Deceptive conservatism of claws: distinct phyletic lineages concealed within Isohypsiboidea (Eutardigrada) revealed by molecular and morphological evidence

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Abstract

Isohypsiboidea are most likely the most basally branching evolutionary lineage of eutardigrades. Despite being second largest eutardigrade order, phylogenetic relationships and systematics within this group remain largely unresolved. Broad taxon sampling, especially within one of the most speciose tardigrade genera, Isohypsibius Thulin, 1928, and application of both comparative morphological methods (light contrast and scanning electron microscopy imaging of external morphology and buccal apparatuses) and phylogenetic framework (18S + 28S rRNA sequences) resulted in the most comprehensive study devoted to this order so far. Two new families are erected from the currently recognised family Isohypsibiidae: Doryphoribiidae fam. nov., comprising all aquatic isohypsibioids and some terrestrial isohypsibioid taxa equipped with the ventral lamina; and Halobiotidae fam. nov., secondarily marine eutardigrades with unique adaptations to sea environment. We also split Isohypsibius into four genera to accommodate phylogenetic, morphological and ecological variation within the genus: terrestrial Isohypsibius s.s. (Isohypsibiidae), with smooth or sculptured cuticle but without gibbosities; terrestrial Dianea gen. nov. (Isohypsibiidae), with small and pointy gibbosities; terrestrial Ursulinius gen. nov. (Isohypsibiidae), with
large and rounded gibbosities; and aquatic *Grevenius* gen. nov. (Doryphoribiidae fam. nov.), typically with rough cuticle and claws with branches of very similar heights. Claw morphology is reviewed and, for the first time, shown to encompass a number of morphotypes that correlate with clades recovered in the molecular analysis. The anatomy of pharynx and cuticle are also shown to be of high value in distinguishing supraspecific taxa in Isohypsibioidea. Taxonomy of all isohypsibioid families and genera is discussed, with special emphasis on the newly erected entities. Finally, a dychotomous diagnostic key to all currently recognised isohypsibioid families and genera is provided.

**Keywords**

Tardigrada – Isohypsibiidae – taxonomy – buccal apparatus – phylogeny – 18S rRNA – 28S rRNA

**Introduction**

Tardigrada are a phylum of microinvertebrates inhabiting almost all environments across the globe (Nelson et al., 2015). Despite the undeniable progress in disentangling tardigrade phylogeny, both tardigrade relationships with other metazoans (e.g., Campbell et al., 2011; Gross & Mayer, 2015) and many within-group affinities remain unclear (e.g., Sands et al., 2008; Bertolani et al., 2014a). One of major tardigrade groups with unresolved phylogeny and taxonomy is Isohypsibioidea Guil et al., 2019. This recently established eutardigrade order is considered problematic because it is based on traits that show high levels of morphological stasis (Marley et al., 2011). In fact, this group was erected relatively late mainly because for decades various taxa with *Isohypsibius* and *Hypsibius* type claws were traditionally grouped into a single order Hypsibioidea Guil et al., 2019. Although the erection of Isohypsibioidea clarified the taxonomy of the Eutardigrada, the few efforts to resolve phylogenetic relationships within the order suffered from insufficient sampling and resulted in prevailing polytomies (Bertolani et al., 2014a; Gąsiorek et al., 2019). So far, only a single study (Cesari et al., 2016) identified a monophyletic lineage within Isohypsibioidea. They found morphological and molecular support to erect the family Hexapodibiidae Cesari et al., 2016, the only other isohypsibioid family apart from Isohypsibiidae Sands et al., 2008.

Thus, in order to elucidate the taxonomy and phylogenetic relationships within the order Isohypsibioidea, here we employ comparative analyses of over fifty species representing eleven isohypsibioid genera. Our study embraces a range of analytical methods that included light and scanning electron microscopy observations of external and internal morphology as well as DNA sequencing of two nuclear markers. We uncovered four distinct phylogenetic lineages, corresponding to two previously identified and two new families. The largest tardigrade genus, *Isohypsibius* Thulin, 1928, as indicated earlier (Bertolani et al., 2014a; Cesari et al., 2016; Gąsiorek et al., 2019), is artificial and comprises at least five distinct evolutionary lineages. For three of these, we propose new formal taxonomic ranks and we demonstrate, for the first time, that even slight qualitative differences in claw anatomy, the number of macroplacoids in the pharynx, and the presence of cuticular gibbosities can be of high taxonomic importance in delineating isohypsibioid genera. This work is, therefore, another step towards making all isohypsibioid families and genera monophyletic.
Materials and methods

Sample processing and comparative material
Tardigrades were isolated from moss, lichen, mixed moss and lichen, or water sediment samples, collected by various persons (see table 1), and processed following the protocol described by Stec et al. (2015). In addition to newly collected material, slides with type specimens of taxa described by Ramazzotti, Maucci, Pilato and Biserov, deposited in the Natural History Museum in Verona, were examined using phase contrast microscope (PCM; see table 1 for details), courtesy of Dr. Roberto Guidetti of the University of Modena and Reggio Emilia, Italy.

Microscopy and imaging
Specimens for light microscopy and morphometry were mounted on microscope slides in Hoyer’s medium according to Morek et al. (2016) and examined under a Nikon Eclipse 50i phase contrast microscope (PCM) fitted with a Nikon Digital Sight DS-L2 digital camera. Specimens for imaging in the scanning electron microscope (SEM) were prepared according to Stec et al. (2015). Buccal apparatuses were extracted following the protocol provided by Eibye-Jacobsen (2001) with modifications described 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 under PCM in a single photograph, a series of 2–6 images were taken every ca. 0.2 μm and then assembled with Corel into a single deep-focus image.

Morphometrics, terminology and classification
All measurements are given in micrometres (μm). Structures were measured only if they were intact and orientated in a flat plane. Terminology for the structures within the buccal apparatus and for the claws follows that of Pilato & Binda (2010) and Gąsiorek et al. (2017). Additionally, in order to avoid misunderstandings and aid comparisons between isohypsiobioid taxa, we propose new terminology describing the details of the oral cavity armature (OCA); see also fig. 1. All sclerified elements in the oral cavity are referred to as ‘teeth’ as their location and shape strongly suggest their function as teeth (see also Hansen & Katholm, 2002, and Michalczyk & Kaczmarek, 2003, for similar proposals in eohypsibiids and macrobiotids, respectively). At the same time, we suggest to abandon the use of unspecific terms such as intrabuccal/infrabuccal ‘baffles’, ‘mucrones’ or ‘ridges’ as they refer to solely shape/appearance rather than to function and therefore may be enigmatic. In all isohypsibioids analysed with SEM, either one or two bands of teeth were observed (Biserov, 1992; Nelson et al., 1999; Jørgensen, 2001, the present study). The first band of teeth was present in all individuals and it was always located on the ring fold (a circular and soft portion of the oral cavity wall; Michalczyk & Kaczmarek, 2003) in the middle of the oral cavity. The second band, if present, was always located behind the first band, at the rear of the oral cavity, just before the buccal tube opening (fig. 1). Therefore, we refer to these bands as ‘the first band of teeth’ and ‘the second band of teeth’, respectively. The numbering of bands of teeth is introduced solely for practical reasons, to allow a concise description of the OCA, and are not for the formulation of hypotheses on homologies. Stylet support insertion point is abbreviated as SSIP, whereas apophyses for the insertion of the stylet muscles – as AISM. Claws were measured following Beasley et al. (2008). In order to quantify the relative difference in height between the secondary and the
| Taxon                                                                 | Locality                      | Coordinates                | Altitude (m asl) | Sample type          | Collector              | Type material | SEM analysis |
|----------------------------------------------------------------------|-------------------------------|----------------------------|------------------|----------------------|------------------------|---------------|---------------|
| **Doryphoribiidae fam. nov.**                                         |                               |                            |                  |                      |                        |               |               |
| *Apodibius confusus* Dastych, 1983                                   | Welzow-South, Germany         | ca. 51°34'N, 14°14'E       | ?                | soil                 | K. Hohberg             | –             | +             |
| *Apodibius nuntius* Binda, 1984                                     | Inhambane, Mozambique         | ?                          | ?                | moss on soil         | G. Costa, A. Petralia  | +             | –             |
| *Doryphoribius bindae* Lisi, 2011                                    | Udzangwa Mountains, Mwanihana | ca. 7°49'S, 36°50'E        | 2100             | moss                 | T. Pape, N. Scharff    | –             | –             |
| *Doryphoribius dawkinsi* Michalczyk & Kaczmarek, 2010                | Heredia, Costa Rica           | 10°00'10"N, 84°06'44"W    | 1161             | moss from tree       | Ł. Kaczmarek          | +             | +             |
| *Doryphoribius korganovae* Biserov, 1994                            | Assumption Island, Seychelles | ca. 9°44'S, 46°38'E        | 5–10             | moss on calcareous slabs | G.A. Korganova       | +             | –             |
| *Doryphoribius monstroosus* (Maucci, 1991)                           | Vegaredonda, Spain            | 43°14'N, 4°59'W           | ?                | moss on soil         | B. Osella             | +             | –             |
| *Doryphoribius polynettae* Biserov, 1988                            | Naryan-Mara, Russia           | ?                          | ?                | moss                 | V.I Biserov           | +             | –             |
| *Doryphoribius taiwanus* X. Li & H. Li, 2008                         | Rio de Janeiro, Brasil        | 22°57'09"S, 43°13'40"W    | 60               | moss                 | Ł. Skoczyzas          | –             | –             |
| *Grevenius baldii* (Ramazzotti, 1945) comb. nov.                     | Lago di Tovel, Italy          | 46°15'N, 10°56'E          | 1180             | sediment             | M. Balsamo            | –             | –             |
| *Grevenius granulifer* (Thulin, 1928) comb. nov.                     | Kraków, Poland                | 50°03'27"N, 19°50'19"E    | 294              | liverworts and algae from stump in temporary pond sediment | P. Gąsiorek, W. Morek| –             | +             |
| *Grevenius longiunguis* (Pilato, 1974) comb. nov.                    | Randazzo, Sicily, Italy       | 37°52'N, 14°56'E          | 710–750          | sediment             | G. Pilato             | +             | –             |

**Table 1** List of isohypsibioid taxa used in presented analyses. All species were examined under PCM, and some also under SEM. In most cases type material was analysed.
List of isohypsibioid taxa used in presented analyses. All species were examined under PCM, and some also under SEM. In most cases type material was analysed

| Taxon                     | Locality                  | Coordinates                              | Altitude (m asl) | Sample type                  | Collector                      | Type material | SEM analysis |
|---------------------------|---------------------------|------------------------------------------|------------------|------------------------------|-------------------------------|---------------|--------------|
| *Grevenius monoicus*     | Corsonna river, Italy     | 44°05’06”N, 10°29’36”E                 | 365              | sediment                    | R. Bertolani                  | +             | –            |
| comb. nov.                |                           |                                          |                  |                              |                               |               |              |
| *Grevenius pushkini*     | Kraków, Poland            | 50°03’45”N, 19°57’27”E                 | 205              | sediment                    | A. Oczkowski, B. Surmacz      | –             | +            |
| comb. nov.                |                           |                                          |                  |                              |                               |               |              |
| *Grevenius sismicus*     | Van, Turkey               | ca. 38°30’N, 43°20’E                   | 1650–1670        | moss on soil                | W. Mauucci                    | +             | –            |
| comb. nov.                |                           |                                          |                  |                              |                               |               |              |
| *Pseudobiotus megalonyx* | Kraków, Poland            | 50°03’27”N, 19°50’19”E                 | 294              | sediment and rotting leaves | P. Gąsiorek                   | –             | +            |
| (Thulin, 1928)           |                           |                                          |                  |                              |                               |               |              |
| *Thulinius ruffoi*       | Zelków, Poland            | 50°09’52”N, 19°48’26”E                 | 368              | sediment                    | M. Wojtaszek                  | –             | +            |
| (Bertolani, 1982)        |                           |                                          |                  |                              |                               |               |              |
| *Thulinius stephaniae*   | Randazzo, Sicily, Italy   | ca. 37°52’N, 14°56’E                   | 710–750          | sediment                    | G. Pilato                     | +             | –            |
| (Pilato, 1974)           |                           |                                          |                  |                              |                               |               |              |
| **Halobiotidae fam. nov.** |                          |                                          |                  |                              |                               |               |              |
| *Halobiotus arcturulius* | Advent Bay, Spitsbergen   | ca. 78°14’N, 15°35’E                     | ?                | sediment                    | L. Kotwicki                   | –             | +            |
| Crisp & Kristensen, 1983 |                           |                                          |                  |                              |                               |               |              |
| *Halobiotus crispeae*    | Nipissat Bay, Greenland   | ca. 69°26’N, 54°12’W                    | ?                | sediment                    | R.M. Kristensen               | +             | –            |
| Kristensen, 1982          |                           |                                          |                  |                              |                               |               |              |
| **Hexapodibiidae**       |                           |                                          |                  |                              |                               |               |              |
| *Hexapodibius micrornyx* | Gela, Sicily, Italy       | ca. 37°04’N, 14°15’E                    | 20–50            | moss on sand                | G. Pilato                     | +             | –            |
| Pilato, 1969              |                           |                                          |                  |                              |                               |               |              |
| *Hexapodibius pseudomicronyx* | Saint-Maur-des-Fossés, France | 48°48’23’N, 2°28’44’E | 38                | moss on tomb                | W. Morek                      | –             | +            |
| Robotti, 1972             |                           |                                          |                  |                              |                               |               |              |
| *Parhexapodibius castrù* | Cerro Pajonal, Chile      | ca. 22°26’S, 68°54’W                    | 4150             | moss                        | ?                            | +             | –            |
| (Ramazzotti, 1964)       |                           |                                          |                  |                              |                               |               |              |
| *Parhexapodibius lagrecai* | Ustica, Italy             | ca. 38°42’N, 13°10’E                    | ?                | moss                         | M.G. Binda, G. Pilato         | +             | –            |
| Species | Location | Coordinates | Collection Method | Author(s) | Status |
|---------|----------|--------------|-------------------|-----------|--------|
| *Parhexapodibius* ramazzottii | Paradiso Pass, Italy | ca. 46°13’N, 10°34’E | soil | Manicardi & Bertolani, 1987 | + – |
| *Parhexapodibius ramazzottii* | Basalovo, Italy | 44°32’22”N, 10°55’55”E | moss on soil | Manicardi & Bertolani, 1987 | – + |
| *Dianea* sattleri | Kamienna Góra, Poland | 50°46’39”N, 16°03’22”E | moss from tree trunk | Durante & Maucci, 1972 | – + |
| *Dianea* vejdovskyi | Carpathians, Czech Republic | ca. 60°57’19”, 19°37’22”E | moss from soil | Říčanov | – + |
| *Eremobiotus* alicatai | Gela, Sicily, Italy | ca. 37°04’N, 14°15’E | moss on sand | Binda, 1969 | – + |
| *Eremobiotus* ovezovae | Kara-Kum Desert, Turkmenistan | ? | moss on sand | Ovezova, 1992 | + – |
| *Eremobiotus* sp. nov. | Gül Baba, Budapest, Hungary | 47°31’01”N, 19°02’06”E | moss on wall | Witwicka, 2004 | + + |
| *Fractonotus* gibus | Mussa-Aci Tara, Russia | ca. 43°36’N, 41°37’E | moss on stump | Biserov, 1986 | + – |
| *Fractonotus* verrucosus | Creag Meagaidh, Scotland | 56°37’03”N, 4°36’09”W | lichen on rock | Blagden, 1991 | + + |
| *Isohypsibius* arbiter | Lucania, Italy | ? | moss on soil | Binda, 1980 | – + |
| *Isohypsibius* ceciliae | Magdalena Bay, Spitsbergen | ca. 79°31’N, 10°42’E | moss on soil | Pilato & Binda, 1987 | + – |
| *Isohypsibius* chiarae | Mount Washburn, Wyoming, USA | ca. 44°47’N, 110°26’W | moss on soil | Maucci, 1987 | + – |
| *Isohypsibius* coulsoni | Hornsund, Spitsbergen | 77°00’40”N, 15°22’20”E | moss from rock | Kaczmarek et al., 2012 | + + |
| *Isohypsibius* dastychi | Modena, Italy | ca. 44°38’N, 10°55’E | moss on soil | Pilato et al., 1982 | – + |
| *Isohypsibius* prosostomus | Tatra Mountains, Poland | 49°14’49”N, 2°39’44”E | moss from tree bark | Thulin, 1928 | – + |
| *Isohypsibius* reticulatus | Simeto river, Sicily, Italy | ca. 37°36’N, 14°56’E | moss from soil | Pilato, 1973 | + – |
| Taxon                                  | Locality                           | Coordinates                  | Altitude (m asl) | Sample type         | Collector            | Type material | SEM analysis |
|----------------------------------------|-------------------------------------|------------------------------|------------------|---------------------|----------------------|---------------|--------------|
| *Isohypsidius sculptus* (Ramazzotti, 1962) | Cordillera de Nahuelbuta, Chile     | ?                            | 1100             | lichen from tree trunk | F. di C. Liviero     | +            | –            |
| *Isohypsidius wilsoni* (Horning et al., 1978) | Taumaka, Open Bay Islands           | 43°51’44”N, 166°52’56”E     | 5–20             | lichen from rock    | D.S. Horning        | +            | –            |
| *Ursulinius dudichi* (Iharos, 1964) comb. nov. | Dauphiné Alps, Ailefroide, France  | 44°53’38”N, 6°19’38”E       | 2500             | moss from rock      | D. Frydryszak       | –            | –            |
| *Ursulinius duranteae* (Maucci, 1978) comb. nov. | Sakçagözü, Turkey                  | ca. 37°10’N, 36°56’E       | ?                | moss on soil        | W. Maucci           | +            | –            |
| *Ursulinius elegans* (Binda & Pilato, 1971) comb. nov. | Catania, Sicily, Italy             | ca. 37°30’N, 15°05’E       | ?                | moss on soil        | M.G. Binda, G. Pilato | +            | –            |
| *Ursulinius lunulatus* (Iharos, 1966) comb. nov. | Chybie, Poland                      | 49°55’36”N, 18°55’34”E     | 260              | moss from concrete wall | A. Witwicka        | –            | +            |
| *Ursulinius pappi* (Iharos, 1966) comb. nov. | Chybie, Poland                      | 49°55’36”N, 18°55’34”E     | 260              | moss from concrete wall | A. Witwicka        | –            | +            |
| *Ursulinius pilatoi* (Durante Pasa & Maucci, 1979) comb. nov. | Kvalsund, Norway                   | ca. 70°30’N, 23°58’E       | ?                | moss from rock      | M.V. Durante Pasa, W. Maucci | +            | –            |
| *Ursulinius ronsisvallei* (Binda & Pilato, 1969) comb. nov. | Dilijan, Armenia                    | ca. 40°44’N, 44°51’E       | ?                | moss on soil        | V.I. Biserov        | –            | –            |
| *Ursulinius silvicola* (Iharos, 1966) comb. nov. | Lago di Doberdò, Italy             | 45°49’60”N, 13°33’10”E     | 3                | moss from tree trunk | P. Gašiorek         | –            | –            |
| *Incertae sedis*                        |                                     |                              |                  |                     |                      |              |              |
| *Ramajendadas dastychi* Kaczmarek et al., 2014 | Antarctic Peninsula, Wilhelm Archipelago | 65°09’56”S, 64°08’47”W | 9                | lichen from rock    | K. Janko            | +            | –            |
| *Ramajendadas renaudi* (Ramazzotti, 1972) | Kerguelen Islands                  | ca. 49°20’S, 70°20’E       | ?                | sediment            | J. Renaud-Mornant   | +            | –            |

*See details in Wiktor et al. (2016).
primary claw branches, we introduce the *br ratio*, i.e., ratio of the height of the secondary claw branch to the height of the primary claw branch (the more the branches are similar in height, the closer to 1.0 the *br* ratio is and the shorter the secondary branch relative to the primary branch, the lower the *br* ratio).

During the review process of this manuscript, the tardigrade phylogeny by Guil et al. (2019) was published. Consequently, we adjusted the taxonomy of the high ranks in our work to the system proposed in Guil et al. (2019), however, in our opinion the new taxonomy is controversial and requires further work: possibly rank shift and taxon membership rearrangements, as the majority of high rank taxa (orders, families) remained in polytomies, compared to the relatively well-resolved relationships from the previous phylogeny by Bertolani et al. (2014). Given that superfamily Isohypsibioidea was elevated to the order level by Guil et al. (2019), according to the article 36.1 of International Code of Zoological Nomenclature (1999), the authorship of the superfamily (Sands et al., 2008) is now superseded by the latest authority.

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**Figure 1** Schematic presentation of the oral cavity armature (OCA) in Isohypsibioidea, the first and/or the second band of teeth are marked by Roman numerals: A – a continuous peribuccal lamina, two bands of teeth (*Apodibius, Grevenius* gen. nov., *Halobiotus, Hexapodibius*); B – a continuous peribuccal lamina, the first band of teeth (*Fractonotus, Isohypsibus, Ursulinus* gen. nov.); C – six convex peribuccal papulae, the first band of teeth (*Eremobiotus*); D – rectangular peribuccal lamellae, the first band of teeth with lateral toothless intervals (*Pseudobiotus*); E – rectangular peribuccal lamellae, two bands of teeth (*Thulinius*); F – six large peribuccal lamellae, two bands of teeth (*Haplomacrobiotus*). Both bands of teeth contain variable number of rows, depending on the genus.
Genotyping
DNA was extracted from individual animals using Chelex® 100 resin (Casquet et al., 2012; Stec et al., 2015). Paragenophores of all sequenced species were mounted on permanent slides and are deposited in the collection of Institute of Zoology and Biomedical Research (Pleijel et al., 2008). We sequenced two DNA fragments: a small ribosome subunit (18S rRNA) and a large ribosome subunit (28S rRNA). All fragments were amplified and sequenced according to the protocols described by Stec et al. (2015), using the primers and specific PCR programmes from: Sands et al. (2008) and Zeller (2010) (18S rRNA) or Mironov et al. (2012) (28S rRNA). 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 7.2.5 of BioEdit (Hall 1999).

Phylogenetics
We aligned all available isohypsibioid and hypsibioid (outgroup taxa) 18S + 28S rRNA sequences from GenBank together with our new sequences (see table 2) using the Q-INS-I strategy, which considers the secondary structure of RNA, in MAFFT version 7 (Katoh et al., 2002; Katoh & Toh, 2008). Currently available partial 28S rRNA sequences for Halobiotus crispa Kristensen, 1982, Hexapodibus micrornyx Pilato, 1969 and Pseudobiotus megalonyx (Thulin, 1928) represent a different region of this marker than the ones sequenced by us, thus they were not included in the dataset. The aligned fragments were edited and checked manually in BioEdit. The best substitution model and partitioning scheme for posterior phylogenetic analysis was chosen under the Akaike Information Criterion (AIC), using PartitionFinder version 2.1.1 (Lanfear et al., 2016). As best-fit partitioning scheme, PartitionFinder suggested to retain two predefined partitions separately and for each of them the best fit model 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 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. The ESS values were greater than 200 and consensus tree was obtained after summarising 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 v1.4.3 available from http://tree.bio.ed.ac.uk/software/figtree. The sequence HQ604951, representing E. alicatai (Binda, 1969), was characterised by highly unstable position in the trees calculated in both methods, and it never clustered with newly sequenced Eremobiotus sp. nov., suggesting it could be a misidentification or a mislabelling. Therefore, we excluded this taxon from the final dataset. Additionally, the aligned fragments were trimmed to the size of the shortest available alignment (i.e., 745 bp for...
### Table 2

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

| Taxon                             | 18S rRNA     | 28S rRNA     | Reference                        |
|-----------------------------------|--------------|--------------|----------------------------------|
| **Isohypsibioida**                |              |              |                                  |
| *Apodibius confusus* Dastych, 1983| KC582830     | KC582834     | Dabert et al. (2014)             |
| *Fractonotus verrucosus* (Richters, 1900) | MG800855 | MG800856     | Gąsiorek et al. (2019)           |
| *Dianea papillifera* (Murray, 1905) | EU266925     | –            | Sands et al. (2008)              |
| *Dianea satleri* (Richters, 1902) | MK675926,    | MK675915,    | present study                    |
|                                  | MK675927     | MK675916     |                                  |
| *Doryphoribius flavus* (Iharos, 1966) | HQ604940     | –            | Bertolani et al. (2014a)         |
| *Doryphoribius macrodon* Binda et al., 1980 | HQ604942 | –            | Bertolani et al. (2014a)         |
| *Eremobiotus* sp. nov.            | MK675928     | MK675917     | present study                    |
| *Grevenius granulifer* (Thulin, 1928) | EF620403,    | –            | Møbjerg et al. (2007), Cesari et al. (2016) |
|                                  | KT778603     |              |                                  |
| *Grevenius pushkini* (Tumanov, 2003) | MK675929     | MK675918     | present study                    |
| *Halobiotus crispe* Kristensen, 1982 | AY582121,    | –            | Jørgensen & Kristensen (2004), Møbjerg et al. (2007) |
|                                  | EF620402     |              |                                  |
| *Haplomacrobiotus utahensis* Pilato & Beasley, 2005 | KT778600 | –            | Cesari et al. (2016)             |
| *Hexapodibius micronyx* Pilato, 1969 | MK675930,    | MK675919     | present study,                   |
|                                  | MK675920,    |              | Bertolani et al. (2014a)         |
|                                  | MK675931     |              |                                  |
| *Isohypsibius arbiter* Binda, 1980 | HQ604915     | –            | Cesari et al. (2016)             |
| *Isohypsibius cambrensis* (Morgan, 1976) | AM500652     | –            | Kiehl et al. (2007)              |
| *Isohypsibius dastychi* Pilato et al., 1982 | HQ604954 | –            | Bertolani et al. (2014a)         |
| *Isohypsibius prosostomus* Thulin, 1928 | EF620404    | –            | Møbjerg et al. (2007)            |
| *Pseudobiotus kathmanaee* Nelson et al., 1999 | HQ604957 | –            | Bertolani et al. (2014a)         |
| *Pseudobiotus megalonyx* (Thulin, 1928) | MK675931,    | MK675920     | present study,                   |
|                                  | MK675935     |              | Bertolani et al. (2014a)         |
|                                  | MK675924     |              |                                  |
| *Thulinius augusti* (Murray, 1907) | KF360230     | –            | Bertolani et al. (2014b)         |
| *Thulinius raffoi* (Bertolani, 1982) | MK675932     | MK675921     | present study                    |
| *Thulinius stephaniae* (Pilato, 1974) | GQ925701     | –            | unpublished                      |
| *Ursulinus lunulatus* (Iharos, 1966) | MK675933     | MK675922     | present study                    |
| *Ursulinus pappii* (Iharos, 1966) | MK675934     | MK675923     | present study                    |
| *Ursulinus silvicola* (Iharos, 1966) | MK675935     | MK675924     | present study                    |
| **Hypsibioida** (outgroup)        |              |              |                                  |
| *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,    | –            | Guil & Giribet (2012), Bertolani et al. (2014a) |
|                                  | HQ604922     |              |                                  |
| *Borealibius zetlandicus* (Murray, 1907) | HQ604924     | –            | Bertolani et al. (2014a)         |
18S rRNA and 756 bp for 28S rRNA), and uncorrected pairwise distances were calculated using MEGA7 (Kumar et al., 2016).

**Results and discussion**

**Molecular phylogeny of Isohypsibioida**

Isohypsibioida were highly supported in our phylogenetic analysis (fig. 2). Isohypsibioida were divided into two major, well-supported clades: clade I (Isohypsibiidae s.s.) embraced exclusively terrestrial, bryophilous or lichenophilous taxa, whereas clade II comprised taxa that inhabit various environments, both aquatic (seas, ponds and rivers) and terrestrial (soil and bryophytes in case of some Doryphoribius Pilato, 1969 spp.).

Clade I embraced Isohypsibiidae s.s. and was further divided into three subclades:

**Table 2**

| Taxon                                      | 18S rRNA | 28S rRNA | Reference                      |
|--------------------------------------------|----------|----------|--------------------------------|
| *Calohypsibius ornatus* (Richters, 1900)   | MH279652 | MK680130 | Gąsiorek et al. (2019),         |
|                                            | HQ604914 |          | Bertolani et al. (2014a)       |
| *Diphascon higginsi* Binda, 1971           | HQ604932 |          | Bertolani et al. (2014a)       |
| *Diphascon pingue* (Marcus, 1936)         | FJ435736, FJ435778 |          | Guil & Giribet (2012),         |
|                                            | HQ604937 |          | Bertolani et al. (2014a)       |
| *Diphascon puniceum* (Jennings, 1976)     | EU266949 |          | Bertolani et al. (2014a)       |
| *Hebesuncus conjungens* (Thulin, 1911)    | AM500646 |          | Kiehl et al. (2007)            |
| *Hebesuncus ryani* Dastych & Harris, 1994 | EU266956 |          | Sands et al. (2008)            |
| *Hypsibius convergens* (Urbanowicz, 1925) | FJ435726, FJ43577 |          | Guil & Giribet (2012)          |
| *Hypsibius exemplaris* Gąsiorek et al., 2018 | MG800327, MG800337 |          | Gąsiorek et al. (2018)         |
| *Hypsibius dujardini* (Doyère, 1840)      | MG777532 | MG777533 | Gąsiorek et al. (2018)         |
| *Hypsibius klebelsbergi* Mihelčič, 1959 | KT901827, KC582835 |          | Dabert et al. (2014, 2015)     |
| *Hypsibius pallidus* Thulin, 1911         | HQ604945 |          | Bertolani et al. (2014a)       |
| *Hypsibius scabropygus* Cuénot, 1929      | AM500649 |          | Dabert et al. (2014)           |
| *Mesocrisata revelata* Gąsiorek et al., 2016 | KU528627, KU528628 |          | Gąsiorek et al. (2016)         |
| *Mesocrista spitzbergensis* (Richters, 1903) | KX347532, KX347533 |          | Gąsiorek et al. (2016)         |
| *Microhypsibius bertolani* 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)       |
| *Pilatobius recamieri* (Richters, 1911)   | KX347526 |          | Gąsiorek et al. (2017)         |
| *Platicrista angustata* (Murray, 1905)    | HQ604948 |          | Bertolani et al. (2014a)       |
| *Ramazzottius oberhaeuseri* (Doyère, 1840) | MG573241, MG573242 |          | Stec et al. (2018),            |
| *Ramazzottius aff. oberhaeuseri*          | AY582122 |          | Jørgensen & Kristensen (2004)   |
| *Ramazzottius subanomalus* (Biserov, 1985) | MF001997, MF001998 |          | Stec et al. (2017)             |
| *Ramazzottius varieornatus* Bertolani & Kinchin, 1993 | HQ604950, MG432818 |          | Bertolani et al. (2014a),      |
|                                            |          |          | Zawierucha et al. (2018)       |
with unresolved phylogenetic relationships: *Isohypsibius* s.s. + *Fractonotus* Pilato, 1998; *Dianea* gen. nov. (including the species hitherto known as *Isohypsibius sattleri* (Richters, 1902) and related spp.); and *Ursulinius* gen. nov. (including the species hitherto known as *Isohypsibius pappi* (Iharos, 1966) and related spp.) + *Isohypsibius dastychi* Pilato et al., 1982 + *Eremobiotus* Biserov, 1992. Clade II also comprised three distinct phyletic lineages in paraphyletic relationships: Halobioidae fam. nov. (marine), Hexapodibiidae (soil-dwelling), and Doryphoribiidae fam. nov. (both freshwater and terrestrial/bryophilous). The latter

**Figure 2** The phylogeny of Isohypsibioida Sands et al., 2008 based on concatenated 18S rRNA and 28S rRNA sequences. New families and genera are marked in bold. Values above branches indicate Bayesian posterior probability values (BI), whereas those under branches show bootstrap values (ML). Branches with support below 0.9 in BI (70% in ML) were collapsed. Scale bar and branch lengths refer to the Bayesian analysis.
family consisted of four subclades (with not fully resolved affinities) that embraced five genera: polyphyletic Doryphoribius and Thu-linius Bertolani, 2003, as well as monophyletic Pseudobiotus Nelson, 1980 (in Schuster et al., 1980), Apodibius Dastych, 1983, and Grevenius gen. nov. (the species hitherto known as Isohypsibius granulifer Thulin, 1928 and kin).

**Head morphology and peribuccal structures in Isohypsibioidea**

The head in all Isohypsibioidea is terminated bluntly, with anterioventral mouth opening (figs. 3–4). The frontal part of the head is smooth or equipped with either of two types of structures: frontal lobes or cephalic papillae (Pilato & Binda, 2010). Cephalic...
FIGURE 4  Cephalic region of various members of Isohypsibioidea (SEM): A – Isohypsibius prosostomus Thulin, 1928 (Isohypsibiidae); B – Ursulinus pappi comb. nov. (Isohypsibiidae); C – Halobiotus crispae Kristensen, 1982 (Halobioidae fam. nov.); D – Doryphoribius dawkinsi Michalczyk & Kaczmarek, 2010 (Doryphoribiidae fam. nov.); E – Apodibius confusus Dastyh, 1983 (Doryphoribiidae fam. nov.); F – Pseudobiotus megalonyx (Thulin, 1928) (Doryphoribiidae fam. nov.); G – Grevenius granulifer comb. nov. (Doryphoribiidae fam. nov.); H – Hexapodibius micronym Pilato, 1969 (Hexapodibiidae). Asterisks indicate frontal lobes or cephalic papillae. Scale bars = 10 μm.
papillae, present only in Halobiota fam. nov. (fig. 4C; Møbjerg et al., 2007), are less clearly delimited from the surrounding cuticle than lobes. Frontal lobes, on the other hand, are present in some remaining Isohypsibioids, although they can vary in size and shape, for example they are smaller and slightly more roundish in Ursulinius pappi (fig. 4B) than in Apodibius confusus Dastych, 1983 (fig. 4E). Paradiphascon Dastych, 1992 has large, dome-shaped frontal lobes (Dastych, 1992). Given that Halobiota fam. nov., in contrast to all remaining Isohypsibioida, secondarily adapted to marine environment, cephalic papillae are most likely a halobiid autapomorphy. If, as hypothesised by Dastych (1992), frontal lobes are homologous remnants of heterotardigrade cephalic papillae, they should be considered an isohypsibioid plesiomorphy. Moreover, in some genera, additional regular circular cuticular wrinkles can be present around the mouth opening (Apodibius and Hexapodibius Pilato, 1969; figs. 4E, H, 5F, J). Mouth ring is smooth (figs. 5A–B, H), or with six either weakly developed or clearly separated peribuccal lobes (figs. 5E, G and figs. 5C–D, F, I–J, respectively). Peribuccal chemosensory organs are present exclusively in Halobiotus crispae (fig. 5D), but our SEM observations showed delicate lobe depressions in Apodibius, Thulinius, and Hexapodibius, which may indicate chemoperception ability in these genera (figs. 5F–G, J). The mouth opening in the majority of genera (Apodibius, Doryphoribius, Fractonotus, Grevenius gen. nov., Halobiotus, Hexapodibius, Isohypsibi, Ursulinius gen. nov.) is surrounded by a continuous peribuccal lamina (figs. 1A–B, 5A–F, I–J). However, in three genera peribuccal lamellae, often fused at their bases or on their entire height, are present (figs. 1D–F, 5G–H; see also Schuster et al., 1980 for the complete fusion of lamellae into a continuous lamina in Thulinius salturus (Schuster et al., 1978)). The number of peribuccal lamellae is considered a generic trait (12 in Thulinius, 30 in Pseudobiotus and an undetermined number in Paradiphascon; Schuster et al., 1980; Bertolani, 1982; Nelson et al., 1999; Dastych, 1992). Finally, in Eremobiotus, and likely in Dastychius Pilato, 2013, six peribuccal papulae are present (Biserov, 1992; Pilato, 2013). The continuous peribuccal lamina is definitely the most widespread morphotype, and likely the ancestral one, which independently evolved into divided or semi-divided peribuccal lamellae in two doryphoribiid genera. Nonetheless, our SEM observations question the validity of peribuccal lamellae as the main trait distinguishing Thulinius and Pseudobiotus (figs. 5G–H), since these structures have variable morphology.

**Oral cavity armature in Isohypsibioida and other Eutardigrada**

In the great majority of isohypsibioid species, OCA is visible only under SEM and all our observations are based on this technique. In all analysed taxa, the oral cavity was equipped with at least one band of conical teeth located on the ring fold, in the central part of the oral cavity (fig. 1). However, in the majority of isohypsibioid genera a second band of teeth was also detected (Apodibius, Grevenius gen. nov., Halobiotus, Hexapodibius, Thulinius; the second band could be present also in Pseudobiotus, see below for details). There are no SEM observations of the oral cavity for Dastychius. The OCA system in Paradiphascon is obscure (Dastych, 1992) and the number or exact shape of peribuccal lamellae are unknown, thus the genus is not included in the present schematic depiction. The first band of teeth, comprising 2–5 rows of teeth, consists either of small and sparse conical teeth in Fractonotus and Halobiotus (figs. 5C–D), medium-sized and more densely arranged conical teeth in Isohypsibi, Eremobiotus, Apodibius, Thulinius, Grevenius gen. nov., Hexapodibius (figs. 5A, F–G, I–J, 13B–C, 14B), or large conical teeth divided into a dorsal and a ventral
FIGURE 5  Peribuccal structures of various members of Isohypsibioidea (SEM): A – Isohypsibius coulsoni Kaczmarek et al., 2012 (Isohypsibiidae); B – Ursulinius pappi comb. nov. (Isohypsibiidae); C – Fractonotus verrucosus (Isohypsibiidae); D – Halobiotus crispae (Halobiidae fam. nov.); E – Doryphoribius dawkinsi (Doryphoribiidae fam. nov.); F – Apodibius confusus (Doryphoribiidae fam. nov.); G – Thulinius ruffoi (Bertolani, 1981) (Doryphoribiidae fam. nov.); H – Pseudobiotus megalonyx (Doryphoribiidae fam. nov.); I – Grevenius granulifer comb. nov. (Doryphoribiidae fam. nov.); J – Hexapodibius micronyx (Hexapodibiidae). Incised arrowheads indicate the first row of teeth, empty incised arrowheads – the second row of teeth, arrowheads – fused peribuccal lamellae, empty arrowheads – peribuccal wrinkles, and the asterisk points the peribuccal chemosensory organ. Scale bars = 1 μm.
row in *Pseudobiotus* (figs. 1D, 5H; Nelson et al., 1999). The second band of teeth, composed of 1–4 rows of teeth, comprises conical teeth that are typically larger than those in the first band, and are located immediately behind the first band and before the buccal tube opening (figs. 5D, F–G, I, 13B–C, 14B). In all examined species, the second band was continuous, except for *Hexapodibius micronyx*, in which the band was divided into a short dorsal and ventral row of irregular teeth (fig. 5J).

In a wider context, so far, greatest attention was paid to OCA in Macrobiotoidea Guil et al., 2019 (Thulin, 1911; Pilato, 1975; Michalczyk & Kaczmarek, 2003; Guidetti et al., 2012) and Eohypsibioidea Guil et al., 2019 (e.g., Hansen et al., 2017), but very little is known about OCA in Hypsibioidea (Gąsiorek et al., 2016, 2018; Stec et al., 2017, 2018) and the topic has not been addressed systematically in Isohypsibioida (OCA was only mentioned occasionally in several species, e.g., in Pilato, 1975; Biserov, 1992; Jørgensen, 2001; Lisi, 2011). The lack of data for Hypsibioidea prevents the formulation of sound hypotheses about the evolution of OCA both within isohypsibioids and in all eutardigrades.

Nevertheless, OCA in isohypsibioids seems to have supra-generic significance. Our observations showed that Isohypsibiidae have only one band of teeth, whereas the three other families exhibit two bands (fig. 1). The only exception in Doryphoribiidae fam. nov.–*Pseudobiotus* with only the first band of teeth visible in the oral cavity – has to be treated with caution as the first band of teeth in this genus is very large and it obscures the view of the posterior part of the oral cavity, therefore it is not possible to say whether the second band of teeth is lacking or it is simply not visible when looking through the mouth opening. However, based on phylogeny, we hypothesise that *Pseudobiotus* exhibits two bands of teeth. Given that the two sister clades constituting Isohypsibiodea (i.e., Isohypsibiidae vs Halobiotidae fam. nov. + Hexapobibiidae + Doryphoribiidae fam. nov.) exhibit one vs two bands of teeth in the oral cavity, it is not possible to state whether the last common ancestor for all isohypsibioids had one or two bands of teeth.

The differences in the location of teeth in the OCA between Isohypsibiodea and both Eohypsibiodea and Macrobiotoidea may suggest that bands of teeth in Isohypsibiodea and in the two latter orders are not homologous. OCA in Eohypsibiodea and Macrobiotoidea consists of three bands of teeth: first (minute cones located in the very anterior of the oral cavity), second (larger cones or ridges parallel to the main axis of the buccal apparatus, located in the rear of the oral cavity just behind the ring fold), and third (a system of ventral and dorsal transverse crest/ridge-shaped teeth, located in the rear of the oral cavity just behind the second band of teeth and before the buccal tube opening in Macrobiontidae Thulin, 1928, Murrayiidae Guidetti et al., 2000 and some Richtersiidae Guidetti et al., 2016 or a band of conical teeth in some Richtersiidae). Thus, Isohypsibiodea do not exhibit the most anterior band of teeth, termed as the first (or anterior) band, that is present in both Eohypsibiodea and Macrobiotoidea in the very anterior of the oral cavity. Moreover, except for *Richtersius* Pilato & Binda, 1989 (which exhibits a highly modified OCA), neither in Eohypsibiodea nor in Macrobiotoidea were the teeth observed on the ring fold. The only congruence in the location of teeth in the oral cavity concerns the most posterior teeth: in Eohypsibiodea and Macrobiotoidea the third band of teeth is located immediately behind the ring fold, i.e., in the same place as the second band of teeth in Isohypsibiodea. Nevertheless, as already mentioned above, the current state of knowledge on the OCA in eutardigrades does not allow to conclude whether the third band in eohypsiids and macrobiotids is homologous with the second band in isohypsibiids.
Interestingly, regardless of phylogenetic relationships and location of teeth in the oral cavity, larger teeth (e.g., in the first band in *Pseudobiotus* and in the third band in *Eohypsibiidae* Bertolani & Kristensen, 1987 and *Macrobioiidae*) tend to be arranged in two rows, ventral and dorsal. We hypothesise that lateral toothless intervals are necessary to allow stylet extrusion through the oral cavity and mouth opening (stylets are positioned laterally, parallel to the buccal tube, and they are extruded in a scissor-like manner; Guidetti et al., 2013).

**Buccal apparatus morphology in the Doryphoribiidae fam. nov. + Hexapodibiidae clade**

Two evolutionary pathways can be recognised in the anatomy of the buccal apparatus in Doryphoribiidae fam. nov.: buccal tube without ventral lamina and with unmodified AISM (*Grevenius* gen. nov., *Pseudobiotus*, *Thulinius*), and the other with the buccal tube enforced with ventral lamina, which is associated with modifications of AISM (*Apodibius*, *Doryphoribius*) (Pilato & Binda, 2010). The buccal apparatus of *Grevenius pushkini* (Tumanov, 2003) comb. nov. and other aquatic "*Isohypsibius*" spp. is generally more similar to that in *Thulinius* spp. than to terrestrial *Isohypsibius* spp. (figs. 13, 14), which is not surprising given the close phylogenetic relationship between the two taxa (fig. 2) and same, aquatic, habitat. Specifically, aquatic "*Isohypsibius*" spp. and *Thulinius* spp. have two rows of teeth in the oral cavity (figs. 13B–C, 14B) and narrow apophyses for the insertion of the stylet muscles (AISM) (figs. 13D, 14C) whereas terrestrial *Isohypsibius* spp. exhibit one row of buccal teeth (fig. 5A) and proportionally broader AISM. The anatomy of buccal apparatus in Hexapodibiidae is more conserved, since only the morphotype with ventral lamina exists (Cesari et al., 2016). Buccal apparatus of *Hexapodibius*, similarly to that of *Haplomacrobiotus*, has reduced AISM due to the developed ventral lamina (figs. 15A–C, 16D). Isohypsibioioid taxa equipped with ventral lamina, i.e., Hexapodibiidae and some Doryphoribiidae fam. nov.: *Doryphoribius* and *Apodibius*, share extreme resemblance of the buccal apparatus anatomy. For example, they all exhibit unmodified *Hypsibius* type furcae (figs. 15D, 16) and two or three short, often almost granular macroplacoids in the pharynx (figs. 15E, 16; Hohberg & Lang, 2016). This is in contrast to taxa without the ventral lamina, *Thulinius*, *Pseudobiotus*, and *Grevenius* gen. nov., which all have elongated macroplacoids. Interestingly, ventral lamina is present in terrestrial but not in freshwater representatives of the Halo-biotidae fam. nov. + Hexapodibiidae + Doryphoribiidae fam. nov. clade (single exceptions can be found in polyphyletic *Doryphoribius*). Ventral lamina in both *Doryphoribius* and Hexapodibiidae has two different morphotypes: a short, delicate lamina reaching no farther than to the half of the buccal tube length in *Apodibius*, *Hexapodibius*, or some *Parhexapodibius* Pilato, 1969, and *Doryphoribius* (figs. 15A–C, 16A, C–D, F); or a long, robust lamina reaching almost the level of the stylet support insertion point in some *Doryphoribius* and *Parhexapodibius* (figs. 16B, E). The presence of ventral lamina in all hexapodibiids but only in some doryphoribiids suggests that either the common ancestor of Hexapodibiidae + Doryphoribiidae fam. nov. exhibited the lamina, which was later independently lost in *Thulinius*, *Pseudobiotus*, and *Grevenius* gen. nov., or lamina evolved independently two or three times: in (1) Hexapodibiidae, (2) ancestor of *Apodibius* and some *Doryphoribius* spp., and (3) in remaining *Doryphoribius* spp. (see fig. 2). The lack of ventral lamina in *Heterotardigrada* Marcus, 1927, *Apotardigra*, Hypsibioidea and many Isohypsibioidea suggests that it is a derived trait that evolved independently in Isohypsibioidea and in the ancestor of Macrobioidea. In other words,
the presence of the ventral lamina should be treated as an example of parallel evolution within Eutardigrada, being at the same time the autapomorphy of Macrobiotoidea as well as of Hexapodibiidae and some genera of Doryphoribiidae fam. nov. (Marley et al., 2011).

**Cuticle morphology in Isohypsibioida**

In contrast to the majority of eutardigrades, isohypsibioids frequently exhibit distinct cuticular sculpturing (figs. 3, 6). Five major kinds of sculpturing can be distinguished within the order: (I) reticulum, (II) circular tubercles of various size, (III) pointy gibbosities, (IV) round gibbosities, and (V) plaques. The most unique type of cuticle morphology characterises *Frac-tonotus*, which has symmetrically arranged dorsal plaques (figs. 3A, 6B) as well as densely arranged smooth tubercles that cover the entire dorsum and limbs (figs. 3A, 6A; Gąsiorek et al., 2019). Isohypsibioid gibbosities can be generally divided into two types: small, weakly demarcated (almost flat in LM) and pointy gibbosities present in *Dianea* gen. nov. (figs. 3B, 6C), or large, mamillose and round gibbosities with developed reticulum or complex ornamentation in *Ursulinus* gen. nov. and many *Doryphoribius* spp. (figs. 3C, 6D–F; Ramazzotti & Maucci, 1983). Gibbosities of *Dianea* gen. nov. are less regular and clearly narrow towards the apex in contrast to hemispherically convex gibbosities in the two latter genera. The usage of dorsal gibbosities as a generic trait was a subject of criticism (Pilato, 1982), as, according to some descriptions, in one species there could be a considerable variation in gibbosity development (e.g., Binda & Pilato, 1971). The same variability was ascribed to cuticular sculpturing in general (Kristensen & Hallas, 1986). However, recent data show that in a single sample, numerous, potentially closely related or pseudocryptic species can be found (e.g., see Faurby et al., 2011; Morek et al., 2019). Therefore, the reports of such profound variability in the development of gibbosities given without genetic data should be taken with caution. In species devoid of *Frac-tonotus* type tubercles or gibbosities, e.g., in *Grevenius* gen. nov., *Thulinius* or *Pseudobiotus* (figs. 3D, 6G), quite often the entire dorsal cuticle is covered with homogenous, rough sculpturing that forms wrinkly epicuticular reticulum or processes (fig. 6H; Bertolani, 1982; Chang et al., 2007; Pilato et al., 2010; Bertolani et al., 2014b). The richness and variability of cuticular sculpturing within Isohypsibioida indicate independent, autapomorphic origin and prevent hypothesising whether the ancestral cuticle state was smooth or sculptured.

**Claw morphology in Isohypsibioida**

Isohypsibioid claws can be divided into six general morphotypes: (I) *Isohypsibius* type, as defined by Ramazzotti & Maucci (1983), the most widespread morphotype, with external and internal claws on the same limb of similar size and with branches forking at a ca. right (90°) angle, (figs. 7A–D, H, K, 8, 9B, 10); (II) *Eremobiotus* type, with all claws with branches forming an obtuse, approaching a straight (ca. 180°) angle, and external and internal claws on the same limb of similar size, but dissimilar branch heights (br < 70%), which is a highly modified *Isohypsibius* type (figs. 7E–F, 9C–D; Biserov, 1992); (III) *Frac-tonotus* type, with all claws with branches forming an obtuse, approaching a straight (ca. 180°) angle, and external and internal claws on the same limb of similar size, but dissimilar branch heights (br < 70%), which could be seen as an intermediate morphotype between the *Isohypsibius* and the *Hypsibius* type claw (figs. 7G, 9A; Gąsiorek et al., 2019); (IV) strongly reduced hexapodibiid claws, with indistinct basal tracts and partly or completely reduced secondary branches (fig. 7Q; Cesari et al., 2016); (V) *Paradiphascon* type, with internal and anterior claws of the *Isohypsibius* type and external and posterior claws of the *Hypsibius* type with very broad bases (Dastych, 1992); (VI) *Pseudobiotus* type
FIGURE 6  Cuticular surface of various members of Isohypsibioidae (SEM): A–B – *Fractonotus verrucosus* (Isohypsibiidae), obtuse tubercles and plaques; C – *Dianea sattleri* comb. nov. (Isohypsibiidae), small wrinkled gibbosities; D – *Ursulinus pappi* comb. nov. (Isohypsibiidae), large reticulated gibbosities; E – *Ursulinus elegans* (Binda & Pilato, 1971) comb. nov. (Isohypsibiidae), large ornamented gibbosities; F – *Doryphoribius dawkinsi* (Doryphoribiidae fam. nov.), large sculptured gibbosities; G – *Grevenius granulifer* comb. nov. (Doryphoribiidae fam. nov.), irregular small tubercles; H – *Grevenius pushkini* (Tumanov, 2003) comb. nov. (Doryphoribiidae fam. nov.), cuticular wrinkles. Scale bars in micrometres.
with secondary and primary branches similar in height (br typically >70%, see table 2), elongated basal tracts, and typically prominent humps on primary branches of internal and anterior claws (figs. 7I–J, L–P, 11). The **Pseudobiotus** type claws are common for the genera **Pseudobiotus**, **Halobiotus**, **Thulinius** and **Grevenius gen. nov.** The peculiar morphology of OCA (see above), together with anatomical modifications related to copulation and parental care in **Pseudobiotus** (hook-like claws on the first pair of legs in males (fig. 7N) and reduced hind claws in females who carry shed exuviae with eggs), seem to be more suitable taxonomic criteria to differentiate **Pseudobiotus** and **Thulinius** rather than the number of peribuccal lamellae, as **Thulinius** is parthenogenetic (or at least does not exhibit sexual dimorphism, however thelytoky was confirmed in *T. augusti* (Murray, 1907) – see Bertolani, 1976, and *T. ruffoi* (Bertolani, 1981) – see Kosztyla et al., 2016) and lacks parental care and associated morphological modifications (Rebecchi & Nelson, 1998).

Morphotypes II–VI are internally homogenous, however **Isohypsibius** morphotype can be further divided into three distinct subgroups: (Ia) *I. prosostomus* type, with secondary branches clearly shorter than primary branches (br ranges from around 40% to 70%, see table 3), claw bases without pseudolunulae, and with single bars under claws (figs. 7A–B, 8A–D); (Ib); *I. dastychi* type, with branches forking at a wide, approaching a 180° angle, present also in *Eremobiotus* (fig. 8E). Interestingly, the topology of the tree indicates the affinity of these two groups as *I. dastychi* and *Eremobiotus* sp. nov. are in a single polytomous clade (that includes also *Ursulinius* gen. nov.). The second morphotype is currently represented only by a single species, **Isohypsibiuss chiarae** Maucci, 1987. Secondary branches in this species are reduced, being short and acute (fig. 8F). Taking into consideration that in the present study, morphological peculiarities of a similar magnitude induced the erections of three new genera, including one representing a different family (**Grevenius gen. nov.**, in Doryphoribiidae fam. nov.), it should be noted that *I. chiarae* does not belong to **Isohypsibius** s.s.
Claw types of various members of Isohypsibioida (SEM): A – *Isohypsibius prosostomus* (Isohypsibiidae); B – *Isohypsibius coulsoni* (Isohypsibiidae); C – *Ursulinius pappi* comb. nov. (Isohypsibiidae), claws I–III; D – *Ursulinius pappi* comb. nov. (Isohypsibiidae), modified claws IV (arrowheads indicate evident pseudolunulae); E – *Eremobiotus* sp. nov. (Isohypsibiidae), external side of claws I–III (incised arrowheads indicate longitudinal internal bar, empty incised arrowheads – the furbelow structure covered with minute granulation, the empty arrowhead – pedal gibbosity); F – *Eremobiotus* sp. nov. (Isohypsibiidae), internal side of claws I–III; G – *Fractonotus verrucosus* (Isohypsibiidae); H – *Dianeasattleri* comb. nov. (Isohypsibiidae); (Cont. on next page)
Systematic position of *Eremobiotus Biserov, 1992*

Both morphological and genetic data undoubtedly show that *Eremobiotus* is a member of Isohypsibiidae (fig. 2). The morphological uniformity of the clade *Eremobiotus* + *Ursulinius* gen. nov. + the *I. dastychi* group (uniting *Isohypsibius* spp. with two macroplacoids and smooth cuticle) is expressed by several traits: two macroplacoids in the pharynx, typically well-developed pseudolunulae (figs. 8E, 10), and evident double bars in *Eremobiotus* and *Ursulinius* gen. nov. (fig. 10) (see also Lisi et al., 2016). However, the monophyly of the genus should be treated with caution since claws in *E. ovezovae* Biserov, 1992, unlike claws of the remaining two described *Eremobiotus* spp., are significantly reduced (compare

**Figure 7 (Cont.)**

I – *Halobiotus arcturulius* Crisp & Kristensen, 1983 (Halobiotidae fam. nov.); J – *Halobiotus crispae* (Halobiotidae fam. nov.); K – *Doryphoribius dawkinsi* (Doryphoribiidae fam. nov.); L – *Thulinius ruffoi* (Doryphoribiidae fam. nov.); M – *Pseudobiotus megalonyx* (Doryphoribiidae fam. nov.); N – *Pseudobiotus megalonyx* (Doryphoribiidae fam. nov.), modified male claws I; O – *Grevenius granulifer* comb. nov. (Doryphoribiidae fam. nov.); P – *Grevenius pushkini* comb. nov. (Doryphoribiidae fam. nov.); Q – *Hexapodibius micronyx* (Hexapodibiidae), reduced claws with undeveloped bases. Scale bars in micrometres.
Fig. 9C–D). Thus, the possibility that *E. ovezo-vae* represents an independent evolutionary line that has convergently evolved claws with widely angled branches must be considered. In fact, it would not be surprising if this claw morphotype evolved more than once in Isohypsibiidae, especially that, for example, claw reduction has been shown to evolve independently in several eutardigrade lineages (Bertolani & Biserov, 1996).

**Taxonomy of Doryphoribiidae fam. nov.**

**Polyphyly of Doryphoribius Pilato, 1969**

In the redesigned systematics of Isohypsibiidae presented herein, the position and composition of *Doryphoribius* appeared as the most problematic. The genus *Doryphoribius* is polyphyletic and this is clearly visible both from earlier studies (Bertolani et al., 2014a) and the current molecular phylogeny.

### Table 3

Secondary/primary claw branch ratios (br) for various *Isohypsibius*-like taxa expressed in percentages. Claws of the fourth pair of legs were measured. Data source: O – original species description, M – measurements performed in the present study.

| Species                                      | Habitat | RANGE | MEAN | SOURCE |
|----------------------------------------------|---------|-------|------|--------|
| *Dianea satleri* (Richters, 1902)            | terrestrial | 58–72 | 64   | M      |
| *Eremobiotus* sp. nov.                       | terrestrial | 50–66 | 58   | M      |
| *Fractonotus verrucosus* (Richters, 1900)    | terrestrial | 54–59 | 56   | M      |
| *Isohypsibius altai* Kaczmarek & Michalczyk, 2006 | terrestrial | 38–67 | 44   | O      |
| *Isohypsibius archangajensis* Kaczmarek & Michalczyk, 2004 | terrestrial | 50–71 | 62   | O      |
| *Isohypsibius condorcanquii* Kaczmarek et al., 2014 | terrestrial | 50–55 | 53   | O      |
| *Isohypsibius coulsoni* Kaczmarek et al., 2012 | terrestrial | 56–68 | 63   | M      |
| *Isohypsibius dastychi* Pilato et al., 1982  | terrestrial | 70–70 | ?    | M      |
| *Isohypsibius prosostomus* Thulin, 1928       | terrestrial | 59–70 | 63   | M      |
| *Ursulinius australicus* (Iharos, 1966)       | terrestrial | 61–61 | ?    | M      |
| *Ursulinius dudichi* (Iharos, 1964)           | terrestrial | 62–70 | 66   | M      |
| *Ursulinius elegans* (Binda & Pilato, 1971)   | terrestrial | 64–64 | ?    | M      |
| *Ursulinius lunulatus* (Iharos, 1966)         | terrestrial | 54–69 | 62   | M      |
| *Ursulinius pappi* (Iharos, 1966)             | terrestrial | 62–70 | 65   | M      |
| *Ursulinius silvicola* (Iharos, 1966)         | terrestrial | 60–66 | 63   | M      |
| *Grevenius baldioides* (Tumanov, 2003)        | aquatic   | 72–75 | ?    | O      |
| *Grevenius granulifer* (Thulin, 1928)         | aquatic   | 83–99 | 91   | M      |
| *Grevenius karenai* (Zawierucha, 2013)        | aquatic   | 70–81 | 76   | O      |
| *Grevenius kotovae* (Tumanov, 2003)           | aquatic   | 68–76 | 71   | O      |
| *Grevenius ladogensis* (Tumanov, 2003)        | aquatic   | 74–79 | 76   | O      |
| *Grevenius laevis* (McInnes, 1995)            | aquatic   | 78–78 | ?    | O      |
| *Grevenius pushkini* (Tumanov, 2003)          | aquatic   | 71–72 | 72   | M      |
**Figure 8** Isohypsibiidae, PCM: A – *Isohypsibius prosostomus*; B – *Isohypsibius arbiter* Binda, 1980; C – *Isohypsibius coulsoni*; D – *Isohypsibius wilsoni* (Horning et al., 1978); E – *Isohypsibius dastychi* Pilato et al., 1982; F – *Isohypsibius chiarae* Mauceri, 1987. Note singular bars (incised arrowheads) and weakly developed or lacking pseudolunulae (empty incised arrowhead). Scale bars = 10 μm.
Specifically, *D. macrodon* Binda et al., 1980 clustered with *A. confusus*, which is not very surprising since both species have the ventral lamina and two short macroplacoids in the pharynx (fig. 2). However, at the same time, another *Doryphoribius*, *D. flavus* (Iharos, 1966), grouped with *Pseudobiotus*, which is unexpected as the two taxa exhibit dissimilar buccal apparatuses and claw morphologies as well as different habitat preferences (terrestrial vs freshwater). This discrepancy between morphology and genetics is not easy to interpret. On one hand, the ventral lamina has most likely evolved independently at least several times within *Doryphoribiidae* fam. nov., which makes the phylogenetic inference based on buccal apparatus anatomy questionable. On the other hand, it cannot be ruled out that the ribosomal markers are too conservative to resolve phyletic affinities within *Doryphoribiidae* fam. nov. and real relationships within *Doryphoribiidae* fam. nov. may
differ from the inferred. Finally, the possibility of misidentification or mislabelling of samples with *D. flavus* with a species representing another genus cannot be excluded. Thus, more *Doryphoribius* spp. and additional genetic markers need to be sequenced to verify phyletic relationships within the family. Nevertheless, the presence of a monophyletic clade characterised by the presence of the ventral lamina (i.e., *Doryphoribius s.l* + *Apodibius*) within *Doryphoribiidae* fam. nov. seems unlikely since this structure may have evolved independently during the natural history of this group (as it has evolved convergently also in other eutardigrades).

Our phylogenetic analysis confirmed that also *Isohypsibius* was polyphyletic (Sands et al., 2009; Guil & Giribet, 2011; Bertolani et al., 2014a and Cesari et al., 2016) and, in order to accommodate different evolutionary lineages, we divided the genus into three isohypsiid terrestrial genera (*Isohypsibius*,...
Claws of aquatic isohypsibioids, *i.e.* Doryphoribiidae fam. nov. (PCM): A – *Grevenius granulifer* comb. nov.; B – *Grevenius pushkini* comb. nov.; C – *Grevenius sismicus* (Maucci, 1978) comb. nov.; D – *Grevenius karenæ* (Zawierucha, 2013) comb. nov.; E – *Grevenius monoicus* (Bertolani, 1981) comb. nov.; F – *Grevenius longiunguis* (Pillato, 1974) comb. nov.; G – *Thulinius ruffoi*; H – *Pseudobiotus megalonyx*. Note singular bars (incised arrowheads) and pseudolunulae (empty incised arrowheads). Asterisks indicate evident internal and anterior claw primary branch widening, the claw curvature forms an obtuse angle (A–C, E) or the expansion is knob-like (D, F). Scale bars = 10 μm.
Figure 12 Hypothesised claw evolution scheme within the class Eutardigrada. Common Eutardigrade Ancestor (CEA) exhibited asymmetric (anisonych/heteronych) claws. Most significant changes in the overall morphology of claws are marked with numerals: (1) – secondary branch elongation; (2) – claw reduction, basal portion indistinctly merged with cuticle; (3) – branch curving; (4) – miniaturisation; (5) – evolution of true lunulae; (6) – claws tripartite; (7) – claw symmetry, claws bipartite; (8) – primary branch elongation, secondary branch reduction, lunulae transformed into longitudinal bars, exclusively aquatic. Drawings are based on SEM and/or PCM microphotographs. Phylogenetic relationships are based on the consensus results from recent published works (Bertolani et al., 2014a; Cesari et al., 2016; Guidetti et al., 2016) and the present study.
Dianea gen. nov., Ursulinius gen. nov.) and one doryphoribiid aquatic genus (Grevenius gen. nov.). Moreover, our study demonstrated that the presence and morphology of gibbosities bear taxonomic importance at the genus level. Thus, it would not be surprising if cuticular gibbosities could be used as diagnostic traits to differentiate new genera in other tardigrade groups. In addition to the presence/absence of cuticular gibbosities, Michalczyk & Kaczmarek (2010) used, in parallel, the number of placoids (2 vs 3) to divide

**Figure 13** Grevenius pushkini comb. nov., the buccal apparatus: A – habitus; B – mouth opening (the incised arrowhead indicates the first band of teeth, whereas the empty incised arrowhead – the second band of teeth); C – oral cavity armature; D – buccal crown (dorsal view); E – buccal crown (lateral view); F – pharynx (dorsal view); G – pharynx (lateral view). Scale bars in micrometres
Doryphoribius into four groups (*doryphorus*, *evelinae*, *vietnamensis*, and *zappalai* group) to aid the taxonomy of the genus. Recent analyses support the hypothesis that the number of placoids in the pharynx is likely to be stable at the genus level (Gąsiorek et al., 2016, 2018; Guidetti et al., 2016; Vecchi et al., 2016), meaning that all the genera with no single pattern of number of placoids are polyphyletic and artificial, e.g., Adropion, Doryphoribius, Microhypsibius, Mixibius, and, still, Isohypsibius. Finally, our study suggests that some lineages may dwell in a specific habitat (e.g., terrestrial or aquatic). Doryphoribius can be divided into taxa with two and three macroplacoids in the pharynx, into taxa with and without gibbosities (Michalczyk & Kaczmarek, 2010), and into terrestrial/xerophilous and strictly aquatic taxa (the present study; see Appendix). Moreover, within the genus, several claw morphotypes can be distinguished (e.g., Bertolani, 1983; Pilato & Lisi, 2004; Beasley et al., 2008; Lisi, 2011; figs. 17B–C) and at least two ventral lamina morphotypes are present (figs. 16B–C, see also Pilato, 2013). This high intrageneric variability clearly suggests that the genus Doryphoribius is polyphyletic. By adding the criterion of habitat to the two morphological criteria proposed by Michalczyk & Kaczmarek (2010), we obtained seven distinct groups of species:

**Figure 14**  *Thulinius ruffoi*, the buccal apparatus: A – habitus; B – mouth opening (the incised arrowhead indicates the first band of teeth, whereas the empty incised arrowhead – the second band of teeth); C – buccal crown (dorsal view); D – furca. Scale bars in micrometres.
terrestrial with gibbosities and two placoids (the Doryphoribius flavus group), aquatic with gibbosities and two placoids (the Doryphoribius evelinae group), terrestrial with two placoids but with no gibbosities (the Doryphoribius doryphorus group = Doryphoribius s.s.), aquatic with two placoids but with no gibbosities (the Doryphoribius koreanus group), terrestrial with gibbosities and three placoids (the Doryphoribius vietnamensis group), terrestrial with three placoids but with no gibbosities (the Doryphoribius bertolanii group), and, finally, aquatic with three placoids but with no gibbosities (the Doryphoribius zapalai group) (see Appendix for species compositions of groups within Doryphoribius s.l.). However, some of these groups are heterogeneous in regards to claw and ventral lamina morphology. Thus, the number of potential genera concealed within this polyphyletic genus may be even higher than seven.

**Systematic position of Grevenius gen. nov.**

Genetic distinctiveness of Grevenius gen. nov. became first apparent in Sands et al. (2008), where close affinities between I. asper (Murray, 1906), I. granulifer, and Thulinius stephaniae (Pilato, 1974) were demonstrated. This clade, although weakly supported in the Maximum Parsimony analysis (MP = 50), was clearly distinct from the well-supported Isohypsibius s.s. clade (represented at the time only by I. prosostomus Thulin, 1928 and I. cambrensis (Morgan, 1976); MP = 99, BI = 1.0; Sands et al., 2008). New sequences for Isohypsibius s.s. and Grevenius gen. nov. confirmed the remote affinity between aquatic Isohypsibius s.l. spp. and Isohypsibius s.s. In addition to distinct genetic divergence, Grevenius gen. nov. also clearly differs from Isohypsibius s.s. morphologically and ecologically. The new genus exhibits distinct differences in the armature of the oral cavity (two rows of teeth vs only one row in Isohypsibius s.s.); see Jørgensen, 2001, where the second row of teeth in G. malawiensis (Jørgensen, 2001) comb. nov. is described as ‘intrabuccal baffles’) and in claw morphology (claws elongated, with a clear hump on the primary branch and with relatively elongated secondary branches (br > 70%) in Grevenius gen. nov., figs. 11A–F vs claws of the Isohypsibius type, without the hump on the primary branch and with a considerable difference in primary and secondary branch height (br ≤ 70%) in Isohypsibius, figs. 8A–D). Moreover, Grevenius gen. nov. inhabits a different ecological niche than in Isohypsibius s.s. (freshwater vs terrestrial).

Claws in Grevenius gen. nov., similarly to those in Pseudobiotus and Thulinius (e.g., see Nelson et al., 1999; Bertolani, 2003), are clearly elongated, which is most likely an adaptation to the aquatic habitat (fig. 12). Moreover, internal claws in the new genus have a clear hump (as in Thulinius) and the cuticle is typically rough (as in Pseudobiotus; e.g., see Bertolani, 1982; Chang et al., 2007 or Pilato et al., 2010). All these similarities suggest a close affinity of the new genus with both Pseudobiotus and Thulinius, which is in agreement with the molecular phylogeny (fig. 2). However, the exact phyletic relationships between the three genera and relationships within Doryphoribiidae fam. nov. are not fully solved. Thus, more DNA sequences, in particular for intertidal Grevenius gen. nov. spp., are needed to better understand its relationships with other doryphoribiid genera.

**Morphology of Hexapodibiidae**

The problematic systematic position of calohypsibiid genera and species (order Hypsibiidea) has been a subject of long debate (Pilato, 1989; Guil et al., 2013; Bertolani et al., 2014a; Gąsiorek et al., 2019). Recently, Cesari et al. (2016) demonstrated the monophyly...
Figure 15  *Hexapodibius micronyx*, the buccal apparatus: A – habitus (ventral view, the arrowhead points the ventral lamina); B – habitus (dorsal view); C – buccal crown and ventral lamina (ventral view); D – furca; E – pharynx. Scale bars in micrometres.
of a clade comprising four of the former five members of Calohypsibiidae (Haplohexapodibius Pilato & Beasley, 1987, Haplomacrobiotus May, 1948, Hexapodibius, Parhexapodibius) and instituted a new family rank for eutar-digrades equipped with the ventral lamina and exhibiting various degrees of claw reduction. All four hexapodibiid genera share the same general morphology of the buccal apparatus, i.e., reduced dorsal AISM, ventral lamina and three granular macroplacoids (compare figs. 15, 16D–F herein and the buccal apparatus of Haplomacrobiotus in Cesari et al., 2016). Interestingly, a similar buccal apparatus morphotype is also present in two doryphoribiid genera: Apodibius and Doryphoribius (although with two macroplacoids in some species), but absent in the remaining doryphoribiid genera (Pseudobiotus, Thulinius and Grevenius gen. nov.). Thus, at the moment, it is not possible to establish whether a similar buccal apparatus morphotype evolved independently in Hexapodibiidae as well as in Apodibius (Hohberg & Lang, 2016) and Doryphoribius (Guidetti et al., 2013) or whether the similarity indicates the ancestral state of
Hexapodibiidae + Doryphoribiidae fam. nov. Nevertheless, an independent (convergent) origin of the ventral lamina within this clade seems more likely as it is a more parsimonious explanation: given that the evolution of the ventral lamina is tightly linked with the reduction of dorsal AISM (same pattern was observed also in other eutardigrades), hypothesising that the ancestor of Hexapodibiidae + Doryphoribiidae fam. nov. had ventral lamina, which was secondarily lost, and ridge-like AISM evolved again, appears less probable. In other words, a plesiomorphic ventral lamina would require a subsequent atrophy of this structure, re-establishing of the dorsal apophysis and the restoration of the overall symmetry of AISM in Grevenius gen. nov., Pseudobiotus and Thulinius.

Despite representing different families, both Hexapodibius and Apodibius exhibit peculiar peribuccal circular wrinkles (figs. 5F, J). Interestingly, it must be noted...
that these structures are found exclusively in soil isohypsibioids (Haplomacrobiotus being an exception (Cesari et al., 2016)). Similarly, frontal lobes are present mainly in soil genera (Apodibius, Haplomacrobiotus, Paradiphascon) and they occur also in Ursulinius gen. nov. Thus, these organs could be another adaptation to the terrestrial habitat.

In accordance with analyses of Guil et al. (2013), claw morphology in Hexapodibiidae represents three levels of reduction: (I) shortened secondary branches and the basal tract being continuous with cuticle surface (reduced pseudolunulae in Parhexapodibius; see Manicardi & Bertolani, 1987), (II) shortened primary branches and lack of claws IV in Hexapodibius (see fig. 7Q), and (III) complete reduction of secondary branches in Haplohexapodibius and Haplomacrobiotus (see Cesari et al., 2016). The reduction is commonly viewed as an adaptation to soil habitat, preferred by hexapodibiids (Bertolani & Biserov, 1996; Hohberg et al., 2011).

**Autapomorphies of Halobiidae fam. nov.**

The erection of Halobiidae fam. nov. is firmly supported both by DNA sequences as well as unique morphology and anatomy, which are most likely the result of secondary adaptation to marine habitat. Traits exclusive to Halobiotus, such as cephalic papillae, peri-buccal chemosensory organs, and gigantic Malpighian tubules, most probably serve in perception of external stimuli and osmoregulation, respectively (Kristensen, 1982; Møbjerg & Dahl, 1996; Møbjerg et al., 2007; Halberg et al., 2013). Claws of Halobiotus (figs. 7I–J) are similar to the most common morphotype of doryphoribiid claws, i.e., with elongated stalks and branches of similar heights, present also in Grevenius gen. nov., Paradiphascon, Pseudobiotus, Thulinius, and some Doryphoribius spp. Therefore, presumably the ancestral claw type of the clade [Halobiidae fam. nov. (Doryphoribiidae fam. nov. + Hexapodibiidae)] was close to this morphotype.

**Incertae sedis: Ramajendas Pilato & Binda, 1991 and Thalerius Dastych, 2009**

Exhibiting a mixture of hypsibioid and isohypsibioid morphological traits, two enigmatic genera, Ramajendas and Thalerius, are a subject of an ongoing debate on their taxonomic affinity. Originally placed in Isohypsibiidae (Marley et al., 2011; Guil et al., 2013), they were later tentatively transferred to Ramazzottiidae Sands et al., 2008 (Bertolani et al., 2014a) and most recently, moved back, also provisionally, to Isohypsibiidae (Zawierucha et al., 2018). On one hand external and posterior claws, by having elongated and flexible primary branch, seem to resemble those in the family Ramazzottiidae (Hypsibioidea). On the other hand, however, the shape of internal and anterior claws is similar to that found in some species representing both Hypsibioidea and Isohypsibioida. Moreover, the two genera lack body pigmentation and paired cephalic elliptical organs (present in Ramazzottiidae), which speak against the close affinity with ramazzottiids (Zawierucha et al., 2018). The body shape and the bucco-pharyngeal apparatus morphology (including AISM shape) in Ramajendas are indeed near those in aquatic doryphoribiid genera. However, this genus comprises both terrestrial taxa and a marine species (R. renaudi (Ramazzotti, 1972]) which strongly indicates that Ramajendas may be polyphyletic, as it was shown above that distinct evolutionary lineages often correspond with the type of environment. Furthermore, Thalerius exhibits the bucco-pharyngeal apparatus similar to many isohypsibioid genera (three granular macroplacoids, widespread in Isohypsibiidea but rare in Hypsibioidea, except for the polyphyletic Mixibius Pilato, 1992 and Diphascon Plate, 1889) and claws with concave bases present in some Itaquasconinae (Hypsibioidea).
In fact, this perplexing mix may indicate a need to create a new higher taxon for *Thalerius*. We are of the opinion that neither buccopharyngeal apparatus nor claw morphology should be given priority (see Schuster et al., 1980, and Pilato, 1982, for opposing views on the relevance of these structures used in the formulation of eutardigrade classification on higher taxonomic levels), making clarification of the status of the two genera impossible without molecular data. To conclude, the mixture of traits exhibited by both *Ramajendas* and *Thalerius* make it difficult to ascribe them to any of the isohypsibioid families distinguished in this work. Therefore, we designate the two genera as *incertae sedis* within Isohypsibioida, pending molecular verification of their taxonomic positions within this or a different eutardigrade order.

**Taxonomic account of the families and genera of Isohypsibioida**

Type genera are underlined with a double line.

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Isohypsibioida Guil et al., 2019

Amended diagnosis (modified from Bortolani et al., 2014a): Double claws asymmetrical with respect to the median plane of the leg (2121), normally with a similar shape and size on each leg; double claws with the external secondary branches inserted perpendicularly on the claw basal tract, or partly reduced (very short, without the common basal tract, with a base as large as the sum of the primary and secondary branch widths, and with an evident suture between the primary and the secondary branch), or elsewhere absent. Buccal tube rigid (apart Paradiphascon) and often relatively large, without the ventral lamina (*Dastychius*, *Dianea* gen. nov., *Eremobiotus*, *Grevenius* gen. nov., *Halobiotus*, *Isohypsibioida*, *Ursulinus*, *Ursulinius* gen. nov., *Paradiphascon*, *Pseudobiotus*, *Ramajendas*, *Thalerius*, *Thu-\[\ldots]\]

Amended diagnosis: Terrestrial eutardigrades with six peribuccal lobes or with a continuous peribuccal ring, and peribuccal lamina. Lacking peribuccal lamellae and ventral lamina on the buccal tube. AISM ridge-like and asymmetrical with respect to the frontal plane (only in *Fractonotus*) or symmetrical (remaining five genera). Stylet furcae of the *Hypsibius* type. Claws with secondary branches clearly shorter than primary branches (\(br \leq 0.70\)).

Composition: *Dastychius* Pilato, 2013, *Dianea* gen. nov., *Eremobiotus* Biserov, 1992, *Fractonotus* Pilato, 1998, *Isohypsibioida* Thulin, 1928, *Ursulinius* gen. nov.

Remarks: Molecular data are not available for representatives of some genera of former Isohypsibiidae s.l., thus their taxonomic assignment may change when the data are obtained. *Dastychius improvisus* (Dastych, 1984) is kept in the family since *Dastychius* type AISM are modified *Isohypsibioida* type AISM (ridged AISM, exceptionally elongated towards the SSIP). Together with peculiar cuticular cavities and typical *Isohypsibioida* type claws, they currently prevent any taxonomic re-shuffling of this genus.

Differential diagnosis: Isohypsibiidae s.s. have no ventral lamina and they retained the plesiomorphic claw morphotype with claw branches of dissimilar heights (\(br \leq 0.70\)). Doryphoribiidae fam. nov., on the other hand, either exhibit the ventral lamina or their claw...
branches are similar in height ($br > 0.70$) or both (i.e., at least one of these two criteria distinguishes any given member of Doryphoriibidae fam. nov. from Isohypsibiidae). Hexapodibiidae are always equipped with the ventral lamina and their claws are strongly reduced. Halobiotidae fam. nov. have dome-shaped cephalic papillae (absent in other isohypsibioids). Moreover, Isohypsibiidae s.s. inhabit terrestrial habitats (mostly mosses and lichens) whereas many Doryphoriibidae fam. nov. inhabit freshwaters, Hexapodibiidae dwell exclusively in soil, and all Halobiotidae fam. nov. are marine.

Genus: *Isohypsibius* Thulin, 1928

Amended diagnosis: Cuticle smooth or sculptured (i.e., reticulated or rugose), but without gibbosities or plaques. Claw bases typically without pseudolunulae. No cuticular bars or only single bars under claws. Only first band of teeth in the oral cavity.

Remarks: The genus *Isohypsibius*, even after the exclusion of species assigned to the three genera erected herein, still remains polyphyletic. For example, *I. dastychi* (the *I. dastychi* group), represents a different evolutionary lineage within Isohypsibiidae. Since the clade *I. dastychi* + *Ursulinius* gen. nov. + *Eremobiotus* lack statistical support (fig. 2), and at least one more representative of this group ought to be sequenced to confirm the monophyly of the *I. dastychi* group, the *dastychi* complex is not erected as a separate genus. Moreover, as recently indicated by Gąsiorek et al. (2019), relationships between *Isohypsibius* and *Fractonotus* need clarification as the only sequenced species, *F. verrucosus* (Richters, 1900), is embedded within the *Isohypsibius* clade (fig. 2).

Type species: *Isohypsibius prosostomus* Thulin, 1928.

Differential diagnosis: *Isohypsibius* is distinguished from *Dastychius* and *Fractonotus* by the AISM shape (symmetric and restricted to the buccal crown vs elongated and extending towards the SSIP and asymmetric, respectively). Moreover, *Dianea* gen. nov. and *Ursulinius* gen. nov. exhibit gibbosities, which are absent in *Isohypsibius*. Finally, *Eremobiotus* has six peribuccal papulae (absent in *Isohypsibius*) and widely forked claw branches (branches forking at a ca. right angle in *Isohypsibius*).

Genus: *Dianea* gen. nov.

Diagnosis: Cuticle with numerous small but distinct, pointy gibbosities covered with reticulum. Claw branches forking at a wide angle; claw bases without pseudolunulae. No cuticular bars under claws.

Etymology: In honour of Diane Nelson, Professor Emerita of the East Tennessee State University, USA, an exquisite American tardigradologist and a great friend.

Type species: *Macrobiotus sattleri* Richters, 1902.

Differential diagnosis: *Dianea* gen. nov. has gibbosities, absent in all isohypsibiids but *Ursulinius* gen. nov. However, gibbosities of *Dianea* gen. nov. are small and pointed, not convex or mamillose as in *Ursulinius* gen. nov. Additionally, *Dianea* gen. nov. has no cuticular bars under claws whereas *Ursulinius* gen. nov. exhibits evident double bars.

Genus: *Ursulinius* gen. nov.

Diagnosis: Cuticle covered with large, mamillose, and sculptured gibbosities. Claw bases with well-developed pseudolunulae. Double cuticular bars under claws.
Etymology: When observed in SEM en face, the first row of dorsal gibbosities look like pointy ears on the head, which results in a teddy bear-like appearance of animals of the new genus (e.g., see fig. 4B). Therefore, the name of the new genus is derived from the Latin word “ursus” (bear), being a diminutive to mean "a small bear”.

Type species: Hypsibius pappi Iharos, 1966.

Differential diagnosis: Ursulinius gen. nov. has gibbosities, absent in all isohypsibiids but Dianea gen. nov. (see above for the distinction with this genus).

Family: Doryphoribiidae fam. nov.

Diagnosis: Freshwater (limnic) or terrestrial eutardigrades with six peribuccal lobes, or with continuous peribuccal ring. Mouth opening surrounded by peribuccal lamellae, often partially or almost completely fused (Paradiphascon, Pseudobiotus, Thulinius) or by a peribuccal lamina (Apodibius, Doryphoribius, Grevenius gen. nov.). Ventral lamina on the buccal tube present (Apodibius, Doryphoribius) or absent (Grevenius gen. nov., Paradiphascon, Pseudobiotus, Thulinius). AISM ridge-like, well-developed and symmetrical in genera with no ventral lamina or greatly reduced and asymmetrical in genera exhibiting the ventral lamina. Flexible pharyngeal tube present (Paradiphascon) or absent (all remaining genera). Two claw types: the dominant type, with secondary branches being similar in height to the primary branches (all genera with the exception of some Doryphoribius spp.); and the second, with secondary branches being clearly shorter than the primary branches (only in some Doryphoribius spp.).

Composition: Apodibius Dastych, 1983, Doryphoribius Pilato, 1969, Grevenius gen. nov., Paradiphascon Dastych, 1992, Pseudobiotus Nelson, 1980, Thulinius Bertolani, 2003.

Remarks: Paradiphascon manningi Dastych, 1992 is transferred from the family Isohypsibiidae primarily on the basis of large peribuccal lamellae. Pilato & Binda (1996) considered lamellae in this taxon as papulae, but the term “papulae” refers to rounded peribuccal structures, present e.g., in Calohypsibius (Gąsiorek et al., 2019). However, extremely peculiar morphological autapomorphies of the genus (highly modified AISM, annulated pharyngeal tube, dorsoposterior apodeme on the border between the buccal and pharyngeal tube, external and posterior claws with wide bases) require molecular and new morphological evidence to verify the tentative affiliation within Doryphoribiidae fam. nov.

Differential diagnosis: For the differences with Halobiotidae fam. nov. and Hexapodibiidae, see the differential diagnoses for these families. There are two clear ecomorphological groups within Doryphoribiidae fam. nov.: (I) aquatic genera with elongated claws (Grevenius gen. nov., Pseudobiotus, Thulinius) (br > 0.70), and (II) terrestrial genera with typical (br ≤ 0.70) or reduced claws, either with the ventral lamina (Apodibius, Doryphoribius) or without the lamina but with the flexible pharyngeal tube (Paradiphascon). In contrast, Isohypsibiidae s.s. do not inhabit limnic habitats (if found in water, they are accidental, ecademic elements) and they have no peribuccal lamellae around the mouth opening or ventral lamina on the buccal tube.

Genus: Grevenius gen. nov.

Diagnosis: Aquatic (limnic or intertidal) or hygrophilic. Cuticle smooth or rough, covered with evident protuberances or wrinkles, sometimes forming reticulum. Claw bases with...
pseudolunulae. Single bars or no cuticular bars under claws. Two rows of teeth in the oral cavity. Stylet furcae of the *Hypsibius* type.

Etymology: Named after Hartmut Greven, Professor Emeritus of the Department of Zoology, Heinrich Heine University Düsseldorf, and an eminent German zoologist, who studied the biology of the type species of the new genus (Greven & Blom, 1977; Wolburg-Buchholz & Greven, 1979).

Type species: *Isohypsibius granulifer* Thulin, 1928.

Differential diagnosis: The presence of peribuccal lamellae distinguishes *Paradiphascon, Pseudobiotus* and *Thulinius* from *Grevenius* gen. nov. (continuous peribuccal lamina). Similarly, the presence of ventral lamina separates *Apodibius* and *Doryphoribius* from *Grevenius* gen. nov. (lack of ventral lamina). *Grevenius* gen. nov. is separated from the former *Isohypsibius* s.l. (= *Isohypsibius* and two other genera established in this work) by the absence of dorsal gibbosities (gibbosities present in *Dianea* gen. nov. and *Ursulinius* gen. nov.), the presence of two bands of teeth in the OCA, and by elongated claws (one band of teeth and, comparatively, markedly shorter claws in *Isohypsibius* s.s., *Dianea* gen. nov. and *Ursulinius* gen. nov.).

Family: *Hexapodibiidae* Cesari et al., 2016

Amended diagnosis: Soil eutardigrades with six peribuccal lobes. Peribuccal lamellae (*Haplohexapodibius, Haplomacrobiotus*) or peribuccal lamina (*Hexapodibius, Parhexapodibius*) around the mouth opening. Ventral lamina on the buccal tube present. AISM reduced. Claws reduced, with indistinct claw bases and no pseudolunulae. Clear sutures between branches. Secondary branches shortened or absent.

Composition: *Haplohexapodibius* Pilato & Beasley, 1987, *Haplomacrobiotus* May, 1948, *Hexapodibius* Pilato, 1969, *Parhexapodibius* Pilato, 1969.

Differential diagnosis: Claws in the family Hexapodibiidae are remarkably reduced (Pilato, 1989; Cesari et al., 2016). The only other example of convergent claw atrophy within Isohypsibioidea is known in *Apodibius*. However, *Apodibius* is completely clawless whereas in Hexapodibiidae entire claws vanished only on the fourth pair of legs (only in *Haplohexapodibius* and *Hexapodibius*).

Family: *Halobiota* fam. nov.

Diagnosis: Marine eutardigrades with six peribuccal lobes equipped with chemosensory organs. Two large, dome-shaped cephalic papillae present. Mouth opening surrounded by the peribuccal lamina. No ventral lamina on the buccal tube. AISM symmetrical, divided into the anterior semilunar hook and the posterior slight thickening. Claws with pseudolunulae.

Composition: *Halobiotus* Kristensen, 1982.

Differential diagnosis: In contrast to exclusively marine Halobiota* fam. nov.*, vast majority of isohypsibioids dwell in limnoterrestrial habitats. Moreover, none of them exhibit peribuccal chemosensory organs and giant Malpighian tubules, both being distinctive features of the new family.

Incertae sedis: *Ramajendas* Pilato & Binda, 1991, *Thalerius* Dastych, 2009.

Remarks: The current data do not allow for assigning these two genera to any of the families. Moreover, it is not certain whether they represent Isohypsibioidea (see above for details).
Evolution of traits within the order in relation to other lineages of Eutardigrada

Isohypsibioida are most likely the most basal lineage in the order Eutardigrada (Sands et al., 2008; Bertolani et al., 2014a). Therefore, unravelling phyletic affinities within this group is of special importance for understanding the evolution of Eutardigrada (Kiehl et al., 2007; Sands et al., 2008). Of the four currently recognised eutardigrade orders, Isohypsibioida, alongside Hypsibioida and Eohypsibioida, exhibit asymmetrical (heteronych) claws and only Macrobiotoidea are characterised by symmetrical (isonych) claws (fig. 12). Given the phylogenetic relationships between the orders (Bertolani et al., 2014a), asymmetrical claws are most likely a plesiomorphy of the Eutardigrada whereas claw symmetry should be considered as a macrobiotid autapomorphy. Nevertheless, in comparison to Hypsibioida, in which a number of claw morphotypes were recognised (e.g., Hypsibius, Ramazzottius or Calohypsibius type), isohypsibioid claws have always been defined as of a single, general “Isohypsibius type”, which suggests prevalent conservatism in their morphology (Pilato & Binda, 2010; Marley et al., 2011). However, our study implies that details of claw shape together with the presence or absence of other pedal structures such as pseudolunulae and cuticular bars, which were often considered as of minor taxonomic significance (e.g., they were omitted in the only comprehensive morphological phylogeny of eutardigrades by Guil et al., 2013), may hold sound phylogenetic signal. Some isohypsibioid taxa seem to have claws intermediate between the Isohypsibius and the Hypsibius types. For example, claws in Fractonotus (Hypsibius-like claw curvature) or Paradiphascon (Hypsibius-like difference in the size of external and internal claws), may signalise a closer affinity between Isohypsibioida and Hypsibioida than with the two remaining orders. As the relationships between the basal families of Hypsibioida, Calohypsibiidae Pilato, 1969 and Microhypsibiidae Pilato, 1998, are not resolved (fig. 12), the plesiomorphic condition for this order remains unknown. However, in the recent phylogenies, the polytomy embraced also Mixibius and Acutuncus Pilato & Binda, 1997, having either hypsibiid-isohypsibiid claws or typical hypsibiid claws, respectively (Bertolani et al., 2014a; Cesari et al., 2016). This suggests that hypsibiid ancestor had claws nearing to the present Hypsibiidae Pilato, 1969, and that the claws of Calohypsibiidae and Microhypsibiidae are considerably modified. Similarly to the hypothesised closer affinity between Isohypsibioida and Hypsibioida, the relationship between Eohypsibioida and Macrobiotoida is well-supported in the development of true, strongly sclerotised lunulae (in contrast to pseudolunulae present in the former), and narrowing of the basal portion of the claw, which became the peduncle (fig. 12).

Aquatic isohypsibioid species are scattered between more numerous terrestrial taxa, and the basal family, Isohypsibiidae s.s., comprises entirely land taxa (fig. 2). Concerning the entire class Eutardigrada, limnic forms occur only in some Doryphoribiidae fam. nov., Microhypsibiidae, some Hypsibiidae and Eohypsibiidae, and Murrayidae, whereas marine—in Halobiotidae fam. nov. and in some Doryphoribiidae fam. nov. (Nelson & Marley, 2000). The current phylogeny indicates all these are examples of independent invasion of aquatic habitats (figs. 2, 12). Maucci (1973–1974) first formulated the hypothesis on the evolution of claw morphotypes in relation to the inhabited ecological niche for Hypsibiidae and Ramazzottiidae: he noted that aquatic species exhibit longer claws compared to terrestrial taxa. The correlation between secondarily aquatic environment and claw morphology is also expressly visible in Isohypsibioida, in which aquatic taxa have elongated claws with branches of almost similar heights, whereas...
terrestrial species exhibit robust claws with markedly shorter secondary branches (table 3; figs. 7–12; see also Bertolani, 1982 and compare limnic taxa depicted therein: the majority of Isohypsibius spp. [now assigned to Grevenius gen. nov.], Pseudobiotus spp., Thulinia Bertolani, 1981 spp. [now Thulinius] with terrestrial taxa: Dianea satleri comb. nov. [Isohypsibius bakonyiensis (Iharos, 1964) then], Eremobiotus alicatai [Isohypsibius alicatai then], Isohypsibius marcellinoi Binda & Pilato, 1971, I. prosostomus, U. pappi comb. nov., U. ronisvallei Binda & Pilato, 1969 comb. nov.).

Concerning buccal apparatus morphology, all isohypsibioid AISM shapes could be seen as derived states of the Isohypsibius type, i.e., ridged AISM (Marley et al., 2011), suggesting this shape as plesiomorphic for the Eutardigrada. Pilato (2013) also hypothesised about the ancestral state of eutardigrade (parachelan then) AISM shape within Isohypsibioida, suggesting however the Dastychius rather than Isohypsibius type (long ridges reaching to the level of SSIP vs short ridges limited to the buccal crown) as a potential candidate. He hypothesised that in the course of evolution, the Dastychius AISM became shorter, which resulted in the Isohypsibius type AISM. Nonetheless, the current state of knowledge does not allow to determine confidently which of these types is plesiomorphic. The highly modified AISM types in Fractonotus and Halobiotus probably evolved by the division of both ventral and dorsal apophyses, and subsequent reduction of lateral AISM portions or by forming hook-like portions. On the other hand, the most modified AISM type, with reduced dorsal apophysis, is present in Hexapodibiidae and some groups within Doryphoribiidae fam. nov. (figs. 15–16). The magnitude of these changes is most likely associated with the parallel evolution of ventral lamina, which constitutes an important stilet muscle attachment and therefore changes the distribution of forces in the buccal apparatus, rendering dorsal apophyses less important for the functioning of the stilet musculature. The pattern of reduction of the dorsal AISM is consistently found in all eutardigrades exhibiting the ventral lamina (i.e., Macrobiotoidea; Pilato & Binda, 2010).

Another instance of parallel evolution, next to the independent origin of the ventral lamina, is the development of dorsolateral gibbosities. Among Eutardigrada, cuticular gibbosities evolved most likely independently in two orders, Isohypsibioida and Hypsibioida. Mamillose, sculptured gibbosities of a very similar appearance are present in four genera representing four families: Ursulinius gen. nov. (Isohypsibiidae), some Doryphoribius spp. (Doryphoribiidae fam. nov.), the majority of Pilatobius Bertolani et al., 2014 spp. (Hypsibiidae), and in Ramazzottius szeptyckii (Dastych, 1979) (Ramazzottiiidae). On the other hand, small, terminated at point and wrinkly gibbosities of Dianea gen. nov. (Isohypsibiidae) are a unique feature of this genus, therefore they should be recognised as its autapomorphy.

**Key to families and genera of Isohypsibioida**

1. Concave claw bases............Thalerius (inc. sed.)
   –. Straight claw bases or lacking claws.............. 2
2(1). Extremely elongated, flexible external claws......................Ramajendas (inc. sed.)
   –. External claws non-flexible or lacking claws........................................ 3
3(2). Exclusively marine, with dome-shaped cephalic papillae.................................
      ..............................................................Halobioidae fam. nov. (Halobiotus)
   –. Limno-terrestrial, rarely marine, without cephalic papillae............................. 4
4(3). Reduced claws with indistinct basal tract.................................................(Hexapodibiidae) 6
   –. Claws with distinct basal tract or claws absent........................................... 5
5(4). Claws always present, with branches of dissimilar heights (br ≤ 70%), mouth opening surrounded with peribuccal lamina, no ventral lamina on the buccal tube

| Claws always present | 5(4) | Claws absent or present, with branches of similar heights (br > 70%), mouth opening surrounded by peribuccal lamellae or lamina, ventral lamina on the buccal tube may be present |
|----------------------|------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| (Isohypsibiidae)      | 9    | (Doryphoriibidae fam. nov.) 14                                                                                               |
| 6(3). Minute claws present on all legs                              | 7    | 7(6). Secondary branches absent or in the form of acute spines                                                                |
| 8(6). Double claws present on legs I–III                           | 10   | 10(9). Dorsal gibbosities present                                                                                               |
| 12(10). Claws present on legs IV having a 180° angle                 | 11   | 11(10). Gibbosities narrow towards apex and with sharp apices, appear flat under PCM                                       |
| 13(12). AISM symmetrical with respect to the frontal plane           | 13   | 13(12). AISM short, typically not exceeding 1/3 of the buccal tube length                                                       |
| 14(5). Claws absent on all legs                                     | 15   | 15(14). Ventral lamina on the buccal tube present                                                                       |
| 16(15). Flexible pharyngeal tube present                            | 17   | 17(16). Lacking peribuccal lamellae                                                                                           |
| 18(17). Twelve peribuccal lamellae, primary claw branches with a clear hump | 19   | 19(20). Thirty peribuccal lamellae, elongated primary claw branches without the hump |

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APPENDIX

Taxonomic composition of isohypsibioid families

Type genera are underlined by a double line, and type species by a single line. Taxa described as species dubiae are either synonyms of other species or their descriptions are too general and do not allow confident identifications; whereas nomina inquirenda embrace most likely valid species, but insufficiently described. This distinction and assessment was done after a careful analysis of the original species descriptions.

Composition of Isohypsibiidae

Genus: Dastychius Pilato, 2013
Species: D. improvisus (Dastych, 1984).

Genus: Dianea gen. nov.
Species: D. acuminata nom. nov. et comb. nov. [Isohypsibius papillifer indicus (Iharos, 1969)], D. basalovoi (Durante & Maucci, 1973) comb. nov., D. belliforma (Mihelčič, 1971) comb. nov. et sp. dub., D. bella (Mihelčič, 1971) comb. nov. et sp. dub., D. brevispinosa (Iharos, 1966) comb. nov. et sp. dub., D. costata (Mihelčič, 1971) comb. nov. et sp. dub., D. effusa (Mihelčič, 1971) comb. nov. et sp. dub., D. franzi (Mihelčič, 1951) comb. nov. et sp. dub., D. helena (Iharos, 1964) comb. nov. et nom. inq., D. indica (Murray, 1907) comb. nov. et nom. inq., D. mammiliosa (Iharos, 1964) comb. nov. et sp. dub., D. papillifera (Murray, 1905) comb. nov., D. sattleri (Richerds, 1902) comb. nov., D. rahmi (Li & Wang, 2006) comb. nov., D. tuberculoides (Mihelčič, 1951) comb. nov. et sp. dub., D. vej dovskyi (Bartoš, 1939) comb. nov. et nom. inq.

Remarks: As stressed by Ramazzotti & Maucci (1983) for Isohypsibius s.l. spp. described by Mihelčič & Iharos, the genus consists mainly of dubious and improperly described species, which very likely are synonyms of the nominal species, D. sattleri comb. nov. Here, I. papillifer bulbosus (Marcus, 1928) is suppressed due to lack of significant differences between the putative subspecies and the nominal subspecies. On the other hand, the subspecies Isohypsibius papillifer indicus (Iharos, 1969) is elevated to the species rank because of significant morphological differences with the nominal subspecies such as two macroplacoids instead of three, larger and more triangular gibbosities, and no cephalic gibbosities. Given that within Dianea gen. nov., the specific name ‘indica’ is already occupied by D. indica (Murray, 1907), a nomen novum D. acuminata comb. nov. is established for I. papillifer indicus (Iharos, 1969) (from Latin acuminatus = acute, sharp, which refers to the elongated gibbosity apices).

Genus: Eremobiotus Biserov, 1992
Species: E. alicatai (Binda, 1969), E. ginevræ Lisi et al., 2016, E. ovezovae Biserov, 1992.

Genus: Fractonotus Pilato, 1998
Species: F. caelatus (Marcus, 1928), F. verrucosus (Richerds, 1900), F. gilvus (Biserov, 1986).

Genus: Isohypsibius Thulin, 1928
Species: I. altai Kaczmarek & Michalczyk, 2006, I. arbiter Binda, 1980, I. archangajensis Kaczmarek & Michalczyk, 2004, I. arcuatus (Bartoš, 1934), I. barbarae Pilato & Binda, 2002, I. borkini Tumanov, 2003, I. brulloii Pilato & Pennisi, 1976, I. cambrensis (Morgan, 1976) comb. nov., I. campbellensis Pilato, 1996, I. canadensis (Murray, 1910) sp. dub., I. ceciliae Pilato & Binda, 1987, I. changbaiensis Yang,
Remarks: *I. cambrensis* is raised to a species rank because of the significant distinctiveness from *I. prosostomus* both in terms of external morphology (finely granulated vs completely smooth cuticle) and genetics (fig. 2). On our phylogenetic tree, the clade *I. dastychi* + *Eremobiotus* + *Ursulinius* gen. nov. can be observed (fig. 2). In our opinion, widely forked claws in the *dastychi* complex (*I. borkini*, *I. dastychi*, *I. jakielii*, *I. marcellinoi*, and *I. panovi*) bear resemblance to *Eremobiotus* type claws. This characteristic, together with only two granular macroplacoids in the pharynx and smooth or weakly reticulated cuticle, which make the *dastychi* complex stand out from *Isohypsisibius* s.s. could warrant erecting a new genus for the *dastychi* complex if new data support the hypothetical monophyly of the complex. As noted by Gąsiorek et al. (2019), species with very pronounced cuticular sculpture (*I. barbarae*, *I. sabellai*) are potentially more closely related to *Fractonotus* than to *Isohypsisibius* s.s., and their systematic position may change when more ample molecular data are available.

Genus: *Ursulinius* gen. nov.

Species: *U. australicus* (Iharos, 1966) comb. nov., *U. bartosi* (Iharos, 1966) comb. nov. et sp. dub., *U. bulbifer* (Mihelčič, 1957) comb. nov. et sp. dub., *U. cameruni* (Iharos, 1969) comb. nov., *U. cyrilli* (Mihelčič, 1951) comb. nov., *U. dudichi* (Iharos, 1964) comb. nov., *U. duranteae* (Mauc-ci, 1978) comb. nov., *U. elegans* (Binda & Pilato, 1971) comb. nov., *U. eplenyiensis* (Iharos, 1970) comb. nov., *U. glaber* (Durante Pasa & Mauc-ci, 1979) comb. nov., *U. gracilis* (Iharos, 1966) comb. nov. et nom. inq., *U. gyulai* (Mihelčič, 1971) comb. nov. et sp. dub., *U. hypostomoides* (Mihelčič, 1971) comb. nov., *U. josephi* (Iharos, 1964) comb. nov., *U. latiunguis* (Iharos, 1964) comb. nov. et nom. inq., *U. leithaicus* (Iharos, 1966) comb. nov., *U. lunulatus* (Iharos, 1966) comb. nov. et nom. inq., *U. mihelicici* (Iha-ros, 1964) comb. nov. et nom. inq., *U. montanus* (Mihelčič, 1938) comb. nov. et nom. inq., *U. neoundulatus* (Durante Pasa & Mauc-cci, 1975) comb. nov., *U. nodosus* (Murray, 1907) comb. nov., *U. novaeguineae* (Iharos, 1967) comb. nov., *U. pappi* (Iharos, 1966) comb. nov., *U. pilatoi* (Durante Pasa & Mauc-cci, 1979) comb. nov., *U. pratensis* (Iharos, 1964) comb. nov. et nom. inq., *U. qinlingensis* (Li et al., 2005) comb. nov., *U. roberti* (Biserov, 1996) comb. nov., *U. ronisvallei* (Binda & Pilato, 1969) comb. nov., *U. rudescri* (Iharos, 1966) comb. nov., *U. septentrionalis* (Thulin, 1928) comb. nov., *U. silviola* (Iharos, 1966) comb. nov. et nom. inq., *U. theresiae* (Iharos, 1964) comb. nov. et nom. inq., *U. torulosus* (Mihelčič, 1959) comb. nov., *U. truncorum* (Iharos, 1964) comb. nov., *U. tucumanensis* (Claps & Rossi, 1984) comb. nov., *U. woodsae* (Kathman, 1990) comb. nov.
Remarks: Within the genus many species need to be revisited, and their diagnoses updated, but, in contrast to Dianea gen. nov., the majority of them seems to be valid. Correct re-analyses of the gibbosity arrangement are crucial for the taxonomy of Ursulinius gen. nov.

Composition of Doryphoribiidae fam. nov.

Genus: *Apodibius* Dastych, 1983

Species: *A. confusus* Dastych, 1983, *A. nuntius* Binda, 1984, *A. richardi* Vargha, 1995

Genus: *Doryphoribius* Pilato, 1969

Species (divided into ecomorphological groups distinguished in this paper):

– two macroplacoids and dorsolateral gibbosities present, terrestrial (the *D. flavus* group): *D. amazzonicus* Lisi, 2011, *D. barbarae* Beasley & Miller, 2012, *D. bindae* Lisi, 2011, *D. dawkinsi* Michalczyk & Kaczmarek, 2010, *D. dupliquaglobulatus* Ito, 1995, *D. flavus* (Iharos, 1966), *D. huangguoshuensis* Wang et al., 2007, *D. maaranguensis* Binda & Pilato, 1995, *D. mcinnesae* Meng et al., 2014, *D. monstruosus* (Maucci, 1991) comb. nov., *D. niedbalai* Zawierucha et al., 2012, *D. picoensis* Fontoura et al., 2008, *D. quadrutuberculatus* Kaczmarek & Michalczyk, 2004, *D. rosanae* Daza et al., 2017, *D. soldiunguis* Lisi, 2011, *D. tessellatus* Meyer, 2011, *D. zyxiglobus* (Horning et al., 1978);

– two macroplacoids and dorsolateral gibbosities present, aquatic (the *D. evelinae* group): *D. evelinae* (Marcus, 1928);

– two macroplacoids present, dorsolateral gibbosities absent, terrestrial (the *D. doryphorus* group = *Doryphoribius* s.s.): *D. doryphorus* (Binda & Pilato, 1969), *D. macrodon* Binda et al., 1980, *D. neglectus* Pilato & Lisi, 2004, *D. pilatoi* Bertolani, 1984, *D. smokiensis* Bartels et al., 2007;

– two macroplacoids present, dorsolateral gibbosities absent, aquatic (the *D. koreanus* group): *D. koreanus* Moon et al., 1994, *D. polymentae* Biserov, 1988, *D. tergumrudis* Bartels et al., 2008;

– three macroplacoids and dorsolateral gibbosities present, terrestrial (the *D. vietnamensis* group): *D. elleneddieei* Haefke et al., 2014, *D. gibber* Beasley & Pilato, 1987, *D. maasaimarenensis* Fontoura et al., 2013, *D. mariae* Pilato & Binda, 1990, *D. minimus* Bartels et al., 2008, *D. vietnamensis* (Iharos, 1969);

– three macroplacoids present, dorsolateral gibbosities absent, terrestrial (the *D. bertolanii* group): *D. bertolanii* Beasley & Pilato, 1987, *D. chetumalensis* Pérez-Pach et al., 2017, *D. korganovae* Biserov, 1994, *D. mexicanus* Beasley et al., 2008, *D. qinlingense* Li et al., 2004, *D. taiwanus* Li & Li, 2008, *D. turkmenicus* Biserov, 1999;

– three macroplacoids present, dorsolateral gibbosities absent, aquatic (the *D. zappalai* group): *D. longistipes* Bartels et al., 2008, *D. zappalai* Pilato, 1971.

Remarks: Currently, *Doryphoribius* comprises the most diverse group of taxa in terms of claw morphology within the entire Isohypsibioidae (see Discussion), which suggests that the genus is polyphyletic (Bertolani et al., 2014a; Cesari et al., 2016; Gąsiorek et al., 2019). In the light of our findings regarding the polyphyly of *Isohypsibioidae*, it seems very likely that *Doryphoribius* may comprise a number of new genera.

Given that *Isohypsibioides monstruosus* Maucci, 1991 exhibits the ventral lamina (fig. 17A), which conforms with the current diagnosis of *Doryphoribius*, the new combination *D.
monstruosus (Maucci, 1991) comb. nov. is proposed. The transfer confirms the supposition of Michalczyk & Kaczmarek (2010), that some Isohypsibius spp. may in fact represent Doryphoribius s.l.

Genus: Grevenius gen. nov.

Species: G. annulatus annulatus (Murray, 1905) comb. nov., G. annulatus minor (Ramazzotti, 1945) comb. nov., G. asper (Murray, 1906) comb. nov., G. baicalensis (Ramazzotti, 1966) comb. nov., G. baldii (Ramazzotti, 1945) comb. nov., G. baldis (Tumanov, 2003) comb. nov., G. brevitubulatus (Rho et al., 1997) comb. nov., G. deconincki (Pilato, 1971) comb. nov., G. deflexus (Mihelčič, 1960) comb. nov. et nom. inq., G. granditintinus (Chang & Rho, 1996) comb. nov., G. granulifer (Thulin, 1928) comb. nov., G. hydrogogianus (Ito & Tagami, 1993) comb. nov., G. irregibilis (Biserov, 1992) comb. nov., G. karenæ (Zawierucha, 2013) comb. nov., G. kenodontis (Kendall-Fite & Nelson, 1996) comb. nov., G. koreanensis (Iharos, 1971) comb. nov. et nom. inq., G. kotovae (Tumanov, 2003) comb. nov., G. kristensenii (Pilato et al., 1989) comb. nov., G. lagodensis (Tumanov, 2003) comb. nov., G. laevis (McInnes, 1995) comb. nov., G. lineatus (Mihelčič, 1969) comb. nov. et nom. inq., G. longiunguis (Pilato, 1974) comb. nov., G. malawiensis (Ito & Tagami, 1993) comb. nov., G. marii (Bertolani, 1981) comb. nov., G. monoicus (Bertolani, 1981) comb. nov., G. myrops (du Bois-Reymond Marcus, 1944) comb. nov., G. nipponicus (Sudzuki, 1975) comb. nov. et nom. inq., G. pulcher (Mihelčič, 1971/72) comb. nov. et nom. inq., G. pushkini (Tumanov, 2003) comb. nov., G. rugosus (Guidi & Grabowski, 1996) comb. nov. et nom. inq., G. rusticus (Pilato et al., 2015) comb. nov., G. sismicus (Maucci, 1978) comb. nov., G. tubereticulatus (Pilato & Catanzaro, 1989) comb. nov., G. verae (Pilato & Catanzaro, 1989) comb. nov., G. zappalai (Pilato et al., 2015) comb. nov.

Remarks: All former aquatic Isohypsibius species are ascribed to the new genus. Although Grevenius gen. nov. comprises taxa dwelling in similar habitats, clear peculiarities can be observed in claw morphology of some limnic and intertidal species (G. brevitubulatus comb. nov., G. deflexus comb. nov., G. granditintinus comb. nov., G. hydrogogianus comb. nov., G. irregibilis comb. nov., G. myrops comb. nov.). Considering that even slight differences in claw anatomy seem to hold very strong phylogenetic signal in Isohypsibioidea, it will not be surprising if these species turn out to represent separate genera when more accurate morphological data and DNA sequences are available.

G. karenæ comb. nov., G. rugosus comb. nov. and G. sismicus comb. nov. were found in hydrophilic substrata, in close vicinities of lakes or ponds, but not strictly in an aquatic habitat. However, their close affinity to Grevenius gen. nov. seems certain, especially that the most similar species to which they were compared in their original descriptions were all exclusively limnic taxa.

Due to morphological differences with G. granulifer comb. nov. (pink body colour and cuticular tubercles of identical size on the whole body in Isohypsibius granulifer koreanensis vs white body colour and cuticular tubercles of different sizes on the dorsum and on the venter in G. granulifer comb. nov.), I. granulifer koreanensis is elevated to a species rank as Grevenius koreanensis comb. nov.

Genus: Paradiphascon Dastych, 1992

Species: P. manningi Dastych, 1992.

Remarks: Given the peculiar apomorphy in the form of flexible pharyngeal tube and disproportionally widened bases of external and posterior claws, the genus requires an integrative redescription to verify its systematic position.

Genus: Pseudobiotus Nelson, 1980
Species: *P. hirsutellus* Pilato et al., 2010, *P. kathmanae* Nelson et al., 1999, *P. longiunguis* (Iha-ros, 1968) sp. dub., *P. matici* (Pilato, 1971), *P. megalonyx* (Thulin, 1928), *P. spinifer* Chang et al., 2007, *P. vladimiri* Biserov et al., 2001.

Remarks: The oldest species in the genus, *P. megalonyx*, requires an integrative redescript- tion to aid species discovery in *Pseudobiotus*.

Genus: *Thulinius* Bertolani, 2003

Species: *T. augusti* (Murray, 1907), *T. itoi* (Tsurusaki, 1980), *T. romanoi* Bertolani et al., 2014, *T. ruffoi* (Bertolani, 1981), *T. saltursus* (Schuster et al., 1978), *T. stephaniae* (Pilato, 1974).

Remarks: The oldest species in the genus, *T. augusti*, requires an integrative redescrip- tion to allow for the verification of alleged nu-

Composition of *Hexapodibidae*

Genus: *Haplohexapodibius* Pilato & Beasley, 1987

Species: *Haplohexapodibius seductor* Pilato & Beasley, 1987.

Genus: *Haplomacrobiotus* May, 1948

Species: *H. hermosillensis* May, 1948, *H. utahensis* Pilato & Beasley, 2005.

Genus: *Hexapodibius* Pilato, 1969

Species: *H. bindae* Pilato, 1982, *H. boothy Dast- tych & McInnes, 1994, *H. christenberryae* Pilato & Binda, 2003, *H. micrornyx* Pilato, 1969, *H. pseudomicrornyx* Robotti, 1972, *H. reginae* Vargha, 1995.

Genus: *Parhexapodibius* Pilato, 1969

Species: *P. bactrianus* Biserov, 1999, *P. castrii* (Ramazzotti, 1964), *P. lagrecai* (Binda & Pilato, 1969), *P. pilatoi* (Bernard, 1977), *P. ramazzottii* Manicardi & Bertolani, 1987

Remarks: Although comparative analyses of *Hallas* (1971) confirmed the validity of *Macro- biotus appelloefi* Richters, 1908 and *Hypsibius geddesi* *Hallas*, 1971, the two species are design- nated as synonyms of *Halobiotus stenostomus* in the current tardigrade species checklist (Degma et al., 2009–18). However, given that both species are insufficiently described, we consider them as awaiting redescriptions to verify the synonymy, staying in agreement with the opinion of the genus authority (R.M. Kristensen, pers. comm.).

*Isohypsibioid genera incertae sedis*

Genus: *Ramajendas* Pilato & Binda, 1990

Species: *R. dastychi* Kaczmarek et al., 2013, *R. frigidus* Pilato & Binda, 1990, *R. heatwolei* Miller et al., 1995, *R. renaudi* (Ramazzotti, 1972).

Genus: *Thalerius* Dastych, 2009

Species: *T. konradi* Dastych, 2009.