# Revisiting Calohypsibiidae and Microhypsibiidae: *Fractonotus* Pilato, 1998 and its phylogenetic position within Isohypsibiidae (Eutardigrada: Parachela)

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## ABSTRACT

Tardigrades constituting the order Parachela are characterised by a generally uniform, worm-like external morphology. Taxa with larger, well-pronounced dorsal cuticular protuberances, tubercles and spines are found in seven genera representing various evolutionary lineages within Hypsibioidea and Isohypsibioidea: *Calohypsibius* Thulin, 1928 (Calohypsibiidae), *Fractonotus* Pilato, 1998 (Microhypsibiidae), *Ramazzottius* Binda & Pilato, 1986 (Ramazzottiidae), *Hypsibius* Ehrenberg, 1848 and *Pilatobius* Bertolani, Guidetti, Marchioro, Altiero, Rebecchi & Cesari, 2014 (Hypsibiidae), and *Doryphoribius* Pilato, 1969 and *Isohypsibius* Thulin, 1928 (Isohypsibiidae). Here, we present the first integrative analysis of a rare eutardigrade species, *Calohypsibius verrucosus* (Richters, 1900), classified until now within the superfamily Hypsibioidea. The species, having a strongly sculptured cuticle, only superficially resembles *Calohypsibius* but the modified *Isohypsibius*-type claws and the morphology of the buccal apparatus place it in a different genus, *Fractonotus*. The genus is currently classified within the family Microhypsibiidae and superfamily Hypsibioidea, but the first molecular data for *Fractonotus* show that it should be transferred to the family Isohypsibiidae within the superfamily Isohypsibioidea. We also show ontogenetic variability in cuticle morphology, which, together with the high intraspecific variability of *F. verrucosus* n. comb., allowed us to designate *Calohypsibius placophorus* (da Cunha, 1943) as a junior synonym of the former species. Furthermore, an analysis of type specimens of *Isohypsibius gilvus* Biserov, 1986 demonstrated its affinity to the revised *Fractonotus*. Finally, the diagnoses for Microhypsibiidae, Calohypsibiidae, *Calohypsibius* and *Fractonotus* are amended and claw morphology is suggested as a key trait for disentangling phyletic affinities within Isohypsibiidae *sensu lato*.

**KEY WORDS**
Bucco-pharyngeal apparatus,
claws,
cuticle,
morphology,
phylogeny,
new synonyms,
new combinations.

**RÉSUMÉ**

*Réexamen des Calohypsibiidae et Microhypsibiidae:* Fractonotus *Pilato, 1998 et sa position phylogénétique au sein des Isohypsibiidae (Eutardigrada : Parachela).*
Les Tardigrades qui forment l'ordre des Parachela sont caractérisés par une morphologie externe vermiforme, généralement uniforme. Des taxons avec des protubérances cuticulaires dorsales de grande taille et très marquées, des tubercules et des épines se trouvent dans sept genres représentant plusieurs lignées évolutives au sein des Hypsibioidea et des Isohypsibioidea : *Calohypsibius* Thulin, 1928 (Calohypsibiidae), *Fractonotus* Pilato, 1998 (Microhypsibiidae), *Ramazzottius* Binda & Pilato, 1986 (Ramazzottiidae), *Hypsibius* Ehrenberg, 1848 et *Pilatobius* Bertolani, Guidetti, Marchioro, Altiero, Rebecchi & Cesari, 2014 (Hypsibiidae), et *Doryphoribius* Pilato, 1969 et *Isohypsibius* Thulin, 1928 (Isohypsibiidae). Dans le présent article, nous présentons la première analyse intégrative d'une espèce rare d'eutardigrade, *Calohypsibius verrucosus* (Richters, 1900), classée jusqu'à présent dans la superfamille des Hypsibioidea. L'espèce ne ressemble que superficiellement à *Calohypsibius* par sa cuticule très ornée, mais ses griffes très modifiées de type *Isohypsibius*, et la morphologie de son appareil buccal la placent dans un genre différent, *Fractonotus*. Ce genre est actuellement classé dans la famille des Microhypsibiidae et la superfamille des Hypsibioidea, mais les premières données moléculaires obtenues pour *Fractonotus* montrent qu'il faudrait le transférer dans la famille des Isohypsibiidae de la superfamille des Isohypsibioidea. Nous montrons également la variabilité ontogénétique dans la morphologie de la cuticule, ce qui, en plus de la très forte variabilité intraspécifique de *F. verrucosus* n. comb., nous permet de désigner *Calohypsibius placophorus* (da Cunha, 1943) comme un synonyme plus récent de l'espèce précédente. Par ailleurs, l'analyse des spécimens types de *Isohypsibius gilvus* Biserov, 1986 montre sa parenté avec *Fractonotus*. Finalement, la diagnose des Microhypsibiidae, des Calohypsibiidae, de *Calohypsibius* et de *Fractonotus* est amendée et nous suggérons que la morphologie des griffes pourrait constituer un caractère clé pour démêler les parentés phylogénétiques au sein des Isohypsibiidae *sensu lato*.

**MOTS CLÉS**
Appareil bucco-pharyngé,
griffes,
cuticule,
morphologie,
phylogénie,
synonymes nouveaux,
combinaisons nouvelles.

## INTRODUCTION

Tardigrades, a phylum of microinvertebrates belonging to the clade Panarthropoda (Campbell *et al.* 2011), are widely distributed in marine, limnic, and terrestrial habitats. Limno-terrestrial species inhabit rivers, lakes, mosses, liverworts, lichens, algae, leaf litter, soil and cryoconite (Nelson *et al.* 2015; Zawierucha *et al.* 2015). Among them, members of the relatively uncommon and poor in species family Calohypsibiidae Pilato, 1969 are found exclusively in mosses and lichens. This group, with adults rarely exceeding 200 μm, comprises particularly small eutardigrades. Pilato (1989) distinguished two main phyletic lineages within the family: one with reduced claws and the other with small, but not reduced claws. The family has probably the most interesting history in terms of taxonomic changes among Eutardigrada. Established in 1969 by Pilato, it comprised four genera, being the second most genus-rich family after Hypsibiidae (with five genera) at that time, a status it held for only a short period. Pilato & Beasley (1987) described the fifth genus, *Haplohexapodibius*, yet at this time Hypsibiidae Pilato, 1969 had become a very speciose and genus-rich family, and the family Macrobiotidae Thulin, 1928 had just started to increase in the number of genera (see Degma & Guidetti 2007; Degma *et al.* 2009-2017). Problems with classifying calohypsibiid genera, partly due to the variable degree of claw reduction, reached its zenith later, when morphological analyses by Guil *et al.* (2013) seemed to confirm the monophyly of Calohypsibiidae, whereas a molecular phylogenetic analysis by Bertolani *et al.* (2014a) resulted in splitting the family into two distinct lineages: Calohypsibiidae *s.s.* within Hypsibioidea, and the remaining four

genera within Isohypsibioidea. The discrepancy between these studies was resolved by Cesari *et al.* (2016), who, having ascertained the phyletic affinities of *Hexapodibius* Pilato, 1969 and *Haplomacrobiotus* May, 1948 within Isohypsibioidea, erected the family Hexapodibiidae.

Thanks to finding abundant populations of *Calohypsibius verrucosus* (Richters, 1900), an otherwise extremely rare eutardigrade species, we were able to – for the first time – analyse the fine morphology and obtain molecular data for this species. Specifically, both phase contrast light microscope (PCM) and scanning electron microscope (SEM) images of external morphology, as well as of the buccal apparatus are presented and compared with that of other calohypsibiids *sensu stricto*. The 18S and 28S rRNA sequences, together with morphological evidence, unequivocally placed the taxon among Isohypsibiidae *sensu lato*, and suggested placing both *Calohypsibius verrucosus* and *Isohypsibius gilvus* Biserov, 1986 within the genus *Fractonotus* Pilato, 1998. The integrative analyses also allowed us to confirm that *C. verrucosus* and *C. placophorus* (da Cunha, 1943) are not separate taxa but represent different ontogenetic stages of a single species. Finally, the transfer of *C. verrucosus* to *Fractonotus*, and of *Fractonotus* to a different superfamily makes both Calohypsibiidae and Microhypsibiidae monophyletic, explicitly underlying the importance of SEM and DNA analyses in tardigrade taxonomy, especially in species with small body size such as calohypsibiids, where light microscope analyses of phylogenetically important traits, such as minute claws, may lead to erroneous conclusions. An integrative approach is, therefore, shown to be a necessary solution for resolving the systematics of this group.

## MATERIAL AND METHODS

### Samples and specimens

We analysed 40 individuals classified as *Calohypsibius placophorus* and *C. verrucosus*, all isolated from three lichen or mixed moss and lichen samples, collected from rocks in mainland Scotland by BB, and from the Outer Hebrides by Shona Morrison in 2014 and 2015 (for exact localities, please see the Results below), and processed following the protocol described by Stec *et al.* (2015). Of the 40 specimens, 26 were examined under PCM in order to study their external and internal morphology as well as morphometry. SEM was used to investigate the fine details of the external morphology of a further four animals and the buccal apparatuses extracted from another five specimens. The remaining five specimens from all three populations were first checked under PCM and then used for DNA extraction (four of these individuals had cuticular plaques – strongly sclerotised structures resembling pebbles – whereas one was devoid of these structures, i.e. the specimens corresponded with original descriptions of *C. placophorus* and *C. verrucosus*, respectively).

Additionally, several calohypsibiid and microhypsibiid taxa were analysed using PCM or differential interference contrast microscope (DIC): a paratype of *C. maliki* Michalczyk & Kaczmarek, 2005; specimens of *C. ornatus* and *Fractonotus caelatus* (Marcus, 1928) collected by Paulina Kosztyła in Bois-le-Roi, France (48°27'45"N, 2°39'36"E; 101 m a.s.l., moss from stone in a forest) on the 6th February 2016; specimens of *C. schusteri* Nelson & McGlothlin, 1996 collected in the Bieszczady Mts., Poland (see Michalczyk & Kaczmarek 2003 for details); two specimens of *C. verrucosus* from the Thulin collection, deposited in the Natural History Museum of Denmark in Copenhagen, collected in Borgholm, Öland, Sweden; and a single female of *Microhypsibius truncatus* Thulin, 1928 from Læsø (Kattegat, Denmark), deposited in the Natural History Museum of Denmark in Copenhagen. Two *Isohypsibius* spp. were used for morphological comparisons (both PCM and SEM*): Isohypsibius coulsoni* Kaczmarek, Zawierucha, Smykla & Michalczyk, 2012 from *locus typicus*, and *Isohypsibius prosostomus* Thulin, 1928 collected by PG in the Tatra Mts., Poland (49°14'49"N, 20°05'42"E; 1037 m a.s.l., moss from tree bark in upper forest zone) on the 22nd May 2016. Moreover, eight paratypes of *Isohypsibius gilvus*, deposited in the Department of Animal Biology (University of Modena and Reggio Emilia, Modena), were examined under PCM. Additionally, *C. ornatus* (Richters, 1900) was analysed by SEM imaging and DNA sequencing.

### Microscopy and imaging

Specimens for light microscopy and morphometry were mounted on microscope slides in Hoyer's medium according to the recipe and mounting protocol in Morek *et al.* (2016a), and then examined under a *Nikon Eclipse 50i* phase-contrast microscope fitted with a *Nikon Digital Sight DS-L2* digital camera. Specimens for imaging by SEM were prepared according to Stec *et al.* (2015). Buccal apparatuses were extracted following a sodium hypochlorite (NaClO) protocol by Eibye-Jacobsen (2001) with modifications described thoroughly in Gąsiorek *et al.* (2016). Both animals and apparatuses were examined under high vacuum in a Versa 3D DualBeam SEM at the ATOMIN facility of Jagiellonian University, Kraków, Poland. For deep structures that could not be fully focused in a single photograph, a series of 2-6 images were taken every *c.* 0.2 µm and then assembled with *Corel* into a single deep-focus image.

### Morphometrics

The sample size for morphometrics was chosen following the recommendations of Stec *et al.* (2016). All measurements are given in micrometres (µm). Structures were measured only if their orientations were suitable. Body length was measured from the anterior to the posterior end of the body, excluding the hind legs. Terminology for the structures within the buccal apparatus and for the claws follows that of Pilato & Binda (2010) and Gąsiorek *et al.* (2017). Gibbosity denotation system by Michalczyk & Kaczmarek (2010) was applied to the plaque arrangement. Claws were measured following Beasley *et al.* (2008). The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube, expressed as a percentage (Pilato 1981) and is presented here in italics. Morphometric data were handled using version 1.2 of the 'Parachela' template, which is available from the Tardigrada Register (Michalczyk & Kaczmarek 2013).

### Genotyping

DNA was extracted from individual animals using Chelex® 100 resin (Casquet *et al.* 2012; Stec *et al.* 2015). We sequenced three DNA fragments that differed in their effective mutation rates: a small ribosome subunit (18S rRNA), a large ribosome subunit (28S rRNA), and internal transcribed spacer (ITS-2). We also attempted to sequence DNA for the cytochrome c oxidase subunit I (COI) but we failed to obtain informative sequences despite trying different primers and their combinations. The three nuclear fragments were amplified and sequenced according to the protocols described by Stec *et al.* (2015); primers and original references for specific PCR programs are listed in Table 1. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Laboratory of the Institute of Environmental Sciences at Jagiellonian University. Sequences were processed using version 7.2.6.1 of BioEdit (Hall 1999).

### Phylogenetic analyses

We aligned all available isohypsibioid, hypsibioid, and two *Milnesium* spp. (outgroup) 18S rRNA sequences (see Table 2) using the default settings of MAFFT version 7 (Katoh *et al.* 2002; Katoh & Toh 2008). The obtained alignment was edited and checked manually in BioEdit and then trimmed to 782 bp. Using PartitionFinder version 2.1.1 (Lanfear *et al.* 2016) under the Bayesian Information Criterion (BIC), the best substitution model was chosen for posterior phylogenetic analysis. First we ran the analysis to test all possible models implemented in the program and the best fit-model was:

TABLE 1. — Primers and references for specific protocols for amplification of the three DNA fragments sequenced in the study.

| DNA fragment | Primer name | Primer direction | Primer sequence (5'-3') | Primer source | PCR program |
|---|---|---|---|---|---|
| 18S rRNA | SSU01_F<br>SSU82_R | forward<br>reverse | AACCTGGTTGATCCTGCCAGT<br>TGATCCTTCTGCAGGTTCACCTAC | Sands *et al.* (2008) | Zeller (2010) |
| 28S rRNA | 28S_Eutar_F<br>28SR0990 | forward<br>reverse | ACCCGCTGAACTTAAGCATAT<br>CCTTGGTCCGTGTTTCAAGAC | Gąsiorek *et al.* (2018)<br>Mironov *et al.* (2012) | Mironov *et al.* (2012) |
| ITS-2 | ITS2_Eutar_Ff<br>ITS2_Eutar_Rr | forward<br>reverse | CGTAACGTGAATTGCAGGAC<br>TGATATGCTTAAGTTCAGCGG | Stec *et al.* (2018) | Stec *et al.* (2018) |

TABLE 2. — List of the GenBank accession numbers for isohypsibioid, hypsibioid and outgroup DNA sequences used for phylogenetic analyses in the present study (new sequences are marked in bold).

| Taxon | 18S rRNA | Reference |
|---|---|---|
| **Isohypsibioidea** | | |
| *Apodibius confusus* Dastych, 1983 | KC582830 | Dabert *et al.* (2014) |
| *Fractonotus verrucosus* (Richters, 1900) n. comb. | **MG800855** | **present study** |
| *Doryphoribius flavus* (Iharos, 1966) | HQ604940 | Bertolani *et al.* (2014a) |
| *Doryphoribius macrodon* Binda, Pilato & Dastych, 1980 | HQ604942 | Bertolani *et al.* (2014a) |
| *Eremobiotus alicatai* (Binda, 1969) | HQ604951 | Bertolani *et al.* (2014a) |
| *Halobiotus crispae* Kristensen, 1982 | AY582121, EF620402 | Jørgensen & Kristensen (2004), Møbjerg *et al.* (2007) |
| *Haplomacrobiotus utahensis* Pilato & Beasley, 2005 | KT778600 | Cesari *et al.* (2016) |
| *Hexapodibius micronyx* Pilato, 1969 | HQ604915–6 | Bertolani *et al.* (2014a) |
| *Isohypsibius arbiter* Binda, 1980 | KT778602 | Cesari *et al.* (2016) |
| *Isohypsibius dastychi* Pilato, Bertolani & Binda, 1982 | HQ604954 | Bertolani *et al.* (2014a) |
| *Isohypsibius granulifer* Thulin, 1928 | KT778603 | Cesari *et al.* (2016) |
| *Isohypsibius papillifer* (Murray, 1905) | EU266925 | Sands *et al.* (2008) |
| *Isohypsibius prosostomus prosostomus* Thulin, 1928 | EF620404 | Møbjerg *et al.* (2007) |
| *Isohypsibius prosostomus cambrensis* (Morgan, 1976) | AM500652 | Kiehl *et al.* (2007) |
| *Pseudobiotus kathmanae* Nelson, Marley & Bertolani, 1999 | HQ604957 | Bertolani *et al.* (2014a) |
| *Pseudobiotus megalonyx* (Thulin, 1928) | HQ604959 | Bertolani *et al.* (2014a) |
| *Thulinius augusti* (Murray, 1907) | KF360230 | Bertolani *et al.* (2014b) |
| *Thulinius stephaniae* (Pilato, 1974) | GQ92570 | unpublished |
| **Hypsibioidea** | | |
| *Acutuncus antarcticus* (Richters, 1904) | EU266943 | Sands *et al.* (2008) |
| *Adropion belgicae* (Richters, 1911) | HQ604925 | Bertolani *et al.* (2014a) |
| *Adropion scoticum* (Murray, 1905) | HQ604927 | Bertolani *et al.* (2014a) |
| *Astatumen trinacriae* (Arcidiacono, 1962) | FJ435733, HQ604922 | Guil & Giribet (2012), Bertolani *et al.* (2014a) |
| *Borealibius zetlandicus* (Murray, 1907) | HQ604924 | Bertolani *et al.* (2014a) |

| Taxon | 18S rRNA | Reference |
|---|---|---|
| *Calohypsibius ornatus* (Richters, 1900) | MH279652 | present study |
| *Diphascon higginsi* Binda, 1971 | HQ604932 | Bertolani *et al.* (2014a) |
| *Diphascon pingue* (Marcus, 1936) | FJ435736, HQ604937 | Guil & Giribet (2012), Bertolani *et al.* (2014a) |
| *Diphascon puniceum* (Jennings, 1976) | EU266949 | Sands *et al.* (2008) |
| *Hebesuncus conjungens* (Thulin, 1911) | AM500646 | Kiehl *et al.* (2007) |
| *Hebesuncus ryani* Dastych & Harris, 1994 | EU266956 | Sands *et al.* (2008) |
| *Hypsibius convergens* (Urbanowicz, 1925) | FJ435726 | Guil & Giribet (2012) |
| *Hypsibius klebelsbergi* Mihelčič, 1959 | KT901827 | Dabert *et al.* (2015) |
| *Hypsibius pallidus* Thulin, 1911 | HQ604945 | Bertolani *et al.* (2014a) |
| *Hypsibius scabropygus* Cuénot, 1929 | AM500649 | Dabert *et al.* (2014) |
| *Mesocrista revelata* Gąsiorek, Stec, Morek, Zawierucha, Kaczmarek, Lachowska-Cierlik & Michalczyk, 2016 | KU528627 | Gąsiorek *et al.* (2016) |
| *Mesocrista spitzbergensis* (Richters, 1903) | KX347532 | Gąsiorek *et al.* (2016) |
| *Microhypsibius bertolanii* Kristensen, 1982 | HQ604992 | Bertolani *et al.* (2014a) |
| *Mixibius saracenus* (Pilato, 1973) | HQ604955 | Bertolani *et al.* (2014a) |
| *Pilatobius nodulosus* (Ramazzotti, 1957) | HQ604934 | Bertolani *et al.* (2014a) |
| *Pilatobius patanei* (Binda & Pilato, 1971) | HQ604935 | Bertolani *et al.* (2014a) |
| *Pilatobius ramazzottii* (Robotti, 1970) | HQ604939 | Bertolani *et al.* (2014a) |
| *Platicrista angustata* (Murray, 1905) | HQ604948 | Bertolani *et al.* (2014a) |
| *Ramazzottius oberhaeuseri* (Doyère, 1840) | AY582122 | Jørgensen & Kristensen (2004) |
| **Apochela (outgroup)** | | |
| *Milnesium berladnicorum* Ciobanu, Zawierucha, Moglan & Kaczmarek, 2014 | KT951660 | Morek *et al.* (2016b) |
| *Milnesium tardigradum* Doyère, 1840 | MG912554 | Morek *et al.* (2019) |

SYM + I + G. Since RAxML (Stamatakis 2014) allows for only a single model of rate heterogeneity (from the GTR family) in partitioned analyses, using PartitionFinder we additionally tested GTR, GTR + I, GTR + G and GTR + I + G. The best fit-model in this analysis was GTR + I + G.

Maximum-likelihood (ML) topologies were constructed using RAxML v8.0.19 (Stamatakis 2014). Strength of support for internal nodes of ML construction was measured using 1000 rapid bootstrap replicates. Bootstrap (BS) support values ≥ 70% on the final tree were regarded as significant statistical
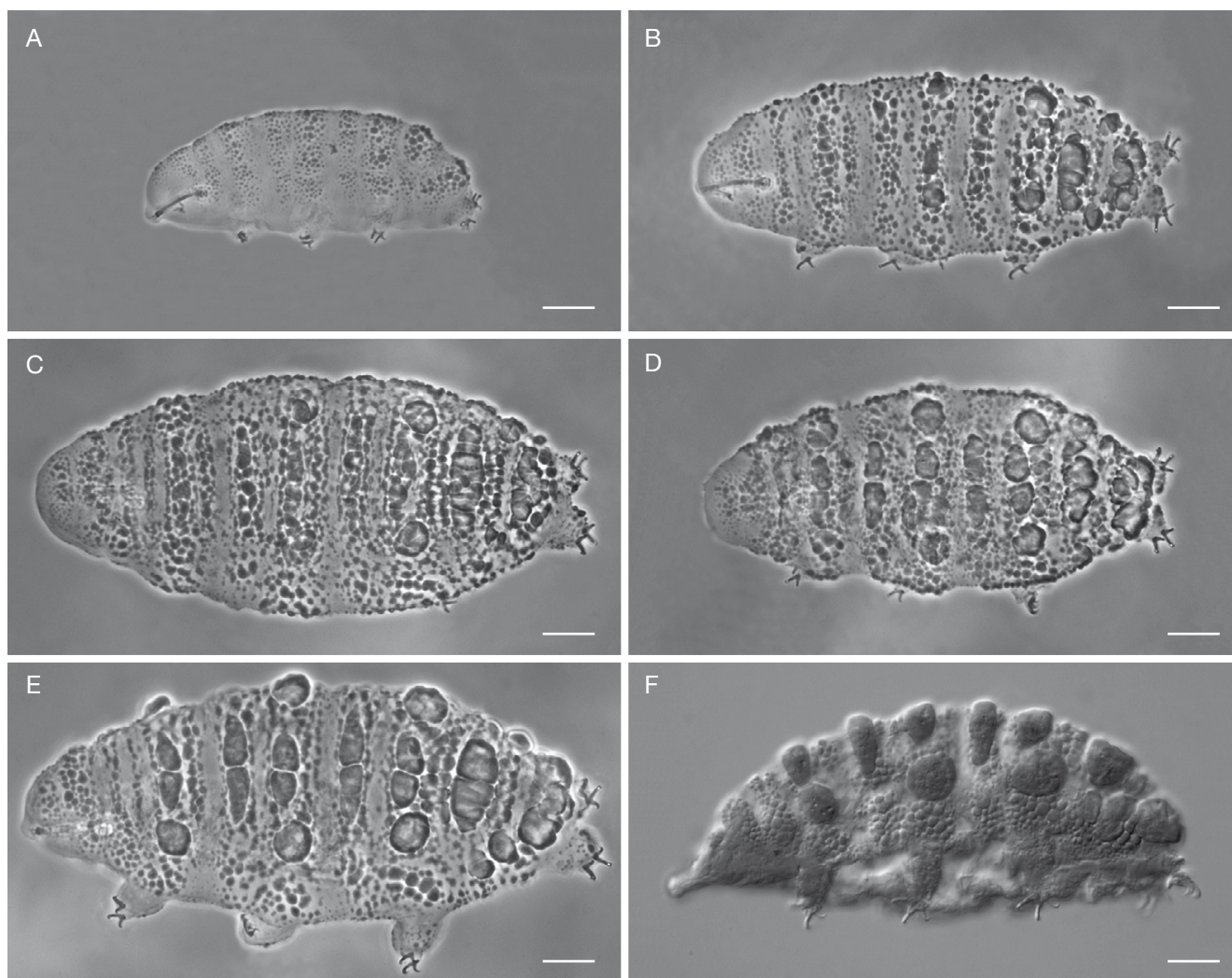
Fɪɢ. 1. — *Fractonotus verrucosus* (Richters, 1900) n. comb., ontogenetic series (Fig. 1F: differential interference contrast [DIC], the remaining photos are phase contrast microscope [PCM]): **A**, juvenile habitus (lateral view); **B**, young adult habitus (dorsal view); **C-F**, old adult habitus, variability in the development of large tubercles (dorsal views); **E**, adult habitus with the symmetrical arrangement of plaques (*Calohypsibius placophorus sensu* da Cunha 1943, dorsolateral view); **F**, adult habitus (a specimen from Borgholm, Öland from the Thulin collection, lateral view, DIC). Scale bars: 20 μm.

support. Bayesian inference (BI) marginal posterior probabilities were calculated using MrBayes v3.2 (Ronquist & Huelsenbeck 2003). Random starting trees were used and the analysis was run for ten million generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of < 0.01 was used as a guide to ensure the two independent analyses had converged. The program Tracer v1.3 (Rambaut *et al.* 2014) was then used to ensure Markov chains had reached stationarity and to determine the correct 'burn-in' for the analysis which was the first 10% of generations. A consensus tree was obtained after summarizing the resulting topologies and discarding the 'burn-in'. The BI consensus tree, clades recovered with posterior probability (PP) between 0.95 and 1.00 were considered well supported, those with PP between 0.90 and 0.94 were considered moderately supported and those with lower PP were considered unsupported. All final consensus tree were viewed and visualized by FigTree v.1.4.3 available from http://tree.bio.ed.ac.uk/software/figtree. Uncorrected pairwise distances were calculated using MEGA7 (Kumar *et al.* 2016).

ABBREVIATIONS

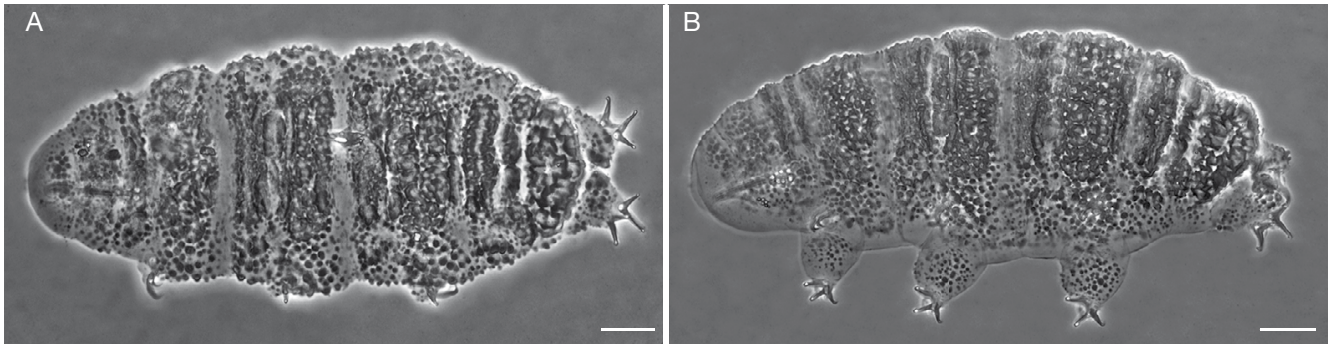| | |
|---|---|
| CU | University of Catania, Sicily; |
| MNHN | Muséum national d'Histoire naturelle, Paris; |
| NHMD | Natural History Museum of Denmark, Copenhagen; |
| UAM | Department of Animal Taxonomy and Ecology, Adam Mickiewicz University, Poznań; |
| UJ | Institute of Zoology and Biomedical Research, Jagiellonian University, Kraków. |

FIG. 2. — *Fractonotus gilvus* (Biserov, 1986) n. comb., adults showing intraspecific variability in cuticular sculpturing (**A**, dorsal and **B**, dorsolateral view, paratypes). Scale bars: 20 μm.

# RESULTS

Phylum TARDIGRADA Doyère, 1840
Class EUTARDIGRADA Richters, 1926
Order PARACHELA Schuster, Nelson,
Grigarick, Christenberry, 1980
Superfamily ISOHYPSIBIOIDEA Sands, McInnes, Marley,
Goodall-Copestake, Convey, Linse, 2008
Family ISOHYPSIBIIDAE Sands, McInnes, Marley, Goodall-
Copestake, Convey, Linse, 2008

Genus *Fractonotus* Pilato, 1998

*Fractonotus* Pilato, 1998: 132.

AMENDED DIAGNOSIS. — Small isohypsibiid (rarely exceeding 200 μm, Fig. 1). Cephalic elliptical organs present (Fig. 7A). Dorsum and limbs covered with densely arranged, blunt protuberances. Six peribuccal lobes present (Fig. 6A). Apophyses for the insertion of stylet muscles (AISM) asymmetrical with respect to the frontal plane – the dorsal apophysis subdivided into two portions: the anterior portion in the shape of a slightly convex longitudinal thickening (and the posterior portion as weakly developed blunt hook); the ventral apophysis in the shape of a mild and long ridge (Fig. 9A). Very large pharyngeal apophyses and placoids in the muscle pharynx. Stylet furcae of the *Fractonotus*-type, i.e. with broad, trapezoid base, thin arms and rounded apices (Figs 8D, 10A). Claws of the modified *Isohypsibius*-type, with triangular bases and strongly curved claw branches (Fig. 12A, B). Accessory points symmetrical or occasionally asymmetrical. Smooth eggs laid in exuviae.

DIFFERENTIAL DIAGNOSIS. — *Fractonotus* shares pronounced cuticular sculpturing with some species of six other parachelan genera: *Calohypsibius* Thulin, 1928, some *Ramazzottius* Binda & Pilato, 1986, *Hypsibius* Ehrenberg, 1848, *Pilatobius* Bertolani, Guidetti, Marchioro, Altiero, Rebecchi, Cesari, 2014, *Doryphoribius* Pilato, 1969 and *Isohypsibius* Thulin, 1928, but it can be readily distinguished from these genera by the morphology of the stylet furcae (square/trapezoid in *Fractonotus* vs narrower and more rectangular in the latter genera; compare Figs 7B, D; 10A-C). Furthermore, *Fractonotus* differs from *Ramazzottius*, *Hypsibius* and *Pilatobius* by having *Isohypsibius*-like claws (claws of the latter genera are of the *Hypsibius* or of the modified *Hypsibius*-type). Moreover, the genus differs specifically from:
– *Calohypsibius* Thulin, 1928 (Hypsibioidea: Calohypsibiidae), by having a different type of cuticular sculpture (roundish or oval tubercles covering the entire dorsum and limbs with smooth dorsal

pebble-shaped plaques in *Fractonotus*, Fig. 5A-D vs multangular or star-like tubercles and occasional spines arranged less densely in *Calohypsibius*, Fig. 5E, F), different structures surrounding the mouth opening (six soft and large peribuccal lobes in *Fractonotus*, Fig. 6A vs six small well defined papulae in *Calohypsibius*, Fig. 6B), a reversed morphology of the dorsal apophysis for the insertion of stylet muscles (an anterior thickening and a tiny posterior hook in *Fractonotus*, Fig. 7E-G vs an anterior large blunt hook and a slight posterior thickening in *Calohypsibius*, Fig. 9A, B), and by claw morphology (modified *Isohypsibius*-type claws with pseudolunulae, triangular bases, and elongated, strongly curved branches with conspicuous accessory points in *Fractonotus*, Figs 11A-D; 12A, B vs very small, rigid, with the base width equal to the sum of the primary and secondary branch widths, with the vertical septum between the two branches, and without pseudolunulae in *Calohypsibius*, Figs 11E; 12C, D).
– *Doryphoribius* Pilato, 1969 (Isohypsibioidea: Isohypsibiidae), by the presence of elliptical organs on the head (absent in *Doryphoribius*), and by the absence of the ventral lamina on the buccal tube (ventral lamina present in *Doryphoribius*).
– *Isohypsibius* Thulin, 1928 (Isohypsibioidea: Isohypsibiidae), by the presence of elliptical organs on the head (absent in *Isohypsibius*), a different shape of AISM (asymmetrical with respect to the frontal plane in *Fractonotus*, Fig. 7E vs ridges symmetrical with respect to the frontal plane *Isohypsibius*, Figs 7H, I; 9A, C), and by the claw morphology (modified *Isohypsibius*-type claws with triangular bases, especially well-marked on the fourth pair of legs, in *Fractonotus* vs *Isohypsibius*-type claws with stalk-like bases in *Isohypsibius*, Figs 11H, I; 12E, F).

## COMPOSITION AND REMARKS

Currently only three species, *Fractonotus caelatus* (the nominal taxon), *F. verrucosus* n. comb. and *F. gilvus* n. comb., are assigned to the genus. The three species are placed in the single genus because they share a number of taxonomically important traits: AISM shape, the presence of elliptical cephalic organs, two granular macroplacoids in the pharynx, and the type of cuticular sculpturing. On the other hand, Pilato (1998) described the claws of *F. caelatus* as of the *Microhypsibius* type, whereas claws in *F. verrucosus* n. comb. and *F. gilvus* n. comb. are closer to *Isohypsibius* type claws. Therefore, given the differences in claw morphology, there is a possibility that *F. verrucosus* n. comb. and *F. gilvus* n. comb. belong to a new isohypsibioid genus, and are only delusively similar to *Fractonotus* due to convergent evolution in the remaining traits. Nevertheless, the majority of traits suggest that all three species should be placed in *Fractonotus*.
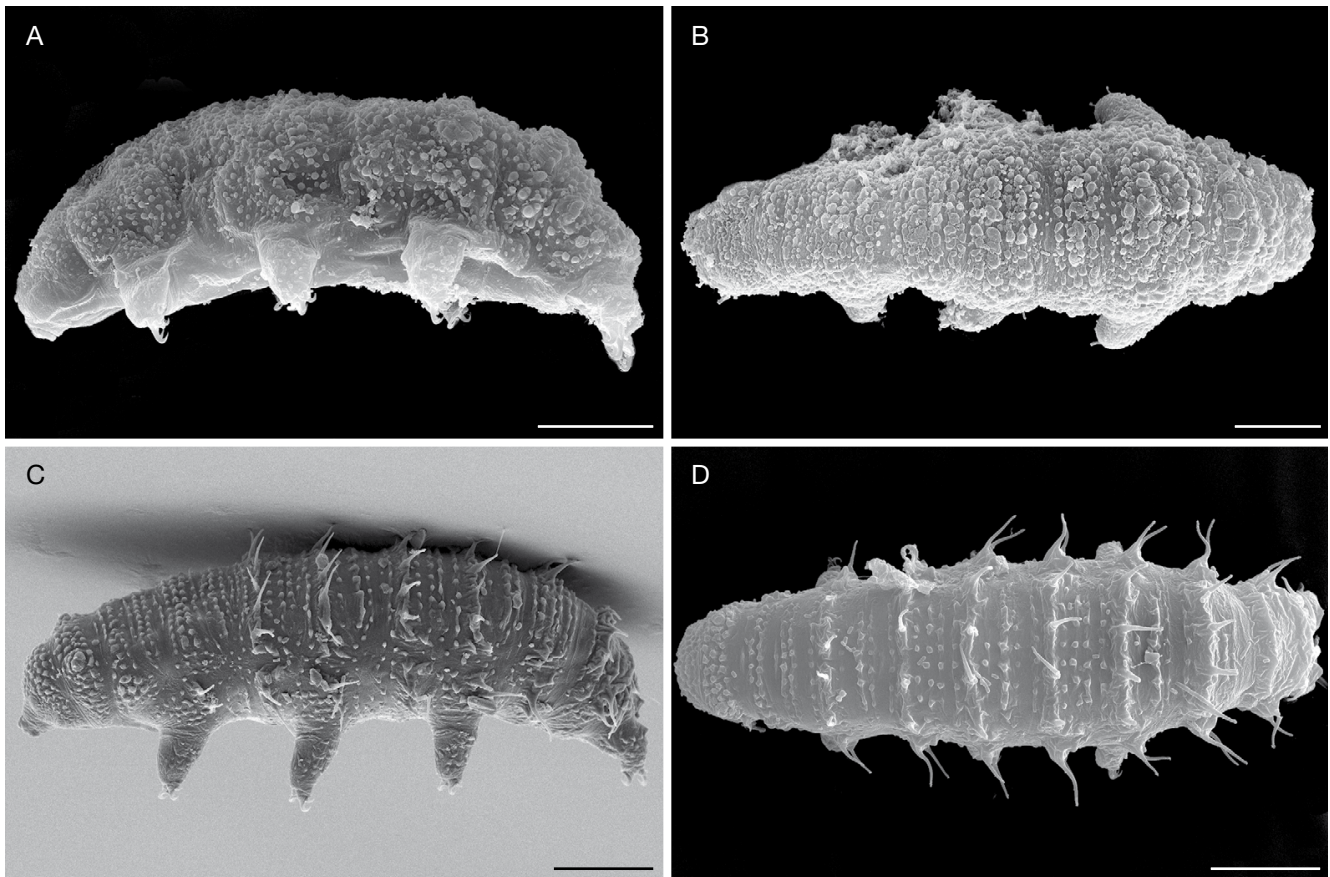
Fig. 3. — *Fractonotus verrucosus* (Richters, 1900) n. comb., habitus (SEM): **A**, lateral view; **B**, dorsal view; *Calohypsibius ornatus* (Richters, 1900), habitus (SEM): **C**, lateral view; **D**, dorsal view. Scale bars: A, 30 µm; B-D, 20 µm.

Biserov (1986) misinterpreted the AISM of *F. gilvus* n. comb. (Fig. 3 therein) as *Isohypsibius*-type AISM, but our observations of the type material confirm that the species has the AISM of the *Fractonotus*-type. However, there are more *Isohypsibius* and *Hypsibius* species, that exhibit cuticular sculpturing similar to that of *Fractonotus*. Thus, they may in fact belong to *Fractonotus* rather than *Isohypsibius* or *Hypsibius*. Nevertheless, we refrained from enacting more transfers, as a careful examination of individuals is needed to confirm whether these species, in addition to cuticular sculpturing, also exhibit other characteristics of *Fractonotus*.

## *Fractonotus verrucosus* (Richters, 1900) n. comb.

*Macrobiotus ornatus* var. *verrucosus* Richters, 1900: 41 (terra typica: Taunus, Germany).

*Macrobiotus scabrosus* Murray, 1911: 10 (locus typicus: Clare Island, Ireland).

*Hypsibius verrucosus* – Thulin 1911: 29 (Kiruna, Lapland, Sweden). — Marcus 1928: 180 (Vannsee, Berlin, Germany). — Cuénot 1932: 77 (the Vosges, France and Switzerland). — da Cunha 1947, 1948: 6, 2 (Serra d'Arga, Serra do Buçaco, Serra da Estrela, Serra da Lousã, Portugal).

*Calohypsibius verrucosus* – Thulin 1928: 239 (Sweden).

*Calohypsibius scabrosus* Thulin, 1928: 239 (Sweden).

*Hypsibius scabrosus* Cuénot, 1932: 77 (the Vosges, France and Switzerland). — da Cunha 1947, 1948: 6, 2 (Serra d'Arga, Serra do Buçaco, Serra da Estrela, Serra da Lousã, Portugal).

*Hypsibius* (*Calohypsibius*) *verrucosus* – Marcus 1936: 285 (Schwarzwald, Germany). — Franceschi 1951-1952: 12 (Val Camonica, Italy). — Mihelčič 1953: 247 (Tirol, Austria). — Fontoura 1981: 18 (Viseu, Arga, Amarante, Portugal).

*Hypsibius placophorus* da Cunha, 1943: 1 (locus typicus: Cabril do Ceira, Portugal); 1947, 1948: 2, 2 (Serra d'Arga, Serra do Buçaco, Serra da Estrela, Serra da Lousã, Portugal) **n. syn.**

LOCALITIES. — **Scotland**. Creag Meagaidh (56°57'10"N, 4°30'35"W; 291 m a.s.l.; collection date: 1.X.2014), lichens from moorland rocks; Scotland, Hebrides, Isle of Lewis, Loch nan Muilne (58°21'08"N, 6°35'14"W; 27 m a.s.l.; collection date: 29.VII.2015), moss and lichen mix from stones on the lakeshores; Invermoriston, Loch Ness (57°12'39"N, 4°35'59"W; 20 m a.s.l.; 25.X.2015; Brian Blagden leg.), moss and lichen mix from stones on the lakeshores.

MATERIAL EXAMINED. — 23 individuals, UJ (19 specimens, including one simplex, on slides GB.005.03-12, GB.008.01-3, GB.028.01-2 and 4 specimens on two SEM stubs); 2 individuals, MNHN (slides GB.005.01-2); 3 individuals, NHMD (slides GB.008.04-5); 2 individuals, UAM (slides GB.028.03-4); 1 individual, CU (slide GB.028.02).
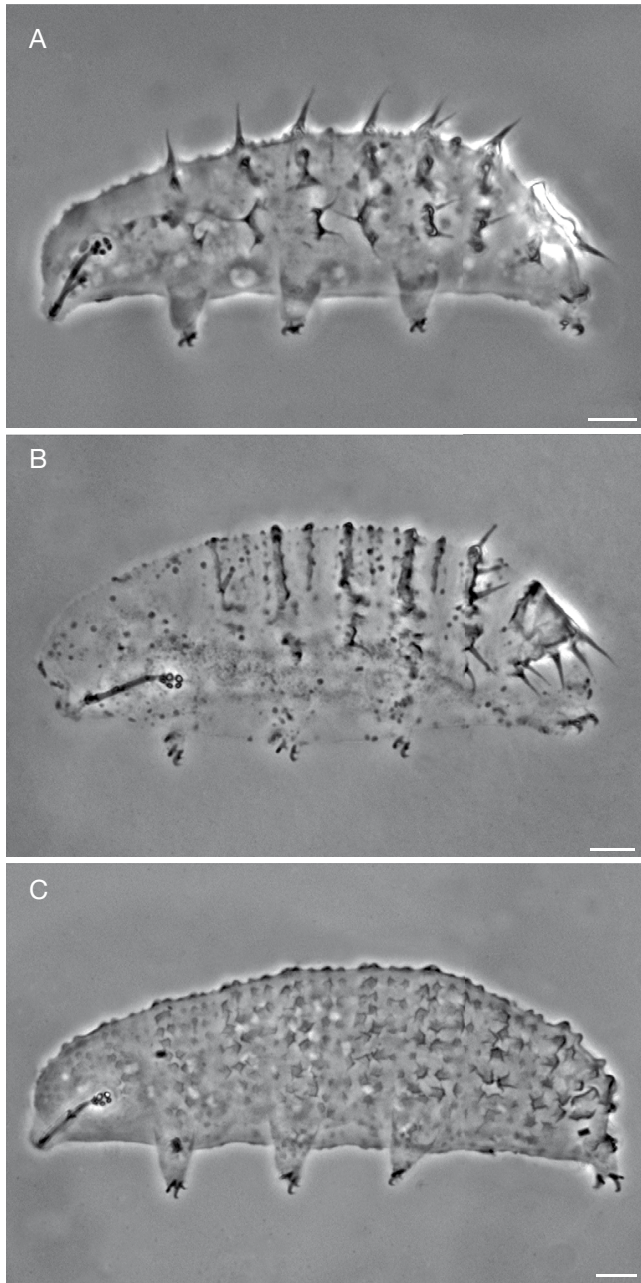
FIG. 4. — Genus *Calohypsibius* Thulin, 1928 *s.s.*, after exclusion of *F. verrucosus* n. comb. (PCM): **A**, *Calohypsibius ornatus* (Richters, 1900); **B**, *Calohypsibius maliki* Michalczyk & Kaczmarek, 2005; **C**, *Calohypsibius schusteri* Nelson & McGlothlin, 1996. Scale bars: 10 µm.

ETYMOLOGY (NOT PROVIDED IN THE ORIGINAL DESCRIPTION). — The name most likely refers to the rugged cuticular surface of the species (from Latin *verruca* = wart).

DIFFERENTIAL DIAGNOSIS. — *Fractonotus verrucosus* n. comb. can be distinguished from *F. caelatus* and *F. gilvus* n. comb. (Fig. 2A, B) by the presence of plaques (absent in the latter species). It also differs from *F. gilvus* n. comb. by shorter, stouter claws (anterior and posterior primary branches 4.1-6.4 µm [N=10] and 4.3-7.4 µm long [N=18], respectively, in *Fractonotus verrucosus* n. comb. vs 7.0-13.0 µm [N=21] and 10.5-16.5 µm long [N=21], respectively, in *F. gilvus* n. comb.; compare Fig. 11A-D).

TABLE 3. — Measurements [in µm] of selected morphological structures of individuals of *Fractonotus verrucosus* (Richters, 1900) n. comb. mounted in Hoyer's medium. Abbreviations: **N**, number of specimens/structures measured; *pt*, the *pt* index; **Range**, refers to the smallest and the largest structure among all measured specimens; **SD**, standard deviation.

| Character | N | Range µm | Range pt | Mean µm | Mean pt | SD µm | SD pt |
|---|---|---|---|---|---|---|---|
| Body length | 26 | 103 - 235 | - | 177 | - | 33 | - |
| Buccal tube | | | | | | | |
| Buccal tube length | 23 | 16.9 - 27.1 | - | 23.1 | - | 2.4 | - |
| Stylet support insertion point | 23 | 9.7 - 15.8 | 56.8 - 62.3 | 13.6 | 59.2 | 1.4 | 1.5 |
| Buccal tube external width | 24 | 1.0 - 1.6 | 4.8 - 6.5 | 1.3 | 5.7 | 0.2 | 0.5 |
| Buccal tube internal width | 24 | 0.2 - 0.7 | 1.2 - 2.6 | 0.4 | 1.9 | 0.1 | 0.4 |
| Placoid lengths | | | | | | | |
| Macroplacoid 1 | 25 | 1.0 - 2.7 | 5.9 - 11.4 | 2.1 | 9.3 | 0.4 | 1.3 |
| Macroplacoid 2 | 25 | 1.3 - 2.3 | 6.5 - 9.5 | 1.8 | 8.1 | 0.3 | 0.7 |
| Macroplacoid row | 25 | 3.3 - 5.3 | 18.2 - 21.8 | 4.5 | 20.0 | 0.5 | 1.0 |
| Claw 1 lengths | | | | | | | |
| External base | 18 | 2.0 - 3.9 | 8.5 - 14.4 | 2.7 | 11.7 | 0.6 | 1.8 |
| External primary branch | 18 | 3.5 - 6.0 | 16.9 - 24.0 | 4.8 | 21.3 | 0.8 | 2.2 |
| External secondary branch | 13 | 2.7 - 4.7 | 12.6 - 17.5 | 3.4 | 14.8 | 0.7 | 1.8 |
| Internal base | 17 | 1.5 - 2.9 | 7.8 - 11.6 | 2.3 | 9.9 | 0.4 | 1.0 |
| Internal primary branch | 12 | 3.0 - 5.5 | 17.7 - 24.2 | 4.5 | 20.5 | 0.8 | 2.0 |
| Internal secondary branch | 9 | 2.3 - 3.9 | 11.9 - 15.6 | 3.1 | 13.6 | 0.5 | 1.1 |
| Claw 2 lengths | | | | | | | |
| External base | 17 | 2.0 - 4.4 | 8.9 - 16.2 | 2.7 | 12.0 | 0.7 | 2.4 |
| External primary branch | 17 | 3.2 - 6.9 | 16.1 - 27.1 | 5.0 | 22.2 | 1.2 | 3.9 |
| External secondary branch | 11 | 2.4 - 5.4 | 11.8 - 19.9 | 3.8 | 16.3 | 0.8 | 2.9 |
| Internal base | 10 | 1.5 - 3.5 | 7.0 - 13.3 | 2.5 | 11.0 | 0.7 | 2.2 |
| Internal primary branch | 8 | 2.7 - 5.8 | 19.0 - 25.1 | 4.9 | 21.6 | 1.0 | 2.2 |
| Internal secondary branch | 6 | 3.0 - 4.3 | 12.1 - 17.5 | 3.7 | 15.2 | 0.5 | 1.8 |
| Claw 3 lengths | | | | | | | |
| External base | 16 | 1.8 - 3.7 | 9.1 - 16.1 | 2.6 | 11.9 | 0.6 | 2.0 |
| External primary branch | 14 | 3.8 - 7.5 | 19.0 - 31.6 | 5.3 | 23.8 | 1.2 | 4.3 |
| External secondary branch | 14 | 2.4 - 5.0 | 11.8 - 21.1 | 3.5 | 26.3 | 0.8 | 2.7 |
| Internal base | 14 | 1.7 - 3.3 | 8.8 - 13.4 | 2.3 | 10.6 | 0.5 | 1.5 |
| Internal primary branch | 11 | 2.8 - 5.4 | 16.6 - 22.4 | 4.5 | 20.1 | 0.9 | 2.1 |
| Internal secondary branch | 10 | 2.2 - 3.9 | 11.4 - 16.0 | 3.1 | 14.4 | 0.7 | 1.5 |
| Claw 4 lengths | | | | | | | |
| Anterior base | 15 | 1.6 - 3.7 | 8.3 - 13.7 | 2.6 | 11.3 | 0.6 | 1.8 |
| Anterior primary branch | 10 | 4.1 - 6.4 | 19.3 - 28.4 | 5.4 | 22.9 | 0.8 | 2.5 |
| Anterior secondary branch | 6 | 2.3 - 5.1 | 15.5 - 20.4 | 4.2 | 18.6 | 1.1 | 2.1 |
| Posterior base | 16 | 2.1 - 4.0 | 8.6 - 15.9 | 2.6 | 11.8 | 0.5 | 1.8 |
| Posterior primary branch | 18 | 4.3 - 7.4 | 20.2 - 30.7 | 5.7 | 25.3 | 0.9 | 2.8 |
| Posterior secondary branch | 8 | 3.1 - 5.5 | 16.1 - 21.9 | 4.3 | 18.4 | 0.7 | 1.9 |

INTEGRATIVE DESCRIPTION
*Animals (see Table 3 for measurements)*
Body stubby, typically slightly rose in live animals, transparent in mounted specimens. Dorsum strongly sculptured from the first instar, although with substantial ontogenetic quantitative and qualitative variability in this trait (Fig. 1A-F). Juveniles with ten transverse bands of numerous tu-
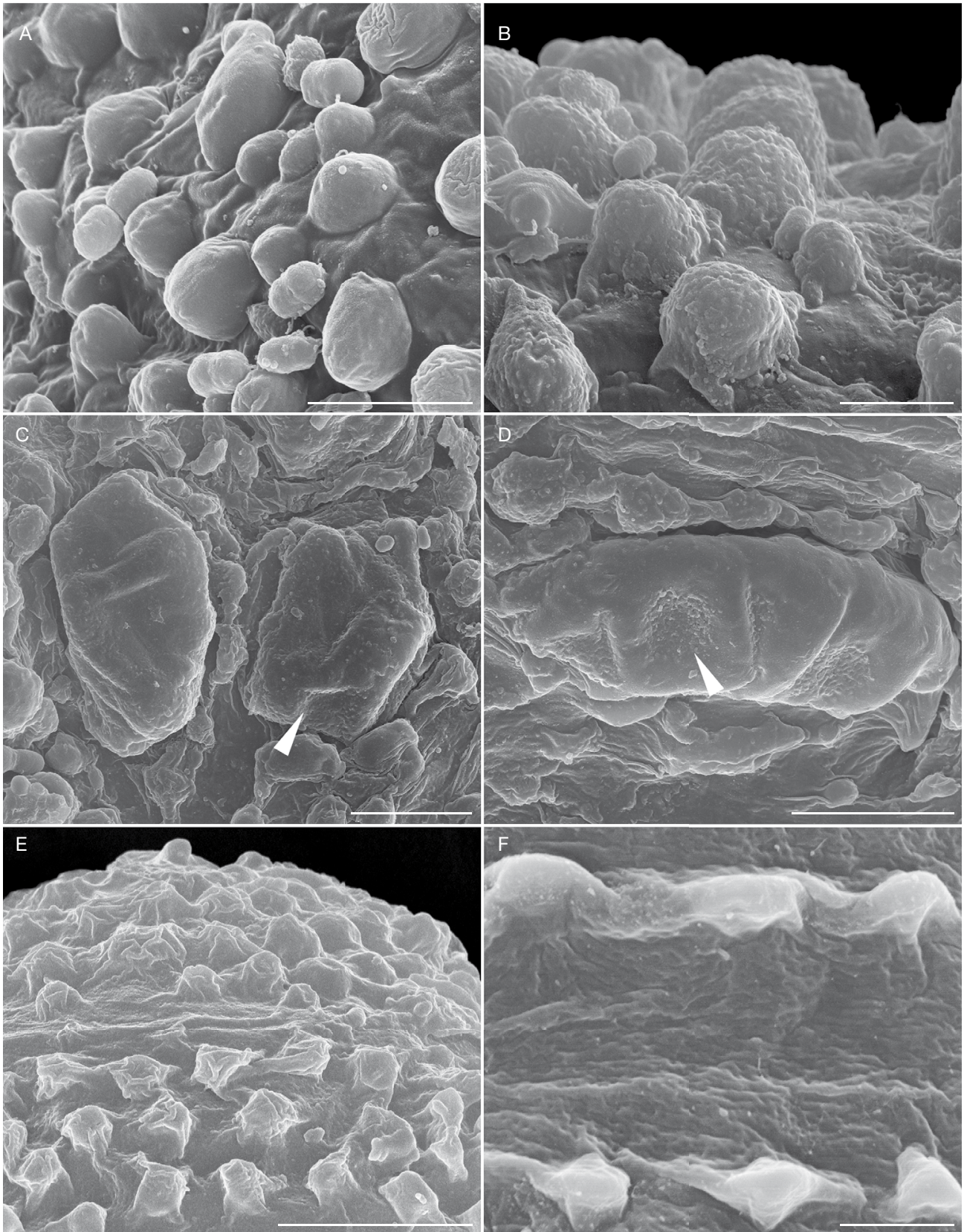
FIG. 5. — **A-D**, *Fractonotus verrucosus* (Richters, 1900) n. comb.; **E**, **F**, *Calohypsibius ornatus* (Richters, 1900), cuticle morphology (SEM): *Fractonotus*-type: **A**, smooth, obtuse tubercles of the smallest specimens (*Calohypsibius scabrosus sensu* Murray 1911, *Calohypsibius verrucosus sensu* da Cunha 1947); **B**, scabrous tubercles occurring in the caudal part of the smallest specimens and on the whole body of adults; **C**, **D**, dorsal plaques of adults (*Calohypsibius placophorus sensu* da Cunha 1943, *Calohypsibius verrucosus sensu* Thulin 1911, Marcus 1928 and Cuénot 1932), arrowheads indicate coarse portions of the plaques; *Calohypsibius*-type: **E**, irregular, multangular tubercles of the cephalic part; **F**, rows of tubercles of the trunk in close-up. Scale bars: A, B, F, 2 µm; C-E, 20 µm.
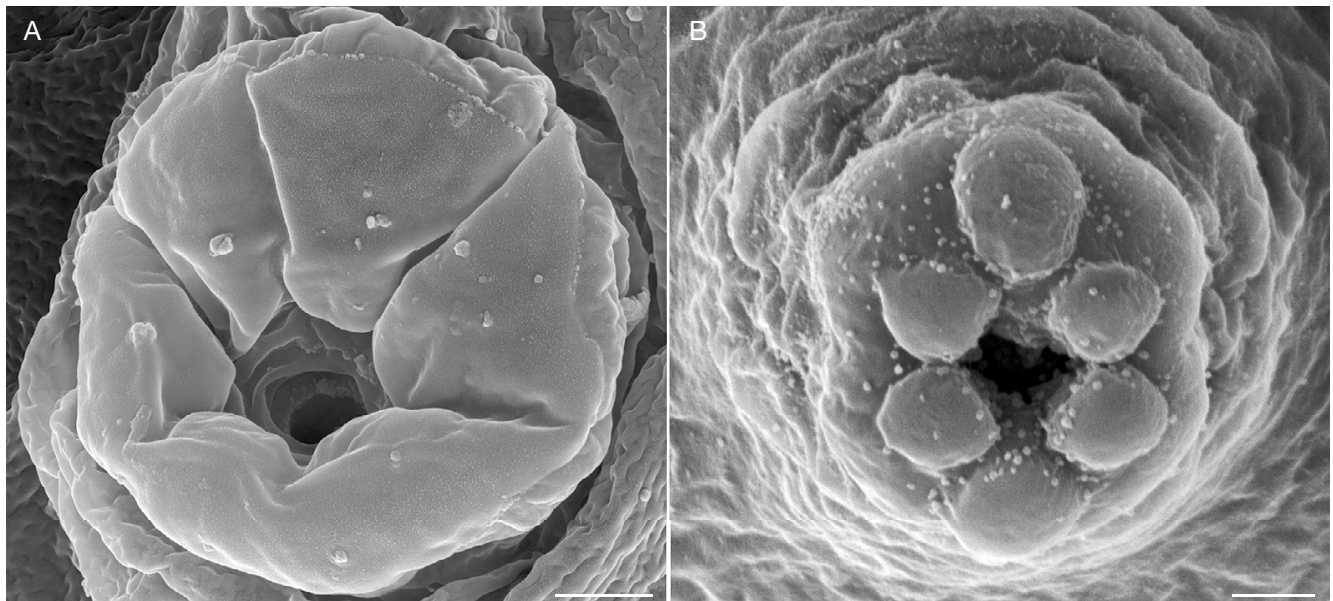
Fig. 6. — *Fractonotus verrucosus* (Richters, 1900) n. comb. and *Calohypsibius ornatus* (Richters, 1900), peribuccal structures (SEM): **A**, *Fractonotus verrucosus* n. comb., with six peribuccal lobes typical for Isohypsibiidae; **B**, *Calohypsibius ornatus* with six small and roundish peribuccal papulae typical for Calohypsibiidae. Scale bars: 1 μm.
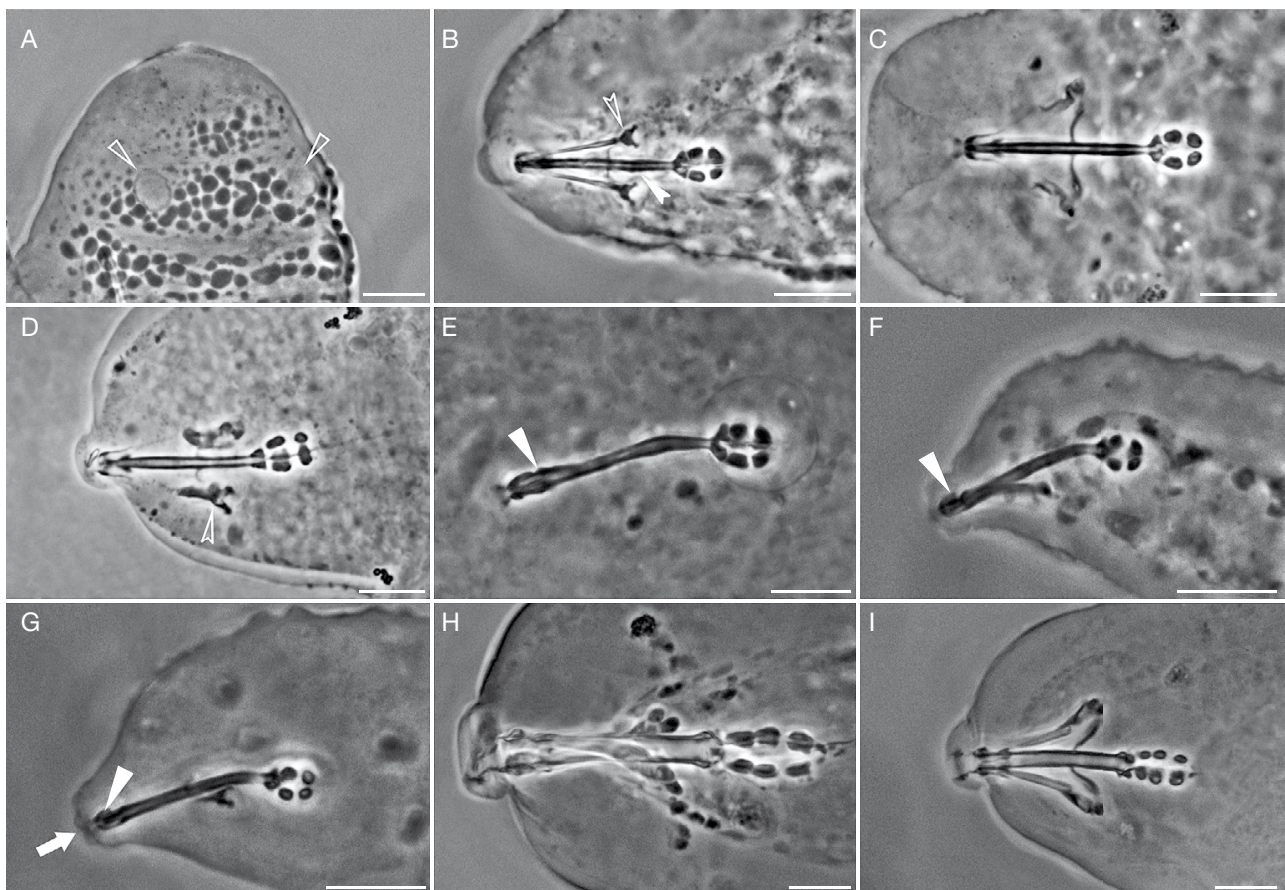


Fig. 7. — **A-E**, *Fractonotus* spp., **F-G**, *Calohypsibius* spp. and **H-I**, *Isohypsibius* spp., elliptical organs and details of the buccal apparatus (PCM): *Fractonotus verrucosus* (Richters, 1900) n. comb.: **A**, cephalic region, empty arrowheads indicate elliptical organs; **B**, dorsal view, incised arrowhead indicates buccal tube thickening, and incised empty arrowhead points to the furca; **C**, ventral view; *Fractonotus gilvus* (Biserov, 1986) n. comb.; **D**, ventral view, incised empty arrowhead points to the furca; *Fractonotus verrucosus* (Richters, 1900) n. comb.; **E**, lateral view, arrowhead indicates the posterior portion of dorsal apophysis in the shape of stumpy hook; **F**, *Calohypsibius ornatus* (Richters, 1900), lateral view, arrowhead indicates the anterior portion of dorsal apophysis in the shape of stumpy hook; **G**, *Calohypsibius schusteri* Nelson & McGlothlin, 1996, arrowhead indicates the anterior portion of dorsal apophysis in the shape of stumpy hook, and arrow points to the visible peribuccal papulae; **H**, *Isohypsibius prosostomus* (Thulin, 1928); **I**, *Isohypsibius coulsoni* Kaczmarek, Zawierucha, Smykla, Michalczyk, 2012. Scale bars: 10 μm.
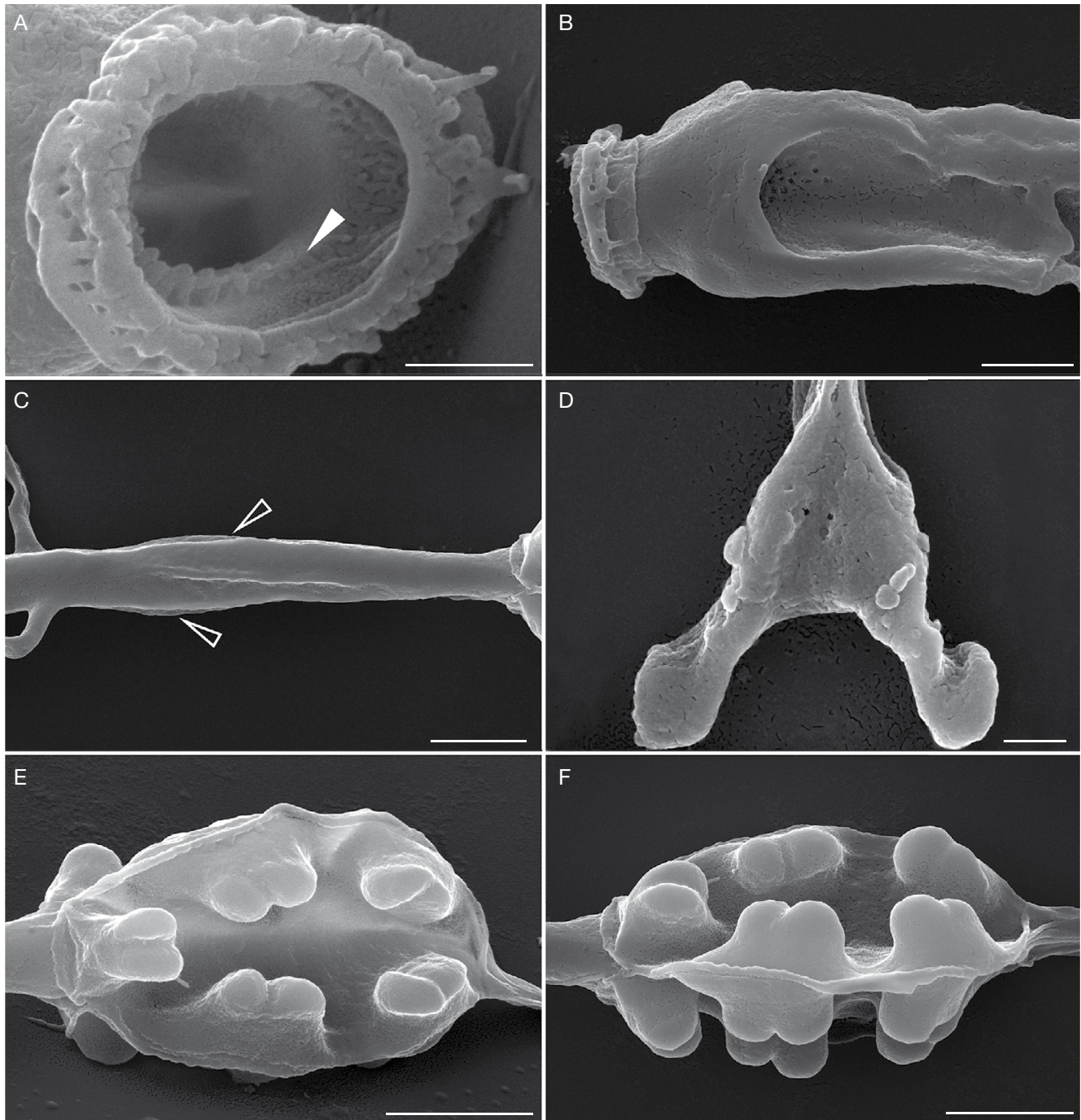
FIG. 8. — Buccal apparatus of *Fractonotus verrucosus* (Richters, 1900) n. comb. (SEM): **A**, oral cavity armature, the arrowhead indicates the row of conical teeth; **B**, the buccal crown and both dorsal and ventral apophyses for insertion of stylet muscles (AISM) in lateral view; **C**, buccal tube, empty arrowheads indicate lateral thickenings; **D**, stylet furca; **E**, pharynx in dorsal view; **F**, pharynx in lateral view. Scale bars: A, D, 0.5 µm; B, 1 µm; C, E, F, 2 µm.

bercles that increase in size towards the caudal end of the body, but fully formed plaques never present, legs covered with fine tubercles (Fig. 1A). All ten bands not always easily identifiable under PCM in juveniles. In young adults, plaques present in bands 6-10, with the most prominent plaques in bands 8-10 (Fig. 1B). In older adults, smooth spaces between the transverse bands becoming narrow and sometimes merge into larger areas (Fig. 1C-F). Plaques larger and more numerous than in young adults, and typically

developping in bands 4-10, but the most evident plaques present in the caudal part of the body (Fig.1C-F). Tubercles more or less round or oval (Figs 3A, B; 5A, B), gradually increasing in size from juveniles to adults, and becoming scabrous with age (compare Figs 1A-F; 5A, B). Plaques, on the other hand, typically smooth and only sometimes slightly rough (Fig. 5C, D, arrowheads); under stereomicroscope strongly opalescent. Plaques arranged symmetrically in respect to the longitudinal body axis, although deviations
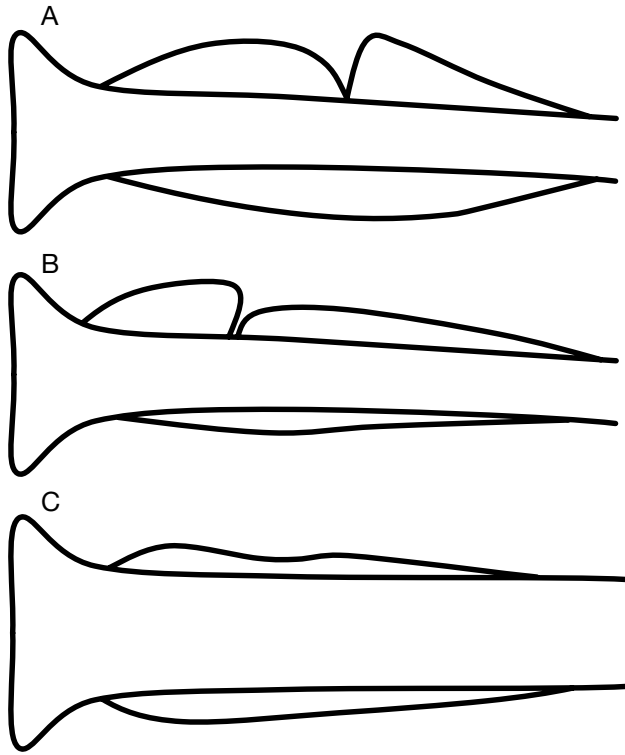
FIG. 9. — Schematic comparison of the AISM (lateral view) in: **A**, *Fractonotus* Pilato, 1998; **B**, *Calohypsibius* Thulin, 1928; **C**, *Isohypsibius* Thulin, 1928.

from symmetry are not rare (Fig. 1C, D). In adults, seven pairs of central plaques and four lateral plaque pairs. Central plaques triangular in shape, with their apices directed laterally and outwards. In rows where only central plaques are present, plaques slimmer and longer than in rows with lateral plaques. Central plaques present in bands aligned with legs I-III as well as in bands between those legs. First three pairs of lateral plaques in line with legs I-III and the last pair of double lateral plaques situated between legs III and IV (Fig. 1E). Plaque configuration VII:4-2-4-2-4-2-6. Cephalic elliptical organs present but not easy to identify, given the rich cuticular sculpturing (Fig. 7A). Eyes absent in live animals.

Buccal apparatus of the *Fractonotus*-type (Fig. 7B, C, E), i.e. with a long ventral AISM, and the dorsal AISM subdivided into the proximal, weakly developed thickening, and the distal, small blunt hook (Fig. 9A). Mouth opening surrounded by six large and soft peribuccal lobes (visible only under SEM, Fig. 6A). Oral cavity armature, visible only under SEM, consisting of a single row of minute conical teeth located on the ring fold (Fig. 8A). Two distinct porous areas on the lateral sides of the buccal crown are visible in SEM only (Fig. 8B). Stylet furcae of the modified *Hypsibius* shape, i.e. with very broad and trapezoid bases, thick arms and rounded apices (Figs 7B, 8D, 10A). Buccal tube with slight lateral thickenings posterior to the stylets supports (Figs 7B, C, E; 8C). Round bulbus with

large pharyngeal apophyses (almost as large as the placoids), and two granular macroplacoids (Figs 7B, C, E; 8E, F). In PCM, macroplacoids without constrictions, however slight central constrictions in both macroplacoids detectable under SEM (Fig. 8E, F).

Claws of the modified *Isohypsibius*-type (Figs 11A-C; 12A, B). Specifically, claw bases triangular, especially pronounced in claws IV (Figs 11C, 12B). Claw branches V-shaped, elongated and strongly curved. Apparent accessory points on the primary branches (Figs 11A-C; 12A, B). Weakly developed pseudolunulae present, particularly visible under the internal and anterior claws (Fig. 11A, C). Claw septa and cuticular bars on legs absent.

*Eggs*
Roundish and smooth, deposited in exuviae (up to two eggs per exuvia recorded).

MOLECULAR MARKERS
The sequences for all DNA markers were of a good quality. The sequenced fragments were of the following lengths: 1.727 bp (18S rRNA; MG800855), 819 bp (28S rRNA; MG800856), and 499 bp (ITS-2; MG800857). All markers, including the specimen without cuticular plaques, were represented by single haplotypes. The p-distances between 18S haplotypes of all available isohypsibioid species and *Fractonotus verrucosus* n. comb. ranged from 2.0% (*I. prosostomus* Thulin, 1928, EF620404 from Denmark) to 7.1% (*Hexapodibius micronyx* Pilato, 1969, HQ604915 from Italy), with an average distance of 5.2%. As our 28S rRNA primers obtain a different gene fragment to the one sequenced by previous authors, comparisons of this gene were not possible. Matrices with p-distances are provided in the Supplementary Material 2.

REMARKS
The vast part of the Richters Collection has been lost, thus the type material (if ever existed) is not available for examination. Moreover, no specimens from Germany were examined in this study, therefore the neotype series is not established. Hence, until the redescription from the *terra typica* in Germany is available, we propose to consider the description of the Scottish specimens only as the current perception of the species.

PHYLOGENETIC POSITION OF *FRACTONOTUS* AMONG OTHER ISOHYPSIBIIDAE
Both Bayesian Inference and Maximum Likelihood methods unreservedly located *Fractonotus* within Isohypsibioidea (Fig. 13), thus corroborating the phenotypic analysis (see above). The genus *Isohypsibius s.s.* (i.e. *I. prosostomus* and its closest relatives) appears paraphyletic with respect to *Fractonotus*. However, in general, all isohypsibioid lineages clearly remain in polytomy, with only the occasional sound Bayesian posterior probabilities characterising clades with morphologically similar taxa. Therefore, the exact relationships between different isohypsibioid clades remain unsolved.
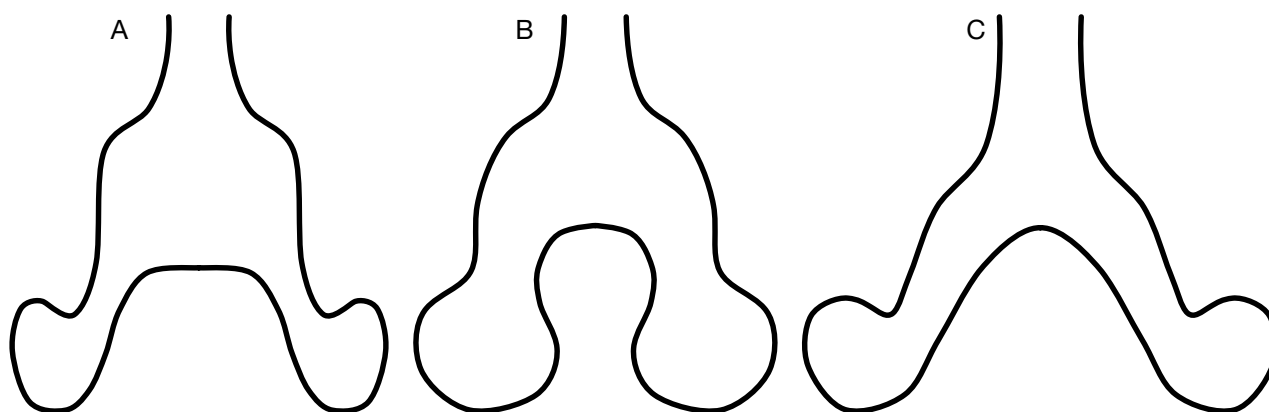
Fig. 10. — Schematic comparison of stylet furcae in: **A**, *Fractonotus* Pilato, 1998; **B**, *Calohypsibius* Thulin, 1928; **C**, *Isohypsibius* Thulin, 1928.

### *Fractonotus gilvus* (Biserov, 1986) n. comb.

*Isohypsibius gilvus* Biserov, 1986: 984.

REMARK
Elliptical organs not always visible due to developed sculpturing in the cephalic portion of the body.

## TAXONOMIC ACCOUNT OF THE CALOHYPSIBIIDAE *SENSU STRICTO*

Superfamily HYPSIBIOIDEA Pilato, 1969
(emended by Bertolani *et al.* 2014a)

Family CALOHYPSIBIIDAE Pilato, 1969
(emended by Bertolani *et al.* 2014a)

EMENDED DIAGNOSIS. — Very small eutardigrades (typically below 150 μm) with elliptical organs on the head. Dorsum covered with irregular, multangular protuberances, and sometimes also with spines (Figs 3C, D; 4; 5E, F). Claws miniaturised, but not reduced, of the *Calohypsibius*-type, i.e. asymmetrical with respect to the sequence of primary and secondary branches (2-1-2-1), but similar in their size, with bases as large as the sum of the primary and secondary branch widths, but devoid of sutures. Pseudolunulae absent. Accessory points symmetrical (Figs 11E; 12C, D). Six peribuccal papulae present (Fig. 6B). AISM asymmetrical with respect to the frontal plane, with the dorsal apophysis subdivided in two portions of different shape (Fig. 9B). Stylet furcae of the *Hypsibius*-type (Fig. 10B). Pharyngeal apophyses smaller than the tiny granular macroplacoids. Smooth eggs laid in exuviae.

COMPOSITION. — A monotypic family, comprising the genus *Calohypsibius*.

### Genus *Calohypsibius* Thulin, 1928

*Calohypsibius* Thulin, 1928: 238.

DIAGNOSIS. — Same as for the family Calohypsibiidae.

ETYMOLOGY (NOT PROVIDED IN THE ORIGINAL DESCRIPTION). — After Schuster *et al.* (1980), from Ancient Greek κάλλος (*kállos*) = beauty; derivatives calli-, callo- mean beautiful, pretty. Thulin most likely wanted to highlight the cuticular sculpturing, which is exceptionally complex, rich and unusual among Eutardigrada.

COMPOSITION AND REMARKS
Currently only three species, namely *C. maliki* Michalczyk & Kaczmarek, 2005 (Fig. 4B), *C. ornatus* (Richters, 1900) (type species; Figs 3C, D, 4A), and *C. schusteri* Nelson & McGlothlin, 1996 (Fig. 4C), are ascribed to the family. Nevertheless, Bartoš (1940) already described the remarkable variability within European records of the *ornatus* complex, which raises justifiable concerns as to whether *C. ornatus* encompasses only a single species. Further, as suggested by Pilato (1998), it is very likely that the genus comprises many more species than currently recognised. However, a systematic integrative study based on extensive sampling is needed to verify this hypothesis.

## TAXONOMIC ACCOUNT OF THE MICROHYPSIBIIDAE *SENSU STRICTO*

Superfamily HYPSIBIOIDEA Pilato, 1969
(emended by Bertolani *et al.* 2014a)

Family MICROHYPSIBIIDAE Pilato, 1998

EMENDED DIAGNOSIS. — Very small eutardigrades (typically below 150 μm in length) without elliptical organs on the head. Cuticle smooth. Claws minute and asymmetrical with respect to the sequence of primary and secondary branches (2-1-2-1), with thin bases continuous with the primary branches. External and internal, and anterior and posterior claws different in shape but similar in size. Pseudolunulae absent. Accessory points symmetrical (Fig. 11G). Peribuccal papulae not visible under PCM. AISM
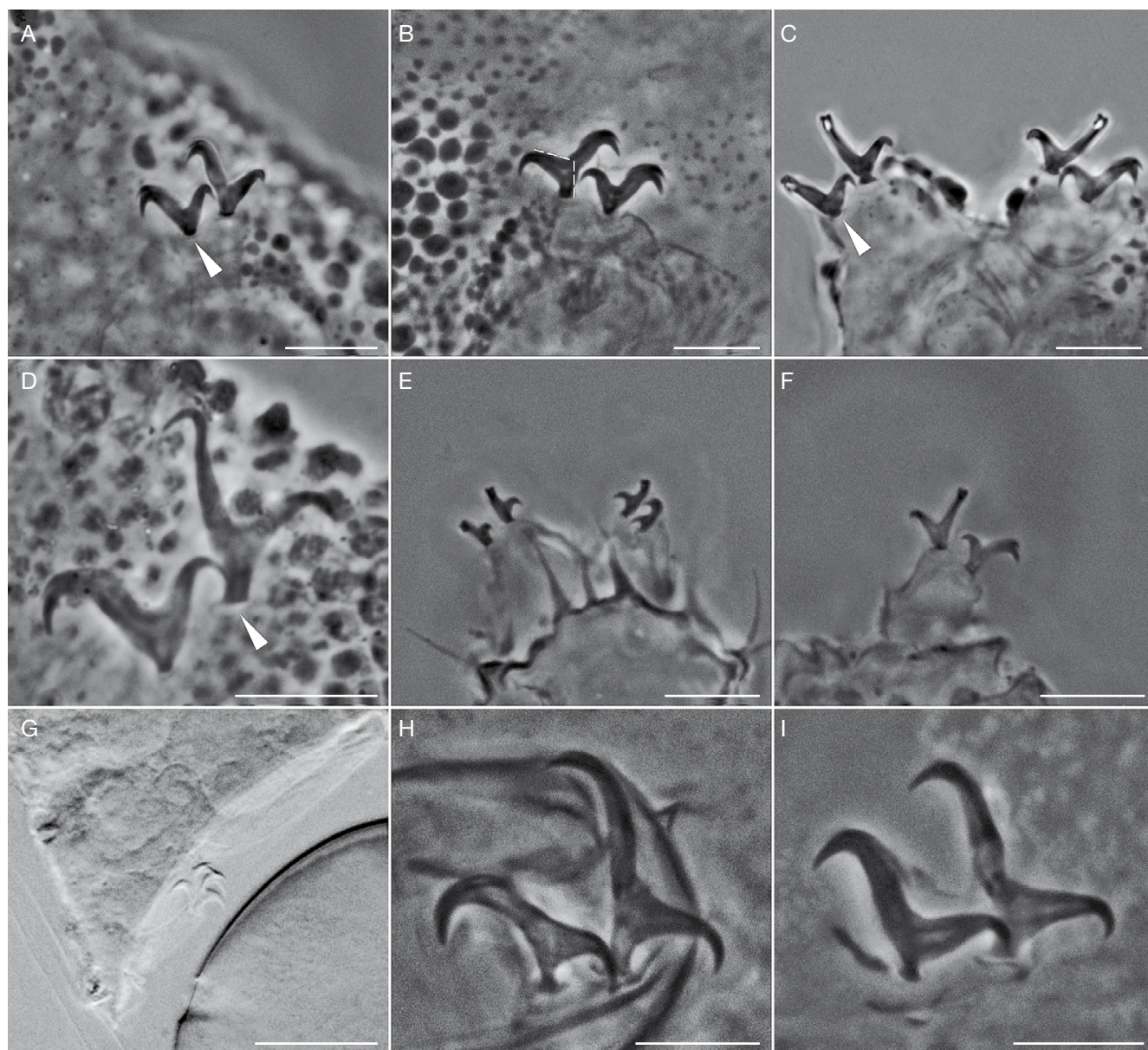
FIG. 11. — Comparison of *Fractonotus*-type (**A-D**, **F**), *Calohypsibius*-type (**E**), *Microhypsibius*-type (**G**), and *Isohypsibius*-type (**H**, **I**) claws (all but Fig. 11F PCM): *Fractonotus verrucosus* (Richters, 1900) n. comb.: **A**, claws I, arrowhead indicates weakly developed pseudolunula; **B**, claws II, interrupted line designates iso-hypsibioid claw curvature; **C**, claws IV, arrowhead indicates weakly developed pseudolunula; *Fractonotus gilvus* (Biserov, 1986) n. comb.; **D**, claws III, arrowhead indicates weakly developed pseudolunula; *Calohypsibius ornatus* (Richters, 1900); **E**, claws IV; *Fractonotus caelatus* (Marcus, 1928); **F**, claws IV; *Microhypsibius truncatus* Thulin, 1928; **G**, claws III (DIC); *Isohypsibius prosostomus* (Thulin, 1928); **H**, claws II; *Isohypsibius coulsoni* Kaczmarek, Zawierucha, Smykla & Michal-czyk, 2012; **I**, claws II. Scale bars: 10 μm.

asymmetrical with respect to the frontal plane, with the dorsal apophysis subdivided in two portions of different shapes (Fig. 9A). Stylet furcae of the *Hypsibius*-type. Pharyngeal apophyses similar in size to macroplacoids. Smooth eggs laid in exuviae.

COMPOSITION. — A monotypic family, comprising the genus *Microhypsibius*.

### Genus *Microhypsibius* Thulin, 1928

DIAGNOSIS. — Same as for the family Microhypsibiidae.

ETYMOLOGY (NOT PROVIDED IN THE ORIGINAL DESCRIPTION). — The name was most likely chosen to underline the minute size of the family members.

COMPOSITION. — *Microhypsibius bertolanii* Kristensen, 1982, *M. japonicus* Ito, 1991, *M. minimus* Kristensen, 1982, *M. truncatus* Thulin, 1928 (type species).

REMARKS
See Kristensen (1982) for the most current depiction of the genus *Microhypsibius*.

Fɪɢ. 12. — **A-B**, *Fractonotus verrucosus* (Richters, 1900) n. comb.; **C-D**, *Calohypsibius ornatus* (Richters, 1900); **E-F**, *Isohypsibius* spp., claws (SEM): *Fractonotus*-type: **A**, claws III; **B**, claws IV; *Calohypsibius*-type; **C**, claws II; **D**, claws IV; *Isohypsibius*-type; **E**, *Isohypsibius prosostomus* (Thulin, 1928), claws I; **F**, *Isohypsibius coulsoni* Kaczmarek, Zawierucha, Smykla & Michalczyk, 2012, claws II. Scale bars: A, B, 3 μm; C, D, 1 μm; E, F, 4 μm.
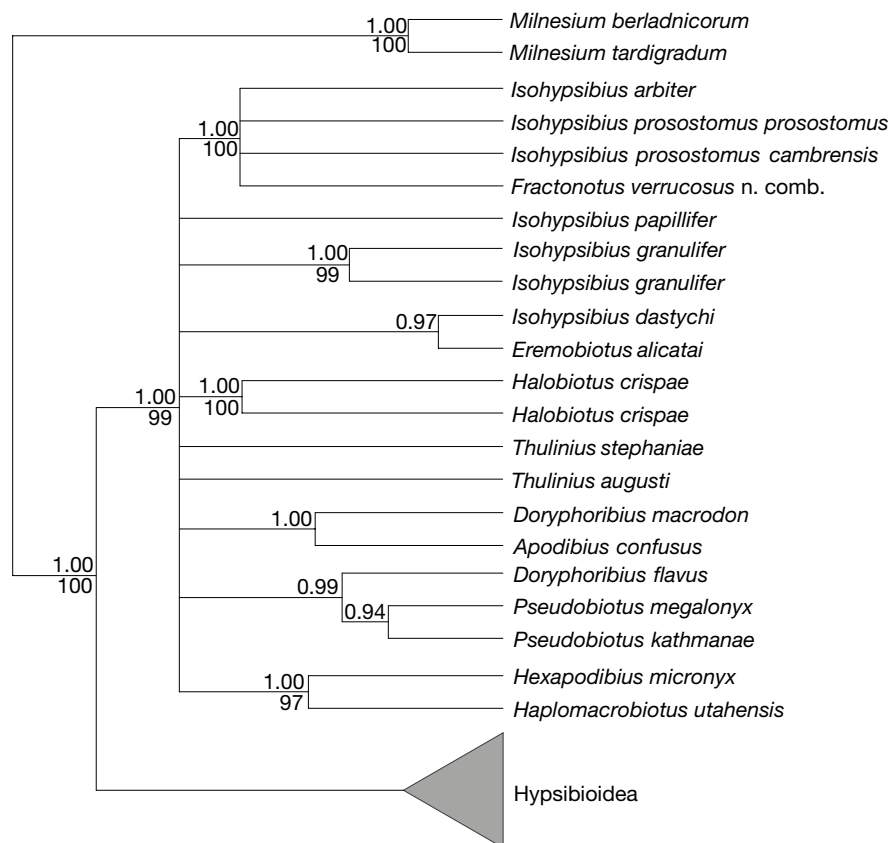
FIG. 13. — The phylogenetic position of *Fractonotus verrucosus* (Richters, 1900) n. comb. on the Bayesian Inference and Maximum Likelihood cladogram constructed from currently available 18S rRNA sequences for Isohypsibioidea and Hypsibioidea, with a *Milnesium* spp. sequence as the outgroup (see also Table 2). Values above branches indicate posterior probability values (BI), whereas values under branches show bootstrap values (ML). Branches with support below 0.9 in BI (70% in ML) were collapsed.

## DISCUSSION

### COMPARISON WITH EARLIER DESCRIPTIONS OF THE *VERRUCOSUS* COMPLEX

The descriptions of *Macrobiotus verrucosus* by Richters (1900), *M. scabrosus* by Murray (1911), and *Hypsibius placophorus* by da Cunha (1943) are brief and affected by opposing opinions on what represents intra- and inter-specific variation. Our molecular analyses confirmed, however, that both corrugated and rugose forms with plaques (i.e. the first instar and an adult instar) belong to the same species, which is in agreement with the prediction by Cuénot (1932), who explicitly expressed doubts about whether *Calohypsibius verrucosus* and *C. scabrosus* truly represent separate species as sometimes they were distinguished only by the presence/absence of eyes. Of the earlier researchers, the most significant observations were made by Murray (1911) who pointed out conspicuously large pharyngeal apophyses and claws with V-shaped branches, which now can be considered as autapomorphies of *Fractonotus*.

It must be noted that the taxonomic obscurities which accrued around the *verrucosus* complex very likely resulted from very small sample sizes. With few individuals, some populations may have comprised only juvenile forms (*F. verrucosus* n. comb.), and other of only young or older adults

(*C. scabrosus* and *C. placophorus* n. syn., respectively). This explicitly demonstrates how important in tardigrade systematics are: 1) a proper sample size; and 2) the examination of both juveniles and adults when describing new species (e.g. see Stec *et al.* 2016 and Morek *et al.* 2016b, respectively).

All above mentioned names are now designated as younger synonyms of *F. verrucosus* (Richters, 1900) n. comb. The original description of this species is limited, and the taxon is in urgent need of redescription, as is *F. caelatus*. Moreover, given that the latter species received a more recent description based on specimens found on different continents (Pilato *et al.* 1989), it is likely that it may comprise multiple taxonomic identities. Furthermore, as noted in the remarks above, the monophyly of *Fractonotus* is uncertain, as *F. caelatus* exhibits slightly different claw morphology from *F. verrucosus* n. comb. and *F. gilvus* n. comb. Therefore, the composition and definition of the genus is likely to be amended in the future, when DNA sequences for *F. caelatus* and *F. gilvus* n. comb. are available.

### *FRACTONOTUS* VS *ISOHYPSIBIUS SENSU LATO*

The obtained Bayesian and Maximum Likelihood phylogenetic trees are generally very similar to the one presented by Cesari *et al.* (2016). However, the genus *Isohypsibius s.s.* should contain the nominal species, *I. prosostomus*, and its

closest congeners, whereas in the present study this hypothetical clade is paraphyletic with respect to *Fractonotus* (Fig. 13). On the other hand, this topology may be an artefact resulting from the generally high conservatism of 18S and 28S rRNA markers, which does not allow for confident separation of the two genera due to their close affinity in respect to other isohypsibiids (see Suppl. Mat.2). Nevertheless, molecular affinity of *Isohypsibius s.s.* and *Fractonotus* is congruent with the modified *Isohypsibius*-type claws in the latter genus, and suggests that claw morphology in the systematics of the isohypsibioid lineage ought not to be neglected.

In the available limited isohypsibiid molecular dataset, *F. verrucosus* n. comb. clusters with *I. prosostomus cambrensis* Morgan, 1976, a species exhibiting distinctly granulated cuticle. This indicates that evidently sculptured cuticle may be an important supraspecific character, and it may have broad phylogenetic implications. Currently, *Isohypsibius* is the most speciose, 'super-polyphyletic' eutardigrade genus (Bertolani *et al.* 2014a, Cesari *et al.* 2016). Given that some of the species currently assigned to *Isohypsibius* and *Hypsibius s.l.* exhibit a high resemblance to *Fractonotus* in cuticle and claw morphology, they may eventually turn out to be more related to *Fractonotus* than to *I. prosostomus* and its immediate kin, thus representing convergently evolving lineages.

## REFERENCES

Bartoš E. 1940. — Über die Variation der Art *Hypsibius ornatus* Richt. (Tardigrada). *Zoologische Jahrbücher abteilung für Systematik Ökologie und Geographie der Tiere* 73: 369-384.

Beasley C. W., Kaczmarek Ł. & Michalczyk Ł. 2008. — *Doryphoribius mexicanus*, a new species of Tardigrada (Eutardigrada: Hypsibiidae) from Mexico (North America). *Proceedings of the Biological Society of Washington* 121: 34-40. https://doi.org/10.2988/07-30.1

Bertolani R., Guidetti R., Marchioro T., Altiero T., Rebecchi L. & Cesari M. 2014a. — Phylogeny of Eutardigrada: New molecular data and their morphological support lead to the identification of new evolutionary lineages. *Molecular Phylogenetics and Evolution* 76: 110-126. https://doi.org/10.1016/j.ympev.2014.03.006

Bertolani R., Bartels P. J., Guidetti R., Cesari M. & Nelson D. R. 2014b. — Aquatic tardigrades in the Great Smoky Mountains National Park, North Carolina and Tennessee, U.S.A., with the description of a new species of *Thulinius* (Tardigrada, Isohypsibiidae). *Zootaxa* 3764 (5): 524-536. https://doi.org/10.11646/zootaxa.3764.5.2

Biserov V. I. 1986. — Terrestrial water bears from the North Caucasus. 2. Eutardigrada. *Zoologicheskii Zhurnal* 65 (7): 981-993.

Campbell L. I., Rota-Stabelli O., Edgecombe G. D., Marchioro T., Longhorn S. J., Telford M. J., Philippe H., Rebecchi L., Peterson K. J. & Pisani D. 2011. — MicroRNAs and phylogenomics resolve the relationships of Tardigrada and suggest that velvet worms are the sister group of Arthropoda. *PNAS* 108 (38): 15920-15924. https://doi.org/10.1073/pnas.1105499108

Casquet J., Thebaud C. & Gillespie R. G. 2012. — Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. *Molecular Ecology Resources* 12: 136-141. https://doi.org/10.1111/j.1755-0998.2011.03073.x

Cesari M., Vecchi M., Palmer A., Bertolani R., Pilato G., Rebecchi L. & Guidetti R. 2016. — What if the claws are reduced? Morphological and molecular phylogenetic relationships of the genus *Haplomacrobiotus* May, 1948 (Eutardigrada, Parachela). *Zoological Journal of the Linnean Society* 178: 819-827. https://doi.org/10.1111/zoj.12424

Cuénot L. 1932. — Tardigrades, *in* Lechevalier P. (ed.). *Faune de France* 24: 1-96.

Cunha A. X. da 1943. — Un Tardigrade nouveau de Portugal: *Hypsibius placophorus* sp. n. *Memórias e estudos do Museu zoológico da Universidade de Coimbra* 155: 1-5.

Cunha A. X. 1947 da . — Tardigrados da Fauna Portuguesa III. *Memórias e estudos do Museu zoológico da Universidade de Coimbra* 177: 1-9.

Cunha A. X. da 1948. — Tardigrados da Fauna Portuguesa IV. *Memórias e estudos do Museu zoológico da Universidade de Coimbra* 188: 1-8.

Dabert M., Dastych H., Hohberg K. & Dabert J. 2014. — Phylogenetic position of the enigmatic clawless eutardigrade genus *Apodibius* Dastych, 1983 (Tardigrada), based on 18S and 28S rRNA sequence data from its type species *A. confusus*. *Molecular Phylogenetics and Evolution* 70: 70-75. https://doi.org/10.1016/j.ympev.2013.09.012

Dabert M., Dastych H. & Dabert J. 2015. — Molecular data support the dispersal ability of the glacier tardigrade *Hypsibius klebelsbergi* Mihelčič, 1959 across the environmental barrier (Tardigrada). *Entomologische Mitteilungen aus dem Zoologischen Museum Hamburg* 17 (194): 233-240.

Degma P. & Guidetti R. 2007. — Notes to the current checklist of Tardigrada. *Zootaxa* 1579: 41-53.

Degma P., Bertolani R. & Guidetti R. 2009-2017. — Actual checklist of Tardigrada species. www.tardigrada.modena.unimo.it/miscellanea/Actual%20checklist%20of%20Tardigrada.pdf, p. 46. Accessed 25.12.2017.

Doyère M. L. 1840. — Mémorie sur les Tardigrades. *Annales des Sciences naturelles, Paris, Series 2* 14: 269-362.

Eibye-Jacobsen J. 2001. — A new method for making SEM preparations of the tardigrade buccopharyngeal apparatus. *Zoologischer Anzeiger* 240: 309-319. https://doi.org/10.1078/0044-5231-00038

Fontoura A. P. 1981. — Contribution pour l'étude des tardigrades terrestres du Portugal, avec la description d'une nouvelle espèce du genre *Macrobiotus*. *Publicaçόes do Instituto de Zoologia 'Dr Augusto Nobre', Faculdade de Ciências do Porto* 160: 1-24.

Franceschi T. 1951-1952. — Contributo alla conoscenza dei Tardigradi d'Italia. *Bollettino dei Musei e degli Istituti Biologici dell'Università di Genova* 24 (149): 5-15.

Gąsiorek P., Stec D., Morek W., Zawierucha K., Kaczmarek Ł., Lachowska-Cierlik D. & Michalczyk Ł. 2016. — An integrative revision of *Mesocrista* Pilato, 1987 (Tardigrada: Eutardigrada: Hypsibiidae). *Journal of Natural History* 50 (45-46): 2803-2828. https://doi.org/10.1080/00222933.2016.1234654

Gąsiorek P., Zawierucha K., Stec D. & Michalczyk Ł. 2017. — Integrative redescription of a common Arctic water bear *Pilatobius recamieri* (Richters, 1911). *Polar Biology* 40: 2239-2252. https://doi.org/10.1007/s00300-017-2137-9

Gąsiorek P., Stec D., Zawierucha K., Kristensen R. M. & Michalczyk Ł. 2018. — Revision of *Testechiniscus* Kristensen, 1987 (Heterotardigrada: Echiniscidae) refutes the polar-temperate distribution of the genus. *Zootaxa* 4472 (2): 261-297. https://doi.org/10.11646/zootaxa.4472.2.3

Guil N. & Giribet G. 2012. — A comprehensive molecular phylogeny of tardigrades – adding genes and taxa to a poorly resolved phylum-level phylogeny. *Cladistics* 28: 21-49. https://doi.org/10.1111/j.1096-0031.2011.00364.x

Guil N., Machordom A. & Guidetti R. 2013. — High level of phenotypic homoplasy amongst eutardigrades (Tardigrada) based on morphological and total evidence phylogenetic analyses. *Zoological Journal of the Linnean Society* 169: 1-26. https://doi.org/10.1111/zoj.12046

Hall T. A. 1999. — BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.

Jørgensen A. & Kristensen R. M. 2004. — Molecular phylogeny of Tardigrada - investigation of the monophyly of Heterotardigrada. *Molecular Phylogenetics and Evolution* 32: 666-670. https://doi.org/10.1016/j.ympev.2004.04.017

Kaczmarek Ł., Zawierucha K., Smykla J. & Michalczyk Ł. 2012. — Tardigrada of the Revdalen (Spitsbergen) with the descriptions of two new species: *Bryodelphax parvuspolaris* (Heterotardigrada) and *Isohypsibius coulsoni* (Eutardigrada). *Polar Biology* 35: 1013-1026. https://doi.org/10.1007/s00300-011-1149-0

Katoh K. & Toh H. 2008. — Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinformatics* 9 (4): 286-298. https://doi.org/10.1093/bib/bbn013

Katoh K., Misawa K., Kuma K. & Miyata T. 2002. — MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* 30 (14): 3059-3066.

Kiehl E., Dastych H., D'Haese J. & Greven H. 2007. — The 18S rDNA sequences support polyphyly of Hypsibiidae (Eutardigrada). *Journal of Limnology: Proceedings of the Tenth International Symposium on Tardigrada* 66(S1): 21-25. https://doi.org/10.4081/jlimnol.2007.s1.21

Kristensen R. M. 1982. — New aberrant eutardigrades from homothermic springs on Disko Island, West Greenland. In: Nelson, D. R. (ed.), *Proceedings of the Third International Symposium on the Tardigrada*, 1980, 203-220.

Kumar S., Stecher G. & Tamura K. 2016. — MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33 (7): 1870-1874. https://doi.org/10.1093/molbev/msw054

Lanfear R., Frandsen P. B., Wright A. M., Senfeld T. & Calcott B. 2016. — PartitionFinder 2: new methods for selecting partitioned models of evolution formolecular and morphological phylogenetic analyses. *Molecular Biology and Evolution* 34 (3): 772-773. https://doi.org/10.1093/molbev/msw260

Marcus E. 1928. — Spinnentiere oder Arachnoidea. IV: Bärtierchen (Tardigrada). *Tierwelt Deutschlands und der angrenzenden Meeresteile Jena* 12: 1-230.

Marcus E. 1936. — Tardigrada. *Das Tierreich, de Gruyter & Co., Berlin and Leipzig* 66: 1-340.

May R.-M. 1948. — Nouveau genre et espèce de tardigrade du Mexique: *Haplomacrobiotus hermosillensis*. *Bulletin de la Société Zoologique de France* 73: 95-97.

Michalczyk Ł. & Kaczmarek Ł. 2003. — Water Bears (Tardigrada) of the Bieszczady Mountains (Poland, West Carpathians). *Zootaxa* 242: 1-24.

Michalczyk Ł. & Kaczmarek Ł. 2005. — The first record of the genus *Calohypsibius* Thulin, 1928 (Eutardigrada: Calohypsibiidae) from Chile (South America) with a description of a new species *Calohypsibius maliki*. *New Zealand Journal of Zoology* 32: 287-292. https://doi.org/10.1080/03014223.2005.9518420

Michalczyk Ł. & Kaczmarek Ł. 2010. — Description of *Doryphoribius dawkinsi*, a new species of Tardigrada (Eutardigrada: Hypsibiidae) from the Costa Rican highlands, with the key to the genus *Doryphoribius*. *Zootaxa* 2393: 46-58. https://doi.org/10.5281/zenodo.193905

Michalczyk Ł. & Kaczmarek Ł. 2013. — The Tardigrada Register: a comprehensive online data repository for tardigrade taxonomy. *Journal of Limnology* 72(S1): 175-181. https://doi.org/10.4081/jlimnol.2013.s1.e22

Mihelčič F. 1953. — Contribucion al conocimiento de los tardigrados con especial consideracion de los tardigrados de Osttirol (II). *Anales de Edafologia y Fisiologia Vegetal* 12 (5): 431-479.

Mironov S. V., Dabert J. & Dabert M. 2012. — A new feather mite species of the genus *Proctophyllodes* Robin, 1877 (Astigmata: Proctophyllodidae) from the Long-tailed Tit *Aegithalos caudatus* (Passeriformes: Aegithalidae): morphological description with DNA barcode data. *Zootaxa* 3253: 54-61.

Morek W., Stec D., Gąsiorek P., Schill R. O., Kaczmarek Ł. & Michalczyk Ł. 2016a. — An experimental test of tardigrade preparation methods for light microscopy. *Zoological Journal of the Linnean Society* 178: 785-793. https://doi.org/10.1111/zoj.12457

Morek W., Gąsiorek P., Stec D., Blagden B. & Michalczyk Ł. 2016b. — Experimental taxonomy exposes ontogenetic variability and elucidates the taxonomic value of claw configuration in *Milnesium* Doyère, 1840 (Tardigrada: Eutardigrada: Apochela). *Contributions to Zoology* 85 (2): 173-200.

Morek W., Stec D., Gąsiorek P., Surmacz B. & Michalczyk Ł. 2019. — *Milnesium tardigradum*: the first integrative study of inter-population variability in a tardigrade species. *Journal of Zoological Systematics and Evolutionary Research* 57: 1-23. https://doi.org/10.1111/jzs.12233

Morgan C. 1976. — Studies on the British tardigrade fauna: some zoogeographical and ecological notes. *Journal of Natural History* 10: 607-632. https://doi.org/10.1080/00222937600770491

Møbjerg N., Jørgensen A., Eibye-Jacobsen J., Halberg K. A., Persson D. & Kristensen R. M. 2007. — New records on cyclomorphosis in the marine eutardigrade *Halobiotus crispae* (Eutardigrada: Hypsibiidae). *Journal of Limnology* 66 (1): 132-140. https://doi.org/10.4081/jlimnol.2007.s1.132

Murray J. 1911. — Clare Island Survey. 37: Arctiscoida. *Proceedings of the Royal Irish Academy* 31 (37): 1-16.

Nelson D. R. & McGlothlin K. L. 1996. — A new species of *Calohypsibius* (Phylum Tardigrada, Eutardigrada) from Roan Mountain, Tennessee-North Carolina, U.S.A. *Zoological Journal of the Linnean Society* 116: 167-174. https://doi.org/10.1006/zjls.1996.0014

Nelson D. R., Guidetti R. & Rebecchi L. 2015. — Phylum Tardigrada. Academic Press. Thorp and Covich's Freshwater Invertebrates, 1: Chapter 17: 347-380.

PILATO G. 1969a. — Evoluzione e nuova sistemazione degli Eutardigrada. *Bolletino di Zoologia* 36: 327-345.

PILATO G. 1969b. — Su un interessante Tardigrado esapodo delle dune costiere siciliane: *Hexapodibius micronyx* n. gen. n. sp. *Bollettino delle sedute dell'Accademia Gioenia di Scienze naturale Catania, Serie IV* 9 (9): 619-622.

PILATO G. 1981. — Analisi di nuovi caratteri nello studio degli Eutardigradi. *Animalia* 8: 51-57.

PILATO G. 1989. — Phylogenesis and systematic arrangement of the family Calohypsibiidae Pilato, 1969 (Eutardigrada). *Zeitschrift für zoologische Systematik und Evolutionsforschung* 27: 8-13.

PILATO G. 1998. — Microhypsibiidae, new family of eutardigrades, and description of the new genus *Fractonotus*. *Spixiana* 21 (2): 129-134.

PILATO G. & BEASLEY C. W. 1987. — *Haplohexapodibius seductor* n. gen. n. sp. (Eutardigrada, Calohypsibiidae) with remarks on the systematic position of the new genus. *Animalia* 14 (1-3): 65-71.

PILATO G. & BINDA M. G. 2010. — Definition of families, subfamilies, genera and subgenera of the Eutardigrada, and keys to their identification. *Zootaxa* 2404: 1-54.

PILATO G., CLAXTON S. & BINDA M. G. 1989. — Tardigrades from Australia II. The evaluation of *Calohypsibius ornatus* (Richters, 1900) *caelatus* (Marcus, 1928) as a valid species and description of *Minibiotus fallax* n. sp. (Eutardigrada). *Animalia* 16: 21-27.

RAMBAUT A., SUCHARD M. A., XIE D. & DRUMMOND A. J. 2014. — Tracer v1.6. Available from http://beast.bio.ed.ac.uk/Tracer

RICHTERS F. 1900. — Beiträge zur Kenntnis der Fauna der Umgebung von Frankfurt a. M. *Bericht der Senckenbergischen Naturforschenden gesellschaft in Frankfurt am Main* 21-44.

RICHTERS F. 1926. — Tardigrada, *in Handbuch der Zoologie, de Gruyter, Berlin and Leipzig*, 3: 58-61.

RONQUIST F. & HUELSENBECK J. P. 2003. — MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19 (12): 1572-1574.

SANDS C. J., MCINNES S. J., MARLEY N. J., GOODALL-COPESTAKE W., CONVEY P. & LINSE K. 2008. — Phylum Tardigrda: an "individual" approach. *Cladistics* 24: 1-18. https://doi.org/10.1111/j.1096-0031.2008.00219.x

SCHUSTER R. O., NELSON D. R., GRIGARICK A. A. & CHRISTENBERRY D. 1980. — Systematic criteria of the Eutardigrada. *Transactions of the American Microscopical Society* 99: 284-303.

STAMATAKIS A. 2014. — RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312-1313. https://doi.org/10.1093/bioinformatics/btu033

STEC D., SMOLAK R., KACZMAREK Ł. & MICHALCZYK Ł. 2015. — An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Kenya. *Zootaxa* 4052 (5): 501-526. https://doi.org/10.11646/zootaxa.4052.5.1

STEC D., GĄSIOREK P., MOREK W., KOSZTYŁA P., ZAWIERUCHA K., MICHNO K., KACZMAREK Ł., PROKOP Z. M. & MICHALCZYK Ł. 2016. — Estimating optimal sample size for tardigrade morphometry. *Zoological Journal of the Linnean Society* 178: 776-784. https://doi.org/10.1111/zoj.12404

STEC D., MOREK W., GĄSIOREK P. & MICHALCZYK Ł. 2018. — Unmasking hidden species diversity within the *Ramazzottius oberhaeuseri* complex, with an integrative redescription of the nominal species for the family Ramazzottiidae (Tardigrada: Eutardigrada: Parachela). *Systematics and Biodiversity* 16 (4): 357-376. https://doi.org/10.1080/14772000.2018.1424267

THULIN G. 1911. — Beitage zur Kenntnis der Tardigradenfauna Schwedens. *Arkiv för Zoologi* 7 (16): 1-60.

THULIN G. 1928. — Über die phylogenie und das system der tardigraden. *Hereditas* 11: 207-266.

WEŁNICZ W., GROHME M. A., KACZMAREK Ł., SCHILL R. O. & FROHME M. 2011. — ITS-2 and 18S rRNA data from *Macrobiotus polonicus* and *Milnesium tardigradum* (Eutardigrada, Tardigrada). *Journal of Zoological Systematics and Evolutionary Research* 49(S1): 34-39. https://doi.org/10.1111/j.1439-0469.2010.00595.x

WHITE T. J., BRUNS T., LEE S. & TAYLOR J. 1990. — *PCR protocols: a guide to methods and application*. Academic Press, San Diego, California, 315-322 p.

ZAWIERUCHA K., KOLICKA M., TAKEUCHI N. & KACZMAREK Ł. 2015. — What animals can live in cryoconite holes? A faunal review. *Journal of Zoology* 295: 159-169. https://doi.org/10.1111/jzo.12195

ZELLER C. 2010. — *Untersuchung der Phylogenie von Tardigraden anhand der Genabschnitte 18S rDNA und Cytochrom c Oxidase Untereinheit 1 (COX I)*. MSc Thesis, Technische Hochschule Wildau, Wildau, 105 p.