangle with 7–12 chaetae, all smooth with no scales (Fig. 16). Post-labial chaetae (8–11)+(8–11), of which (1–2)+(1–2) most proximal ones are small, serrated and others are smooth, without interspersed scales.

**Head and body** (Fig. 18B). Head dorsally with stable macrochaetae arrangement as in Fig. 18B; antennal mac 5+2; laterally with scales mixed with medium-sized serrated mes; ventrally with smooth and serrated mes, scales absent.

Dorsal chaetotaxy of tergites and head illustrated in Fig. 18B. Ms formula 1,0/1,0,1,0,0; sens formula 2,2/1,3,3,3,3; macrochaeta formula (3+0+4),(3+3)0,2,3,(2+0+3+3). Macrochaeta arrangement stable. Th. II with three anterior and four posterior mac; Th. III with three posterior and three lateral mac; Abd. IV with two medial (A6 is mes), three antero-lateral and three postero-lateral mac (F2 and E3 are mes); Abd. V with 11+11 mac; Abd. VI with 13–14 mac. On Abd. IV 28–34+28–34 S-like chaetae. Thin serrated mes associated with sens in tandem on Abd. II and Abd. III, absent on Th. III.

**Legs** (Fig. 19, 20). Subcoxa I with three dorso-posterior serrated mac. Subcoxa II with four dorso-anterior serrated

chaetae ranging in size from mac to mes and four dorso-posterior serrated mac. Subcoxa III with group of four dorso-posterior serrated mac and some serrated mes.

Coxae with pseudopores as 2, 2, 6 on coxae I, II and III. Smooth mac as 2, 0, 0 on dorso-anterior side of coxae I, II and III; multiplet of five dorso-posterior serrated chaetae on coxa I, two multiplets (dorso-anterior one and dorso-posterior one) of 5–8 serrated chaetae on coxa II. Dorsally on coxa III, a transversal line of three mac sockets and five mes sockets.

Trochanter I with 10–13 smooth chaetae, trochanter II with 8–10, trochanter III with 6–7 and trochanteral organ consisting of 35–40 shorter, straight, spine-like mes. Scales in large number on trochanter III, absent on trochanters II and III.

Femur with many serrated mes, serrated mac, smooth mes and smooth mac. Few scales present on whole length of femur I, numerous on whole length of femora II and III. Ventrally with 2, 2, 1 smooth macrochaetae on femora I, II and III, with additional smooth mes on femur I. Largest smooth mac 0.38–0.48× length of femur. Group of ventro-proximal smooth mes without interspersed scales on femur III.

Distal whorl of Tita with 11–15 chaetae including 4–5, 3–4, 3–4 smooth mes and one tenent hair on legs I, II and III (Fig. 20E). Serrated mac as 2, 2–3, 3 on Tita I, II and III (Fig. 19E1, E2, E3). Besides the apical ones, smooth mes present ventrally on Tita I, II, III arranged in four rows on Tita I, three or four rows on Tita II and in three rows on Tita III. In total, 12–17 smooth mes in four rows on Tita I, 10–16 smooth mes in three or four rows on Tita II, 12–18 smooth mes in three rows on Tita III (Fig. 19E1, E2, E3). Few scales present proximally on Tita III, absent on Tita I and II. Tita of leg III longer than those of legs I and II; length 0.31, 0.35, 0.44 mm for Tita I, II and III for holotype.

Unguis with one inner pair of equal basal teeth at 14, 16, 19% from its base for unguis I, II and III ( $n=3$ ) (Fig. 20E), inner unpaired tooth absent. Unguis (internal edge) 1.5, 1.5, 1.4× longer than unguiculus for claws I, II and III ( $n=3$ ). Unguis (internal edge) 3.4× longer than width of unguis at basis for claw I ( $n=3$ ), 3.5× in holotype. Unguiculus robust, pointed, 0.66–0.67× as long as inner edge of unguis for claws I and II, 0.72× for claw III ( $n=3$ ) (Fig. 20E). Small outer tooth, sometimes difficult to see at 32% from its base ( $n=3$ ) on antero-external lamella of unguiculus I, II and postero-external lamella of unguiculus III (Fig. 20E). Unguiculus 6× longer than its width at basis for claw I ( $n=3$ ). Tenent hair 1.2× longer than width of empodium and 0.5× the length of unguis (internal edge) for claw I ( $n=2$ ).

Ventral tube posteriorly with 40–45 serrated and smooth mes, with large smooth mes and no scales. Anteriorly with 20–26+20–26 smooth mes (sometimes some weakly serrated present), 2+2 smooth mac distally and several large smooth mes. Each latero-distal flap with 23–30 smooth mes (one serrated mes observed in one specimen).

**Flwca** (Fig. 21E, E1). Pseudopores as 4–8 dorso-distally on each side of manubrium, among distal group of chaetae. Manubrium dorsally with two longitudinal stripes of serrated mes, each one interspersed with 14–15 smooth pointed mac (Fig. 21E); scales absent between these stripes. Manubrium ventrally without chaetae, except an unpaired ventro-basal serrated and thick mac, and 1+1 apical serrated mes near the

axis. Distal manubrial thickening with one large and 2–3 smaller teeth on each side.

Dens non-annulated distal part  $\sim 5\times$  as long as mucro (Fig. 21E). All dental chaetae serrated except 3–4 smooth mac dorsally on basal part of each dens (Fig. 21E). Ventrally dense cover of medium-size elongated scales, small and rounded on basal part of dens, increasing in size laterally to large, fusiform, pointed apically. Dens thickening with two faint transversal structures. Ratio manubrium: dens:mucro = 22:31:1. Mucro + dens  $7.9\times$  longer than unguis III (internal edge) and  $1.4\times$  longer than Tita III. Mucro bidentate with one basal spine reaching the apex of the proximal tooth, distal tooth elongated (Fig. 21E).

**Ecology.** The type locality Velja peč is a cave with a bat colony and therefore bat guano: entrance 630 m ASL; cave length  $\sim 350$  m; air temperature in deeper parts of the cave  $8.1\text{--}9.5^\circ\text{C}$ . Numerous specimens were collected on guano, but also in other parts of the cave.

#### Discussion

With its body length of 2.2–2.5 mm, *Verhoeffiella milosi*, sp. nov. is one of the smaller species of the genus. Its antennae too are short (antenna: body ratio = 0.6:1), with a low ratio Ant. II:(III+IV) (1:3.87), only comparable to that of *V. absoloni* (1:3.5) and *V. constantinellus* (Čurčić and Lučić in Lučić *et al.* 2007b) (1:3.62, measurement possibly dubious as Ant. III and IV of holotype seem fused or damaged). Dorsal macrochaetotaxy of Abd. IV is identical to that of *V. cavicola* with A6, F2, E3 on Abd. IV mes, but the two species can be clearly distinguished on distal organ of Ant. IV, which is present in *V. milosi*, but absent in *V. cavicola*. *Verhoeffiella milosi* differs from all species of the genus by the following combination of characters: reduced sensory fields of Ant. Ib, II, III with lowest number of S-chaetae of type i (10–12, 8–12, 10–12); absent S-chaetae (type h) on Ant. Ib; presence of both subapical papilla and distal organ of Ant. IV; sens in tandem with serrated mes absent on Th. III; 2, 2–3, 3 serrated mac on Tita I, II and III; 2, 2, 1 smooth mac on femora I, II and III. For other diagnostic characters see Tables 1–3.

#### Remarks

In the species description, only characters that differed and were variable among *Verhoeffiella* species have been given. All other characters are the same as in type species of the genus, *V. cavicola* (see ‘Materials and methods’ for more details).

#### Etymology

The species is named after Miloš Pavićević, a friend and speleobiologist from Podgorica and one of the founders of Biospeleological Society of Montenegro, for locating and exploring the type locality of this species and invaluable help during many field trips in Montenegro.

#### *Verhoeffiella polychaetosa* Lukić & Deharveng, sp. nov.

(Fig. 1–3, 5–6, 10–16, 18–21)

ZooBank lsid:3964806C-FBD3-4B7A-9736-B88DF7D61CD5

#### Material examined

**Holotype.** Adult ♀ on slide: Bosnia and Herzegovina: Sanski Most: Hrustovo, Hrustovača (cave) (WGS84  $44^\circ40'20.6''\text{N}$ ;  $16^\circ41'56.2''\text{E}$ ; 270 m ASL), M. Lukić leg., 26.xi.2013 (CLL4958).

**Paratypes.** The same data as holotype, 3 subadult ♀ on slides, 20 ex. in 96% alcohol, M. Lukić leg., 26.xi.2013. (CLL4769); 1 subadult ♀ and 1 subadult of unknown sex on slides, 13 ex. in 96% alcohol, 2 ex. for DNA (CA003, CA004), M. Lukić leg., 27.iii.2012 (CLL4202).

**Material deposition.** 1 subadult ♀ on slide, 3 ex. in 96% alcohol (CLL4769); 1 subadult of unknown sex on slide (CLL4202) deposited in MNHN. All other material, including holotype deposited in CBS.

#### Diagnosis

Head and body length without antennae: 3.5–3.6 mm. Colour pale orange. Scales present on dorsal and ventral side of body, dorsal side of head and Ant. II, on legs, ventrally on manubrium and dens. Antennae 1.1–1.2 $\times$  as long as (head and body) length; ratio Ant. II: (III+IV) 1:4.77. Pseudopores as 3–4, 3–5, 3–4 distally on Ant. I, II and III. Ventral sensory field of Ant. Ia with 12–15 S-chaetae of type l and 2–3 S-chaetae of type k; 3–7 S-chaetae (type h) on Ant. Ib, 6–10 on Ant. II; subapical papilla of Ant. IV present, distal organ of Ant. IV present. Labial triangle with 9–17 smooth chaetae. Post-labial chaetae without interspersed scales, (2–4)+(2–4) proximal post-labial chaetae are serrated. Dorsal macrochaetotaxy as (3+4),(3+4)/0,2,3,(2+0+3+3). Trochanteral organ with 40–45 chaetae. Smooth mac on femora I, II and III as 3, 2, 1. Serrated mac on Tita I, II and III as 2, 2, 2; on Tita I 29–31 smooth mes in 3–4 rows, on Tita II and II 33–38 and 32–39 smooth mes respectively, in five rows. Tenent hair acuminate, smooth; ratio tenent hair: width of empodium for leg I 1:1. Unguis thin and elongated, with one pair of inner basal teeth ratio unguis I length: unguis I width at basis 3.2:1. Manubrium with 10–12 dorso-distal psp, dorsally without scales and 33–40 smooth pointed mac, 1+1 ventro-apical serrated mes; 7–9 basal smooth mac dorsally on dens; mucro tridentate or bidentate with one basal spine.

#### Description

Head and body length combined without antennae: 3.5 to 3.6 mm ( $n=3$ ), 3.6 mm in holotype. Habitus as in Fig. 1B, 2B. Abd. IV 1.4–1.7 $\times$  as long as Abd. III ( $n=3$ ), 1.4 in holotype. Antennae slightly longer than length of (head and body) combined. Colour pale orange, pigment scattered as orange dots ventrally and dorsally on body, ventral tube, manubrium, coxa, paler on Ant., trochanter and femur. Intersegment area, mouth parts, antennae, other leg segments, and dens unpigmented. Body dorsally and laterally with white regular patches on an orange background (Fig. 1B, 2B). Eyes absent, no pigment patch in their place.

**Antennae** (Fig. 3, 5, 6, 10–13). Antennae 1.1–1.2 as long as (head and body) length ( $n=3$ ), 1.2 in holotype. Antennae 5.5–5.7 $\times$  longer than head diagonal ( $n=2$ ), 5.7 in holotype. Ant. segment ratio as: Ia: Ib: II: III: IV = 0.09:0.63:1:2.12:2.96 ( $n=5$ ), for holotype 0.09:0.64:1:2.26:2.98; Ant. II: (III+IV) = 1:5.08 ( $n=5$ ), for holotype 1:5.24. Ant. III and IV annulated, except the 1/10 proximal part of Ant. III and the tip of Ant. IV. Pseudopores present ventro-apically on Ant. Ib (3–4), Ant. II (3–5) and Ant. III (3–4).



Scales numerous dorsally on Ant. Ib, in proximal and medial part of Ant. II (in distal part absent), absent on Ant. Ia, III, IV and ventrally on Ant. Ib.

Ant. Ia dorsally with a group of centro-dorsal chaetae, two of which are probably mic (type g) and the others slightly serrated; ventrally with acuminate smooth mes (type f) and sensory field consisting of 12–15 S-chaetae of type l and 2–3 S-chaetae of type k (Fig. 3F).

On Ant. Ib dorsally with one small basal thick slightly serrated mes (type b), dorso-latero-internal with 3–4 serrated mac (type a). Externo-apical 3–7 small swollen S-chaetae (type h). Ant. Ib ventrally with large number of pointed smooth mes (type e), ventro-external sensory field consisting of S-chaetae of type k and 20–25 S-chaetae of type i. A few S-chaetae of type k always present extero-apically to sensory field (Fig. 5F). S-chaetae of type l proximally to sensory field present, located just above Ant. Ia sensory field.

Ant. II dorsally with thin serrated mes (type c) in larger number distally, apical row of serrated mac (type a), with an extero-apical group of 6–10 swollen S-chaetae of type h (AIIIO) (Fig. 6F). Ant. II ventrally with pointed smooth mes (type e), distal row of small smooth mac (type d) and serrated mes (type b), and extero-apical sensory field consisting of 20–25 S-chaetae of type i, 1–3 chaetae of type j and of S-chaetae of type k (2–4 short S-chaetae of type k always present extero-distally between sensory field and AIIIO) (Fig. 6F). Basal mic present (one dorsal, three ventral, one latero-external, one latero-internal).

AIIIO located dorso-externo-apically, with two swollen h-type S-chaetae side by side, surrounded internally by one thin short k-type S-chaeta and externally by two S-chaeta, one pointed and bent j-type and one thin k-type (Fig. 10F); a few additional S-chaetae of k-type below AIIIO absent (Fig. 10F). Ant. III ventro-externo-distally with sensory field consisting of 20–30 S-chaetae of i-type, 2–3 small pointed j-type chaetae and of k-type chaetae (Fig. 11F). Ant. IV with at least three thick subcylindrical i-type S-chaetae in medio-distal part and one large subapical j-type S-chaeta (Fig. 12F). On Ant. IV except in distal part some S-chaetae of k-type are larger and intermediate with i-type. Ant. IV tip with subapical papilla devoid of 'pin seta' (Fig. 12F, 13F). Distal organ of Ant. IV well developed with several cuticular channels opening to the surface cuticle just behind papilla (Fig. 13F).

**Mouthparts** (Fig. 14F, 16F). Clypeus with 40–50 smooth mes (Fig. 14F). Ventro-distally on labrum two unequal combs, each with one apical row of ~12 and 15 teeth respectively, and a pair of simuous rods. Labial triangle with 9–17 chaetae, all smooth and no scales (Fig. 16F). Post-labial chaetae (11–13) + (11–13), of which (2–4) + (2–4) most proximal ones are small, serrated and others smooth, without interspersed scales.

**Head and body** (Fig. 18C). Head dorsally with stable macrochaetae arrangement as in Fig. 18C; antennal mac (5–6) + (2–3); laterally with scales mixed with medium-sized serrated mes; ventrally with smooth and serrated mes, scales absent.

Dorsal chaetotaxy of tergites and head illustrated in Fig. 18C. Ms formula 1,0/1,0,1,0,0; sens formula 2,2/1,3,3,3–4,3; macrochaeta formula (3+0+4), (3+4)/0,2,3, (2+0+3+3). Macrochaeta arrangement stable. Th. II with three anterior and four posterior

mac; Th. III with four posterior and three lateral mac; Abd. IV with two medial (A6 is mes), three antero-lateral and three postero-lateral mac (E2 and E3 are mes); Abd. V with (10–11) + (10–11) mac; Abd. VI with 11–13 mac. On Abd. IV 35–40 + 35–40 S-like chaetae. Thin serrated mes associated with sens in tandem on Abd. II and Abd. III, possibly present on Th. III.

**Legs** (Fig. 19, 20). Subcoxa I with three dorso-posterior serrated mac. Subcoxa II with 3–5 dorso-anterior serrated chaetae ranging in size from mac to mes and 3–7 dorso-posterior serrated mac. Subcoxa III with group of four dorso-posterior serrated mac and some serrated mes.

Coxa II with 5–11 pseudopores, at least four on coxa III, not observable for coxa I. Smooth mac as 1, 1, 0 on dorso-anterior side of coxae I, II and III; multiplet of four dorso-posterior serrated chaetae on coxa I, two multiplets (dorso-anterior one and dorso-posterior one) of 5–7 serrated chaetae on coxa II. Dorsally on coxa III, a transversal line of three mac sockets and 5–7 mes sockets.

Trochanter I with 30–35 smooth chaetae, trochanter II with 30–35, trochanter III with 30–35, and trochanteral organ consisting of 40–45 shorter, straight, spine-like mes. Scales absent on trochanter I, few scales on trochanter II and in large number on trochanter III.

Femur with many serrated mes, serrated mac, smooth mes and smooth mac. Scales numerous on femora I, II and III. Ventrally, with 3, 2, 1 smooth macrochaetae on femora I, II and III, with additional smooth mes on femur I. Largest smooth mac 0.41–0.51 × length of femur. Group of ventro-proximal slightly serrated mes without interspersed scales on femur III.

Distal whorl of Tita with 13–15 chaetae including 5–7, 5–6, 4–5 smooth mes and one tenent hair on legs I, II and III (Fig. 20F). Serrated mac as 2, 2, 2 on Tita I, II and III (Fig. 19F1, F2, F3). Besides the apical ones, smooth mes present ventrally on Tita I, II and III arranged in 3–4 rows on Tita I and in five rows on Tita II and III, with several smooth mes interspersed between rows. In total, 29–31 smooth mes in 3–4 rows on Tita I, 33–38 smooth mes in five rows on Tita II, 32–39 smooth mes in five rows on Tita III (Fig. 19F1, F2, F3). Scales present in proximal and medial part of Tita III, absent on Tita I and II. Tita of leg III longer than those of legs I and II; length 0.62, 0.65, 0.84 mm for Tita I, II and III ( $n=3$ ).

Unguis with one inner pair of equal basal teeth at 18, 20, 24% from its base for unguis I, II and III ( $n=3$ ) (Fig. 20F), inner unpaired tooth absent. Unguis (internal edge) 1.5, 1.4, 1.3 × longer than unguiculus for claws I, II and III ( $n=3$ ). Unguis (internal edge) 3.2 × longer than width of unguis at basis for claw I ( $n=4$ ), 2.9 in holotype. Unguiculus robust, pointed, 0.69–0.71 × as long as inner edge of unguis for claws I and II, 0.79 × for claw III ( $n=3$ ) (Fig. 20F). Small outer tooth, sometimes difficult to see at 35% from its base ( $n=3$ ) on antero-external lamella of unguiculus I, II and postero-external lamella of unguiculus III (Fig. 20F). Unguiculus 7 × longer than its width at basis for claw I ( $n=3$ ). Tenent hair long as width of empodium and 0.4 × the length of unguis (internal edge) for claw I ( $n=3$ ).

Ventral tube posteriorly with 120–150 serrated and smooth mes, with large smooth mes and no scales (one observed in

one specimen). Anteriorly with 28–30+28–30 smooth mes (sometimes some weakly serrated present), 2+2 smooth mac distally and several large smooth mes. Each latero-distal flap with 55–60 mes, of which 6–8 are serrated and others smooth.

*Furca* (Fig. 21F, FI). Pseudopores as 10–12 dorso-distally on each side of manubrium, among distal group of chaetae. Manubrium dorsally with two longitudinal stripes of serrated mes, each one interspersed with 33–46 smooth pointed mac (Fig. 21F); scales absent between these stripes. Manubrium ventrally without chaetae, except an unpaired ventro-basal serrated and thick mac, and 1+1 apical serrated mes near the axis. Distal manubrial thickening with one large and 2–3 smaller teeth on each side.

Dens non-annulated distal part  $\sim 3\times$  as long as mucro (Fig. 21FI). All dental chaetae serrated except 7–9 smooth mac dorsally on basal part of each dens (Fig. 21F). Ventrally dense cover of medium-sized elongated fusiform scales, pointed apically. Dens thickening with three faint transversal structures. Ratio manubrium : dens : mucro = 18 : 25 : 1. Mucro + dens  $6.7\times$  longer than unguis III (internal edge) and  $1.2\times$  longer than Tita III. Mucro usually tridentate, sometimes bidentate (Fig. 20FI, F2). Four of the six observed specimens have tridentate mucro. Mucro with one basal spine reaching the apex of the proximal tooth, distal tooth elongated (Fig. 21FI).

*Ecology*. The type locality Hrustovača is a cave with a bat colony and therefore bat guano: entrance 270 m ASL; cave length:  $\sim 2500$  m; air temperature in deeper parts  $11.5^{\circ}\text{C}$ . Specimens were very abundant in the deeper parts of the cave, on decaying wood or on guano.

#### Discussion

*Verhoeffiella polychaetosa*, sp. nov. is similar to *V. longicornis* in having four posterior mac on Th. III. It differs from all species of the genus described in detail by the following combination of characters: presence of both subapical papilla and distal organ of Ant. IV; E2 chaetae on Abd. IV as mes; larger number of smooth mac dorsally on manubrium (33–46) and dorso-basally on dens (7–9); 2, 2, 2 serrated mac on Tita I, II and III; 29–31 smooth mes on Tita I arranged in 3–4 rows, 33–38, 32–39 smooth mes on Tita II, III in five rows. For other diagnostic characters see Tables 1–3.

#### Remarks

In species description, only characters that differed and were variable among *Verhoeffiella* species have been given. All other characters are the same as in type species of the genus, *V. cavicola* (see 'Materials and methods' for more details).

#### Etymology

The species name, originating from Greek, refers to its numerous smooth mesochaetae dorsally on the manubrium.

Additions to the redescription of *Verhoeffiella absoloni*  
Kseneman, 1938 in Lukić *et al.* (2015)

#### Material examined

*Neotype*. Montenegro: Boka Kotorska: Risan, Golodražnica (cave), adult ♀ on slide, 4.xii.2014, M. Lukić leg. (CLL 4557).

Sensory field on Ant. Ia consists of S-chaetae of type l and i according to observation on light microscope in Lukić *et al.* (2015). According to SEM images (Fig. 4A) of sensory field on Ant. Ia in *V. cavicola* S-chaetae of type i are in fact S-chaetae of type k.

In Lukić *et al.* (2015), AIII in *V. absoloni* is described and figured to have externally two pointed and bent S-chaetae of type j. According to observation of other *Verhoeffiella* species in this study and re-examination of the neotype of *V. absoloni*, it is determined that second S-chaeta of type j in fact belongs to sensory field of Ant. III. Therefore, it is considered that *V. absoloni* has one S-chaetae of type j in AIII as do most other *Verhoeffiella* species.

Other observations: on Ant. Ib small, basal, thick, serrated mes of type b is present; on Ant. Ib dorso-latero-internal serrated mac of type a are absent; ventrally on Ant. Ib several smooth mac of type d present; 14–17 S-chaetae of type i in sensory area of Ant. Ib; many S-chaetae of type k present proximally from sensory field of Ant. Ib to ventro basal part of Ant Ib; thin serrated mes of type c on Ant. II dorsally are absent; 2–3 small smooth mac of type d present on Ant. II ventrally; distal row of chaetae on Ant. II ventrally consisting of small smooth mac (type d) or large smooth mes (type e) and serrated mes; scales on Ant. II are present on the whole length, but absent in most distal part; two ventral basal mic present on Ant. III; large subapical S-chaetae of type j of Ant. IV present; distal organ of Ant. IV absent; papilla E of the labial palp with five guard chaetae; Abd. VI with 11–12 serrated mac in two rows; two smooth mac present on each anal valves; sens in tandem with thin serrated mes on Th. III present; trochanter III with small number of smooth chaetae, overall number difficult to observe; scales numerous on whole length of femora I, II and III; serrated mac on femora I, II and III present, overall number difficult to observe; ratio unguis I length : unguis I width at basis = 2.7 : 1; ratio unguiculus I length : unguiculus I width at basis = 5.1 : 1; ratio tenent hair : width of empodium for leg I = 1.2 : 1.

#### Results

##### Morphological character assessment

Thorough morphological study enabled more detailed insight into diversity and variability of the morphological characters of *Verhoeffiella*. First assessment of differential and other characters is given below, with notes on troglomorphic traits and possibly adaptive values of certain characters.

##### Body length

Body length is the most obvious difference between some species of *Verhoeffiella* (Fig. 2; Table 1). Adult specimens of the largest species – *V. constantinellus* (5.06 mm), *V. anagastumensis* Lučić, 2008 (in Lučić *et al.* 2008) (4.35 mm) and *V. longicornis* (4.2 mm) – have body lengths double the size of the smallest species – *V. margusi* (2.1 mm) and *V. media* (2.1 mm). Together with their remarkably long antennae, *V. anagastumensis* and *V. longicornis* reach the total length of more than 1 cm, probably representing two of the largest cave Collembola. Increase in body size is one of the common troglomorphic characteristics documented for Collembola and several other groups (Christiansen 2012).



#### *Morphology of chaetae on SEM*

Scanning electron microscopy images of *Verhoeffiella* enabled more detailed insight into morphology of diversified ordinary and S-chaetae. Swollen S-chaetae of type h present on Ant. Ib, II and III are strongly longitudinally striated with wide socket ring always in the depression of the cuticle (Fig. 4C, D, 7A, B). Thick subcylindrical S-chaetae of type i present in sensory fields of Ant. Ib, II and III and distally on Ant. IV are longitudinally striated with smaller socket rings, never in the depression of the cuticle (Fig. 4B, 7D). Smaller S-chaetae of type i are sometimes present in the proximal or lateral parts of sensory fields and are also longitudinally striated (Fig. 7F). Under optical microscopy, striation cannot be seen, and S-chaetae of type h and i appear to be smooth or just slightly rugose (Fig. 8B; Lukić *et al.* 2015, fig. 11, 12). Chaetae of type h and i appear to be rather similar on SEM, but under an optical microscope chaetae of type h are always more inflated and bound exclusively to the externo-apical part of Ant. II and III and in some species of Ant. Ib. S-chaetae of types j, k and l on SEM images are smooth to just slightly rugose (Fig. 4A, B, C, 7C, D, E, 9A, B). Sens on tergites are smooth with basal rings (Fig. 9C, D). S-like chaetae present only on Ant. IV are smooth and appear to be without basal rings on SEM (or the ring is inserted in the cuticle and not observable); thus, sockets of S-like chaetae on SEM are similar to those of scales (Fig. 9C). However, under an optical microscope S-like chaetae appears to have basal rings (Fig. 8C). Thick serrated mes of type b are strongly ciliated along the whole length with well-marked socket rings (Fig. 9A). Thin serrated mes of type c have shorter cilia along the whole length with smaller socket rings (Fig. 9A). Acuminate smooth mes of type f are smooth with socket rings slightly protruding above integument level (Fig. 9A).

#### *Scales distribution*

Distribution of scales on most body parts seems to be stable in studied species. Detailed distribution and abundance of scales are given in Table 3. Owing to frequent loss of scales during specimen mounting, it is sometimes difficult to observe presence and distribution of scales especially on leg segments if the scales are present in small numbers. Differences of distribution on antennae and leg segments among species comprise relatively minor differences in distribution within one segment (e.g. few present proximally, present in proximal half or numerous along the entire length of segment) or absence of scales on certain segments. Surprisingly, differences in scale distribution among species are recorded on the ventral side of the head and dorsally on the manubrium. *Verhoeffiella verdemontana* and *V. absoloni* have numerous scales present ventrally on the head, including the post-labial area and dorsally on the manubrium where scales are distributed between two stripes of serrated mes (Fig. 21D) (Lukić *et al.* 2015). *Verhoeffiella longicornis* has numerous scales also on the ventral side of the head, but only one scale is present on the proximal part of the post-labial area, and there are no scales dorsally on the manubrium. All other species are devoid of scales ventrally on the head and dorsally on the manubrium. Ventral tube is usually devoid or with only few scales, with the exception of *V. longicornis* with 15–20 scales present posteriorly.

#### *Pseudopores*

Pseudopores are recorded ventro-apically on Ant. Ib, II and III, dorsally on tergites from Th. II to Abd. IV, on coxae, and dorso-distally on the manubrium. The numbers of pseudopores on antennae and the manubrium are expressed as ranges in Table 2. The number of pseudopores on antennae varies within species, but the ranges are narrow with the exception of their number on Ant. II in *V. cavicola*, *V. verdemontana* and *V. absoloni*, in which ranges are slightly larger. Larger numbers of pseudopores (up to 14) are usually recorded on the dorso-distal part of the manubrium with interspecific variation (Fig. 15E). Pseudopore formula on tergites is 1,1/1,1,1,1,0,0 from Th.II to Abd.VI as in most Entomobryoida, while their range on coxae is difficult to count for several species.

#### *Antenna length and segment ratio*

In collected specimens, antennae are often damaged or fused, and collecting adult specimens with unfused antennae can be challenging. Indeed, species descriptions have been published on specimens with fused antennae, such as in *V. longicornis* and *V. constantinellus* (Absolon 1900; Lukić *et al.* 2007b). Most variation in antennae relative length relies on different degrees of elongation of Ant. III and IV, as seen from antennal segment ratio (Table 1). Ant. Ia, Ib and II do not differ significantly in relative length among species. Therefore, ratio Ant. II length : Ant. (III+IV) length is calculated to rank the species more easily. The most remarkable species in terms of antenna elongation is *V. longicornis* with antennae twice as long as body plus head length. Conversely, *V. absoloni* has the shortest antennae, approximately half of the body plus head length (Table 1). Calculated ratios revealed differences in degree of antenna elongation, a classical troglomorphic character. Ant. length : head diagonal ratio should only be used as indicative because of common wrinkling of the cuticle and deformation of the head on slides. This ratio is given for the new species and redescriptions in this study as for some species with poor original descriptions this is one of the very few morphological characters measured.

#### *Antennal chaetae*

Mic are present basally on Ant. Ia, II and III. Number of mic is stable and uniform across species (two dorsally and three ventrally on Ant. Ia; one dorsal, two ventral, one latero-external, one latero-internal on Ant. II and two ventral on Ant. III), although it is rather difficult to distinguish mic from other small basal chaetae on Ant. Ia in *V. longicornis*, *V. margusi*, *V. polychaetosa* and ventrally on Ant. II in *V. longicornis*.

Distribution of ordinary antennal chaetae follows fuzzy but obvious patterns in arrangement, which is roughly retrieved across the different species with the following exceptions:

- (i) on Ant. Ia ventrally acuminate smooth mes of type f are absent only in *V. longicornis*,
- (ii) on Ant. Ib dorso latero-internal serrated mac of type a are absent only in *V. milosi*,
- (iii) on Ant. II the number of thin dorsal serrated mes of type c vary between species and they are absent in *V. absoloni*,



- (iv) on Ant. II ventrally pointed smooth meses of type e are absent only in *V. longicornis*,
- (v) pattern of the apical row of chaetae dorsally and ventrally on Ant. II is rather stable within populations, but vary between several of the species,
- (vi) differences of chaetae distribution on Ant. IV tip are recorded for *V. longicornis* (only few thin serrated meses of type c present) and for *V. margusi* (only few thick serrated meses of type b present) (Fig. 12B, C).

S-chaetae distribution and abundance are also stable across studied species with the following differences:

S-chaetae of type l proximal to sensory field of Ant. Ib are absent only in *V. cavicola* and *V. margusi*. The overall number and distribution of distal, thick, subcylindrical S-chaetae of type i in the distal part of Ant. IV is sometimes difficult to observe, but numbers vary usually from two to four. In some species S-chaetae of this type are not observed in the very distal part of Ant. IV but more proximally (*V. longicornis*), or they are present along the whole length of Ant. IV (*V. margusi*, *V. milosi*). *Verhoeffiella longicornis* has two large subapical S-chaetae of type j on the Ant. IV tip (one in all other species) and no S-chaetae of type k in the apical part of Ant. IV (present in all other species). Patterns of other S-chaetae on antennae (in sensory fields and antennal organs) are discussed in the following paragraph.

#### Sensory fields on antennae

Sensory fields are present on Ant. Ia, Ib, II and III. They have been recorded by Bonet (1931) for Ant. Ib, II, III and figured by Mari Mutt (1986) for Ant. III in *V. hispanica*. Difference in size of sensory fields and number and size of S-chaetae might in fact represent new troglomorphic traits within Collembola, and have been documented for several cave species of genus *Pseudosinella* (Lepidocyrtinae) (Deharveng and Gouze 1986; Kováč and Rusek 2012) and *Troglopedetes* (Troglopedetinae) (Fanciulli et al. 2003; Soto-Adames et al. 2014).

Sensory fields on Ant. Ia differ from others in types of chaetae and ventro-apical position, not being homologous to sensory fields on other segments because of subdivision of Ant. I. S-chaetae of type l and k are present in the sensory field of Ant. Ia and their number is usually within a relatively narrow range, with the exception of *V. cavicola* (Table 2; Fig. 3).

Externo-apical sensory fields on Ant. II and III contain S-chaetae of type i, j and k, while the field on Ant. Ib contains S-chaetae of type i and k. The number of S-chaetae of type i in sensory fields varies in relatively large overlapping ranges for most of the species (Table 2); however, in two species they differ significantly from others by their thickness and number: the smallest species, *V. margusi*, has numerous and relatively thicker S-chaetae of type i despite its small body size; the second smallest species, *V. milosi*, has a smaller number of S-chaetae of type i on both antennal segments. The number of S-chaetae of type j in sensory fields on Ant. II and III varies, usually within the range of 1–4 (2–7 in *V. cavicola* and 2–8 in *V. absoloni* for the Ant. II sensory field). A few S-chaetae of type k are

always present extero-apically to the sensory fields on Ant. Ib and II, while there are many present from the sensory field of Ant. Ib to the ventro-basal part of the segment, and laterally and proximally to the sensory fields on Ant. II and III.

#### Antennal organs (*AlbO*, *AIIO* and *AIII0*)

Swollen S-chaetae of type h are recorded on Ant. Ib (in some species), II and III (Table 2). Groups of these chaetae on Ant. Ib and II might be homologous to that of AIII0. Presence of such swollen S-chaetae on Ant. Ib and II is rather unusual and might represent a troglomorphic character. S-chaetae of type j and k of AIII0 are not observed on Ant. Ib and II. AIII0 itself is located dorso-externo-apically, with two swollen S-chaetae of type h side by side, surrounded by two thin short S-chaetae of type k, with an additional one of type j externally. This standard pattern is different only in *V. longicornis*, which sometimes has two external S-chaetae of type k and in *V. margusi*, which does not have external S-chaetae of type j in AIII0 (Fig. 10). These variations are unusual as the pattern of these five chaetae of AIII0 is usually stable in most Collembola (Chen and Christiansen 1993, 1997). Below the AIII0 there are often several additional S-chaetae of type k, as is observed in some other Entomobryoida (Chen and Christiansen 1997).

#### Ant. IV papilla and distal organ

A subapical papilla of Ant. IV, always devoid of 'pin seta', is present in all species except *V. margusi*, but the size of the papilla differs among species. Just behind the papilla, in three of the studied species, there are invaginations of the cuticle, named here distal organ of Ant. IV (Fig. 12, 13). Such a structure is recorded for the first time in Collembola. The distal organ looks like branched invaginations of the cuticle with a complex network of channels. It is present in juveniles too. Except for the granulated-like channels, no special structures have been observed by optical microscope inside or around the organ. In the studied species the distal organ varies in size: in *V. milosi* it is the smallest with the shortest channels; in *V. polychaetosa* and *V. longicornis* the channels are longer and seem to have multiple openings to the cuticle surface; in *V. margusi* the distal organ is the largest and most deeply inserted into the antennae with only one channel opening to the cuticle surface. Two species lack the distal organ, *V. cavicola* and *V. verdemontana*, which both have large subapical papillae. The development of the distal organ might be correlated with the reduction of papillae, as in *V. polychaetosa*, *V. longicornis* and *V. milosi* the papillae are smaller in size while in *V. margusi*, which has the most developed distal organ, papillae are absent. This correlation needs to be further studied in a larger number of *Verhoeffiella* species. In position and general appearance, the distal organ resembles the olfactory chemoreceptor called the cupuliform organ, described in soil- and cave-dwelling species of Campodeidae (Diplura) (Juberthie-Jupeau and Bareth 1980; Sendra et al. 2004, 2017). The size of the cupuliform organ and the number of folds and sensilla increases in troglomorphic Campodeidae compared with soil-dwelling species. Similarly, there are differences in size and complexity of distal organs in *Verhoeffiella*. It may have an

olfactory function, but further ultrastructure study is needed to establish its role in *Verhoeffiella*.

#### Maxilla

The maxilla is similar to the figures given in Fjellberg (2007) for *H. nitidus*, but lamellae 1 and 2 are longer, overpassing the claw, and lamellae 4 and 5 are more strongly ciliated. The presence of a long thin rod has already been mentioned in the redescription of *V. absoloni* (Lukić *et al.* 2015).

#### Labial triangle and mentum

The number of chaetae in the labial triangle ranges from seven to 12 and fluctuates within a narrow range in all species with the exception of *V. polychaetosa*, in which it varies between nine and 17 chaetae. All chaetae in studied species are smooth and differ only in size. In all species the five chaetae of the mentum (A3–5, L1–2) are smooth.

#### Labial palp

The labial palp is similar in all studied species with five proximal chaetae and five papillae. The guard chaeta of papilla A is integrated in the papilla B complex, which thus appears to have five guard chaetae (Fjellberg 1999, p. 325); papilla C lacks guard chaetae; papilla D has four guard chaetae; papilla E has five guard chaetae and well-developed labial process. Five guards on papilla E are recorded also in *H. nitidus*, *H. major* (Moniez, 1889) and *Dicranocentrus* sp. (Fjellberg 1999). A main hypostomal chaeta (H) with two accessory chaetae (h1 and h2) are present in all species.

#### Post-labial chaetae

The number of post-labial chaetae usually varies within a narrow range (6–13) (Table 2; Fig. 16). In several species a few proximal chaetae are serrated, while in others all chaetae are smooth (Table 2). In *V. verdemontana* and *V. absoloni*, chaetae are interspersed with scales, while in *V. longicornis* there is only one scale present in the proximal part. The post-labial area in other species is devoid of scales.

#### Dorsal macrochaetotaxy

Only macrochaetae have been considered in this study, as patterns for mesochaetae are difficult to reconstruct (their sockets are rather similar to those of scales in our species); however, they may bring interesting information (Szeptycki 1979; Hüther 1983).

Dorsal macrochaetotaxy of the head is stable and similar in all studied species. Differences concern only the numbers of antennal chaetae, which are within the range (5–6)+(2–3), with the exception of *V. margusi*, which has (3–4)+1 antennal mac (Fig. 17).

Dorsal macrochaetotaxy of tergites from Th. II to Abd. IV are usually stable. Most interspecific and intraspecific variations are observed on Abd. IV (Table 1; Fig. 17, 18). Th. II has three anterior and four posterior mac, with the exception of *V. longicornis*, which has one additional medial mac (m2?). Th. III has three lateral and three posterior mac (four posterior

mac present in *V. longicornis* and *V. polychaetosa*). Abd. I is always devoid of mac, Abd. II has two mac (only one mac in *V. margusi*) and Abd. III has three mac.

The most complete mac pattern of Abd. IV is observed in *V. absoloni* and *V. verdemontana*, which have three medial, four antero-lateral and four postero-lateral mac. Certain mac on Abd. IV of these species are replaced by mes in some other species, such as *V. cavicola* and *V. milosi*, in which A6 in median, F2 in antero-lateral and E3 in postero-lateral groups are always mes, or like in *V. polychaetosa*, in which A6 in median, E2 in antero-lateral and E3 in postero-lateral groups are always mac. Significant displacement of mac is observed only on Abd. IV in *V. longicornis* where B5 of the median group is placed more proximally and where E2 of the antero-lateral group are displaced more laterally (Fig. 17B). Further, on some specimens there are 2–3 mac in antero-lateral and postero-lateral groups, while others are present as mes.

Interspecific variation of macrochaetotaxic patterns is therefore rather low. Two pairs of the studied species have exactly the same pattern: *V. cavicola* and *V. milosi*, and *V. verdemontana* and *V. absoloni*. Variation in number and position of mac on Abd. V and VI is greater than that observed on other tergites.

#### S-chaetae on body

Sens and ms are difficult to observe as they are often detached or hidden by scales. Number and distribution of ms is the same across all studied species (ms formula 1,0/1,0,1,0,0). The strange presence of a ms on Th. III was mentioned in two specimens of *V. absoloni* (Lukić *et al.* 2015), but has not been observed in the species studied here. Sens formula is usually 2,2/1,3,3,3,3, with the exception in *V. margusi*, which has 2–3 sens on Abd. V (usually two), and *V. polychaetosa*, which has 3–4 sens on Abd. IV. Sens are often in 'tandem', that is, associated with thin serrated mes on Th. III, Abd. II and III. In all examined specimens of *V. cavicola*, tandems are absent on all tergites, while in *V. milosi* they are absent on Th. III.

#### Ventral tube

The ventral tube bears smooth and serrated mes, smooth mac and sometimes scales. Number of mes posteriorly, anteriorly and on latero-distal flap usually varies within a narrow range for each species, with significant interspecific differences (Table 2). Scales are present in larger numbers only in *V. longicornis* (Table 3).

#### Claw

The unguis is elongated in all species with one inner pair of equal basal teeth and 1+1 small latero-basal teeth. An unpaired tooth on the unguis is present only in *V. absoloni* and *V. verdemontana*. The unguises of claws II and III are less elongated than that of claw I, and therefore the ratio 'unguis I length : unguis I width at basis' is the best way to rank the species according to unguis elongation (Table 2). *Verhoeffiella margusi* has the most slender and elongated unguis among studied species. The unguiculus is slender and pointed with a small outer tooth and is generally the same



shape in all species. Strange internal structure of the unguis (Lukić *et al.* 2015, fig. 37) is not observed in studied species. Modifications of the unguis and unguiculus represent a classical, well-documented adaptive troglomorphic character among Collembola (Christiansen 1965).

#### Tita

The Tita bears a large number of serrated mes, few serrated mac and smooth mes, and a thin distal tenent hair. The total number of smooth and serrated mes in the distal whorl varies from 10 to 16 in studied species and is variable within species. The number of smooth mes in the distal whorl varies from four to seven and is also variable within species, but it fluctuates within more narrow ranges. Tenent hair is thin, acuminate and decreases in length from Tita I to III. The ratio of tenent hair: width of empodium for leg I reveals differences in the relative length of tenent hair among species (Table 2). The ratio ranges from 1.0 in *V. longicornis* and *V. polychaetosa* to 1.8 in *V. verdemontana*. Shortened tenent hair is a classical troglomorphic trait among Collembola (Christiansen 1965). Number and pattern of thick serrated mac on Tita I, II and III are stable within species, ranging from one to three. Thick serrated mac are absent only on Tita I in *V. cavicola*. Besides the ones in distal whorl, smooth mes arranged in longitudinal rows are present ventrally. Usually, there are 2–5 longitudinal rows, and their number as well as the total number of smooth mes show relatively stable patterns with low intraspecific variation (Table 2). Smooth mes outside of the rows are present in small numbers. *Verhoeffiella cavicola* is the only species deviating from the described pattern with a large number of smooth mes present ventrally and dorsally on Tita I, II and III. In this species, chaetae arrangement in rows can be reconstructed only ventrally on Tita I and II where at least four rows are recognised. Smooth mes dorsally on Tita I, II and III and ventrally on Tita III are not arranged in rows. *Verhoeffiella cavicola* has the largest number of smooth mes on Tita among studied species.

#### Femur

The number of smooth mac ranges from one to four, decreasing in number from femur I to III (Table 2), and stable within species. Distribution and presence of smooth mes show some interspecific variations: absent in *V. verdemontana* and *V. margusi*; present only on femur I in *V. milosi* and *V. absoloni*; and present on femora I, II and III in other studied species.

#### Manubrium and dens

The number of smooth mac dorsally on the manubrium and dorso-basally on the dens shows interspecific differences, while it fluctuates within narrow ranges at intraspecific level (Table 2). The total number of smooth mac on one side ranges from 9–12 in *V. cavicola* to 33–46 in *V. polychaetosa*, and they are usually not completely symmetrical on both sides of the manubrium. Distal manubrial thickening has 1–5 teeth on each side. The total number of smooth mac dorso-basally on the dens ranges from one to nine, usually symmetrical on both dens. A short and thick interno-basal spine is present on each dens in all species. This spine is also present in *H. major* and *H. nitidus* (pers. obs.).

#### Mucro

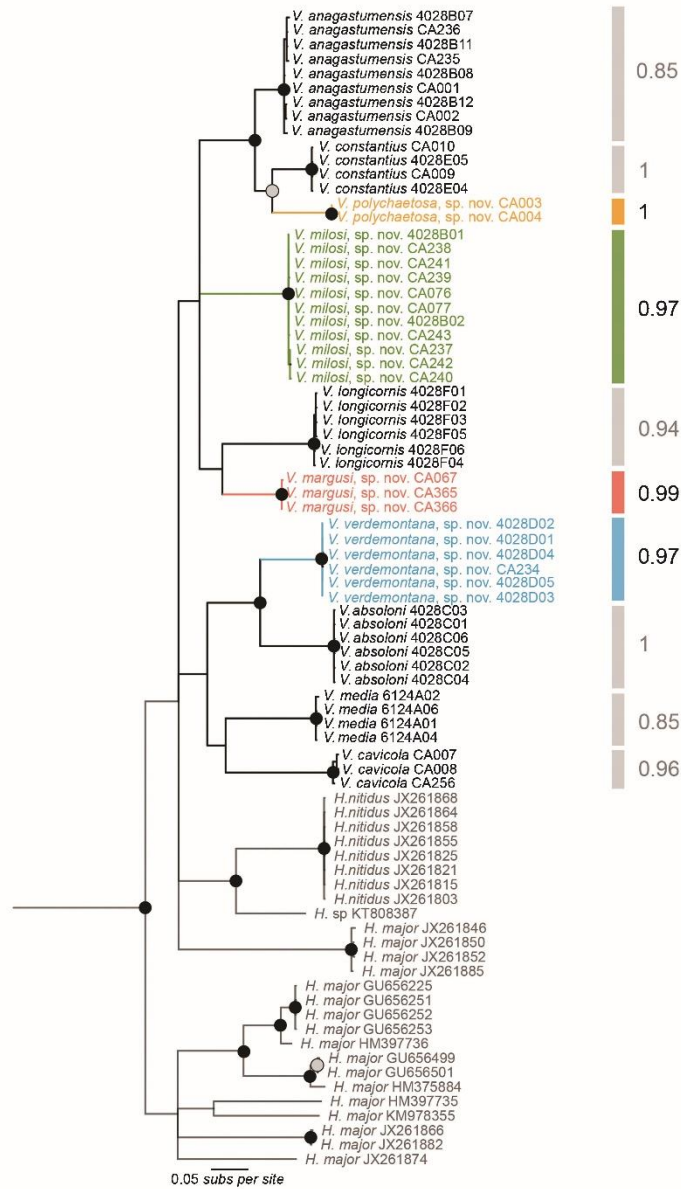
The mucro is usually bidentate with the distal tooth elongated compared with that of *Heteromurus nitidus*. Elongation of the distal tooth is considered to be a troglomorphic character among Collembola (Christiansen 1988). A tridentate mucro is recorded in three species: *V. longicornis*, *V. margusi* and *V. polychaetosa*. In *V. longicornis*, the third tooth is smaller in size and was observed only in two of the 10 specimens (Fig. 21B1, B2). In *V. margusi* and *V. polychaetosa*, two proximal equally large teeth are side by side and have been observed in three of the six and two of the six specimens respectively (Fig. 21C1, C2, F1, F2). Tridentate mucro have been observed among Entomobryidae only in the enigmatic and monotypic Bessoniellinae, where the mucro have very weak teeth with the two proximal not side by side but one below the other. Proximal equally large teeth that are side by side are recorded for the first time among Entomobryidae.

#### Phylogenetic analyses, species delimitation and genetic distances

The tree topology largely corresponds to the morphologically characterised *Verhoeffiella* species and many branches received maximum Bayesian posterior probabilities, PP=1 (Fig. 22). However, many 'basal' clades representing interspecific or even intergeneric relations received low Bayesian support, emphasising the uncertainty of relations at the genus level. The inability to resolve the basal relations may be related to saturation of the *COI* 3rd codon position (results not shown). Species delimitation procedures, no matter which approach was used, gave identical results (Fig. 22). The same putative species were recognised by the tree-based PTP and three alternative evolutionary models available in the distance-based ABGD method. Moreover, the results of the molecular species delimitations supported the results of morphological species discrimination. Within species, nucleotide divergence, measured by uncorrected p-distances, reached a maximum value at 0.005 in *V. anagastumensis* (results not shown). Minimal interspecific divergence was noted among the closely related *V. anagastumensis* and *V. constantus* species pair (0.096), while the maximum value was noted between the geographically and phylogenetically distant *V. polychaetosa* and *V. absoloni* species pair (0.2). Values of mean interspecific distances are presented in Table 4.

#### Discussion

Both species delimitation procedures PTP and ABGD supported the morphological species limits and yielded identical results. Concordance of morphological and genetic data, including relatively high values of uncorrected p-distances between the species, unambiguously supports the conclusion that all analysed populations, being both morphologically and genetically well characterised, should be treated as separate species. Therefore, with newly described species, *Verhoeffiella* now encompasses 10 species in caves of the Dinaric karst (Fig. 23). Still, low support for basal relations in the *COI* gene tree suggests the need for the inclusion of additional genetic markers, which would enable resolving all phylogenetic relationships.



**Fig. 22.** Cytochrome *c* oxidase I gene tree of the nominal *Verhoeffiella* species from the Dinaric karst. Posterior probabilities for nodes are shown as dots: grey >0.95; black = 1. Results of the ABGD and bPTP species delimitation, including support for bPTP putative species, is presented on the right. Outgroup taxa, genus *Alloscopus*, are removed from the figure.

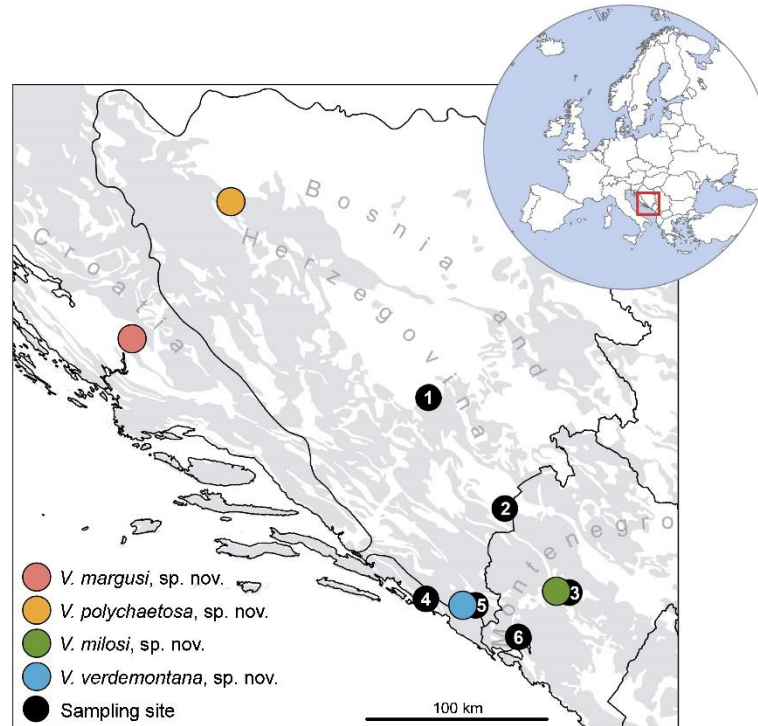


Fig. 23. Collection sites for specimens of *Verhoeffiella* used in this study. All collecting sites are type localities of the species. Type localities of newly described species are colour coded. Sampling sites of the previously described species are represented by the black dots and numbered: (1) *V. cavicola* Absolon, 1900; (2) *V. constantius* Čurčić and Lučić (in Lučić et al. 2007a); (3) *V. anagastumensis* Lučić, 2008 (in Lučić et al. 2008); (4) *V. media* Loksa & Bogojević, 1967; (5) *V. longicornis* (Absolon, 1900); (6) *V. absoloni* Kseneman, 1938. Grey areas on the map mark karst surface.

Table 4. Mean uncorrected  $p$ -values between the *Verhoeffiella* species of the Dinaric karst

Species									
<i>V. verdemontana</i> , sp. nov.									
<i>V. absoloni</i>	0.129								
<i>V. media</i>	0.166	0.151							
<i>V. cavicola</i>	0.157	0.161	0.156						
<i>V. anagastumensis</i>	0.157	0.177	0.167	0.174					
<i>V. constantius</i>	0.181	0.192	0.185	0.180	0.096				
<i>V. polychaetosa</i> , sp. nov.	0.168	0.200	0.184	0.162	0.110	0.105			
<i>V. milosi</i> , sp. nov.	0.185	0.195	0.168	0.173	0.155	0.159	0.166		
<i>V. longicornis</i>	0.171	0.174	0.184	0.184	0.147	0.165	0.169	0.166	
<i>V. margusi</i> , sp. nov.	0.150	0.164	0.174	0.172	0.139	0.164	0.165	0.151	0.140

The described species inhabit caves with different altitudinal and temperature characteristics, encompassing oligotrophic and cutrophic habitats, an altitudinal range spanning from 220 to 1320 m ASL and temperatures in caves ranging between

4.6 and 13.8°C. When combined, data on species abundances and amount of organic matter present could establish preferences of certain species to oligotrophic or cutrophic habitats. For example, *V. longicornis* and *V. margusi* were always found in



oligotrophic parts of the caves, while *V. milosi* was found in great numbers on bat guano. However, further targeted sampling and specifically designed studies are needed to explore this interesting pattern.

#### *Taxonomic characters of specific value*

As chaetotaxic patterns are widely used to separate species in Entomobryidae and are relatively stable across *Verhoeffiella* species, robustness of species delimitations relies on other discriminant characters. One of the most obvious differences among studied species of *Verhoeffiella* is the variation in body size (Table 1; Fig. 2). There are no characters that we could unambiguously mark as size-dependent across species; however, there are several that correlate with body size to a certain extent: number of S-chaetae of type I in the sensory field on Ant. Ia, S-chaetae of type h on Ant. II, post-labial chaetae, chaetae of the trochanteral organ, dorso-distal pseudopores on the manubrium and smooth mac dorsally on the manubrium. Consequently, some of the observed characters might be size-dependent, a hypothesis that could be tested in the future when more new species become available.

All characters that have traditionally been used to distinguish *Verhoeffiella* species are summarised in Table 1. The use of some ratios, such as antenna:head diagonal, manubrium:(mucro+dens), abdominal segment III:abdominal segment IV, is questionable, as head diagonal, length of the manubrium and sometimes abdominal tergite lengths are difficult to measure accurately. Seven Dinaric species that have been described in detail using an updated descriptive framework (Lukić *et al.* 2015) enable the assessment of new discriminant characters of specific value compiled in Table 2. The discriminant value of each character is indicated in Table 2 as follows: (I) high differential value, (II) characters with low interspecific and intraspecific variation but informative because of the exceptions in a few species, (III) characters with large overlapping ranges among species, which cannot be unambiguously used to delineate species but are informative in species characterisation. In addition to the characters listed in Table 2, some classical characters listed in Table 1 have also discriminant value at level I (antennal segment ratio, dorsal body macrochaetotaxy, inner unpaired tooth on unguis), at level II (distribution pattern of ordinary chaetae on Ant.) or at level III (body length).

Most of the characters noted above have been used to various extents in the taxonomy of Entomobryidae at specific or generic level. Some are new or rarely used for Entomobryidae and have been shown to be useful in species delimitation within *Verhoeffiella*: pattern of S-chaetae in sensory fields on antennae, number of S-chaetae of type h on Ant. Ib and II, distribution pattern of ordinary chaetae on antennal segments, presence and form of distal organ on Ant. IV tip, number and arrangement of serrated mac and smooth mes on Tita, and variation in number of dorso-distal pseudopores on the manubrium.

#### *Taxonomic characters of generic value*

We have identified some characters that might only be found in *Verhoeffiella* within Heteromurinae. These include number of

S-chaetae in sensorial fields of Ant. Ia, Ib, II and III, number of small teeth in distal manubrial thickening and number of dorso-distal pseudopores on the manubrium. While sensory fields are found in some Lepidocyrtinae (cave species of *Pseudosinella*) (Deharveng and Gouze 1986; Kováč and Rusek 2012) and Troglapedetinae (cave species of *Troglapedetes*) (Fanciulli *et al.* 2003; Soto-Adames *et al.* 2014), they have not been reported in Heteromurinae other than *Verhoeffiella*. Presence of distal manubrial thickening with small teeth has been observed in all the studied species of *Verhoeffiella*. This character is absent or rarely mentioned in other Entomobryidae; therefore, its generic value should be further studied. On the other hand, a large number of pseudopores on the dorso-distal part of the manubrium, ranging from four to 14 side by side (except in *V. margusi*), is unusual, considering that other Entomobryidae have 2–3 + 2–3 pseudopores (Gisin 1964).

Another character used at generic and suprageneric levels in the classification of Entomobryidae is the distribution and morphology of scales (Yoshii and Suhardjono 1989; Zhang *et al.* 2014). However, the presence of scales on antennae and legs used by the first authors as the major discriminant character between *Lepidocyrtus* and *Lanocyrtus* has been until recently given specific value in the taxonomy of North American and European Lepidocyrtinae (Mateos and Greenslade 2015). In *Verhoeffiella*, scale distribution on antennae, leg segments and ventral tube is congruent with other characters in species characterisation, but so far cannot serve as generic criteria. Surprisingly, differences in presence of scales ventrally on the head and dorsally on the manubrium, expected to be uniform within the genus, represent also a very useful character in species delimitation.

*Verhoeffiella* resembles *Heteromurus* in numerous characters used at generic level within Heteromurinae (see table 1 in Cipola *et al.* 2016). Our detailed morphological study of *Verhoeffiella* has introduced several new characters of possible discriminant value, but they are insufficiently documented among *Heteromurus*. The annulation of Ant. III remains therefore the only undisputable differential character between the two genera and we shall use *Verhoeffiella* as defined in Cipola *et al.* (2016) as far as antennal annulation morphologies intermediate between the two genera have not been disclosed.

#### *Lack of correlation between troglomorphic traits*

Collembola have served as model arthropod organisms to describe convergent adaptations in subterranean animals. Species that presented such morphology were called 'troglomorphes' by Christiansen (1962), while associated characters are classically qualified as troglomorphic (Christiansen 2012). *Verhoeffiella* live exclusively in caves, and they exhibit a wide range of morphological characters that are traditionally considered to be troglomorphic features of adaptive value (Christiansen 1965, 1988, 2012). Examples are elongation of antennae, increase of body size, elongation of unguis, reduction of teeth on unguis and tenent hair. High morphological diversity of these characters as well as others documented in this study could represent different levels and patterns of troglomorphy ('gradients of troglomorphy' in the sense of Christiansen 2012): number of S-chaetae in sensory fields on

Ant. Ia, Ib, II and III, and number of S-chaetae of type h on Ant. Ib, II. But we observed a large decoupling in the development of these traits within a species, meaning that not all troglomorphic characters are strongly expressed in all 'troglomorphic' species. For example, the smallest species with moderately short antennae, *V. margusi*, has the most slender and elongated claw and a large number of thick S-chaetae in sensory fields on Ant. Ib, II and III. Yet, another species, *V. longicornis*, the largest in the genus with the most elongated antennae and shortest tenent hair, has only moderately elongated claws.

For the first time, we report on the two cases of co-occurring pairs of *Verhoeffiella* species. The small species *V. milosi* with short antennae is described from a cave that is the type locality of one of the largest species, *V. anagastumensis*, which has elongated antennae. Similarly, the large species *V. verdemontana* with moderately long antennae is described from a cave that is the type locality of one of the largest species with the longest antennae, *V. longicornis*. Co-occurrence of two congeneric species with strong differences in troglomorphic traits, particularly body size and antennal elongation, has been previously reported in South-east Asian caves for different genera of Paronellidae and Lepidocyrtinae (Deharveng 1988, 1990; Deharveng and Bedos 2000; Jantari et al. 2013). Such patterns raise questions about the evolution of disconnected morphological traits, and their adaptive value in subterranean environments. Indeed, there are factors that influence the phenotypes of cave species, for example, physical characteristics of microhabitats and presence of potentially competing species, which can potentially trigger changes in appendage length and body size. This has been recently shown for the aquatic cave genus *Niphargus* (Trontelj et al. 2012; Delić et al. 2016) and mentioned for cave beetles in the region (Trontelj and Polak 2012). Testing hypotheses on the evolutionary forces that have produced the remarkable morphological diversity of troglomorphic traits in *Verhoeffiella* will require wider taxonomic sampling and a broader phylogenetic framework.

#### Conflicts of interest

The authors declare that they have no conflicts of interest.

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**Appendix 1.**

**Table A1. List of oligonucleotide primers used in the study**

Gene	Primer pair	Reference
<i>COI</i>	LCO1490–HCO2198	Folmer <i>et al.</i> 1994
<i>COI</i>	Lep-F1–Lep-R1	Hebert <i>et al.</i> 2004
<i>COI</i>	LCO1490col–HCO2198col	Greenslade <i>et al.</i> 2011

**Table A2. List of samples and GeneBank accession numbers used in the analyses**

All samples of *Verhoeffiella* species are from type localities

Species	Locality/reference	wgs 84 longitude	wgs 84 latitude	BOLD sample ID	Voucher	GenBank accession number
<i>V. absoloni</i>	Lukić <i>et al.</i> 2015	18.69962	42.50282	4028C01_MNECLL2278		JN275273
<i>V. absoloni</i>	Lukić <i>et al.</i> 2015	18.69962	42.50282	4028C02_MNECLL2278		JN275274
<i>V. absoloni</i>	Lukić <i>et al.</i> 2015	18.69962	42.50282	4028C03_MNECLL2278		JN275275
<i>V. absoloni</i>	Lukić <i>et al.</i> 2015	18.69962	42.50282	4028C04_MNECLL2278		JN275276
<i>V. absoloni</i>	Lukić <i>et al.</i> 2015	18.69962	42.50282	4028C05_MNECLL2278		JN275277
<i>V. absoloni</i>	Lukić <i>et al.</i> 2015	18.69962	42.50282	4028C06_MNECLL2278		JN275278
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177	4028B07_MNECLL2260		JN275267
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177	4028B08_MNECLL2260		JN275268
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177	4028B09_MNECLL2260		JN275269
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177	4028B11_MNECLL2260		JN275271
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177	4028B12_MNECLL2260		JN275272
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177		CA001	MG459463
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177		CA002	MG459464
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177		CA235	MG459465
<i>V. anagastumensis</i>	Montenegro, Nikšić, Budoš, Velja peć (cave)	18.95209	42.73177		CA236	MG459466
<i>V. cavicola</i>	Bosnia and Herzegovina, Sarajevo, Laništa, Bjelašnica Mt., Megara (cave)	18.08447	43.71463		CA007	MG459445
<i>V. cavicola</i>	Bosnia and Herzegovina, Sarajevo, Laništa, Bjelašnica Mt., Megara (cave)	18.08447	43.71463		CA008	MG459446
<i>V. cavicola</i>	Bosnia and Herzegovina, Sarajevo, Laništa, Bjelašnica Mt., Megara (cave)	18.08447	43.71463		CA256	MG459468
<i>V. constantius</i>	Bosnia and Herzegovina, Gacko, Avtovac, Mulje (cave)	18.59653	43.14651	4028E04_BHCLL2320		JN275286
<i>V. constantius</i>	Bosnia and Herzegovina, Gacko, Avtovac, Mulje (cave)	18.59653	43.14651	4028E05_BHCLL2320		MG220419
<i>V. constantius</i>	Bosnia and Herzegovina, Gacko, Avtovac, Mulje (cave)	18.59653	43.14651		CA009	MG459461
<i>V. constantius</i>	Bosnia and Herzegovina, Gacko, Avtovac, Mulje (cave)	18.59653	43.14651		CA010	MG459462
<i>V. longicornis</i>	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028F01_BHCLL2283		JN275293
<i>V. longicornis</i>	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028F02_BHCLL2283		MG220420
<i>V. longicornis</i>	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028F03_BHCLL2283		JN275294
<i>V. longicornis</i>	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028F04_BHCLL2283		JN275295
<i>V. longicornis</i>	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028F05_BHCLL2283		JN275296
<i>V. longicornis</i>	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028F06_BHCLL2283		JN275297

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**Table A2.** (continued)

Species	Locality/reference	wgs 84 longitude	wgs 84 latitude	BOLD sample ID	Voucher	GenBank accession number
<i>V. margusi</i> , sp. nov.	Croatia, Dmiš, Oklaj, Punjane, Jazinka (cave)	16.02971	43.97589		CA067	MG459467
<i>V. margusi</i> , sp. nov.	Croatia, Dmiš, Oklaj, Punjane, Jazinka (cave)	16.02971	43.97589		CA365	MG459457
<i>V. margusi</i> , sp. nov.	Croatia, Dmiš, Oklaj, Punjane, Jazinka (cave)	16.02971	43.97589		CA366	MG459458
<i>V. media</i>	Croatia, Dubrovnik, Osojnik, Močiljska špilja (cave)	18.07101	42.68989	6124A01_CROCLL2240		JN275235
<i>V. media</i>	Croatia, Dubrovnik, Osojnik, Močiljska špilja (cave)	18.07101	42.68989	6124A02_CROCLL2240		JN275236
<i>V. media</i>	Croatia, Dubrovnik, Osojnik, Močiljska špilja (cave)	18.07101	42.68989	6124A04_CROCLL2240		JN275237
<i>V. media</i>	Croatia, Dubrovnik, Osojnik, Močiljska špilja (cave)	18.07101	42.68989	6124A06_CROCLL2240		JN275239
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177	4028B01_MNECLL2270		MG220422
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177	4028B02_MNECLL2270		MG220421
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA076	MG459449
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA077	MG459450
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA237	MG459455
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA238	MG459451
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA239	MG459452
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA240	MG459454
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA241	MG459453
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA242	MG459456
<i>V. milosi</i> , sp. nov.	Montenegro, Nikšić, Budoš, Velja peč (cave)	18.95209	42.73177		CA243	MG459448
<i>V. polychaetosa</i> , sp. nov.	Bosnia and Herzegovina, Sanski Most, Hrustovo, Hrustovača (cave)	16.69895	44.67240		CA003	MG459459
<i>V. polychaetosa</i> , sp. nov.	Bosnia and Herzegovina, Sanski Most, Hrustovo, Hrustovača (cave)	16.69895	44.67240		CA004	MG459460
<i>V. verdemontana</i> , sp. nov.	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028D01_BHCLL2282		JN275279
<i>V. verdemontana</i> , sp. nov.	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028D02_BHCLL2282		JN275280
<i>V. verdemontana</i> , sp. nov.	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028D03_BHCLL2282		JN275281
<i>V. verdemontana</i> , sp. nov.	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028D04_BHCLL2282		JN275282
<i>V. verdemontana</i> , sp. nov.	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638	4028D05_BHCLL2282		JN275283
<i>V. verdemontana</i> , sp. nov.	Bosnia and Herzegovina, Trebinje, Bihovo, Pavlova pećina (cave)	18.30778	42.66638		CA234	MG428989
<i>Alloscopus</i> sp.	Vietnam, Kien Giang, Kien Luong, Ba Hon, Nui Chau Hang				CA340	MG459447
<i>Alloscopus</i> sp.	Zhang <i>et al.</i> 2015					KM978344
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					JX261874
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					JX261882
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					JX261866
<i>Heteromurus major</i>	Zhang <i>et al.</i> 2015					KM978355
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					HM397735
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					HM375884
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					GU656501
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					GU656499
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					HM397736
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					GU656253

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**Table A2.** (continued)

Species	Locality/reference	wgs 84 longitude	wgs 84 latitude	BOLD sample ID	Voucher	GenBank accession number
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					GU656252
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					GU656251
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					GU656225
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					JX261885
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					JX261852
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					JX261850
<i>Heteromurus major</i>	Porco <i>et al.</i> 2012					JX261864
<i>Heteromurus</i> sp.	Shaw and Benfer 2015					KT808387
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261803
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261815
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261821
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261825
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261855
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261858
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261864
<i>Heteromurus nitidus</i>	Porco <i>et al.</i> 2012					JX261868

**Table A3.** Codon specific evolutionary models used in the *COI* phylogenetic analysis

	Selected sites	Selected evolutionary model
1st codon position	1–657/3	SYM+G
2nd codon position	2–657/3	HKY+Y
3rd codon position	3–657/3	GTR+G

### 2.3 DISTRIBUTION PATTERN AND RADIATION OF THE EUROPEAN SUBTERRANEAN GENUS *VERHOEFFIELLA* (COLLEMBOLA, ENTOMOBRYIDAE)

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Ena najbolj zanimivih značilnosti podzemnih vrst živali so majhna območja razširjenosti. Ta prevladujejo ne le na vrstnem, ampak pogosto tudi na rodovnem nivoju. Vendar pa imajo nekateri podzemni rodovi kontinentalno, a disjunktno razširjenost, kar postavlja pod vprašaj njihovo monofilijo in terja razlago njihovega izvora in kolonizacije. V naši študiji smo raziskali rod skakačev *Verhoeffiella*, ki je trenutno znan iz petih oddaljenih kraških regij v Evropi. Evolucijsko zgodovino rodu smo proučili z analizo zaporedij štirih jedrnih genov in enega mitohondrijskega gena. Testirali smo monofilijo rodu, raziskali njegov odnos z domnevnimi površinskimi sorodniki in časovne vzorce molekulske diverzifikacije. Filogenetska analiza je razkrila kompleksen odnos rodu *Verhoeffiella* s površinskimi *Heteromurus nitidus* in deloma razrešila biogeografsko vprašanje disjunktne distribucije. Nadalje, v Dinaridih smo prepoznali več filogenetskih linij, kar kaže na poprej zelo podcenjeno diverziteteto. Število odkritih samostojnih filetskih linij je skoraj sedemkrat večje od števila opisanih vrst. Radiacija je relativno mlada, povezana z geološkimi dogodki v času Mesinijske slanostne krize in v času pleistocenskih klimatskih nihanj. Rod *Verhoeffiella* ima zaradi kombinacije izjemne podzemne radiacije in bližnjih evolucijskih povezav s površinskimi sorodniki posebno mesto pri raziskovanju sicer slabo poznanega filogenetskega izvora troglobiontov v zmernih klimatih.

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ORIGINAL ARTICLE

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## Distribution pattern and radiation of the European subterranean genus *Verhoeffiella* (Collembola, Entomobryidae)

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### Abstract

One of the most striking features of obligate subterranean species is their narrow distribution ranges. These prevail not only at specific, but often also at generic level. However, some subterranean genera have continental scale and disjunct distribution, which challenges their monophyly and questions the scenarios of their origin and colonization. In our study, we investigated the subterranean collembolan genus *Verhoeffiella*, currently known from five remote karst regions of Europe. Four nuclear and one mitochondrial genes were assembled to reveal the evolutionary history of the genus. We tested the monophyly of the genus, explored its relationship with putative surface relatives, and its temporal patterns of molecular diversification. The phylogeny revealed a complex relationship of *Verhoeffiella* with surface species *Heteromurus nitidus* and partially disentangled the biogeographical question of its disjunct distribution. Further on, several lineages of *Verhoeffiella* were recognized in the Dinarides, showing highly underestimated diversity and, compared with the number of described species, a sevenfold increase in the number of MOTUs. The radiation is relatively recent, with the events triggering the diversification linked to the Messinian salinity crisis and Pleistocene climatic shifts. The combination of this extensive subterranean radiation and close evolutionary links with epigeal relatives makes *Verhoeffiella* an exceptional case within the subterranean fauna of temperate areas, which significantly contributes to our understanding of subterranean colonization and diversification patterns.

### KEYWORDS

colonization, hidden diversity, molecular phylogeny, Pleistocene, troglobionts, Western Balkans

Marko Lukić and Teo Delić contributed equally to this work.

Louis Deharveng and Maja Zagnmajster share senior authorship.



## 1 | INTRODUCTION

Understanding processes of colonization, speciation and dispersal in subterranean habitats is one of the main challenges in evolutionary studies of subterranean species around the globe (Culver & Pipan, 2019; Moldovan, Kováč, & Halse, 2018). One of the most striking features of obligate subterranean species (also termed troglobionts) is their narrow distribution ranges (Niemiller & Zigler, 2013; Trontelj et al., 2009; Zagmajster et al., 2014). In extreme cases, the proportion of species known from single or only a few localities can represent more than half of the species pool in a region (Bregović, Fišer, & Zagmajster, 2019; Niemiller & Zigler, 2013; Trontelj et al., 2009). Small ranges are often related to limited dispersal abilities, due to species morphological and physiological adaptations (Christiansen, 1961, 1965; Polak, Delić, Kostanjšek, & Trontelj, 2016; Porter, 2007; Thibaud & Vannier, 1986; Vannier & Thibaud, 1984). Range sizes of subterranean taxa have so far been explained via the fragmented karstic landscapes, complex paleogeographic events and/or historic climatic variability (Deharveng & Gers, 1993; Eme et al., 2014; Fresneda, Valenzuela, Bourdeau, & Faille, 2019; Rizzo, Comas, Fadrique, Fresneda, & Ribera, 2013; Trontelj et al., 2009; Zagmajster et al., 2014). Small distribution ranges of troglobionts prevail at specific level, but are also frequent at the generic level. However, some genera exhibit large distribution ranges (Baratti, Filippelli, Nardi, & Messana, 2010; Faille et al., 2010; Gibert & Deharveng, 2002; Isaia, Mammola, Mazzuca, Arnedo, & Pantini, 2017; Trontelj et al., 2009; Zakšek, Sket, & Trontelj, 2007), especially among Collembola (Christiansen, da Gamma, & Bellinger, 1983; Lukić, 2019).

The most intriguing cases of widespread and exclusively subterranean genera are the ones with disjunct distributions—occurring in geographic regions that can be hundreds or thousands of kilometres apart. Among European terrestrial troglobionts, such examples have been reported in different taxonomic groups, from gastropods to insects (Deharveng & Bedos, 2018; Duchae, 2001; Faille, Casale, Hernando, Ait Mouloud, & Ribera, 2018; Fanciulli, Inguscio, Rossi, & Dallai, 2003; Weigand et al., 2013). Some of them, known from the Iberian Peninsula, the Alps and the Dinarides, even share similar distribution patterns (Inäbnit et al., 2019; Lukić, Houssin, & Deharveng, 2010; Sendra, Lara, Ruiz Aviles, & Tinaut, 2004; Taiti et al., 2018). Among the subterranean taxa with disjunct distribution, only spiders and terrestrial gastropods were studied with molecular approaches (Pavlek & Ribera, 2017; Ribera, Elverici, Kunt, & Özkütük, 2014; Weigand et al., 2013), while for the others, the taxonomical assignments of geographically disjunct populations rely on morphology alone. Due to striking morphological convergences common among troglobionts (Culver & Pipan, 2019),

disjunct distribution patterns challenge the validity of extant monophylies, while the scenarios of origin and colonization call for further testing within molecular phylogenetic frameworks.

With more than 500 troglotrophic species worldwide, Collembola are among the most common inhabitants of the subterranean realm (Deharveng & Bedos, 2018; Lukić, 2019). They have been traditionally used as evolutionary models for the study of morphological adaptations to subterranean habitats (Christiansen, 1961, 1965, 2012), but their potential for testing evolutionary hypotheses using molecular approaches has rarely been exploited (Katz, Taylor, & Davis, 2018). Among the taxa with disjunct distributions, one of the most interesting examples to study is the exclusively subterranean European genus *Verhoeffiella* Absolon of the family Entomobryidae.

The genus *Verhoeffiella* embodies several features of interest: (a) the genus has a wide disjunct distribution, occurring in five karst regions of Europe; (b) its centre of diversity is in the Dinarides; and (c) it has a putative surface relative, *Heteromurus nitidus* (Templeton, 1835). Currently, the genus encompasses 14 nominal species, ten of which are described from caves in the Dinarides (Lukić, Delić, Zagmajster, & Deharveng, 2018). Four other karst regions contain one species each and are from 150 to 1,000 km apart: Jakupica (North Macedonia), south-eastern Calcareous Alps (Italy), southern Catalonia and Cordillera Cantabrica (Spain) (Lukić, Porco, Bedos, & Deharveng, 2015). This intriguing distribution, coupled with diversity of morphological traits, has already led to recent taxonomic revisions of the existing and descriptions of new species of the genus (Lukić et al., 2018, 2015). As stated in previous works, there is a need to resolve the relationship of *Verhoeffiella* and *Heteromurus* s.str. Wankel, as both genera share most characters used at the generic level within Heteromurinae (Cipola, Oliveira, Morais, & Bellini, 2016; Mari Mutt, 1980b). Remarkable morphological similarity of the genus *Verhoeffiella* and the epigeal species *H. nitidus* (Bonet, 1931; Lukić et al., 2018, 2015; Mari Mutt, 1980a) sets the latter as the most likely candidate for a surface relative of *Verhoeffiella*.

The close relationship of the two taxa may offer an insight into the origin of disjunct distribution of *Verhoeffiella* and contribute to a better understanding of colonization and diversification dynamics within the subterranean habitats in general. Therefore, we first tested the monophyly of *Verhoeffiella* populations from remote European regions and determined the position of the genus within the Entomobryidae family. We then explored the relationship of *Verhoeffiella* to its putative surface relative *H. nitidus* from different geographical areas. Further, we estimated hidden diversity within the genus *Verhoeffiella* with a special emphasis on the Dinarides. Finally, we explored the relationship between different populations of the genus *Verhoeffiella*, in order to reconstruct the

evolutionary scenarios of diversification in relation to paleogeographic events.

## 2 | MATERIAL AND METHODS

### 2.1 | Design of the study

The basis of the study was a set of 411 individuals of *Heteromurus nitidus*, *Heteromurus major* (Moniez, 1889) and *Verhoeffiella* species. The data set predominantly included newly sequenced populations of *H. nitidus* and *Verhoeffiella*, but also publicly available sequences.

The study proceeded via a number of steps. Depending on the specific scope of each of the steps, we used an appropriate data set and methodology. Details on each of the steps are given in the paragraphs below and are summarized in Table 1.

### 2.2 | Samples collection

Specimens of *Verhoeffiella* and *Heteromurus* were collected from 105 sites altogether (81 and 31, respectively; including 7 sites where they occur sympatrically), throughout the *Verhoeffiella* distributional range, including the Dinarides, south-eastern Calcareous Alps, Catalonia and Cordillera Cantabrica (Figure 1a; Figure S1). Samples were hand or pitfall trap collected in caves, while samples of litter and soil from surface habitats were extracted using Berlese funnels. All samples were preserved in 96% ethanol. In addition to own fieldwork data, several collections were examined for specimens of targeted genera, including the collections

of Croatian Biospeleological Society (Zagreb), University of Ljubljana's SubBioLab Zoological collection, Museum of Natural Sciences of Barcelona, Torres Sala entomological collection (Valencia), Cave Fauna Collection of the Department of Zoology of the University from the Basque Country. Cuticles and DNA extracts were deposited in the Collection of Croatian Biospeleological Society, Zagreb, Croatia. Topotypic specimens of nominal species were used whenever possible.

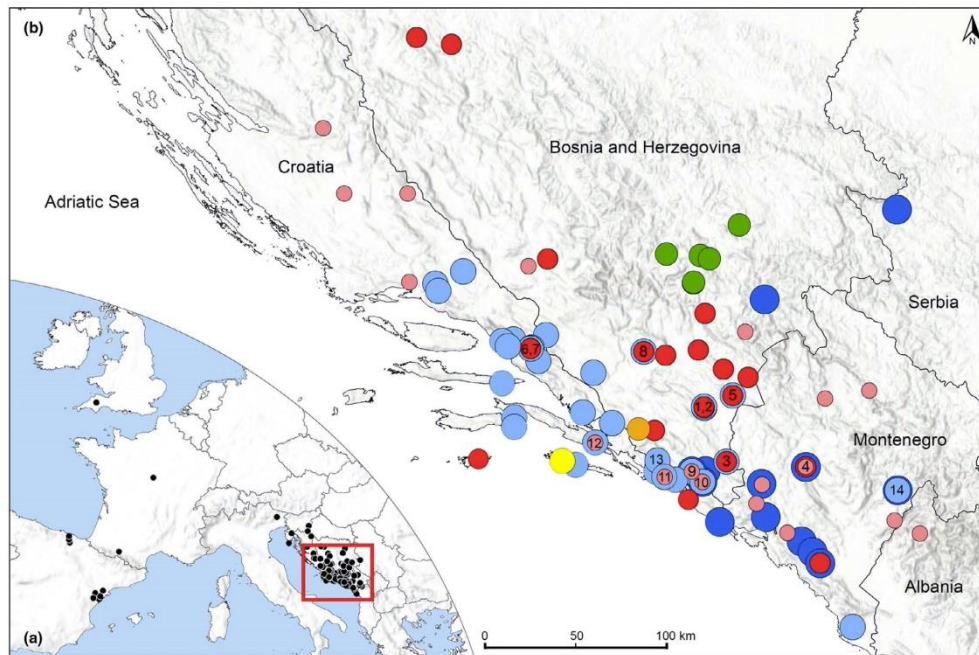
### 2.3 | DNA isolation, amplification and sequencing

Genomic DNA was isolated from the whole specimens, by opening a minute aperture between the abdominal segments to preserve the specimen's cuticle for the subsequent morphological analyses. DNA isolation was performed using the GenElute Mammalian Genomic DNA (Sigma-Aldrich, United States) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). All specimens used for the molecular analyses were, whenever possible, identified to species level using morphological characters thoroughly described in Lukić et al. (2018). We amplified both nuclear DNA—28S rRNA, 18S rRNA, histone H3 (H3), internal transcribed spacer I and II (ITS)—and mitochondrial DNA—cytochrome oxidase I (COI). Different combinations of amplified genetic markers were used to assemble alternative data sets, as is explained further on. A list of primers and PCR amplification protocols used is available in Appendix S1. PCR products were purified using Exonuclease I and FastAP (Thermo Fisher Scientific Inc., United States) according to the manufacturer's

TABLE 1 Overview of the study design with the data and samples used in each step

Analytical step	Method and software used	Type of data	Samples used	Specific results
Family-level phylogenetic analysis	Bayesian and maximum-likelihood phylogenetic analysis	Concatenated sequences: 18S rRNA, 28S rRNA, COI, 16S rRNA (4,147 bp)	Sequences of <i>H. major</i> , <i>H. nitidus</i> , nominal <i>Verhoeffiella</i> species and published Entomobryidae sequences (Table S1)	Phylogenetic trees (Figure S2, S3)
Species delimitation	Molecular species delimitation: ABGD, PTP	COI sequences (658 bp)	411 individuals of <i>H. major</i> , <i>H. nitidus</i> and <i>Verhoeffiella</i> species (Table S3)	Molecular operational taxonomic units (MOTUs) (Table 2; Table S4)
Phylogenetic backbone analysis of the <i>Verhoeffiella</i> - <i>H. nitidus</i> clade	Bayesian and maximum-likelihood phylogenetic analysis	Concatenated sequences: 18S rRNA, 28S rRNA, H3, ITS, COI (3,876 bp)	132 individuals: representative of each MOTU of <i>H. nitidus</i> and <i>Verhoeffiella</i> , <i>H. major</i> and out-group representatives (Table S2)	Phylogenetic trees (Figure 2; Figure S4)
Estimation of divergence times	BEAST analysis	Concatenated sequences: 18S rRNA, 28S rRNA, H3, ITS, COI (3,876 bp)	132 individuals: the same data set as for phylogenetic backbone analysis	Time-calibrated phylogeny (Figure 3)
Lineage through time (LTT) plots	Phytools	Maximum credibility tree, BEAST output	132 individuals: the same data set as for phylogenetic backbone analysis	Pattern of lineage temporal diversification (Figure 3)





**FIGURE 1** (a) Map of all *Heteromurus nitidus* and *Verhoeffiella* samples included in the analysis. (b) Distribution of the genus *Verhoeffiella* in the Dinarides (Western Balkans). Sampling sites are colour-coded and numbered as per Figure 2. Numbered circles represent localities with co-occurring MOTUs (details are available in Table S2) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

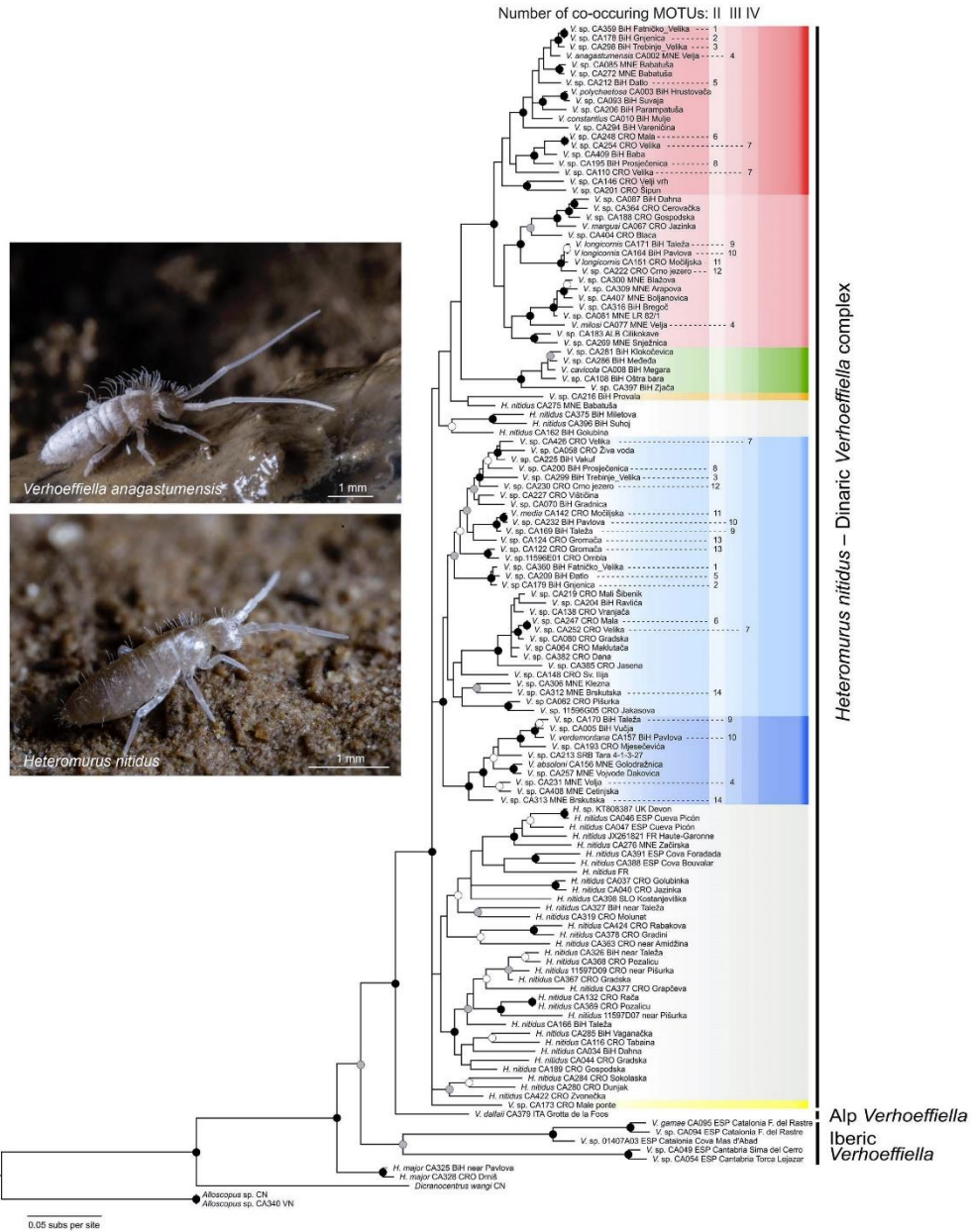
instructions. Successfully amplified fragments were sequenced bidirectionally using the same PCR amplification primers by Macrogen Europe (Amsterdam, Netherlands), and the resulting chromatograms were assembled and edited using Geneious 9.1.8. (Biomatters, New Zealand). All the information on the specimens used, including GenBank accession numbers, voucher codes, sampling localities, etc., is available in the Tables S1–S3.

#### 2.4 | Family-level phylogenetic analysis

In order to test the monophyly of *Verhoeffiella* and determine its position within the Entomobryidae family, we combined previously published data set of Entomobryidae phylogeny (Zhang, Chen, et al., 2014; Zhang, Sun, Yu, & Wang, 2015), publicly available sequences and own genetic data of *Verhoeffiella* and *Heteromurus* from different karst regions. Altogether, we compiled a data set of 64 Entomobryidae representatives (Table S1). Three distantly related collembolan species, namely *Tomocerus ocreatus* Denis, 1948, *Folsomia quadrioculata* (Tullberg, 1871) and *Folsomia candida* Willem, 1902, were used to root the tree. The molecular data used included two nuclear (18S rRNA and 28S rRNA) and

two mitochondrial (COI and 16S rRNA) genes. All the sequences were aligned using MAFFT v. 7 (Katoh & Standley, 2013) Q-INS-i algorithm and concatenated into the final, 4,147-bp-long alignment. The best partitioning scheme and the optimal substitution models for the codon positions were obtained using PartitionFinder 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017). Both, optimal substitution models and the best partitioning scheme, including lengths of specific gene alignment, are available in Appendix S1.

Phylogenetic relationships were reconstructed on a concatenated data set using two alternative approaches: Bayesian inference with partition-specific settings in MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003) and maximum likelihood with partition-specific setting and ultrafast bootstrapping in IQ-tree 1.6.7 (Nguyen, Schmidt, von Haeseler, & Minh, 2014). Bayesian MCMC tree in MrBayes was inferred after two independent runs with four chains each for 20 million generations. The trees were sampled every 1,000 generations, and the first 25% of trees were discarded after checking for stationary phase in Tracer v1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). The remaining trees were used to calculate a 50% majority rule consensus tree. Alternatively, the nodal supports for the maximum-likelihood analyses in



**FIGURE 2** Bayesian phylogeny of genus *Verhoeffiella* and *Heteromurus nitidus* on the concatenated data set of five genes (18S rRNA, 28S rRNA, H3, ITS, COI). Each terminal taxon represents a single MOTU derived from species delimitation methods. Dots indicate posterior probabilities: black circles = 1; grey circles  $\geq 0.95$ ; white circles  $\geq 0.90$ . Lineages are colour-coded and numbers match localities with co-occurring MOTUs as per Figure 1b. In situ photographs: M. Lukić [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



IQ-tree were assessed after performing 1,000 ultrafast bootstrap replicates (Hoang, Chernomor, von Haeseler, Minh, & Vinh, 2018).

## 2.5 | Defining molecular operational taxonomic units (MOTUs)

In order to explore the hidden diversity and minimize the amount of missing data for the fine-scale phylogenetic analysis, we first ran species delimitation on barcoding region of 411 COI sequences (Table S3). The data set included publicly available and our own sequences of *H. nitidus* and *H. major*, most nominal *Verhoeffiella* species and a large number of newly sequenced *Verhoeffiella* populations. Usually, three specimens per locality (up to 16) were sequenced to minimize the sequencing error. We used two alternative approaches: a tree-based Poisson tree processes (PTP) model (Zhang, Kapli, Pavlidis, & Stamatakis, 2013) and a distance-based species delimitation approach, the Automatic Bridge Gap Discovery (ABGD) (Puillandre, Lambert, Brouillet, & Achaz, 2012).

Poisson tree processes is a phylogeny-based species delimitation method, based on the assumption that intra- and interspecific nucleotide substitution levels notably differ and can be modelled as two independent Poisson processes. The PTP analyses were performed on 211 unique COI haplotypes, assessed after removing duplicate sequences using a custom Perl script (Eme, Malard, Konecny-Dupré, Lefébure, & Douady, 2013). Phylogenetic relationships within the COI sequences were estimated in a separate PhyML (Guindon et al., 2010) analysis, using a GTR + I + G substitution model and a gamma shape parameter and proportion of invariant sites estimated by the algorithm itself. The resulting tree was then used to run the Poisson tree processes (PTP) analysis on the species delimitation server <http://species.h-its.org/>. Bayesian posterior probabilities for tentative species were acquired after running 500,000 generations, sampling every 100 generations, and discarding the first 20% of the samples as a burn-in.

Automatic Bridge Gap Discovery is an automated procedure that clusters sequences into MOTUs based on pairwise distances by detecting differences between intra- and interspecific variation (i.e. barcoding gap) without a priori species hypothesis (Puillandre et al., 2012). ABGD analyses were performed at the ABGD web server <http://www.wabi.snv.jussieu.fr/public/abgd/abgdweb.html> and analysed separately on the full COI sequences data set using the Kimura K80, with a default value of relative gap width ( $X = 1.5$ ) and Pmax value (0.1).

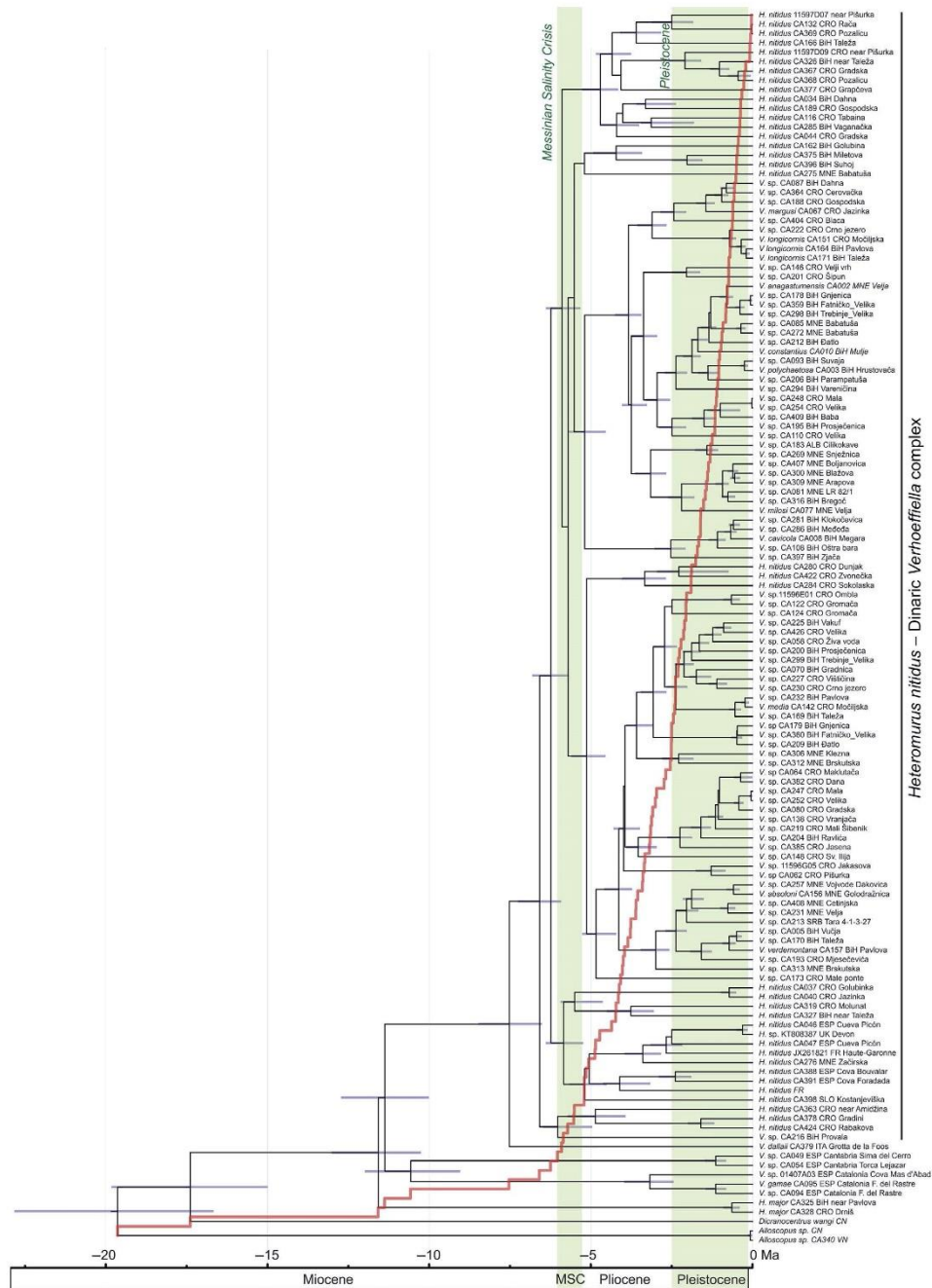
## 2.6 | Phylogeny of the *Verhoeffiella*–*Heteromurus nitidus* clade

Due to limited coverage of genetic markers between previously published data set of Entomobryidae phylogeny (Zhang, Chen, et al., 2014; Zhang et al., 2015) and our data set, the phylogenetic backbone analyses were run on a different, 3,876-bp concatenated data set. The data set contained four nuclear (18S rRNA, 28S rRNA, H3, ITS) and one mitochondrial (COI) gene. The data set comprised of 132 specimens and included a single representative per previously defined *Verhoeffiella*–*Heteromurus* MOTUs, individuals from geographically remote populations which could not be included in species delimitation analysis, due to failure in COI gene sequencing, and the distantly related *Alloscopus* Börner and *Dicranocentrus* Schött (Heteromurinae) to root the tree. Phylogenetic relations were reconstructed using the tree inferring procedure as described in the family-level phylogenetic analysis section. All family-level and clade-level phylogenetic analyses were run on the CIPRES Science Gateway (Miller et al., 2015; accessible at [www.phylo.org](http://www.phylo.org)). Optimal substitution models and the best partitioning scheme are available in Appendix S1.

## 2.7 | Estimation of divergence times

To set the time frame on the genus phylogeny hypothesis, we used the same backbone analyses data set and Bayesian inference as implemented in BEAST v 2.5.1 (Bouckaert et al., 2014). The BEAST analysis was run using the following settings: a model of nucleotide evolution for each of the partitions was selected using bModelTest (Bouckaert & Drummond, 2017); the uncorrelated relaxed lognormal molecular clock with a gamma prior for each partition separately; and a linked Yule tree prior for all defined partitions. The Yule tree model prior was selected after comparing the alternative tree priors in Path Analyser (results not shown). Hereafter, the most suitable analysis was repeated four times for 30 million generations each, sampling every 3,000 generations, and excluding 20% of the trees as a burn-in. The results of the four independent runs were analysed for convergence and adequate sample size (effective sample size above 200) using Tracer v1.7 (Rambaut et al., 2018) and merged using LogCombiner (Bouckaert et al., 2014). The maximum credibility tree was then summarized and annotated using TreeAnnotator (Bouckaert et al., 2014). Time of the splits within the *Verhoeffiella*–*H. nitidus* clade was assessed using previously employed approach, using data

**FIGURE 3** Time-calibrated phylogeny of genus *Verhoeffiella* and *Heteromurus nitidus*, corresponding to the Bayesian relaxed-clock analysis based on the clock rate values for COI and 28S rRNA (Papadopoulou et al., 2010), and estimated values for 18S rRNA, H3 and ITS. The ages of the major paleogeographic events, Messinian salinity crisis (MSC) and Pleistocene, are shown in light green. 95% confidence intervals are shown as blue node bars, and lineage through time accumulation as a red line [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



on molecular rates of distantly related Tenebrionid beetles (Papadopoulou, Anastasiou, & Vogler, 2010), due to the absence of available fossil calibrations in Collembola. Molecular rates, which were not accessible from the original article, were estimated based on the published pairwise divergence per million years for COI and 28S rRNA, 3.36% and 0.12%, respectively. To visually examine the pattern of lineage temporal diversification, lineage through time (LTT) plots were reconstructed using phytools v.0.6-60 (Revell, 2012) with the mean diversification value generated based on the BEAST Maximum credibility tree. In addition, observed diversification value was compared to 1,000 simulations of pure-birth LTTs, and gamma statistics (Pybus & Harvey, 2000), explaining speciation trends was extracted.

### 3 | RESULTS

#### 3.1 | Family-level phylogenetic analysis

All the groups, recognized in Zhang et al. (2015), were also inferred in our analysis and were supported with high Bayesian posterior probabilities (p.p.  $\geq 0.95$ ) (Figure S2) and maximum-likelihood fast bootstrap support ( $\geq 95$ ; Figure S3). In both analyses, similar tree topologies, showing minimal differences, were inferred. The subfamily Heteromurinae, including the genera *Alloscopus*, *Dicranocentrus*, *Heteromurus* and *Verhoeffiella*, received the highest support (p.p. = 1) for its monophyly. *Heteromurus major* was shown to be the sister taxon of *Verhoeffiella*-*H. nitidus* clade, while the geographically distant *Verhoeffiella* from Catalonia and Cordillera Cantabrica were positioned at the basis of the latter (Figure S2). This relation received low support (p.p. = 0.66) in this analysis, but was highly supported in the extended data set analysis (p.p. = 0.99) (Figure 2). Next, the *Verhoeffiella* from south-eastern Calcareous Alps, *V. dallaii* Nosek & Paoletti, 1985, was shown to be in a sister relation with the complex of *H. nitidus* and Dinaric *Verhoeffiella* species. Within the complex, genera *Verhoeffiella* and *Heteromurus* turned out to be paraphyletic and some populations exhibited unresolved relationships (Figure S2).

#### 3.2 | Hidden diversity within *Verhoeffiella* and *Heteromurus*

Species delimitation methods on a large data set of 411 COI sequences enabled us to estimate the hidden diversity within *Verhoeffiella* and *Heteromurus*. Number of MOTUs identified with both delimitation methods, PTP and ABDG, was consistent, with differences in one or two MOTUs (Table 2; Table S4). Our analysis revealed high molecular divergence between MOTUs, reflecting hidden diversity within both genera.

**TABLE 2** Number of nominal species and MOTUs in two *Heteromurus* and all *Verhoeffiella* species in Europe, as identified using two species delimitation methods on barcoding region of 411 COI sequences. Number of *Verhoeffiella* species and MOTUs, referring to populations in the Dinarides, are written in parentheses

Species	Nominal species	PTP MOTUs	ABDG MOTUs
<i>Heteromurus major</i>	1	11	12
<i>Heteromurus nitidus</i>	1	33	31
<i>Verhoeffiella</i> spp. (Dinaric populations)	14 (10)	79 (74)	79 (73)

#### 3.3 | Phylogeny of the *Verhoeffiella*-*Heteromurus nitidus* clade

Phylogenetic backbone analyses showed the same relationship within Heteromurinae (Figure 2; Figure S4) as on the family-level analysis (Figure S2). *Heteromurus major* had sister relationship to *Verhoeffiella*-*Heteromurus nitidus* clade. Populations from Catalonia and Cordillera Cantabrica, including already described *V. gamae* Lukić et al., 2015 and several undescribed MOTUs, were positioned basally. Interestingly, the populations from the two mountain ranges are molecularly highly differentiated, suggesting a long-lasting separation. Species *V. dallaii* from the south-eastern Calcareous Alps kept the position of a sister taxon to the most extensive part of *Heteromurus nitidus*-Dinaric *Verhoeffiella* complex (Figure 2), as in family-level analysis (Figure S2).

Within the complex, *Verhoeffiella* and *H. nitidus* lineages were shown to be paraphyletic, with a basal polytomy preventing the more exact resolution of their relationship (Figure 2). At least three different and spatially separated lineages of *H. nitidus* can be recognized. The lineage richest in MOTUs is broadly distributed over Europe and includes specimens from France, Great Britain, Cantabria, Catalonia and the Dinarides. The possible fourth lineage of *H. nitidus*, consisting of a single population, could not be undoubtedly phylogenetically positioned.

Two large, one smaller and at least two isolated *Verhoeffiella* lineages were recognized in the Dinarides (Figure 2). Ranges of the two large lineages overlap to a great extent, with the smaller one situated separately at the northern parts of south-eastern Dinarides (Figure 1b). Two isolated lineages are known only from a single cave, nested within the ranges of larger lineages (Figure 1b). Such a large magnitude of spatial overlap among different lineages and sublineages of *Verhoeffiella* and *H. nitidus* generated a vivid pattern of co-occurring MOTUs, especially in south-eastern Dinarides (Figures 1b, 2). Ten caves with two, three caves with three and even one cave with four co-occurring *Verhoeffiella* MOTUs were recognized (Figures 1b, 2; Table S2).



### 3.4 | Divergence times of *Verhoeffiella*–*Heteromurus nitidus* clade

Onset of the diversification of the whole *Verhoeffiella*–*Heteromurus nitidus* clade was estimated to have occurred in middle Miocene, approximately 11.3 MA, with a 95% confidence interval ranging from 10 to 12.7 MA (Figure 3). The most extensive part of the diversification, covering all the Dinaric representatives, was estimated to 6.58 MA (5.91–7.26 MA). All the major cladogenetic events, including origins of all the major *Verhoeffiella* and *H. nitidus* lineages, were estimated to an age corresponding to the Messinian salinity crisis (5.96–5.33 MA). In contrast, most of the speciation events within *Verhoeffiella* were estimated to have occurred during the Pleistocene (2.5 MA–11.7 k years ago). This pattern was also demonstrated by the Pybus and Harvey gamma statistics, which supported a trend of increase in diversification towards the present ( $\gamma = 0.2741$ ,  $p = .74$ ) (Figure 3).

## 4 | DISCUSSION

### 4.1 | Phylogenetic analysis dispute the disjunct distribution of *Verhoeffiella*

Our results show that the exclusively subterranean genus *Verhoeffiella*, together with lineages traditionally ascribed to the epigeal species *Heteromurus nitidus*, forms a well-supported monophyletic clade nested within Heteromurinae. Populations of *Verhoeffiella* from the Iberian Peninsula and the Alps are positioned basally to the complex of *H. nitidus* and Dinaric *Verhoeffiella*. Therefore, *Verhoeffiella* cannot be regarded as monophyletic, but rather paraphyletic, and not only closely related, but even intermixed with lineages of *H. nitidus*. Differently from *Verhoeffiella*, which is known from the subterranean habitats in Southern Europe only, *Heteromurus nitidus* is widespread in surface habitats across the majority of Palearctic and Nearctic regions. In Europe, it is also known from numerous caves within the range of *Verhoeffiella* (Jordana, Arbea, & Ariño-Plana, 1990; Lukić & Deharveng, 2008; Thibaud, 2017). Therefore, our results dispute the biogeographical enigma of disjunct distribution of exclusively subterranean genus *Verhoeffiella*, as it is closely related to surface and generally widespread *H. nitidus* species.

Disjunct distributional patterns encompassing records from the Iberian Peninsula, the Alps and the Dinarides are based on morphological comparisons for terrestrial subterranean taxa of several groups: Pseudoscorpiones (Duchae, 2001); Isopoda (Taiti et al., 2018); Collembola (Fanciulli et al., 2003; Lukić et al., 2018, 2010); and Diplura (Sendra et al., 2004). Only two genera, the terrestrial snail *Zospeum* (Weigand et al., 2013) and the spider *Typhlonesticus* (Ribera et al., 2014), were subjected to molecular phylogenetic studies and found to be monophyletic. Therefore, for both genera

peculiar continental scale, disjunct distributions were confirmed. Although our results did not support the monophyly of *Verhoeffiella*, the pattern of disjunction is still interesting, as it largely coincides with the spatial and even molecular patterns recognized in the terrestrial snail *Zospeum*. Molecular phylogeny of *Zospeum* showed that Iberian populations are in a sister relationship to Alpine, and Alpine to Dinaric populations (Inäbnit et al., 2019; Weigand et al., 2013), reflecting the pattern revealed in *Verhoeffiella*. Further research is needed to elucidate the origin and the possibly congruent colonization scenarios of these remote areas by taxa with presumably limited dispersal possibilities and different natural histories.

### 4.2 | Limits of *Heteromurus* and *Verhoeffiella*

Originally, *Verhoeffiella* was described as a subgenus of *Heteromurus*, but treated later as a separate genus or even as a part of *Heteromurus* s. str. (see Lukić et al., 2018). Changes in taxonomic status were usually not thoroughly justified by authors and merely reflected a subjective appreciation of the value of antennae III annulation, the only character used to discriminate the two genera (Cipola et al., 2016; Lukić et al., 2018; Mari Mutt, 1980b, 1980a).

Molecular phylogeny of *Verhoeffiella* and *Heteromurus* confirmed their perplex status, where *Verhoeffiella* is related with one of the *Heteromurus* species, namely *H. nitidus*. Revealed diversification pattern indicates that the current taxonomy does not reflect the phylogenetic relations, but rather the ecology of the group. Across Europe, all troglomorphic and subterranean lineages were traditionally assigned to the genus *Verhoeffiella*. At the same time, the non-troglomorphic subterranean or epigeal lineages were assigned to the species *H. nitidus*. This long-lasting taxonomic dilemma originates from the use of antennae III annulation as a discriminant character at the generic level, while it could be a troglomorphic feature connected to antennae elongation, associated with life in subterranean habitats (Christiansen, 2012; Cipola et al., 2016). Some additional features have been recognized in Dinaric *Verhoeffiella*, which can be used to separate *Verhoeffiella* and *Heteromurus* (Lukić et al., 2018). However, we refrain from new generic definitions as they are out of the scope of this study. At the same time, our results support the need for an integrative revision of the genus *Heteromurus*, that would combine morphology and molecular phylogeny and would also encompass the redescription of *H. nitidus*.

### 4.3 | Remarkable hidden diversity within the Dinarides

Dinarides in the Balkans have been recognized as a world hotspot of subterranean biodiversity (Culver et al., 2006;



Zagmajster et al., 2014; Zagmajster, Malard, Eme, & Culver, 2018). High species diversity in the area was confirmed also when morphological and molecular species determinations were compared on a continental scale (Eme et al., 2017). Cryptic diversity within the region was shown in different aquatic groups (Delić, Trontelj, Rendoš, & Fišer, 2017; Trontelj et al., 2009; Zakšek, Sket, Gottstein, Franjević, & Trontelj, 2009), but also in terrestrial troglobionts (Inábnit et al., 2019; Weigand et al., 2013), where only a few taxonomic groups were subjected to phylogenetic studies (Bedek, Taiti, Bilandžija, Ristori, & Baratti, 2019; Lukić et al., 2018; Murienne, Karaman, & Giribet, 2010; Njunjić et al., 2018; Njunjić, Perreau, Kasper, Schilthuizen, & Deharveng, 2016; Pavlek & Ribera, 2017; Polak et al., 2016; Ribera et al., 2014). Based on the geographic extent and the number of specimens used, our study represents the most extensive molecular phylogeny of terrestrial troglobionts in the region, unveiling the hidden diversity within the Dinarides.

The extent of increase in diversity, as based on MOTU delimitations, was still very surprising. Two different species delimitation methods supported the morphological species limits of the 10 nominal species, but also revealed a much higher number of *Verhoeffiella* MOTUs in Dinarides (PTP = 74 and ABGD = 73). It turns out that the number of MOTUs is about seven times higher than the number of nominal species, representing remarkable subterranean radiation within a relatively narrow geographical range. This finding additionally supports and even reinforces the Dinarides as a centre of high diversification of terrestrial troglotrophic fauna.

Strong diversification of collembolan genera within subterranean habitats has already been documented, but on a larger geographical scale (Lukić, 2019). The most extreme example is that of the genus *Pseudosinella* encompassing more than 100 troglotrophic species, distributed across Europe and the Mediterranean (Jordana et al., 1990; Thibaud, 2017). High cryptic diversity within Collembola was already noted (for an overview see Zhang, Jantarit, et al., 2018), but not to the extent discovered in *Verhoeffiella* and rarely within subterranean habitats (Katz et al., 2018). If most of the discovered MOTUs will be proven to equate morphological species, *Verhoeffiella* will be listed among the most diversified terrestrial subterranean genera in the region.

#### 4.4 | On possible colonization scenarios of subterranean habitats

Paraphyletic relationship of the genera *Verhoeffiella* and *Heteromurus*, differing in ecology and distributional patterns, enables discussions on colonization hypotheses as one of the fundamental questions in subterranean biology (Culver & Pipan, 2019; Moldovan et al., 2018). The basal

position of troglotrophic populations of *Verhoeffiella* from the Iberian Peninsula and the Alps, in relation to the intermixed populations of epigeal *H. nitidus* and Dinaric *Verhoeffiella*, suggests differing hypotheses on a continental and regional level. It is important to note that disjunct pattern of troglotrophic *Verhoeffiella* populations could hardly be a result of sampling bias, as no *Verhoeffiella* species has ever been recorded in thoroughly sampled regions in between the known distribution range, namely Western Alps, French southern Massif Central and the Pyrenees (Dallai & Malatesta, 1982; Dallai, Malatesta, & Ramellini, 1995; Jordana et al., 1990; Thibaud, 2017).

On a continental scale, the most plausible hypothesis would be that a common, epigeal *H. nitidus*—like ancestor or even a group of closely related species, independently colonized subterranean habitats in different geographic regions. Such an ancestor(s) would have had a role of a surface vector dispersing into geographically distant karst regions, and further evolving to subterranean forms—commonly referred to as *Verhoeffiella*. This hypothesis might explain the *Verhoeffiella* relationship on a continental scale: sister relationship of Catalanian and Cantabrian populations and sister relationship of Iberian to Alpine and Alpine to Dinaric populations. A similar role of surface vector was documented in the *Trechus fulvus* group and in the genus *Duvalius* of the Trechinae beetles (Faille, Casale, Balke, & Ribera, 2013). An alternative hypothesis, implying continental scale distance dispersal of already adapted subterranean lineages, would have to be rejected. Especially as the distance itself, coupled with the geographical barriers, such as rivers or areas of unsuitable habitats, represent an impassable obstacle for taxa with limited dispersal abilities. Passive dispersal was proposed to explain the disjunct distribution of subterranean terrestrial snail *Zospeum* (Weigand et al., 2013), known also from the Iberian Peninsula, the Alps and the Dinarides. However, this seems implausible for Collembola species due to different lifestyle and ecology of the two groups. Another complementary process that might have taken part in shaping the extant distribution pattern of troglotrophic *Verhoeffiella* populations is extinction of a part of subterranean populations, possibly due to climate changes in Pleistocene, particularly in the Alps (Mammola, Goodacre, & Isaia, 2018; Mammola, Schönhofer, & Isaia, 2019; Stoch & Galassi, 2010).

Radiation of *Verhoeffiella* within the Dinarides and the complex relationship with *H. nitidus* in this region offer further possibilities for interpretation of colonization scenarios on the regional scale. As at continental scale, Dinaric *Verhoeffiella* lineages might originate from many simultaneous colonizations in different areas of Dinarides by a group of closely related epigeal *H. nitidus*—like ancestor. An alternative hypothesis, that could get more support at the

regional scale, is dispersal of the already adapted subterranean lineages. In that case, *Verhoeffiella* lineages would have originated from a few separated colonization events, followed by dispersal via more or less continuous karst area and subsequent speciation. This hypothesis is in line with the recent studies of Dinaric Leiodidae beetles of genera *Anthroherpon* and *Hadesia*, which suggest a long-distance dispersal of the already adapted subterranean taxa (Njunjić et al., 2018; Polak et al., 2016). With the data set at hand, it is not possible to rule out one of the alternative hypotheses. However, the large magnitude of the spatial overlap of the five recognized *Verhoeffiella* lineages supports the multiple colonization hypothesis. This is further corroborated by many co-occurring species and MOTU pairs belonging to different *Verhoeffiella* lineages. As much as four highly divergent MOTUs from two lineages were found in a single cave.

Extinction of ancestor or related surface-dwelling populations is almost a universal rule for troglolithic taxa of temperate regions (Culver & Papan, 2019; Humpreys, 2000). It is possible that surface ancestor of Iberian *Verhoeffiella* population became extinct (climate relict hypothesis) (Peck & Finston, 1993), as Iberian lineages of *H. nitidus* included in our analysis did not cluster with Iberian *Verhoeffiella* populations. Instead, they were nested within *H. nitidus*–Dinaric *Verhoeffiella* complex, suggesting that extant *H. nitidus* populations might represent direct descendants of an ancestor of Dinaric *Verhoeffiella*. This hypothesis is supported by several lineages of *H. nitidus* intermixed with lineages of Dinaric *Verhoeffiella*. However, we did not find direct evidence, for example *Verhoeffiella*–*H. nitidus* sister species pairs, which would unambiguously identify the *H. nitidus* lineage as a direct surface relative to *Verhoeffiella*. Nevertheless, they are undoubtedly closely related. In any case, all colonization scenarios have to be treated with caution, especially due to low support for some relations within *H. nitidus*–Dinaric *Verhoeffiella* complex. With data at hand, we cannot undoubtedly exclude even the possibility that *H. nitidus* could present a monophyletic group. If proven to be true, such a scenario would imply that unsampled *H. nitidus* population, or an extinct epigeal species, is ancestral to the whole radiation.

The comparison of colonization scenarios with other Dinaric terrestrial subterranean taxa is limited as their possible ancestors remain largely unknown. Evolutionary studies on beetles of the genera *Anthroherpon* and *Hadesia* have shown that their common ancestor probably already had a similar subterranean lifestyle (Njunjić et al., 2018; Polak et al., 2016). The primarily systematic studies on *Zospeum* (Gastropoda) (Inäbnit et al., 2019) and *Alpioniscus* (Isopoda) (Bedeck et al., 2019) demonstrated a large spatial overlap of divergent lineages, possibly suggesting similar complex colonization and speciation scenarios as in *Verhoeffiella*. However, their possible ancestor or extant surface relative remains unknown. Therefore, the discovery of a close relationship

between the subterranean radiation of *Verhoeffiella* and the epigeal lineage of *H. nitidus* represents an exceptional study system within the Dinarides, and even subterranean fauna of temperate areas.

#### 4.5 | Estimations of divergence times

In the absence of reliable fossil calibrations for Collembola, we have employed estimates of substitution rates for distantly related tenebrionid beetles (Papadopoulou et al., 2010), as already used in several studies on Collembola (Katz et al., 2018; Zhang, Yu, et al., 2014; Zhang, Yu, Stevens, & Ding, 2018). Our results and the results of Katz et al. (2018), that also studied cave Collembola and used substitution rates for tenebrionid beetles, coincide well with the paleogeographic events in the respective geographical regions, Dinarides in Europe and the Salem Plateau in the United States. This suggests that the use of substitution rates for tenebrionid beetles is likely appropriate for estimating Collembola divergence times.

Onset of the whole *Verhoeffiella*–*Heteromurus nitidus* clade, including divergence time of *Verhoeffiella* sister populations from Cantabria and Catalonia, is estimated to middle Miocene. This estimation coincides with the origin of other Pyrenean subterranean fauna, for example Trechini beetles (Faille et al., 2010). Estimation of divergence times within the *Heteromurus nitidus*–Dinaric *Verhoeffiella* complex enabled us to link the cladogenetic and speciation events to major paleogeographic events. Estimated origin of all the major lineages of this complex is dated in the late Miocene (5–7 MA), corresponding to the age of Messinian salinity crisis (5.96–5.33 MA). This is also the timeframe that corresponds to high orogenic activity in the development of the Dinarides (Aliaj, 2006; Vrabec & Fodor, 2006). In addition, during the middle part of the Lower Miocene, extensive parts of Dinarides were covered by an ancient lake (Jiménez-Moreno et al., 2009), which later disintegrated into a complex lacustrine environment (de Leeuw, Mandić, Krijgsman, Kuiper, & Hrvatić, 2011). Lake area largely overlaps with the extant distribution of Dinaric *Verhoeffiella* populations. Therefore, the diversification pattern of *Verhoeffiella* is likely to have been affected by the orogenic activity and the lake desintegration dynamics. Most of the speciation events, that include continent–island sister species pairs, were estimated to the Pleistocene (2.5 MA–11.7 k years ago), an age characterized by repetitive cycles of marine regressions and transgressions (Krijgsman, Hilgen, Raffi, Sierro, & Wilson, 1999), as well as glaciations in the Dinarides (Hughes, Woodward, van Calsteren, & Thomas, 2011; Žebre & Stepišnik, 2014).

Very few studies of other subterranean taxonomic groups with divergence time estimation are available for comparison within the Dinarides. In the case of two sister taxa of subterranean Leiodidae beetles, genera *Hadesia* and *Anthroherpon*,



there is some disagreement. Njunjić et al. (2018) states that speciation of both genera corresponds to the Miocene, while Polak et al. (2016) concluded that speciation within *Hadesia* occurred in Pleistocene. Messinian and Pleistocene sea-level changes were already evoked as triggers for inter- and intraspecific diversification within amphipods and some terrestrial isopods of the Dinarides (Bedeck et al., 2019; Delić, Švara, Coleman Charles, Trontelj, & Fišer, 2017). Further, the origin of most Dinaric taxa, whose ranges fully or partially corresponds to those of Dinaric *Verhoeffiella*, are generally estimated to be of early or middle Miocene age (Bedeck et al., 2019; Delić, Švara, et al., 2017; Njunjić et al., 2018; Trontelj et al., 2007). Our data on *Verhoeffiella* showed a somewhat different pattern, with relatively young colonization of subterranean habitats dated in the late Miocene (5–7 MA), which produced remarkable subterranean radiation within the Dinarides. Linking the subterranean radiation of *Verhoeffiella* to major paleogeographic events provides important insight into processes that drove invertebrate diversification within Dinaric terrestrial subterranean habitats.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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### 3 DISCUSSION AND CONCLUSIONS

#### 3.1. DISCUSSION

##### 3.1.1. Taxonomy and phylogenetics

###### 3.1.1.1. Generic level

The results of our molecular phylogeny revealed that the genus *Heteromurus* is paraphyletic and the genus *Verhoeffiella* is polyphyletic. This explains the ‘floating’ taxonomic status of *Verhoeffiella* throughout the history of its study. It was treated as a separate genus, as subgenus of *Heteromurus* or even as *Heteromurus* sensu stricto (see Lukić et al., 2018). The status changes reflected different authors’s appraisal of the importance of the single diagnostic character – the annulation of third antennal segment. The annulation refers to the successive subtle cuticular invaginations that form annuli, each of them bearing a whorl of chaetae (see Lukić et al., 2018: Fig 9.A). All *Verhoeffiella* species have third and fourth antennal segment annulated, while *Heteromurus* have only fourth antennal segment annulated. However, it is not always easy to observe this difference. Even more, our study revealed that the third antennal segment is not completely annulated in *Verhoeffiella*. Different species have 1/3 to 1/10 non-annulated proximal part of this segment and sometimes chaetae in annuli are arranged in spirals (Lukić et al., 2015, 2018). A population of an epigeal *Heteromurus* from US had even fourth antennal segment is non-annulated, but the impression of the annulation under lower magnification is there due to the chaetae arranged in whorls (Mari Mutt, 1980b). Another example is a cave species *Heteromurus peyerimhoffi* Denis, 1937 from Algeria, having the fourth antennal segment annulated, but the chaetae of the third antennal segment arranged in whorls, even though the actual segment is not annulated (Mari Mutt, 1980a). A further problem with the use of annulation as a generic diagnostic character is that it could be a troglomorphic feature related to antennae elongation, associated with life in subterranean habitats (Christiansen, 2012). In collembolan genera of different families, antennal segments are sub-segmented bearing whorls of chaetae (e.g. *Arrhopalites*, *Pygmarrhopalites*, *Disparrhopallites*, *Tomocerus*, *Tritomurus*), with increased number of these subsegments in cave species (Vargovitsh, 2017). Therefore, the annulation of third antennal segment within *Verhoeffiella* species might be a mechanism of antennae elongation. It is important to note that sub-segmentation and annulation is not the universal rule of antennae elongation in Collembola, as there are many examples of cave species whose antennal segments are simply lengthened without annulation, segmentation or specially arranged chaetae e.g. genera *Coecobrya* and *Sinella* of family Entomobryidae (Zhang and Deharveng, 2011; Nilsai et al., 2017). It seems that within the *Heteromurus*–*Verhoeffiella* complex, the annulation repeatedly occurred during the colonization of cave habitats, but due to partially resolved relationship it is not possible to identify exactly how many times it occurred (Lukić et al., 2020). Although antennae within Entomobryomorpha

usually bear strong generic diagnostic characters, e.g. subdivision in the case of genera *Dicranocentrus*, *Orchesella* and *Troglopedetes* (Mari Mutt, 1979, 1980b; Deharveng and Gers, 1993), the use of antennae annulation as a diagnostic generic character in *Heteromurus* and *Verhoeffiella* was misleading. When looking at other morphological characters used at generic level within Heteromurinae, *Heteromurus* and *Verhoeffiella* species are very similar (see Cipola et al., 2016), with the exception of several Iberian populations. Described dorsal body macrochaetotaxic pattern of *V. gamae* from Catalonia (Lukić et al., 2015) and *V. cf. longicornis* from Cantabria (da Gama, 1984) depart from the standard macrochaetotaxic pattern of Heteromurinae, while in *V. gamae* labial triangle, tibiotarsus, femur and manubrium chaetotaxy, and claw morphology differ from the ones recognized in Dinaric *Verhoeffiella* and *H. nitidus* (Lukić et al., 2015). These morphological differences are also corroborated by phylogeny as Iberian *Verhoeffiella* phyletic line is molecularly highly divergent (Lukić et al., 2020).

The result of the molecular phylogeny implicates the need for taxonomic re-arrangements at the generic level of Heteromurinae, with two possibilities. One would be to establish the monophyly, by synonymizing *Verhoeffiella* with *Heteromurus*. In that case, the genus *Heteromurus* would comprise several molecularly highly divergent phyletic lineages: *H. major*, Iberian *Verhoeffiella*, Alpine *Verhoeffiella*, and Dinaric *Verhoeffiella* + *H. nitidus* complex. Another possibility would be to divide the clade in three genera: (1) *H. major* lineages, (2) Iberian *Verhoeffiella*, (3) Alpine and Dinaric *Verhoeffiella* + *Heteromurus nitidus* complex. However, we restrain so far from new taxonomic re-arrangements due to several reasons. First, at this moment there are no hard morphological data that would support either of the two options. As *Heteromurus* is the only genus among Heteromurinae that still lack detailed modern species description, it is important to collect topotypes, retrieve molecular data and redescribe type species of the genus, *H. margaritarius* Wankel 1860 described from Czech Republic. It is also important to study *Heteromurus nitidus* described from Ireland under the name *Podura (Ptenura) nitida* Templeton, 1836. It is important to re-evaluate the taxonomic status of these two populations as *H. margaritarius* has been synonymized with *Podura (Ptenura) nitida*, but considering the number of disclosed *nitidus* lineages (Lukić et al., 2020) we cannot rule out that they might belong to separate species. It is important to note that generic name *Heteromurus* has been placed on the Official List of the Generic Names in Zoology, while generic name *Ptenura* have been placed on Official Index of Rejected and Invalid Generic Names in Zoology by the Opinion 1064 (ICZN, 1976), which adds to the complexity of the case. Second, it is necessary to include more Iberian *Verhoeffiella* species in the analysis. Important differences in characters that have been observed in *V. gamae* from Catalonia (Lukić et al., 2015) and are possibly less convergent should be checked on other Iberian species and their possible suprageneric importance should be evaluated. Third, large polytomy of *H. nitidus*–Dinaric *Verhoeffiella* complex conceals their true relationship, and we cannot rule out that *H. nitidus* represents a monophyletic group (Lukić et al., 2020). This could be possibly resolved by adding more populations of *H. nitidus* from other European sites,



more *Verhoeffiella* populations and/or additional genes to the analysis. Further, two stenoendemic species of the *nitidus* group sensu Mari-Mutt 1980 (*H. noseki* Mari Mutt & Stomp, 1980 from cave and MSS in Romania, and *H. peyerimhoffi* Denis, 1937 from caves in Algeria), and species of the *H. major* group sensu Mari-Mutt 1980 (*H. gigans* Mari Mutt & Stomp, 1980 from Portugal, and the Eurasian *H. sexoculatus* Brown, 1926), should be included in the study, as they could help resolve the uncertain relationships. Fourth, several genera are still missing in the molecular phylogeny of Heteromurinae: the pantropical genus *Heteromurtrella*, the genus *Pseudodicranocentrus* from Mexico and Guatemala, and the recently described genus *Falcomurus* from India. Their inclusion might not alter the paraphyly of *Heteromurus* but could result in a more robust phylogeny of Heteromurinae.

Morphological and molecular phylogenies are powerful tools for improving a taxonomical hierarchy. Preferably, they should be combined to test the hypotheses on taxa relationships. Robustness of morphological phylogenies greatly depends on the choice of the morphological characters used. Chaetotaxy is today the most important morphological character in the modern taxonomy of Entomobryoidea, following the seminal papers of (Yosii, 1956; Gisin and da Gama, 1962; Gisin, 1963). Chaetotaxic system involves the nature, number, and position of the chaetae, and is suitable for character coding, but only if there is unequivocal recognition of chaetotaxic homology. As Entomobryidae are strongly plurichaetotic (Szeptycki, 1979), chaetae-to-chaetae homologies are often disputable. Plurichaetosis starts to appear from the second instar onwards. The pioneering work of (Szeptycki, 1969, 1972, 1979) on the first instar chaetotaxy and its development during ontogenesis gave the basic criteria of homology recognition and related nomenclature system. Chaetotaxy of the first instar is therefore of special interest and has already been studied and proven useful in Entomobryidae (Szeptycki, 1972; Barra, 1975; Szeptycki, 1979; Soto-Adames, 2008; Zhang et al., 2011; Zhang and Deharveng, 2015b; Zhang et al., 2019) and other Collembolan families (see Deharveng, 2004). Dorsal body macrochaetotaxic and S-chaetae patterns were well documented among *Verhoeffiella* species studied in this thesis (Lukić et al., 2015, 2018). However, mapping and assigning homologies of chaetae for the formation of a morphological data matrix turned out as very problematic. First, we had problems with recognizing chaetotaxic homology, which is of crucial importance for accurate species comparison and phylogenetic reconstruction. Although the groundwork of Szeptycki, (1969, 1972, 1979) sets the basis for homology recognition, it was only very recently that homology hypotheses have been tested and corrected on a wide range of Entomobryoidea taxa (Zhang et al., 2019). This chaetotaxic homologization will ensure more accurate mapping and naming of chaetae in future studies on *Verhoeffiella*. Second, head and body mesochaetae were often detached during slide preparation. The sockets of these detached chaetae remain visible but were very similar to S-like chaetae what hampered the mapping, particularly on the fourth abdominal tergite. Third, we observed that the number and position of the dorsal body mesochaetae were sometimes variable within the populations. Fourth, in our study we had no first instars, that are important for studying chaetae-to-chaetae homologies, available for *Verhoeffiella*.

Difficulties in collecting the first instar are common also in many other species of cave Collembola. Further efforts are needed to collect them, which should include targeted sampling technique, like Berlese funnels, washing and floatation or laboratory breedings. Fifth, many samples in our study had a low number of adults. Most of the collected specimens were juveniles and subadults, even in the samples with hundreds of individuals as was the case in *V. absoloni* (Lukić et al., 2015). These problems were a limiting factor for inclusion of populations in the morphological study, and affected the possibility to evaluate the variability of the characters that could potentially be coded. In our study, we focused on the antennal chaetotaxy and discovered a number of new diagnostic characters (Lukić et al., 2018). Regarding chaetotaxy, we recognized twelve categories of antennal chaetae, arranged in obvious but polychaetotic and fuzzy patterns (Lukić et al., 2015, 2018). These patterns are retrieved across the different *Verhoeffiella* species, but chaeta to chaeta homologies, hence coding, are impossible in most cases. The aforementioned issues therefore strongly limited the possibilities to build a significant data matrix for morphological phylogeny.

#### 3.1.1.2. Specific level

The poor and sketchy original descriptions of *Verhoeffiella* species hampered progress on the taxonomy of *Verhoeffiella*. It was even difficult to decide whether any newly collected taxon was identical or different from a previously described one. Therefore, we made a detailed re-description of two species *V. absoloni*, and *V. cavicola*, the type species of the genus (Lukić et al., 2015, 2018). These descriptions were very detailed and lengthy, encompassing all characters used in the modern taxonomy of Entomobryidae. In order to shorten descriptions of other species we described, rather than describing all characters, we presented only characters that differ or are variable among *Verhoeffiella* species (Lukić et al., 2018). Altogether, we described five new species and re-described three known ones, and set the groundwork for future comparative morphological studies (Lukić et al., 2015, 2018). Data gathered on diversity and variability of the morphological characters made it possible to evaluate their taxonomic value and to use them in an extensive list of diagnostic characters (Lukić et al., 2018: see Tables 2, 3). The list mainly encompassed distribution and number of specific chaetae types on antennae, head, body, legs, ventral tube and furca, foot complex morphology, ratios of antennae elongation and distribution of scales (Lukić et al., 2015, 2018). Some characters were variable within species, so their values have been given as ranges of values. Overlapping ranges of some characters bring further problems in characterizing species. Nevertheless, our comparative study and newly established morphological framework resulted in set of characters that will facilitate future studies of *Verhoeffiella*, but also of other species within Heteromurinae (Jantarit and Sangsiri, 2020). Similar frameworks have already been proven as very useful tools in the taxonomy of several collembolan groups (Szeptycki, 1979; Deharveng, 1983; Christiansen et al., 1990;

Potapov, 2001; Jordana and Baquero-Martin, 2005; Zhang and Deharveng, 2011; Zhang and Deharveng, 2015a; Queiroz and Zeppelini, 2017; Schneider, 2017; Cipola et al., 2018).

The morphological framework and the subset of diagnostic characters can also allow quick species identifications, and preliminary characterization of species. Turbo-taxonomic papers, an approach combining DNA barcoding with short taxonomic descriptions of morphological characters (Butcher et al., 2012; Riedel et al., 2013) may be prepared based on the selection of diagnostic characters instead of lengthy and time-consuming species descriptions. But this approach is more difficult in groups like Collembola where most species lack sexual characters and have a taxonomy based on numerous independent characters on various body parts. By combining shorter morphological descriptions and molecular characterization, a faster taxonomic progress on the *Verhoeffiella* species could be nevertheless achieved. However, there are some drawbacks to the use of molecular approach in *Verhoeffiella* species description, at least at current state of the knowledge. It is highly likely that the description of a larger number of *Verhoeffiella* species based on molecular diagnoses would not be reflected in morphological diagnoses, as intervals of values for a number of characters may overlap greatly (Lukić et al., 2018: Table 2). But we managed to identify some stable morphological characters with almost no interspecific and intraspecific variation, that are limited to only one or two species and have turned out as useful in species determination. Without a detailed morphological study of the new species, novel morphological characters might remain undetected. Considering the level of revealed taxonomic incompleteness and the number of new species from different lineages that are awaiting morphological descriptions, it is very likely that we currently have a limited view on the diversity and variability of the morphological characters. Therefore, future advances in the taxonomy of *Verhoeffiella* should not rely only on this reduced subset of characters. Our descriptions of *Verhoeffiella* species have been pushed far in terms of morphological resolution, and of time spent on studying the specimens, but there is still room to improve our knowledge. In particular, mapping the complete dorsal body chaetotoaxy, with focus on the homology and variability of the mesochaetae pattern would probably bring a lot of information and new characters.

### 3.1.1.3. Cryptic species

It has already been documented, that in many Collembola families there is strong speciation within some genera (Deharveng, 2004). One of the most remarkable cases is the genus *Pseudosinella* of family Entomobryidae, having more than 300 species (Bellinger et al., 2019), of which half are cave restricted (Lukić, 2019). This immense diversity has been documented on morphology alone, while the genus has never been molecularly studied. Molecular studies done so far on Collembola revealed that populations are highly structured, with a high genetic divergence between and low divergence within populations (von Saltzwedel et al., 2016). This high intraspecific divergence is sometimes comparable to interspecific divergence values in congeneric species (Porco et al., 2012; Sun et al.,

2018.), suggesting the existence of cryptic species. With DNA-based approaches cryptic diversity has increasingly been reported among many collembolan families: e.g. Onychiuridae, Tomoceridae, Isotomidae, Entomobryidae and Neelidae (Soto-Adames, 2002; Cicconardi et al., 2010; Felderhoff et al., 2010; Schneider et al., 2011; Lukić et al., 2012; Cicconardi et al., 2013; Porco et al., 2014; Zhang et al., 2014; Katz et al., 2015; von Saltzwedel et al., 2016, 2017; Sun et al., 2017, 2018; Zhang et al., 2019).

In the genus *Heteromurus*, seven cryptic lineages have been detected on a small sample of European populations of *H. major* (Lukić et al., 2012). In our analyses, we have discovered three or four more molecular operational taxonomic units (MOTUs) of *H. major* (PTP = 11, ABGD = 12) (Lukić et al., 2020). Following a larger sampling, we discovered an even higher number of MOTUs in another species of the genus, *H. nitidus* (PTP=33, ABGD=31) (Lukić et al., 2020). *Heteromurus nitidus* is a well-defined species based on several important morphological characters (Mari Mutt, 1980a), although some characters are given as rather variable in the literature (Bonet, 1931; Denis, 1931; Delamare Deboutteville, 1944; da Gama, 1962; Mari Mutt, 1980a). On the other hand, many forms, varieties and subspecies have been described within *H. nitidus*, and are considered as synonyms on very light grounds (Mari Mutt, 1980a). During our preliminary examinations of the *H. nitidus* material, we observed that specimens of one MOTU from Croatia differed notably in dorsal macrochaetotoaxy from all other species of the genus. It likely represents a new, morphologically easily recognizable species, or may correspond to another of the subspecies synonymized with *H. nitidus*. We did not perform any detailed morphological studies on other *H. nitidus* populations, which were included in molecular analyses. But, even the few observations we have made indicate that cryptic diversity within *H. nitidus* needs to be further studied, and taxonomically evaluated using the established morphological framework (Lukić et al., 2018).

Cryptic diversity among Collembola in subterranean habitats has been reported only for two genera: *Pygmarrhopalites* (Arrhopalitidae) (Katz et al., 2018) and *Coecobrya* (Entomobryidae) (Zhang et al., 2018). The case of *Verhoeffiella* is therefore the third case, where cryptic diversity was established – we delimited 79 different MOTUs of *Verhoeffiella*, most of them coming from the Dinarides (Lukić et al., 2020). This supports the previous observations of high subterranean cryptic biodiversity in the region, which was shown mostly on different aquatic (Trontelj et al., 2009; Zakšek et al., 2009; Eme et al., 2017), but also terrestrial troglobionts (Weigand et al., 2013; Bedek et al., 2019; Inäbnit et al., 2019). However, it can be questioned whether all cases in *Verhoeffiella* really reflect cryptic diversity, in the sense that there are no differences in taxonomically relevant characters. All nominal species of *Verhoeffiella*, that belongs to separate MOTUs, are morphologically well characterized. A closer look at some other MOTUs revealed a number of diagnostic morphological characters. Therefore, many additional species of the genus await to be morphologically described (Lukić et al., 2020). This may be easy for species of distantly related lineages, but it may be a bigger challenge for closely related



species. If we cannot separate them morphologically, we would regard them as cryptic species, which call for further evaluation using multilocus delineation methods, and further morphological studies under high-resolution differential interference contrast (DIC) and scanning electron microscope. Formal morphological descriptions of such closely related species would have to be complemented with molecular diagnoses (Eme et al., 2017). Current extent of the cryptic diversity within *Verhoeffiella* and also within *H. nitidus* present exceptional case among Collembola analysed so far. Great amount of work would be needed for the taxonomical evaluation of all the discovered MOTUs, and it remains very likely that not all of them will ever be formally described. For sure the established morphological framework would play a key role in facilitating this work and enhancing species descriptions.

### 3.1.2. Multiple origin and diversification of *Verhoeffiella*

Molecular phylogeny showed that the exclusively subterranean genus *Verhoeffiella*, together with lineages traditionally ascribed to the species *H. nitidus*, forms a well-supported clade nested within Heteromurinae (Lukić et al., 2020). Epigeal *Heteromurus major* turned out to be the sister taxon of the clade. Within the clade, geographically distant populations of *Verhoeffiella* from Catalonia and Cantabria branch at the basis. The *Verhoeffiella dallaii* from south-eastern Calcareous Alps is in a sister relationship with the *H. nitidus*–Dinaric *Verhoeffiella* complex. Within this complex, lineages of *H. nitidus* and Dinaric *Verhoeffiella* seem to be intermixed but a large basal polytomy conceals their true relationship (Lukić et al., 2020).

Although only partially resolved, phylogenetic relationship enables setting up the hypotheses on colonization of subterranean habitats. On a continental scale, the most plausible hypothesis would be that there were at least four separate colonization events, in Catalonia, Cantabria, the Alps, and the Dinarides. Single colonization and subsequent dispersal underground is a less probable scenario at this scale, due to large geographical distances among the regions, as well as important geographical barriers (like for example non karstic areas and sedimentary soils). This last hypothesis would also require the extinction of populations due to Pleistocene climate oscillations, as speculated for other taxonomic groups in the Alps (Stoch and Galassi, 2010; Mammola et al., 2018; Mammola et al., 2019), but it would have occurred at a larger scale, and would have concerned large karstic areas like Pyrenees.

On a regional scale, within the Dinarides, single colonization of subterranean habitats, followed by dispersal and speciation could have resulted in several *Verhoeffiella* lineages. But, alternatively, it would be possible that multiple colonizations and subsequent dispersals occurred, which would explain the high number of *Verhoeffiella* lineages and their spatial overlaps. Our results suggest that Dinaric *Verhoeffiella* is closely related to European populations of *H. nitidus* (Lukić et al., 2020), but their unresolved relationship

hinders the support to any of the alternative hypotheses. Resolving large polytomy on the basis of *H. nitidus*–Dinaric *Verhoeffiella* complex is the only way to know whether Dinaric *Verhoeffiella* and *H. nitidus* represent two monophyletic sister groups, or whether the *Verhoeffiella*–*H. nitidus* sister species pairs would indicate multiple colonization events. As both colonization processes may have been concomitant, the issue promises to be complex.

The comparison of colonization scenarios of *Verhoeffiella* with those of other terrestrial troglotaxa is limited due to relatively few targeted studies. Multiple colonizations of subterranean habitats were documented for two Trechinae genera, *Trechus* (*fulvus* group) and *Duvalius*, whose epigeal species are widely distributed (Faille et al., 2013). Passive dispersal was proposed (Weigand et al., 2013) to explain the disjunct distribution of subterranean terrestrial snail *Zospeum*, known also from Cantabria, the Alps, and the Dinarides. *Verhoeffiella* and *Zospeum* share even pattern of molecular lineage distribution, where snail populations from the Iberian Peninsula are in a sister relationship to Alpine, and Alpine to Dinaric populations (Weigand et al., 2013; Inäbnit et al., 2019). However, it seems implausible for troglotaxa species to passively disperse due to different lifestyles and ecology compared to microsnails.

Within the Dinarides, few studies have discussed hypotheses on colonization of subterranean habitats. In beetles of the genera *Anthroherpon* and *Hadesia* the phylogenetic trees imply that their common ancestor had a subterranean lifestyle, and that adapted subterranean taxa dispersed over large distances in the continuous karst (Polak et al., 2016; Njunjić et al., 2018). Similarly to *Verhoeffiella*, *Zospeum* (Inäbnit et al., 2019) and *Alpioniscus* (Bedeck et al., 2019) include molecularly highly divergent lineages and exhibited large spatial overlaps, suggesting complex colonization and speciation scenarios. However, these studies were primarily taxonomic and did not provide colonization hypotheses.

Even though different European karst regions were colonized by *Verhoeffiella*, they differ extensively in diversification rates. A great number of MOTUs and species were found in numerous caves of the Dinarides (Lukić et al., 2020). After successful colonization there were speciation events that led to an outstanding diversity in the Dinarides. Contrary to this, very few *Verhoeffiella* records are known for the Alps and the Iberian Peninsula, although these regions have been well sampled for Collembola (Dallai and Malatesta, 1982; Jordana et al., 1990; Dallai et al., 1995; Thibaud, 2017). We have studied the collections of cave Collembola from Catalonia and Cantabria but found only a few more localities with *Verhoeffiella* species, always with only a few specimens in the samples. For example, we have found two co-occurring MOTUs in the cave Forat del Rastre in Catalonia (Jantarit and Sangsiri, 2020), but no additional MOTU were found in the limited number of samples from surrounding caves. In Cantabria, we could not include several new localities of *Verhoeffiella* in our analyses, as we failed to amplify target genes. Therefore, it is possible that we did not detect complex speciation events in other regions

due to limited availability of populations for molecular studies. Targeted sampling is needed to provide stronger evidence. Another interesting difference is that *Verhoeffiella* is not so ubiquitous and abundant in the Iberian Peninsula and the Alps, as it is in the Dinarides, where it is even the dominant cave collembola in subterranean communities (Lukić and Deharveng, 2008).

We have assessed the approximate timeframe of the diversification processes of *Verhoeffiella* and *Heteromurus*, by estimating divergence times. Estimation of divergence times for Collembola is challenging and has been conducted only in few studies (Zhang et al., 2014; Katz et al., 2018; Zhang et al., 2018). In all of them, estimate of substitution rates for distantly related tenebrionid beetles (Papadopoulou et al., 2010) was used, due to absence of reliable fossil calibrations for Collembola. Even though it may be argued that the substitution rate for distantly related taxa might result in the erroneous estimates, we used the same approach due to convincing results in Katz et al. (2018). They studied cave Collembola on the Salem Plateau in the United States, and their estimations of divergence times coincided well with the paleogeographic events in the region. Using this approach, we discovered that the onset of the whole *Verhoeffiella*–*H. nitidus* clade, including divergence time of *Verhoeffiella* sister populations from Cantabria and Catalonia, occurred in middle Miocene. Within the *H. nitidus*–Dinaric *Verhoeffiella* complex, the origin of all the major lineages is dated to the late Miocene (5–7 MA), while most of the speciation events were dated to the Pleistocene (2.5 MA–11.7 k years ago) (Lukić et al., 2020). Both time frames coincide well with several paleogeographic events within the region. Late Miocene corresponds to a high orogenic activity in the development of the Dinarides (Aliaj, 2006; Vrabec and Fodor, 2006), to the disintegration of the Dinarid Lake System (Jiménez-Moreno et al., 2009; de Leeuw et al., 2011) and to the Messinian salinity crisis (5.96–5.33 MA; Krijgsman et al., 1999). Pleistocene is characterized by climatic oscillations, marine regressions and transgressions (Krijgsman et al., 1999), as well as glaciations in the Dinarides (Hughes et al., 2011; Žebre and Stepišnik, 2014). We can speculate that events in the Late Miocene caused major cladogenetic events, e.g. that formation of the newly emerged landmasses during the disintegration of the Dinarid Lake System and orogeny potentially enabled colonization of the ancestral lineages. Marine regressions and transgressions during Pleistocene might have affected the speciation of the coastal lineages. The estimated ages of splits of most continent–island sister species pairs corresponds to the Pleistocene (e.g. *Verhoeffiella* species pairs CA146 Lastovo island–CA201 Dubrovnik mainland; CA058 Hvar island–CA426 Biokovo mainland; Lukić et al., 2020: Figure 3). Still, the exact mechanism of some of these paleogeographic events remains unknown. With current dataset, we were not able to date the colonization of caves, yet from the time-calibrated phylogeny we can suggest that Dinaric *Verhoeffiella* species are of late Miocene age or younger. It is different from what was assessed for some other subterranean taxa in the Dinarides, the origins of which were estimated to be older, of an early or middle Miocene age (Trontelj et al., 2007; Delić et al., 2017; Njunjić et al., 2018; Bedek et al., 2019).

### 3.1.3. Biogeography

The first important biogeographical issue we addressed was the widely disjunct distribution of two troglobiotic species of *Verhoeffiella* (da Gama, 1984): *V. absoloni* described from the Dinarides, and recorded from Catalonia, and *V. longicornis* described from the Dinarides, and recorded from Cantabria. A detailed morphological comparison between *V. absoloni* specimens from the type locality in Dinarides and specimens from Catalonia have shown unambiguously that the specimens from Catalonia belong to new species. We described it as *V. gamae* (Lukić et al., 2015). Molecular phylogeny confirmed that *V. absoloni* and *V. gamae* are distantly related (Lukić et al., 2020). The claim on large disjunct distribution of *V. absoloni* was based on poor morphological descriptions of the species which led to misidentifications (Lukić et al., 2015). The same can be expected for Cantabrian records of *V. longicornis*, as we found many important morphological differences between type population from the Dinarides (Lukić et al., 2018) and the Cantabrian population (da Gama, 1984).

The second interesting problem was the validity of the disjunct distribution of the genus *Verhoeffiella*. Currently, ten species of *Verhoeffiella* are known from the Dinarides, while four species come from one karst region each: Jakupica (North Macedonia, following the transfer of *Heteromurus constantinellus* Ćurčić & Lučić in Lučić, Ćurčić & Mitić 2007 into *Verhoeffiella*), south-eastern Calcareous Alps (Italy), southern Catalonia and Cordillera Cantabrica (Spain) (Lukić et al., 2015, 2018). It is intriguing that some other terrestrial subterranean taxa have a similar distribution to that of *Verhoeffiella*. Taxa with records from the Iberian Peninsula, the Alps, and the Dinarides are snail *Zospeum* (Weigand et al., 2013), false-scorpion *Neobisium* (*Blothrus*) (Duchae, 2001), spider *Typhlonesticus* (Ribera et al., 2014), isopod *Alpioniscus* (*Illyrionethes*) (Taiti et al., 2018), and Collembola *Tritomurus* (Lukić et al., 2010), *Troglopedetes* (Fanciulli et al., 2003) and *Ongulonychiurus* (own unpublished data). Among them, only the snail *Zospeum* and the spider *Typhlonesticus* were subjected to molecular phylogenetic studies. In addition, *Zospeum* shares even the pattern of molecular lineage distribution recognized in *Verhoeffiella* (Weigand et al., 2013; Inäbnit et al., 2019). Genus *Typhlonesticus* was found to be monophyletic (Ribera et al., 2014), however low support values between the disjunctly distributed species prevent the comparison with phylogeographical patterns revealed for *Verhoeffiella* and *Zospeum*. The status of the other taxa needs to be checked through an integrative taxonomic approach, as it is possible that current disjunct distributions are results of erroneous taxonomy. So the question of possible congruent colonization scenarios, timeframes and paleogeographic events that generated such patterns remains open.

*Verhoeffiella* has been recorded in numerous caves in the south-eastern part of the Dinarides, its range spanning from north-western Bosnia and Herzegovina to the border of Montenegro and Albania (Lukić et al., 2020). The limit of its distribution in the eastern



part of the range remains uncertain due to lack of speleobiological research in these regions. The most north-western records are those in the caves near Sanski Most in Bosnia and Herzegovina and Gračac in Croatia. Extensive sampling in central Croatia and Lika region did not yield additional records of *Verhoeffiella*, suggesting the north-western limit of *Verhoeffiella*. We discovered a complex geographical pattern of five *Verhoeffiella* lineages (Lukić et al., 2020). Two lineages with the highest number of MOTUs spread over an area of more than 300 km straight line distance, and greatly overlap. Each of the two lineages is divided into two sub-lineages, which also largely overlap. Within their range, two more isolated lineages are known from a single cave, while the fifth lineage is located separately at the northern parts of south-eastern Dinarides (Lukić et al., 2020: Figure 1). The large magnitude of spatial overlap among different lineages and sublineages reflects phylogeographical replicates. These replicates, combined with the high species diversity and diversity of troglomorphic traits, makes *Verhoeffiella* a new and interesting model for the study of subterranean evolution.

### 3.2 CONCLUSIONS

In this dissertation, we have explored evolutionary pathways and diversification in subterranean environments using the collembolan cave genus *Verhoeffiella* as a study model. We have advanced the knowledge on systematics and biogeography of this taxon through morphological study and in a molecular phylogenetic framework. We can draw the following conclusions:

1. We set up an operational morphological framework for describing new species of the genus *Verhoeffiella* and identified new diagnostic characters at specific level. Morphological species characterization was corroborated by molecular species delimitation, supporting the robustness of morphological framework. The framework will be used for *Verhoeffiella* species descriptions, and beyond for the taxonomy of other genera of Heteromurinae.
2. Morphological study brought significant contributions to the general knowledge of Heteromurinae morphology, in particular for antennal chaetae patterns. This complex pattern and classification of antennal chaetotaxy was already used in the study of distantly related genus *Alloscopus* (Jantarit and Sangsiri, 2020) and will have to be compared to the pattern of other Heteromurinae.
3. An extensive subterranean diversification of the genus *Verhoeffiella* was discovered. Five new species were described out of a total number of 79 *Verhoeffiella* MOTUs. This suggests that total number of 14 nominal *Verhoeffiella* species still represent only a fraction of total genus diversity.
4. Molecular phylogeny revealed that exclusively subterranean genus *Verhoeffiella*, together with lineages of *Heteromurus nitidus*, forms a well-supported clade nested within Heteromurinae. Within the clade, *Verhoeffiella* is polyphyletic and *Heteromurus* is paraphyletic implicating the need for taxonomic re-arrangements at the generic level of

Heteromurinae. The use of the annulation of the third antennal segment, a single diagnostic character between the two genera was shown to be misleading.

5. Partially resolved relationship of *Heteromurus* and *Verhoeffiella*, differing in ecology and distributional patterns, enables setting up the hypotheses on colonization of subterranean habitats. On a continental scale, the most plausible hypothesis would be that there were at least four separate colonization events, in Catalonia, Cantabria, the Alps, and the Dinarides. On a regional scale, unresolved relationship of *H. nitidus* and Dinaric *Verhoeffiella* hinders the support to any of the alternative hypotheses – single or multiple colonization. Resolving this relationship in future studies might provide more robust data on colonization scenarios.

6. Revealed pattern of molecular lineage distribution recognized in *Verhoeffiella* is shared with the troglobiotic snail *Zospeum*, also known from the Iberian Peninsula, the Alps, and the Dinarides. Several troglobiotic taxa of false-scorpions, spiders and isopods have a similar disjunct distribution. The status of these taxa needs to be checked through an integrative taxonomic approach to eliminate the possibility of erroneous taxonomy. Further research might elucidate the possibly congruent colonization scenarios of these remote regions among different taxonomic groups.

7. We speculate that within the Dinarides paleogeographic events in the Late Miocene caused major cladogenetic events, while those of Pleistocene have increased the speciation rate within the *H. nitidus*–Dinaric *Verhoeffiella* complex (Lukić et al. 2020), although the exact mechanism remains unknown. From the time-calibrated phylogeny we can infer that Dinaric *Verhoeffiella* species are of late Miocene age or younger. The origin of most other subterranean taxa in Dinarides is generally estimated to be older than *Verhoeffiella*, of an early or middle Miocene age.

8. Molecular phylogeny revealed a complex geographical pattern of *Verhoeffiella* lineages within Dinarides. The large magnitude of spatial overlap among different lineages and sublineages reflects phylogeographical replicates. These replicates, combined with the high species diversity and diversity of troglomorphic traits, makes *Verhoeffiella* a new and interesting model for the study of subterranean evolution.

## 4 SUMMARY

### 4.1 SUMMARY

Subterranean habitats differ from the surface ones in several features: constant darkness, the paucity of food, relatively stable abiotic parameters e.g. temperature and humidity. Species adapted to these conditions (also called troglobionts) live permanently in the subterranean realm. The phylogenetic origin of these troglobiotic taxa in most cases remains poorly known. This might be due to strong morphological differences of troglobiotic species and their epigean relatives that conceals their true relationship, or to the extinction of these epigean relatives. It is a common view that most of the subterranean taxa originate from independent colonization events, sometimes followed by dispersal and different speciation mechanisms (Trontelj, 2018; Culver and Pipan, 2019). Most of these taxa have small distribution ranges, that have been usually explained by karstic landscape fragmentation, complex paleogeographic events and/or historic climatic variability (Deharveng and Gers, 1993; Trontelj et al., 2009; Rizzo et al., 2013; Eme et al., 2014; Zigmajster et al., 2014). Exceptionally, some are widely distributed and among them the most intriguing are those occurring in geographic regions that can be hundreds or thousands of kilometers apart. Within Europe, the distribution of only a few terrestrial troglobionts illustrates this case. Interestingly, several troglobionts share similar disjunct distribution pattern, being known from the Iberian Peninsula, the Alps and the Dinarides (Lukić et al., 2010; Weigand et al., 2013; Ribera et al., 2014; Pavlek and Ribera, 2017; Taiti et al., 2018). The disjunct distribution patterns of troglobionts suggest possible taxonomic misidentifications, challenge the validity of their monophylies as well as the colonization scenarios.

In this dissertation we addressed these questions by exploring distribution patterns and diversification of the collembolan genus *Verhoeffiella*. The genus has a disjunct distribution, occurring in four karst regions of Europe – the Dinarides, South-eastern Calcareous Alps, southern Catalonia and Cordillera Cantabrica (Lukić et al., 2015). It had ‘floating’ taxonomic status throughout the history. Status change was a direct consequence of the poor original descriptions of almost all the species of the genus and reflected subjective appreciation of the value of the third antennal segment annulation, the only character used to separate *Verhoeffiella* from *Heteromurus* s.str. Species of the genus are very similar to the non-troglobiotic and non-troglomorphic species *Heteromurus nitidus* (Lukić et al., 2015). Their possible close relationship may offer an insight into the origin of the disjunct distribution of genus *Verhoeffiella*. Thesis specific aims address four issues: (i) morphological and taxonomical study of *Verhoeffiella*; (ii) test of the monophyly of *Verhoeffiella* and its phylogenetic position within Entomobryidae; (iii) relationship of *Verhoeffiella* and *Heteromurus nitidus*; (iv) diversification of *Verhoeffiella* in the Dinarides. In the following paragraphs we give an overview of methodology and results.

(i) A need for new diagnostic characters and re-assessment of *Verhoeffiella* position within subfamily Heteromurinae has been stressed several times since Mari Mutt, (1980a). To address these taxonomic questions we sampled a large number of populations and initiated a detailed morphological and taxonomical study of the genus. Phase contrast, differential interference contrast, and scanning electron microscopy were used. For stabilizing the genus taxonomy, we re-described its type species, *V. cavicola* and the species *V. longicornis* and *V. absoloni*, and we described five new ones (Lukić et al., 2015, 2018). Currently, the genus encompasses 14 nominal species. Special attention was given to the search for new diagnostic characters with focus on chaetotaxy, diversity of chaetae and troglomorphic characters. We have explored the variability of the morphological characters, and evaluated which ones are taxonomically relevant on a generic and specific level.

(ii) Taxonomic position of *Verhoeffiella* was always changing, from being a separate genus to being a subgenus of the genus *Heteromurus* or even *Heteromurus* s.str. Unfortunately, *Verhoeffiella* was not included in molecular phylogenetic studies of the family Entomobryidae and its taxonomic status remained uncertain (Zhang et al., 2014, 2015). As the intriguing disjunct distribution pattern of the genus strongly challenge the validity of its monophyly, it was necessary to include populations of *Verhoeffiella* from remote geographical regions in the molecular phylogeny of Entomobryidae. We combined a previously published dataset of Entomobryidae phylogeny (Zhang et al., 2014, 2015), publicly available sequences and our genetic data of *Verhoeffiella* and *Heteromurus*. Altogether, we compiled a dataset of 64 Entomobryidae representatives, including three distantly related collembolan species to root the tree. The molecular data used included two nuclear (18S rRNA and 28S rRNA) and two mitochondrial (COI and 16S rRNA) genes. Phylogenetic relationships were reconstructed on a concatenated dataset using two alternative approaches: Bayesian inference with partition-specific settings in MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) and maximum likelihood with partition-specific setting and ultrafast bootstrapping in IQ-tree 1.6.7 (Nguyen et al., 2014). Our results showed that genus *Verhoeffiella*, together with lineages ascribed to the species *H. nitidus*, forms a well-supported clade nested within Heteromurinae. *Heteromurus major* was shown to be the sister taxon of the clade. Within the clade, geographically distant populations of *Verhoeffiella* from Catalonia and Cantabria branch at the basis. The *Verhoeffiella dallaii* from south-eastern Calcareous Alps is in a sister relationship with the *H. nitidus*–Dinaric *Verhoeffiella* complex. Within this complex, lineages of *H. nitidus* and Dinaric *Verhoeffiella* seem to be intermixed but a large basal polytomy conceals their true relationship (Lukić et al., 2020).

(iii) *Heteromurus nitidus* is a widely distributed species in epigeal habitats across the almost entire Palearctic and Nearctic. Populations of this species also often colonize subterranean habitats, and many troglophilic populations have been recorded in Europe and the United States. Species of *Verhoeffiella* are very similar to *H. nitidus* in important



morphological characters so we hypothesize that *H. nitidus* is closely related to the genus *Verhoeffiella* and that they might be in a sister relationship. We explored the relationship of *Verhoeffiella* species from remote European regions to populations of *H. nitidus* from the same areas. In order to reduce the dataset for the phylogenetic analysis, we first ran the species delimitation of a large number of COI sequences (see point iv below). A single specimen per MOTU was chosen for further analysis. Then the main phylogenetic analyses were run on this reduced concatenated dataset of 132 specimens of *Verhoeffiella*, *Heteromurus* and the distantly related *Alloscopus* and *Dicranocentrus* (Heteromurinae) to root the tree. The dataset contained sequences of four nuclear (18S rRNA, 28S rRNA, H3, ITS) and one mitochondrial (COI) gene. Phylogenetic relations were reconstructed using the tree inferring procedure as for the phylogeny of family Entomobryidae (point ii above). Generally, the results showed the same relationship between *Verhoeffiella* populations and *H. nitidus* as in the previous analysis (point ii above). In addition, the analysis revealed five lineages of *Verhoeffiella* in south-east Dinarides and at least three different lineages of *H. nitidus*. These lineages seem to be intermixed but a large polytomy still prevents the resolution of their relationship (Lukić et al., 2020).

(iv) Special emphasis was given to *Verhoeffiella* populations from the Dinaric region, the center of species diversity of the genus. We compiled an extensive molecular dataset to explore cryptic diversity in the genus. We have also assessed the approximate timeframe of the diversification processes, in order to reconstruct evolutionary scenarios of diversification in relation to paleogeographic events. Specimens of *Verhoeffiella* and *Heteromurus* from 105 sites were included in the analysis. We ran species delimitation on barcoding region of 411 COI sequences of *H. nitidus*, *H. major*, most nominal *Verhoeffiella* species and a large number of newly sequenced *Verhoeffiella* populations. We used two alternative approaches: a tree-based Poisson tree processes (PTP) model (Zhang et al., 2013) and a distance-based species delimitation approach, the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012). PTP method is a phylogeny-based species delimitation method, based on the assumption that intra and inter-specific nucleotide substitution levels differ notably and can be modelled as two independent Poisson processes. ABGD is based on assumption that interspecific distances are larger than intraspecific distances, with notable disparity between pairwise genetic distances. We have discovered 79 MOTUs of *Verhoeffiella*, 33 or 31 MOTUs of *H. nitidus*, and 11 or 12 MOTUs of *H. major* (PTP and ABGD method, respectively) revealing a great taxonomic incompleteness. For estimation of divergence times we used the same phylogenetic analysis used in the point (iii) above and Bayesian inference as implemented in BEAST v 2.5.1 (Bouckaert et al., 2014). Time of the splits was assessed using data on molecular rates of distantly related Tenebrionid beetles (Papadopoulou et al., 2010). Due to absence of Collembola fossils, and due to lack of estimations of molecular clocks for the groups, we used the molecular rates estimated for beetles: 3.36% pairwise divergence per million years for mitochondrial COI, and 0.12% pairwise divergence per million years for the nuclear 28S rRNA gene. Temporal diversification of *Verhoeffiella* and *Heteromurus* were

generated using phytools v.0.6-60 (Revell, 2012) based on the BEAST Maximum credibility tree. In addition, observed diversification value was compared to 1,000 simulations of pure-birth LTTs, and gamma statistics (Pybus and Harvey, 2000) explaining speciation trends were extracted. Our estimation showed that the onset of the diversification of the whole *Verhoeffiella*–*Heteromurus nitidus* clade occurred in the middle Miocene. Within the *H. nitidus*–Dinaric *Verhoeffiella* complex, the origin of all the major lineages was dated in the late Miocene (5–7 MA), while most of the speciation events were estimated to the Pleistocene (2.5 MA–11.7 k years ago) (Lukić et al., 2020). This pattern was also demonstrated by the Pybus and Harvey gamma statistics, which supported a trend of increase in diversification towards the present ( $\gamma = 0.2741$ ,  $p = .74$ ) (Lukić et al., 2020).

Molecular phylogeny disclosed novel information and contributed towards the resolution of the taxonomic status of the genera *Verhoeffiella* and *Heteromurus*. The analysis revealed that genus *Heteromurus* is paraphyletic and genus *Verhoeffiella* is polyphyletic (Lukić et al., 2020). This explains the ‘floating’ taxonomic status of *Verhoeffiella* throughout the history of its study and showed that the use of the annulation of a third antennal segment, the single diagnostic character between the two genera, was misleading. Antennae annulation, in this case, could be a troglomorphic feature connected to antennae elongation, associated with life in subterranean habitats. The result of the molecular phylogeny, therefore, implicates the need for taxonomic re-arrangements at the generic level of Heteromurinae. To establish the monophyly *Verhoeffiella* could be synonymized with *Heteromurus*, alternatively, the clade could be divided into three genera. However, we refrain from new taxonomic re-arrangements at this step due to several reasons. At this moment there are no hard morphological data that would support either of the two options. As *Heteromurus* is the only genus among Heteromurinae that still lacks detailed modern species description it is important to collect topotypes, retrieve molecular data and redescribe type species of the genus *H. margaritarius*, but also *H. nitidus*, the species with which *H. margaritarius* has been synonymized. It is also necessary to include more Iberian *Verhoeffiella* species in the analysis, to taxonomically evaluate the differences in several characters observed so far in one species from Catalonia (Lukić et al., 2015). Further, large polytomy on the basis of *H. nitidus*–Dinaric *Verhoeffiella* complex conceals their true relationship. This could be possibly resolved by adding more populations of *H. nitidus*, *Verhoeffiella*, and several stenoendemic *Heteromurus* species and/or additional genes to the analysis. In the course of this study construction of morphological phylogeny was hampered by several factors, but in the future it could be also used to support taxonomic re-arrangements. Although we have advanced our taxonomic knowledge in the course of this study prior to taxonomic re-arrangements within Heteromurinae an integrative revision of genus *Heteromurus* is needed. The basis for this revision is given through the proposed morphological framework and molecular phylogeny of *H. nitidus*–*Verhoeffiella* clade (Lukić et al., 2018, 2020).

Although only partially resolved, phylogenetic relationship enables setting up the hypotheses on colonization of subterranean habitats that would explain disjunct distribution of *Verhoeffiella*. On a continental scale, the most plausible hypothesis would be that there were at least four colonization events, in Catalonia, Cantabria, the Alps, and the Dinarides. On a regional scale, within the Dinarides, single colonization, dispersal and speciation could have resulted in several *Verhoeffiella* lineages. But, alternatively, it would be possible that multiple colonizations and subsequent dispersals occurred, which would explain the high number of *Verhoeffiella* lineages and their spatial overlaps. Our results suggest that Dinaric *Verhoeffiella* is closely related to European populations of *H. nitidus* (Lukić et al., 2020), but their unresolved relationship hinders the support to any of the alternative hypotheses. Resolving large polytomy on the basis of *H. nitidus*–Dinaric *Verhoeffiella* complex is the only way to know whether Dinaric *Verhoeffiella* and *H. nitidus* represent two monophyletic sister groups, or whether the *Verhoeffiella*–*H. nitidus* sister species pairs would indicate multiple colonization events. The comparison of *Verhoeffiella* colonization scenarios with those of other terrestrial troglobiotic taxa is limited due to relatively few targeted studies. It is intriguing that several troglobiotic taxa from other groups (snails, false-scorpions, spiders, isopods) have a disjunct distribution similar to that of *Verhoeffiella* being known from the Iberian Peninsula, the Alps, and the Dinarides. But very few were subjected to molecular phylogenetic studies. Among them, the most interesting is the snail *Zospeum* which shares even pattern of molecular lineage distribution recognized in *Verhoeffiella* (Weigand et al., 2013; Inäbnit et al., 2019). The status of the other taxa needs to be checked through an integrative taxonomic approach, as it is possible that current disjunct distributions are results of erroneous taxonomy. On regional level, within the Dinarides, it was shown that snail *Zospeum* (Inäbnit et al., 2019) and isopod *Alpioniscus* (Bedeck et al., 2019) include molecularly highly divergent lineages and exhibited large spatial overlaps similarly to *Verhoeffiella*. This suggests complex colonization and speciation scenarios but these studies were primarily taxonomic and did not provide colonization hypotheses. So the question of possible congruent colonization scenarios, timeframes and paleogeographic events that generated such patterns, either on regional or continental scale, cannot be currently answered.

We have assessed the approximate timeframe of the diversification processes of *Verhoeffiella* and *Heteromurus*, by estimating divergence times using substitution rates for distantly related tenebrionid beetles (Papadopoulou et al., 2010). Even though it may be argued that the substitution rate for distantly related taxa might result in the erroneous estimates, our time frames coincide well with several paleogeographic events in the region. Origin of all the major lineages is dated in the late Miocene (5–7 MA), while most of the speciation events were dated to the Pleistocene (2.5 MA–11.7 k years ago) (Lukić et al., 2020). Late Miocene corresponds to a high orogenic activity in the development of the Dinarides (Aliaj, 2006; Vrabc and Fodor, 2006), to the disintegration of the Dinarid Lake System (Jiménez-Moreno et al., 2009; de Leeuw et al., 2011) and to the Messinian salinity crisis (5.96–5.33 MA; Krijgsman et al., 1999). Pleistocene is characterized by climatic

oscillations, marine regressions and transgressions (Krijgsman et al., 1999), as well as glaciations in the Dinarides (Hughes et al., 2011; Žebre and Stepišnik, 2014). We can speculate that events in the Late Miocene caused major cladogenetic events, e.g. that formation of the newly emerged landmasses during the disintegration of the Dinarid Lake System and orogeny potentially enabled colonization of the ancestral lineages. Marine regressions and transgressions during Pleistocene might have affected the speciation of the coastal lineages as the estimated ages of splits of most continent–island sister species pairs corresponds to the Pleistocene. Still, the exact mechanism how these paleogeographic events triggered the speciation remains unknown. With current dataset, we were not able to date the colonization of caves, yet from the time-calibrated phylogeny we can suggest that Dinaric *Verhoeffiella* species are of late Miocene age or younger. The origin of most other subterranean taxa in Dinarides are generally estimated to be older than *Verhoeffiella*, of an early or middle Miocene age (Trontelj et al., 2007; Delić et al., 2017; Njunjić et al., 2018; Bedek et al., 2019).

Our study significantly broadened our knowledge on the diversity, taxonomy, and biogeography of the genus *Verhoeffiella* within the Dinarides. We have shown that after successful colonization in the Dinarides subsequent speciation led to an outstanding diversity of *Verhoeffiella* in this region (Lukić et al., 2020). Current extent of the cryptic diversity within *Verhoeffiella* present exceptional case among Collembola analysed so far. In the course of this study we have formally named and described only a fraction of the discovered diversity. An important milestone in the study of this group is the congruence of our morphological species characterization with molecular species delimitation. This confirmed the robustness and usefulness of the proposed morphological framework that will serve as a tool for future morpho-taxonomic studies for the genus *Verhoeffiella* (Lukić et al., 2018) but could also be valuable for advances in the taxonomy of other genera of Heteromurinae (Jantarit and Sangsiri, 2020). Within the south-east Dinarides, we have disclosed a complex geographical pattern of five *Verhoeffiella* lineages. Two largest lineages spread over an area of more than 300 km straight line distance. Their sublineages also have wide distribution and largely overlapping ranges. The large magnitude of spatial overlap among different lineages and sublineages reflects phylogeographical replicates. These replicates, combined with the high species diversity and diversity of troglomorphic traits, makes *Verhoeffiella* a new and interesting model for the study of subterranean evolution.



## 4.2 POVZETEK

Podzemni habitati se od površinskih razlikujejo v več ozirih: stalni temi, pomanjkanju hrane, ter relativno stabilnih abiotskih parametrov, kot sta npr. temperatura in vlažnost. Vrste, ki so se prilagodile na stalno življenje v podzemnem okolju, imenujemo troglobionti. Filogenetski izvor troglobiontov ostaja v številnih primerih neznan. To je posledica težav pri odkrivanju odnosov med troglobionti in njihovimi površinskimi sorodniki, saj so lahko morfološke razlike med njimi zelo velike, ali pa so površinski sorodniki celo izumrli. Splošno sprejeto pa je, da večina podzemnih taksonov izhaja iz neodvisnih kolonizacijskih dogodkov, ki so jim lahko sledili disperzija in različni mehanizmi speciacije (Trontelj, 2018; Culver and Pipan, 2019). Večina teh taksonov ima majhna območja razširjenosti, kar najbolje razložijo fragmentacija kraške krajine, kompleksni paleogeografski dogodki in/ali historična klimatska variabilnost (Deharveng and Gers, 1993; Trontelj et al., 2009; Rizzo et al., 2013; Eme et al., 2014; Zagamajster et al., 2014). Izjemoma so nekateri taksoni širše razširjeni. Med takimi so najbolj zanimivi tisti, ki se pojavljajo v regijah, ki so oddaljeni stotine in tisoče kilometrov. Znotraj Evrope je le nekaj kopenskih troglobiontov, ki predstavljajo take primere. Pri tem je zanimivo, da ima več skupin troglobiontov podobno disjunktne vzorce, in sicer so znani z Iberskega polotoka, iz Alp in iz Dinaridov (Lukić et al., 2010; Weigand et al., 2013; Ribera et al., 2014; Pavlek and Ribera, 2017; Taiti et al., 2018). Disjunktne distribucijske vzorce nakazujejo na morebitno napačno taksonomsko identifikacijo, pod vprašaj postavljajo monofilijo taksonov kot tudi scenarije kolonizacij.

V tej disertaciji smo ta vprašanja naslovili s proučevanjem vzorcev razširjenosti in diverzifikacije jamskega rodu skakačev *Verhoeffiella*. Ta rod je disjunktno razširjen v štirih kraških območjih v Evropi: Dinaridih, jugovzhodnih Apeniških Alpah, v južni Kataloniji in Kantabrijskem gorovju (Lukić et al., 2015). Rod je imel vselej nestabilen taksonomski status, kar je posledica pomanjkljivih opisov skoraj vseh vrst. Poleg tega pa je odražal tudi subjektivno vrednotenje anulacije tretjega člena antene, kar je edini znak, ki je razlikoval rod *Verhoeffiella* od rodu *Heteromurus* s.str. Vrste rodu *Verhoeffiella* so zelo podobne netroglobiotski in netroglobiomorfni vrsti *Heteromurus nitidus* (Lukić et al., 2015). Domnevno bližnje sorodstvo menjanih rodov omogoča vpogled v izvor disjunktne razširjenosti rodu *Verhoeffiella*. Specifični cilji te naloge obravnavajo štiri tematske sklope: (i) morfološka in taksonomska študija rodu *Verhoeffiella*; (ii) testiranje monofilije rodu *Verhoeffiella* in njegovega filogenetskega položaja znotraj družine Entomobryidae; (iii) raziskovanje filogenetskih odnosov med rodom *Verhoeffiella* in vrsto *Heteromurus nitidus*; (iv) diverzifikacija rodu *Verhoeffiella* v Dinaridih. V naslednjih odstavkih podajamo pregled metod in rezultatov.

(i) Da bi bilo treba poiskati nove diagnostične znake in ponovno proučiti položaj rodu *Verhoeffiella* znotraj poddružine Heteromurinae je bilo poudarjeno že večkrat, začeniši z delom Mari Mutt, (1980a). To taksonomsko vprašanje smo naslovili tako, da smo uporabili veliko število populacij in izvedli podrobno morfološko in taksonomsko študijo. Uporabili

smo fazno kontrastno, diferenciacijsko interferenčni kontrast in vrstično elektronsko mikroskopijo. Za stabilnejšo opredelitev rodu smo ponovno opisali tipsko vrsto, *V. cavicola*, vrsti *V. longicornis* in *V. absoloni*, ter opisali pet novih vrst (Lukić et al., 2015, 2018). Trenutno rod obsega 14 vrst. Posebno pozorni smo bili na diagnostične znake, s poudarkom na razporeditev het (hetotaksija), njihovo raznolikost in troglomorfne lastnosti. Raziskali smo morfološko variabilnost znakov, in presodili, kateri so taksonomsko pomembni na rodovnem ali vrstnem nivoju.

(ii) Taksonomski status rodu *Verhoeffiella* se je vseskozi spreminjal, od samostojnega rodu, do podrodu znotraj rodu *Heteromurus* ali pa celo *Heteromurus* s str. Žal je rod *Verhoeffiella* bil izključen iz molekularno filogenetskih študij družine Entomobryidae, tako da je njegov taksonomski status ostal nejasen (Zhang et al., 2014, 2015). Nenavaden disjunkten vzorec razširjenosti tega rodu postavlja pod vprašaj njegovo monofilijo, zato smo populacije rodu *Verhoeffiella* iz oddaljenih geografskih regij umestili v širšo filogenijo družine Entomobryidae. Zbrali smo že objavljene podatke iz filogenije družine Entomobryidae (Zhang et al., 2014, 2015), javno dostopne sekvence in lastne genetske podatke za rodova *Verhoeffiella* in *Heteromurus*. Zbrali smo podatke za 64 predstavnikov družine Entomobryidae, kot tudi za tri predstavnike daljno sorodnih vrst za koreninjenje drevesa. Za računanje filogenetskih dreves smo uporabili dva jedrna (18S rRNA in 28S rRNA) in dva mitohondrijska (COI in 16S rRNA) gena. Filogenetske odnose smo rekonstruirali na združenem genskem naboru podatkov, z uporabo dveh alternativnih pristopov: Bajezijanske inference s particijsko-specifičnimi nastavitvami v MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) in metodo maksimalnega verjetja s particijsko specifičnimi nastavitvami in ultrahitrim bootstrapom v IQ-tree 1.6.7 (Nguyen et al., 2014). Naši rezultati kažejo, da rod *Verhoeffiella*, skupaj z linijami, ki so obravnavane kot vrsta *H. nitidus*, oblikuje dobro podprt monofiletski klad, ki je vgnezen v poddružino Heteromurinae. Sestrška temu kladu je površinska vrsta, *Heteromurus major*. Znotraj klada se geografsko oddaljene populacije *Verhoeffiella* iz Katalonije in Kantabrije odcepijo že na bazi. Nadalje, *Verhoeffiella* iz Jugovzhodnih Apneninških Alp, *V. dallaii*, je sestrška kompleksu »*H. nitidus*-dinarske *Verhoeffiella*«. Znotraj tega kompleksa se linije obeh rodov izmenjujejo, velika bazalna politomija pa onemogoča razrešitev njunega dejanskega odnosa (Lukić et al., 2020).

(iii) *Heteromurus nitidus* je splošno razširjena vrsta v površinskih habitatih Palearktike in Nearktike. Populacije te vrste pogosto naseljujejo tudi podzemne habitate; tako so v Evropi kot tudi ZDA našli številne troglafilne populacije *H. nitidus*. Vrste rodu *Verhoeffiella* so morfološko zelo podobne vrsti *H. nitidus*, zato smo domnevali, da je *H. nitidus* najbližji sorodnik rodu *Verhoeffiella* in da bi lahko bila v sestrskem odnosu. Raziskali smo filogenetske odnose vrst znotraj rodu *Verhoeffiella* iz različnih evropskih regij in populacij *H. nitidus* iz istih regij. Da bi zmanjšali delež manjkajočih podatkov za filogenetske analize, smo najprej naredili delimitacijo vrst na podlagi COI sekvenc (Glej točko iv spodaj). Za nadaljnje analize smo izbrali po en osebek na, v delimitacijah določen, MOTU.

Glavne filogenetske analize smo izvedli na zmanjšanem in ujemajočem se podatkovnem setu 132 domnevnih vrst *Verhoeffiella*, *Heteromurus* in daljnje sorodnih rodov *Alloscopus* in *Dicranocentrus* (Heteromurinae) za koreninjenje drevesa. Podatki so vključevali sekvence štirih jedrnih (18S rRNA, 28S rRNA, H3, ITS) in enega mitohondrijskega gena (COI). Filogenetske odnose smo rekonstruirali z uporabo statističnih metod, enakih kot pri filogeniji družine Entomobryidae (točka ii zgoraj). V splošnem so rezultati razkrili enake odnose med populacijami rodu *Verhoeffiella* in *H. nitidus* kot pri prejšnji analizi (točka ii zgoraj). Dodatno je analiza pokazala pet linij *Verhoeffiella* v jugovzhodnih Dinaridih in vsaj tri različne linije *H. nitidus*. Te linije se zdijo povezane, a velika politomija onemogoča, da bi odnose med njimi odnose natančneje raziskali.

(iv) Posebno pozornost smo namenili populacijam rodu *Verhoeffiella* v Dinaridih, kot centru vrstne pestrosti rodu. Zbrali smo obsežen nabor molekulskih podatkov, da bi raziskali pojav kriptične diverzitete znotraj rodu. Ocenili smo tudi približni časovni okvir diverzifikacije, ter rekonstruirali evlucijske scenarije glede na znane paleogeografske dogodke. Vključili smo osebke rodov *Verhoeffiella* in *Heteromurus* s 105 lokalitet. Delimitacijo vrst smo zagnali na 411 COI sekvenc *H. nitidus*, *H. major*, večine nominalnih vrst rodu *Verhoeffiella* kot tudi velikega števila na novo sekvenciranih populacij rodu *Verhoeffiella*. Uporabili smo dva alternativna pristopa. Prvi model domnevne vrste definira glede na optimalno razporeditev znotraj in med-vrstnih substitucij, ki jih opiše z dvema različnima Poissonovima procesoma glede na filogenetsko-hierarhične odnose med osebki (PTP – *Poisson Tree Process*) (Zhang et al., 2013). Drugi pristop predpostavlja, da so genetske razlike med vrstami znatno večje od razlik znotraj vrst, ter da imajo znotraj- in medvrstne genetske razlike vsaka svojo, jasno ločeno distribucijo. Osebke umesti v vrste glede na genetske razdalje z drugimi osebki (ABGD – *Automated Barcoding Gap Distribution*) (Puillandre et al., 2012). Odkrili smo 79 MOTU-jev znotraj rodu *Verhoeffiella*, 33 ali 31 MOTU-jev znotraj vrste *H. nitidus*, in 11 ali 12 MOTU-jev znotraj vrste *H. major* (Metodi PTP in ABGD), kar razkriva veliko taksonomsko nepopolnost. Da bi cepitve umestili v čas, smo uporabili enake filogenetske analize kot v točki (iii) zgoraj in Bayesijsko inferenco, kot je vgrajena v BEAST v 2.5.1 (Bouckaert et al., 2014). Čas cepitev je bil ocenjen na podlagi podatkov o molekulskih stopnjah za daljnje sorodne hrošče Tenebrionidae (Papadopoulou et al., 2010). Ker ne poznamo fosilov skakačev, oz., ker za samo skupino niso ocenili molekularnih ur, smo se v kalibracijskih analizah oprli na molekularne ure, ki so jih izračunali za hrošče: za mitohondrijski COI (3.36% na milijon let) in jedrni 28S rRNA (0.12% na milijon let) gen. Časovni potek diverzifikacije rodov *Verhoeffiella* in *Heteromurus* smo generirali z uporabo phytools v.0.6-60 (Revell, 2012), ki temelji na BEAST drevesu maksimalne verodostojnosti kladov. Dodatno smo opaženo vrednost diverzifikacije primerjali s tisto, ki smo jo dobili po 1000 simulacijah kumulativnega kopičenja filogenetskih linij skozi čas (angleško Lineage through time, LTT) in gama stasiko (Pybus and Harvey, 2000). Starost celotnega klada *Verhoeffiella* - *H. nitidus* je ocenjena na obdobje srednjega miocena. Znotraj kompleksa *H. nitidus*-dinarski *Verhoeffiella*, je izvor vseh glavnih linij ocenjen na obdobje poznega miocena (5-7

milijonov let nazaj). Večina speciacijskih dogodkov med sestrskimi vrstami je domnevno potekla v pleistocenu (2,5 milijonov–11,700 let nazaj) (Lukić et al., 2020). Ta vzorec je bil pokazan tudi s strani Pybus & Harvey opisne gamma statistike, ki podpira trend naraščanja diverzifikacije do danes ( $\gamma = 0.2741$ ,  $p = .74$ ) (Lukić et al., 2020).

Z molekulsko filogenijo smo zbrali nove informacije in s tem pomembno prispevali k razreševanju taksonomskega statusa rodov *Verhoeffiella* in *Heteromurus*. Ugotovili smo, da je rod *Heteromurus* parafiletski in rod *Verhoeffiella* polifiletski (Lukić et al., 2020). Rod *Verhoeffiella* ima nestabilen taksonomski status že vso zgodovino njegovega proučevanja. Izkazalo se je, da je anulacija tretjega člena anten, ki velja za edini razločevalni znak med dvema rodovoma, zavajajoč. Anulacija anten bi lahko bila troglomorfna značilnost, povezana s podaljševanjem anten zaradi življenja v podzemnih habitatih. Rezultati molekulske filogenije so pokazali, da bodo potrebne taksonomske spremembe na rodovnem nivoju znotraj Heteromurinae. Da bi vzpostavili monofilijo rodu, bi rod *Verhoeffiella* morali sinonimizirati z rodom *Heteromurus*, ali pa bi klad morali razdeliti v tri rodove. Takim taksonomskim premestitvam v našem delu se iz več razlogov izogibamo. V tem trenutku ni dobrih morfoloških podatkov, ki bi podpirali katerokoli od dveh možnosti. Ker je rod *Heteromurus* edini rod v poddružini Heteromurinae, ki še nima modernega opisa vrst, bi bilo najprej treba zbrati topotipe. Zbrati bi bilo treba molekulske podatke in narediti redeskripcije tipskih vrst rodu, *H. margaritarius*, a tudi *H. nitidus*, s katerim je bil *H. margaritarius* sinonimiziran. V analize bi bilo treba vključiti tudi več iberskih populacij rodu *Verhoeffiella*, da bi lahko taksonomsko ovrednotili razlike v več znakih, ki smo jih opazili pri vrsti iz Katalonije (Lukić et al., 2015). Nadalje, velika politomija na bazi kompleksa »*H. nitidus* - dinarski *Verhoeffiella*« onemogoča razkritje njunega pravega odnosa. To bi se morda lahko razrešilo z dodajanjem več populacij *H. nitidus*, *Verhoeffiella*, in več stenoendemnih vrst rodu *Heteromurus*; in/ali z dodajanjem novih genov v analize. Tekom te študije je bila konstrukcija morfološke filogenije otežena zaradi več faktorjev, v prihodnje pa bi lahko bila uporabljena, da bi podprla taksonomske spremembe. Čeprav nam je uspelo izboljšati taksonomsko znanje, pa bi bilo treba narediti integrativno revizijo rodu *Heteromurus*, preden bi se šlo v taksonomske spremembe znotraj poddružine Heteromurinae. Osnova za tako revizijo je podana ravno z našim predlogom morfološkega okvira in molekulske filogenije klada *H. nitidus-Verhoeffiella* (Lukić et al., 2018, 2020).

Čeprav so filogenetski odnosi le delno razrešeni, pa omogočajo postavljanje hipotez o kolonizaciji podzemnih okolij in o disjunktni razširjenosti rodu *Verhoeffiella*. Na kontinentalnem nivoju je najverjetnejša hipoteza, da so potekali vsaj štirje dogodki kolonizacije, v Kataloniji, Kantabriji, Alpah in Dinaridih. Na regionalnem nivoju, znotraj Dinaridov, pa je mogoče, da bi več *Verhoeffiella* linij izviralo iz ene kolonizacije, ki sta ji sledila disperzija in speciacija. Temu alternativnemu pogledu je, da je prišlo do več kolonizacij, ki jim je sledila disperzija. S tem bi pojasnili več linij rodu *Verhoeffiella* in prostorska prekrivanja razširjenosti. Čeprav naši rezultati nakazujejo, da so dinarske linije rodu



*Verhoeffiella* bližnje sorodne evropskim populacijam *H. nitidus* (Lukić et al., 2020), pa zaradi nerazrešenih odnosov med njima nobene od alternativnih hipotez ni mogoče podpreti. Razrešitev politomije na bazi kompleksa »*H. nitidus* - dinarski *Verhoeffiella*« bi bila potrebna, da bi lahko ugotovili, ali sta dinarska *Verhoeffiella* in *H. nitidus* dve monofiletski sestrski skupini, ali pa gre za pare vrst *Verhoeffiella* - *H. nitidus* in večkratne kolonizacije. Možnost primerjav scenarijev kolonizacije rodu *Verhoeffiella* z drugimi kopenskimi troglobionti je omejena zaradi pomanjkanja usmerjenih študij. Zanimivo je, da ima več troglobiontov drugih skupin (polži, paščipalci, pajki, enakonožci) podobno disjunktno distribucijo kot *Verhoeffiella*, ki je znana z Iberskega polotoka, Alp in Dinaridov. Le malo med navedenimi pa je bilo molekularno raziskanih. Med temi so najbolj zanimivi polži rodu *Zospeum*, ki imajo podoben vzorec distribucije molekularskih linij kot rod *Verhoeffiella* (Weigand et al., 2013; Inäbnit et al., 2019). Šele uporaba integrativnih taksonomskih pristopov tudi pri drugih taksonih bo lahko pokazala, ali so disjunktne distribucije rezultat napačne taksonomije. Na regionalnem nivoju znotraj Dinaridov, tako polži rodu *Zospeum* (Inäbnit et al., 2019) kot enakonožci rodu *Alpioniscus* (Bedek et al., 2019) vključujejo visoko divergentne molekularne linije, ki se prekrivajo v razširjenosti, podobno kot pri rodu *Verhoeffiella*. Čeprav gre za študije, ki so primarno taksonomske, in ne podajajo hipotez za kolonizacije, pa te nakazuje kompleksne scenarije kolonizacije in speciacije. A trenutno ne moremo ugotoviti morebitnega ujemanja scenarijev kolonizacij, časovnih okvirjev in paleogeografskih dogodkov, ki so take vzorce generirali. Ne na regionalni kot tudi ne na kontinentalni skali.

Ocenili smo časovni okvir poteka diverzifikacije rodov *Verhoeffiella* in *Heteromurus*. Ker ne poznamo fosilov skakačev in ker za samo skupino še niso ocenili molekularnih ur, smo se v kalibracijskih analizah oprli na molekularne ure, ki so jih izračunali za hrošče (Papadopoulou et al., 2010). Čeprav bi bilo mogoče, da stopnja substitucije za daljnje sorodne taksone poda napačne ocene časovnih okvirjev, pa se te v našem primeru ujemajo s nekaterimi paleogeografskimi dogodki v regiji. Izvor vseh večjih linij je umeščen v čas poznega miocena (5 - 7 milijonov let nazaj), medtem ko se večina speciacijskih dogodkov umešča v pleistocen (2,5 milijonov – 11, 700 let nazaj) (Lukić et al., 2020). V poznem miocenu je potekala visoka orogenetska aktivnost v razvoju Dinaridov (Aliaj, 2006; Vrabc and Fodor, 2006), razpad dinarskega sistema jezer (Jiménez-Moreno et al., 2009; de Leeuw et al., 2011) in Messinijska slanostna kriza (5.96–5.33 milijonov let nazaj; Krijgsman et al., 1999). Za pleistocen so značilna klimatska nihanja, morske regresije in transgresije (Krijgsman et al., 1999), kot tudi poledenitve v Dinaridih (Hughes et al., 2011; Žebre and Stepišnik, 2014). Predvidevamo lahko, da so dogodki v poznem miocenu sprožili glavne kladogenetske dogodke, saj sta orogeneza in oblikovanje novih kopenskih mas med razpadom dinarskega sistema jezer omogočila kolonizacijo predniških linij. Morske regresije in transgresije med pleistocenom so lahko vplivale na speciacijo obalnih linij, saj se ocenjena starost razcepitev večine kontinentalno-otoških parov ujema z obdobjem pleistocenom. Še vedno pa ostajajo neznani mehanizmi speciacije, ki so jih sprožili ti paleogeografski dogodki. Naši podatki niso omogočili datiranja kolonizacije

jam, a iz časovno kalibrirane filogenije lahko domnevamo, da so dinarske vrste rodu *Verhoeffiella* iz obdobja poznega mioцена ali mlajše. Izvor večine drugih podzemnih taksonov v Dinaridih je ocenjen kot starejši, iz časa zgodnjega in srednjega miocena (Trontelj et al., 2007; Delić et al., 2017; Njunjić et al., 2018; Bedek et al., 2019).

Z našo študijo smo bistveno izboljšali poznavanje raznolikosti, taksonomije in biogeografije rodu *Verhoeffiella* v Dinaridih. Uspešni kolonizaciji Dinaridov je sledila speciacija, ki je vodila do izredne raznolikosti rodu v tej regiji (Lukić et al., 2020). Obseg kriptične diverzitete rodu *Verhoeffiella* je izjemen znotraj vseh doslej analiziranih predstavnikov skakačev. Nekaj vrst smo formalno opisali, a to predstavlja le del prepoznane diverzitete rodu. Kot pomemben dosežek smatramo ugotovitev, da smo pokazali veliko ujemanje med morfološki opisi in vrstami, ki so bile določene z molekulsko delimitacijo. Tako smo potrdili robustnost in uporabnost predlaganega morfološkega okvirja, ki lahko služi kot orodje za prihodnje morfo-taksonomske študije rodu *Verhoeffiella* (Lukić et al., 2018), kot tudi pri proučevanju taksonomije drugih rodov znotraj Heteromurinae (Jantarit and Sangsiri, 2020). Na območju jugovzhodnih Dinaridov smo odkrili kompleksen geografski vzorec petih molekulskih linij rodu *Verhoeffiella*. Dve največji liniji sta razširjeni na območju večjem od 300 km največje linearne dolžine. Tudi linije znotraj dveh glavnih linij imajo široko razširjenost in prekrivajoče areale. Veliko prostorsko prekrivanje med različnimi linijami predstavlja filogeografske replike. Te v kombinaciji z visoko vrstno pestrostjo in raznolikostjo troglomorfnihih znakov, postavljajo rod *Verhoeffiella* za nov in zanimiv model v študijah podzemne evolucije.

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