An upgraded comprehensive multilocus phylogeny of the Tardigrada tree of life
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INTRODUCTION

Animals’ phylogenies, our understanding of their evolution and implications in our scheme of relationships within the tree of life have increased in the latest decades, due to vast advances in molecular information for phylogenetic studies. On the one hand, exploration of new genetic information has opened the possibility to solve several uncertainties within the tree of life at different node levels (Boeckmann et al., 2015; Burki, 2014; Giribet, 2016a). On the other hand, the inclusion of new organisms’ diversity in selected phyla has returned in novel hypothesis hardly considered before the molecular
era (Aguinaldo et al., 1997; Dunn, Giribet, Edgecombe, & Hejnol, 2014; Giribet, 2016a, 2016b). However, uncertainties do still prevail specially regarding small invertebrates, probably caused by low and/or unappropriate diversity included (Guil & Giribet, 2012). Improvements within phylogenies of neglected phyla will help in inclusion of appropriate representation of internal diversity for each phylum (see Discussions, e.g., in Rokas, Kruger, & Carroll, 2005; Roeding et al., 2007; Dunn et al., 2008; Hejnol et al., 2009; Pick et al., 2010; Giribet, 2016a).

Among those neglected phyla is Tardigrada. Tardigrades comprise ca. 1,200 species (Degma, Bertolani, & Guidetti, 2018) that inhabit terrestrial, freshwater and marine environments in all altitudes and latitudes, from the North Pole to the South Pole, and from the highest peaks to the deepest ocean trenches (Nelson, Guidetti, & Rebecchi, 2015). Three classes organize the phylum classification: Heterotardigrada with ca. 41% of tardigrade diversity, Eutardigrada with ca. 59% of total diversity and Mesotardigrada, a monospecific class which validity has been repeatedly questioned (Grothman et al., 2017; Ramazzotti & Maucci, 1983). The most remarkable characteristic of tardigrades is their ability to survive under extreme terrestrial and extraterrestrial conditions (Guidetti, Altiero, & Rebecchi, 2011; Jönsson, Rabbow, Schill, Harms-Ringdahl, & Retberg, 2008; Møbjerg et al., 2011; Persson et al., 2011; Rebecchi et al., 2009). Their biological and physical characteristics (dispersal and cryptobiotic capabilities, physiological mechanisms, resistance of cuticle for new materials) bestowed a model organism in several fields on them (such as the use of substances and mechanisms involved in their cryptobiosis in Biomedicine; their survival in extreme conditions searching for life in other planets; and solving evolutionary questions; see, e.g., Erdmann & Kaczmarek, 2017; Guil, 2011; Horikawa et al., 2008; Hashimoto et al., 2016). In spite of those potential uses, fundamental questions about tardigrades, such as internal phylogenetic relationships, are still hardly understood. The phylum has been included within the superphylum Ecdysozoa, closely related to arthropods and onychophorans in the majority of more recent molecular phylogenies (Dunn et al., 2014, 2008; Hejnol et al., 2009), although heterotardigrades are poorly represented on those phylogenies (Guil & Giribet, 2012). Both analysed classes (Heterotardigrada and Eutardigrada) have been supported in many studies (Bertolani et al., 2014; Garey, Nelson, Mackey, & Li, 1999; Marley, McInnes, & Chester, 2011; Sands et al., 2008), even though class monophyly has been proven to be outgroup dependent (Guil & Giribet, 2012). In addition, modifications towards a natural classification of tardigrades have been proposed based on molecular phylogenies (Bertolani et al., 2014; Dabert, Dastych, Hohberg, & Dabert, 2014; Guil & Giribet, 2012; Guil, Machordom, & Guidetti, 2013; Marley et al., 2011; Sands et al., 2008).

The main objective of this study is better understanding internal relationship within the Tardigrada phylogeny through a more comprehensive analysis. Secondary objectives will be: (a) evaluate monophyletic status from orders to genera considering classification changes, if needed; (b) provide tardigrade taxa selection for future metazoans’ phylogenies; and (c) infer evolutionary traces of claws in the clawless genus Apodibius and claw reduction by means of the upgraded Tardigrada phylogeny.

2 | MATERIAL AND METHODS

2.1 | Specimens’ collection and identifications

Specimens for this study were obtained from Reinhardt M. Kristensen collection of mosses and lichens housed in the Natural History Museum of Denmark (University of Copenhagen), and Noemi Guil collection of mosses and lichens deposited at the National Museum of Natural History in Madrid (CSIC, Spain), where voucher samples are deposited. Dry moss samples were soaked in water overnight, washed, squeezed and filtered through a 32-μm mesh-size sieve. The filtered product was transferred to a Petri dish for examination under a stereomicroscope. Each specimen was then isolated, and mounted in temporary microscopy slides with distilled water, and identified by light microscopy at the highest possible magnification (100 × objective) using phase contrast and following current taxonomic standards and specific keys (Bertolani et al., 2014; Cesari et al., 2016; Degma et al., 2018; Fontoura & Pilato, 2007; Guidetti & Bertolani, 2005; Guidetti et al., 2016; Guidetti, Schill, Bertolani, Dandekar, & Wolf, 2009; Kaczmarek & Michalczyk, 2017; Kaczmarek, Gawlak, Bartels, Nelson, & Roszkowska, 2017; Kaczmarek, Goldyn, Prokop, & Michalczyk, 2011; Marley et al., 2018, 2011; Michalczyck, Welnicz, Frohme, & Kaczmarek, 2012; Michalczyk & Kaczmarek, 2005, 2010; Tumanov, 2006; Vecchi et al., 2016). In addition, taxonomically relevant structures (cuticle, claws, buccopharyngeal apparatus, eggs when available, etc.; Ramazzotti & Maucci, 1983; Guidetti & Bertolani, 2005; Pilato & Binda, 2010) for each specimen were photographed, recorded and stored.

2.2 | Molecular analyses

Two nuclear ribosomal genes 18S rRNA and 28S rRNA were chosen because they have been proven informative for tardigrade phylogenies in previous analyses (Bertolani et al., 2014; Cesari et al., 2016; Dabert et al., 2014; Guidetti et al., 2016; Guil & Giribet, 2012; Jørgensen, Møbjerg, & Kristensen, 2011; Marley et al., 2011; Sands et al., 2008; Vecchi et al., 2016). DNA was extracted from 45 individuals...
**TABLE 1** List of species and specimens newly sequenced for this study

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<th>Locality</th>
<th>Coordinates</th>
<th>Collection Year</th>
<th>Species</th>
<th>Code</th>
<th>Genbank accession number</th>
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</table>

(Continues)
products were directly sequenced using an automated ABI PRISM 310 Genetic Analyzer. Chromatograms obtained from the sequencer were read, and contigs assembled using the sequence editing software SEQUENCER version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI). Assembled sequences were edited with BioEdit version 2007 (Hall, 1999), to identify fragments based on internal primers and conserved regions, as in a previous work (Guil & Giribet, 2012). All new sequences have been deposited in GenBank under accession numbers MH079453 to MH079475 for 18S rRNA, and MH079494 to MH079516 for 28S rRNA (Tables 1 and Supporting information Table S1).

### 2.3 Phylogenetic analyses

We used available tardigrade sequences in GenBank, coincident with fragments analysed in the present study (Supporting information Table S1), to perform a more comprehensive analysis. We used four outgroups as in Guil and Giribet (2012) (Table 2). Disparity of genetic markers used for phylogenetic analyses of the Tardigrada phylum and taxa with those markers made us to perform three parallel analyses with: (a) 18S rRNA (fragment delimited by primers 18Sa2.0 and 18S 9R), (b) 28S rRNA (fragment delimited by primers 28Sa and 28S 5b) and (c) a combined analysis with specimens where both genes, 18S and 28S, were successfully sequenced (Table 1).

Parallel analyses of maximum likelihood (ML) and Bayesian analyses (BI) were performed. Prior to likelihood analysis, jModeltest 2.1.1 (Darriba, Taboada, Doallo, & Posada, 2012) was executed to choose the best-fit model of nucleotide substitution for each gene (18S and 28S) and combined matrices, under the Akaike information criterion (AIC). For the 18S data set, the model 012343+I+G+F was obtained (with corrections for gamma distributions, proportion of invariable unchanging sites and the equilibrium base frequencies in the sequences are estimated by observing the occurrence in the data). For 28S, the model TIM2+I+G (transition model)

### Table 1 (Continued)

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<th>Locality</th>
<th>Coordinates</th>
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<th>Species</th>
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<td>Tar787 MH079475</td>
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<td><em>Zackenberg, Sydkæret</em></td>
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<td>2004</td>
<td><em>Macrobiotus sp.</em></td>
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</table>

Note. Localities, coordinates, year of collection, species, code in analyses and Genbank accession numbers for each individual and gene are specified.

'aSequences obtained from an embryonated egg.'
was resulted (with corrections for gamma distributions and proportion of invariable unchanging sites). Combined analyses were performed with partition data and their respective model described for each one. ML analyses were conducted using the program IQ‐Tree (Nguyen, Schmidt, Haeseler, & Minh, 2015) in the web server version (https://iqtree.cibiv.univie.ac.at/), adapting model obtained with jModeltest. Nodal support was evaluated with 100 bootstrap replicates.

BI was performed with MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Substitution model was specified in each case with parameters specifications as obtained with MrModeltest (Nylander, 2004) and separated models configured in combined analyses. Burn‐in times were assessed by first running shorter analyses and graphing the Bayesian log likelihoods (LnL); these burn‐in times were subsequently confirmed by comparison with the complete log likelihood graphs of all analyses after 15,000,000 generations. Using Tracer version 1.5, burn‐in times in a log likelihood graphs of all analyses were visualized, discarding 50,000 trees in each analysis. Support for nodes is expressed as posterior probabilities, calculated on a maximum clade credibility tree of the post‐burn‐in sample.

3 | RESULTS

We have sequenced 45 specimens from 26 taxa, obtained from moss and lichen samples collected in 18 localities widely distributed (Table 1 and Supporting information Table S1). This study included a large tardigrade diversity, as it covered over 80% of tardigrade families and subfamilies and 53% of genera (Austeruseus; Trygvadóttir & Kristensen, 2001) were newly sequenced for these molecular analyses (Table 1 and Supporting information Table S1).

ML and BI analyses have been congruent between them irrespective genes used, being BI support stronger than ML bootstraps (Figures 1–3 for 18S; Figures 4,5 for 28S). Analyses combining 18S and 28S complete data sets agreed with analyses including one gene (18S or 28S) (Figure 6). Information from the 18S rRNA solved nodes at different levels within the phylogeny (from classes to genera), while 28S rRNA solved deep (classes) and terminal nodes (genera and groups of genera) but not middle nodes. The two classes (Heterotardigrada and Eutardigrada) were supported with 18S, 28S and combined phylogenies, as well as eutardigrade orders Apochela and Parachela (Figures 1–6). Within Heterotardigrada, only family

### TABLE 2 Genbank accession numbers for outgroups used in analyses

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<th>Genes</th>
<th>18S a2.0-9R</th>
<th>28S a-5b</th>
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<table>
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<th>Taxa level</th>
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<td>Superfamilies</td>
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</table>

| Percentage respect global tardigrade diversity | Genera | 53 | 47 | 59 |
| Subfamilies | 81 | 73 | 100 |
| Families | 85 | 50 | 92 |
| Superfamilies | 100 | 0 | 100 |
Echiniscidae was supported by the three analyses (18S, 28S and combined), and order Echiniscoidea was only recovered with combined analysis (Figures 1–6). Families Halechiniscidae, Echiniscooidae and Echiniscidae were monophyletic. The family Echiniscidae was divided into five phylogenetic lineages despite the data used: (a) *Hypechiniscus*, *Testechiniscus*, *Diploechiniscus* and *Echiniscus*; (b) *Bryodelphax* and *Bryochoerus*; (c) *Acanthechiniscus*, *Antechiniscus*, *Cornechiniscus* and *Proechiniscus*; (d) *Pseudechiniscus* (*P. novaezeelandiae*, *P. facettlais* and *P. suillus*); and (e) *Parechiniscus* (Figures 1, 4 and 6). *Mopsechiniscus* remained in a doubtful position within the family Echiniscidae.

The family Milnesiidae (Apochela, Eutardigrada) showed two phyletic lines (Figure 6): (a) *Milnesium eurystomum* (Spain) with *Milnesium tardigradum* from Denmark, Greenland and Spain, and (b) *Milnesium tardigradum* from Spain. Within parachelans, four phylogenetic lineages corresponding to superfamilies were supported (by 18S rRNA and combined analyses; Figures 2, 3 and 6): Hypsibioidea, Eohypsibioidea, Macrobioitoidea and Isohypsibioidea. At the level of parachelan superfamilies and families, 28S rRNA information showed no resolution (Figure 5). The family Eohypsibiidae confirmed its monophyly incorporating a new genus, *Austeruseus* (Figures 3, 5 and 6). Within

**FIGURE 1** Bayesian phylogram obtained with the nuclear 18S a2.0-9R data set (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information Table S1. Parachelan superfamilies are represented in detail in Figures 2–3. Classes, orders, families, superfamilies, genus and group of genera are indicated. Squares in different grey scales and dot limited squares highlight supported clades at different node levels. Scale bar = number of substitutions/site
FIGURE 2  Bayesian phylogram obtained with the nuclear 18S a2.0-9R for the superfamilies Hypsibioidea and Isohypsibioidea (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information Table S1. Orders, families, subfamilies genus and group of genera are indicated when monophyletic. Squares in different grey scales and dot limited squares highlight supported clades at different node levels. Scale bar = number of substitutions/site
FIGURE 3  Bayesian phylogram obtained with the nuclear 18S a2.0-9R for the superfamilies Macrobiotoidea and Eohypsibioidea (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information Table S1. Orders, families, genus and group of genera are indicated when monophyletic. Squares in different grey scales and dot limited squares highlight supported clades at different node levels. Scale bar = number of substitutions/site.
Macrobiotoidea, three phylogenetic lineages can be detected corresponding to families with the combined analysis (Figure 6): (a) Murrayidae, (b) Adorybiotus (maybe representing the family Richtersiidae, also supported with 28S; Figure 5) and (c) Macrobiotidae. Information with 18S rRNA only showed support to family Murrayidae but not to Richtersiid (Richtersius and Adorybiotus) and Macrobiotidae (due to the inclusion of Eohypsibiidae; Figure 3). The family Macrobiotidae can be subdivided into four phyletic lines (Figure 6): (a) Macrobiotus hufelandi group and Mesobiotus, (b) Macrobiotus furcatus, (c) Minibiotus gumersindoi and (d) Paramacrobiotus with Macrobiotus pallarii. Analysis with 18S data included more biodiversity of species and genera, and five similar phyletic lines were supported (Figure 3): (a) Macrobiotus hufelandi group, (b) Mesobiotus, (c) Macrobiotus furcatus, (d) Paramacrobiotus with Macrobiotus pallarii and (e) Adorybitus with Macrobiotus echinogenitus.

The superfamily Hypsibioidea and the family Isohypsibiidae were divided into five phyletic lines (Figure 6): (a) Doryphoribius zyxiglobus, (b) Isohypsibius prosostomus, (c) Halobiotus with Isohypsibius sp., (d) Apodibius and (e) Eremobiotus. Within Isohypsibioidae, 18S information exhibited low resolution (Figure 2). Contrary, 28S data showed seven phyletic lines similar to those obtained in combined analysis (Figure 5): (a) Doryphoribius zyxiglobus; (b) Doryphoribius flavus; (c) Doryphoribius macrodon; (d) Isohypsibius granulifer; (e) Eremobiotus and Isohypsibius prosostomus; (f) Halobiotus, Isohypsibius species (including I. granulifer), Eremobiotus, Apodibius, Pseudobiotus kathmana and Doryphoribius macrodon; and (g) Thulinius, Isohypsibius species (including I. dastychi), Pseudobitus megalonyx, Haplonacrobiotus and Hexapodibius.

**FIGURE 4** Bayesian phylogram obtained with the nuclear 28S a-5b for the class Heterotardigrada (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information Table S1. Classes, orders, families, genus and group of genera are indicated. Squares in different grey scales and dot limited squares highlight supported clades at different node levels. Scale bar = number of substitutions/site.
Monophyletic status of several genera was questioned (Figures 1–6): Mopsechiniscus, Isohypsibius, Doryphoribius, Richtersius, Macrobiotus, Minibiotus, Hypsibius, Hebesuncus, Acutuncus.

### 4 | DISCUSSION

#### 4.1 Towards a natural classification of Tardigrada

The main purpose of Tardigrada phylogenies has been supporting, modifying or rejecting current tardigrade classification on the phylogenetic basis. We present a more comprehensive Tardigrada phylogeny, which reliability relies on the inclusion of 63 tardigrade genera out of the 119 described (Tables 3 and Supporting information Table S1).

The three classes within Tardigrada (i.e., Heterotardigrada, Mesotardigrada and Eutardigrada) were created at the beginning of the XX century, being Mesotardigrada questioned in several occasions (Grothman et al., 2017; Ramazzotti & Maucci, 1983). Eutardigrada monophyly has also been examined resulting dependent on the selection of out-groups for analyses (Guil & Giribet, 2012). In that study, the order Apochela was independent of class Eutardigrada.
Morphological differences among Tardigrada classes included: presence of appendages over the body, and morphology of claws and buccopharyngeal apparatuses (Bertolani, et al., 2014; Kristensen, 1987; Ramazzotti & Maucci, 1983). Heterotardigrada includes heterotardigrade (marine and terrestrial) claws and buccopharyngeal apparatus (Figure 7a,b) with a great variety of appendages in head and body, while Mesotardigrada shows heterotardigrade (Echiniscoidea) claws (Figure 7a), eutardigrade buccopharyngeal apparatus and cirrus A on head (Kristensen, 1987; Pilato & Binda, 2010; Ramazzotti & Maucci, 1983). Contrary, within the class Eutardigrada can be found claws and buccopharyngeal apparatuses of apochelan and parachelan types (Figure 7c–f), while head appendages are present only in apochelans (peribuccal and cephalic papillae; Figure 8 and Schuster, Nelson, Grigarick, & Christenberry, 1980) (parachelans showed in some cases sense organs but not appendages). So, differences between orders Apochela and Parachela include head appendages and claw morphology used to differentiate classes within Tardigrada. In addition, phylogenetic evidences show strong support to class Heterotardigrada, and current orders Apochela and Parachela (Figure 6). If considering class level as indicated in Figure 6, a new configuration with three classes (and doubtful Mesotardigrada) is evidenced as in other studies (Bertolani et al., 2014; Guidetti et al., 2009; Guil & Giribet, 2012). So, two groups of evidences support the creation of a new class for the current order Apochela: (a) a unique morphology for claws and buccopharyngeal

**FIGURE 6** Bayesian phylogram obtained combining nuclear genes 18S rRNA and 28S rRNA data set (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information Table S1. Classes, orders, families, superfamilies, genus and group of genera are indicated. Squares in different grey scales and dot limited squares highlight supported clades at different node levels. New node level for classes proposed is indicated with a vertical line. Scale bar = number of substitutions/site
apparatus (Figure 7e,f) together with the presence of cephalic appendages (peribuccal and cephalic papillae; Figure 8) and (b) molecular support from Bayesian and likelihood analyses with 18S rRNA and 28S rRNA information (Figure 6). Consequently, we propose a new tardigrade class named Apotardigrada, following the former order name (Apochela) that indicates separate primary and secondary branches on claws. Within this new class Apotardigrada, the order Apochela is included, containing the family Milnesiidae, and genera and species composing this family as specified in Degma et al. (2018). Consequently, the class Eutardigrada diagnosis is amended excluding the cephalic appendages and claws with main and secondary branches separated. Since only parachelans remain within Eutardigrada, we propose to erect current superfamilies (Eohypsibioidae, Macrobiotoidea, Hypsibioidae, Isohypsibioidae, Ramazzottiidae and Calohypsibiidae; Sands et al., 2008; Guil & Giribet, 2012; Bertolani et al., 2014; Guidetti et al., 2016; Vecchi et al., 2016), but also remain open questions that need of further data and analyses to be solved. As an example, Eohypsibiidae confirmed its monophyly, but not Eohypsibioidae (Figure 6), being probably caused by differential biodiversity analysed (Bertolani and Eohypsibius in Bertolani et al., 2014; and Austeruseus with Bertolanius in the present study; Figures...
3, 5 and 6). A second example refers to Adorybiotus, which was tentatively located within Richtersiidae by Guidetti et al. (2016), but its inclusion within Richtersiidae is questioned by their and our results (Figures 3 and 6). Another issue is a possible polyphyletic status of Hypsibioidea (found when analysing individual genes, but not when they are combined) (as previously hypothesized: Kiehl, Dastych, D’Haese, & Greven, 2007; Marley et al., 2011), even when five phylogenetic lineages can be distinguished within Hypsibioidea (Ramazzottiidae, Diphasconinae, Calohypsibiidae, Itaquasconinae with Hypsibus, and Acutuncus; Figure 6).

And finally, two lines can be detected within Isohypsibiidae: the family Isohypsibiidae and Doryphoribius zyxiglobus (maybe a new family). The status of families and subfamilies of the five/seven phyletic lines within Isohypsibiidae (Doryphoribius zyxiglobus, Isohypsibius prosostomus, Halobiotus, Thulinus with Isohypsibius sp. and Apodibius with Ereomobiotus, Pseudobiotus, Doryphoribius macrodon, Isohypsibius sp. and Isohypsibius granulifer; Figures 5 and 6) has to be evaluated.

Heterotardigrada internal classification has been problematic since the first molecular phylogenies, as they did not support the classical classification based on morphological similarities (Bertolani et al., 2014; Fujimoto, Jorgensen, & Hansen, 2017; Guil & Giribet, 2012; Guil, Machordom, et al., 2013; Jorgensen et al., 2011). Few attempts to organize the heterotardigrade classification have been done (Møbjerg, Kristensen, & Jorgensen, 2016) despite to recent phylogenies that contradicted arthrotardigrade and echiniscoidean classifications (Fujimoto et al., 2017; Guil, Machordom, et al., 2013; Jørgensen, Faurby, Hansen, Møbjerg, & Kristensen, 2010). Our results supported five phylogenetic lineages ((a) Hyperchiniscus, Testechiniscus, Diploechiniscus and Echiniscus; (b) Bryodelphax and Bryochoerus; (c) Acanthechiniscus, Cornechiniscus and Proechiniscus; (d) Pseudochiniscus with Mopsechiniscus; and (e) Parechiniscus; Figure 6), also found by other authors with morphological and/or molecular information (Guil & Giribet, 2012; Guil, Machordom, et al., 2013; Jørgensen, 1999; Jørgensen et al., 2011; Kristensen, 1987; Vecchi et al., 2016). Characters differentiating heterotardigrade families included place where claws were inserted (discs, toes, papillae, etc.), presence of certain cephalic appendages and presence of cuticular plates over dorsal and ventral surface (Kristensen, 1987; Møbjerg et al., 2016; Ramazzotti & Maucci, 1983).

Here, we propose a new internal classification for the family Echiniscidae, with subfamilies and tribes (named after type genera) based on plates’ presence and composition and shape of buccal sensory organs. We propose to create three subfamilies (Echiniscinae subfam. n., Pseudoechiniscinae subfam. n. and Parechiniscinae subfam. n.) supported by molecular (Figure 6) and morphological information based on the presence of pseudosegmental and neck plates (see Systematic section for details). Subfamily Echiniscinae subfam. n. is divided into two tribes on the basis of the shape of cirri A, external and internal buccal cirri and phylogenetic information with molecular data (Figure 6): Echinisci tribe n. and Bryodelphaxini tribe n. Three tribes organize internally the subfamily Pseudoechiniscinae subfam. n. based on specific presence of pseudosegmental plates and phylogenetic support with molecular information (Figure 6): Cornechiniscini tribe n., Pseudochiniscini tribe n. and Anthechiniscini tribe n. And two tribes are described within the subfamily Parechiniscinae subfam. n. on the basis of the presence of third median and/or head plate and phylogenetic support with molecular data (Figure 6): Parechiniscini tribe n. and Novechiniscini tribe n. Detailed taxonomic information, composition and diagnosis are available in the Systematics section.

4.2 | Tardigrada representation in broader studies

The use of tardigrades in animal phylogenies is broad but biased towards eutardigrades (especially from Milnesium, Macrobiotus and Hypsibus, see, e.g., Giribet et al., 1996; Dunn et al., 2008; Dunn et al., 2014; Laumer et al., 2015) with scarce use of heterotardigrades (from Pseudochiniscus, Echiniscus, Testechiniscus and Batillipes; Peterson & Eernisse, 2001; Ryu et al., 2007; Yamasaki, Fujimoto, & Miyazaki, 2015). Artefacts obtained with biased diversity (as well as misidentifications) included in phylogenetic analyses, despite molecular data (from fragments to phylogenomics), and its relation with long-branch attraction (LBA) have been previously established (Pick et al., 2010). We propose, based on genetic diversity and our phylogenetic results, at least four biodiversity groups to be included on Metazoan and Ecdysozoa phylogenies: (a) heterotardigrades from the marine order Arthropodaria; (b) heterotardigrades from another more easy-to-find genera, such as the terrestrial Echiniscus (order Echiniscoidea); (c) one apotardigrade (newly created class Apodibida, formerly order Apochela, e.g., Milnesium); and (d) an eutardigrade (e.g., from the new created order Macrobiotoidea, formerly superfamiliy).

4.3 | Evolution of the Clawless Apodibius

Claw morphology is crucial in the tardigrade taxonomy and evolution, in contrast to buccopharyngeal apparatus, used in taxonomy and ecology but of homoplasic evolution (Guil & Sanchez-Moreno, 2013; Guil, Jorgensen, Giribet, & Kristensen, 2013). Evolution of claw reduction within Eutardigrada was proposed from morphology to evolve into two different lineages (former families Calohypsibiidae and Necopinatidae) being strongly criticized (Bertolani & Bisero, 1996; Guil, Jørgensen, et al., 2013; Pilato & Binda, 2010; Pilato, 1969a, 1969b, 1989).
Originally, Calohypsibiidae included five genera and was created on the basis of the calohypsibi type of claw with two phyletic lines: one with normally developed claw (Calohypsibi) and the other with different grades of claw reduction (Parhexapodibi, Hexapodibi, Halomacrobiotus and Halophlobodibi) (Bertolani & Biserov, 1996; Pilato, 1989; Pilato & Binda, 2010). Phylogenetically, it has been demonstrated that former Calohypsibiidae was polyphyletic, with Calohypsibi within Hypsibiidea (Figure 6), and the claw reduced genera within Isohypsibiidea (creating a new family Hexapodibiidae; Cesari et al., 2016). The other lineage, Necopinatidae, was composed by Necopinatum and Apodibi, two claw reduced and clawless genera, respectively (Dabert et al., 2014; Degma et al., 2018; Pilato & Binda, 2010). The assertion of the homoplastic evolution of claw reduction within Eutardigrada was confirmed when supported by redundant information of claw reduction in the eutardigrade morphological phylogeny (Guil, Machordom, et al., 2013).

In this sense, Apodibi inclusion, a clawless genus, within Isohypsibiidea (Figure 6; Dabert et al., 2014) allows hypothesizing its claw evolution from an original iso-hypsibi claws from an iso hypsibiidean ancestor until claw lost in current Apodibi. Claws’ modification in the soil-dwelling Apodibi could be related to its association with soil and related environments, with tiny spaces between soil grains, where a worm-like shape would favour their movement. Hofberg and Lang (2016) related Apodibi to Doryphoribi and Hexapodibi based on ventral lamina presence. However, Apodibi shares phylogenetic lineage with genera without ventral lamina, that is, Pseudobi, Eremobi, Isohypsibi and Thulini within Isohypsibiidea (Figures 2 and 6). Then, ventral lamina presence (Doryphoribi, Hexapodibi, Halomacrobiotus, Apodibi) or absence (Eremobi, Halobi, Isohypsibi, Pseudobi, Thulini) is homoplastic within the Isohypsibiidea clade (Figure 6), confirming a homoplastic evolution of the buccopharyngeal apparatus and its structures (Guil, Machordom, et al., 2013). Maybe, diversification to different feeding habits within distinct phylogenetic lineages, and so homoplastic evolution of the buccopharyngeal apparatus, can be related to guarantee of food roles execution within ecosystems (Guil & Sanchez-Moreno, 2013; Guil, Jørgensen, et al., 2013). These hypotheses, relating claw and buccopharyngeal apparatus evolution with ecology, open a new research line within tardigrades that need of further genetic, developmental, taxonomical and ecological information to be clarified.

5 | SYSTEMATICS

Tardigrada Doyère, 1840

Class Mesotardigrada Rahm, 1937 nomen dubium (diagnosis as in Ramazzotti & Maucci, 1983 and Grothman et al., 2017)


Class Apotardigrada (Schuster et al., 1980) comb. n.

Diagnosis: Papillae around the mouth (peribuccal papillae) and two lateral papillae on the head (cephalic papillae) are present. Claws with completely separated primary and secondary branches. Elongated pharyngeal bulb without placoids.

Composition:

Order Apochela Schuster et al., 1980 (same description as the class)

Family Milnesiidae Ramazzotti, 1962
Type genus: Milnesium Doyère, 1840
Other genera: Bergtrollus, Limmenius, Milnesioides

Class Eutardigrada Marcus, 1927

Diagnosis (amended): Cephalic appendages are absent. Claws with primary and secondary branches fused, very rarely claws are reduced or lost. Pharyngeal bulb has placoids that very rarely are reduced or lost.

Composition: superfamilies elevated to orders; descriptions and composition of orders as in Bertolani et al., 2014; Cesari et al., 2016; Guidetti et al, 2016; Vecchi et al., 2016:

Order Eohypsibiidea Bertolani & Kristensen, 1987 comb. n.
Order Hypsibiidea Pilato, 1969 comb. n.
Order Macrobioidea Thulin, 1928 comb. n.
Order Isohypsibiidea Sands et al., 2008 comb. n.

Class Heterotardigrada Marcus, 1927 (description as in Kristensen, 1987)

Diagnosis: Tardigrada with cephalic, trunk and leg appendages. Gonopore separated from anus. Malpighian tubules lacking. Placoids consisting of three CaCO₃ elements or three delicate, bar-shaped cuticular structures.


Order Arthrotardigrada Marcus, 1927 (classification as in Degma et al., 2018)
Order Echiniscoidea Richters, 1926 (description as in Kristensen, 1987)

Diagnosis: Heterotardigrada without toes on the legs. Median cirrus absent.

Family Echiniscidae Thulin, 1928 (description as in Kristensen, 1987).

Diagnosis: Echiniscoidea without seminal receptacles. Dorsal plates present. Adults with four claws on each leg. Semi-aquatic and terrestrial. Cryptobiosis exhibited by most genera.

Composition:

Subfamily Echiniscinae subfam. n.
Diagnosis: Echiniscidae without pseudosegmental plates.

Tribe Echiniscini tribe n.
Diagnosis: Cirri A are filaments with cirrophores. External and internal buccal cirri with cirrophores.

Tribe Bryodelphaxini tribe n.
Diagnosis: Cirri A are filaments with cirrophores. External and internal buccal cirri without cirrophores.
Composition: Bryodelphax Thulin, 1928 (type genus), Bryochoerus Marcus, 1936.

Subfamily Pseudechiniscinae subfam. n.
Diagnosis: Echiniscidae with pseudosegmental plates.

Tribe Cornechiniscini tribe n.
Diagnosis: Unpaired pseudosegmental plates I’ and III’.

Tribe Pseudechiniscini tribe n.
Diagnosis: Only pseudosegmental plate IV’ present.
Composition: Pseudechiniscus Thulin, 1911 (type genus), Mopschechiniscus du Bois-Reymond Marcus, 1944 (tentatively located in this tribe, waiting for more molecular analyses that will clarify its monophyletic status).

Tribe Anthechiniscini tribe n.
Diagnosis: Present (Paired or unpaired) pseudosegmental plates II’, III’ and IV’.
Composition: Anthechiniscus Kristensen, 1987 (type genus), Multipseudechiniscus Schulte & Miller, 2011.

Subfamily Parechiniscinae subfam. n.
Diagnosis: Neck dorsal plate absent.

Tribe Novechiniscini tribe n.
Diagnosis: Median plate m3 absent.
Composition: Novechiniscus Kristensen, 1987 (type genus).

Tribe Parechiniscini tribe n.
Diagnosis: Head plate absent.
Composition: Parechiniscus Cuénot, 1926 (type genus).

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**SUPPORTING INFORMATION**

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

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