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An upgraded comprehensive multilocus phylogeny of the Tardigrada tree of life

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Abstract

Providing accurate animals' phylogenies rely on increasing knowledge of neglected phyla. Tardigrada diversity evaluated in broad phylogenies (among phyla) is biased towards eutardigrades. A comprehensive phylogeny is demanded to establish the representative diversity and propose a more natural classification of the phylum. So, we have performed multilocus (18S rRNA and 28S rRNA) phylogenies with Bayesian inference and maximum likelihood. We propose the creation of a new class within Tardigrada, erecting the order Apochela (Eutardigrada) as a new Tardigrada class, named Apotardigrada comb. n. Two groups of evidence support its creation: (a) morphological, presence of cephalic appendages, unique morphology for claws (separated branches) and wide-elongated buccopharyngeal apparatus without placoids, and (b) phylogenetic support based on molecular data. Consequently, order Parachela is suppressed and its superfamilies erected as orders within Eutardigrada, maintaining their current names. We propose a new classification within the family Echiniscidae (Echiniscoidea, Heterotardigrada) with morphological and phylogenetic support: (a) subfamily Echiniscinae subfam. n., with two tribes Echiniscini tribe n. and Bryodelphaxini tribe n.; (b) subfamily Pseudechiniscinae subfam. n., with three tribes Cornechiniscini tribe n., Pseudechiniscini tribe n. and Anthechiniscini tribe n.; and (c) subfamily Parechiniscinae subfam. n., with two tribes Parechiniscini tribe n. and Novechiniscini tribe n. Reliable biodiversity selection for tardigrades in broad phylogenies is proposed due to biased analyses performed up to now. We use our comprehensive molecular phylogeny to evaluate the evolution of claws in the clawless genus *Apodibius* and claw reduction across the Tardigrada tree of life. Evolutionary consequences are discussed.

KEYWORDS

classification, clawless evolution, molecular phylogeny, new class, new heterotardigrade subfamilies, Tardigrada

1 | 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, & Hejnal, 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; Hejnal 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, & Rettberg, 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; Hejnal 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- μ m 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 \times 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; Michalczyk, 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

Locality	Coordinates		Collection		Species	Code	Genbank accession number	
							18S rRNA	28S rRNA
Arctic Station, Disko Island, Greenland	N 69°19'	W 54°04'	2004		<i>Dactylobiotus ambiguus</i>	TarCPH_7		MH079500
			2004		<i>Diaforobiotus islandicus</i>	TarCPH_23		MH079486
			2004		<i>Hypsibius cf. dujardini</i>	TarCPH_13		MH079506
			2004		<i>Macrobiotus echinogenitus</i>	TarCPH_16	MH079460	MH079513
			2004		<i>Macrobiotus grandis</i>	TarCPH_17		MH079490
			2004		<i>Mesobiotus harmsworthi</i>	TarCPH_18	MH079462	MH079489
			2004		<i>Murrayon pullari</i>	TarCPH_29		MH079499
			2009		<i>Milnesium cf. tardigradum</i>	TarCPH_27	MH079453	MH079477
			2009		<i>Milnesium cf. tardigradum</i>	Tar758	MH079455	MH079478
2009		<i>Milnesium cf. tardigradum</i>	Tar767	MH079456	MH079480			
Bear cave, Crete	N 35°35'	E 24°08'	2004		<i>Paramacrobiotus richtersi</i>	TarCPH_25	MH079461	MH079493
Bergen, Norway	N 60°23'	E 05°19'	2009		<i>Milnesium cf. tardigradum</i>	Tar793	MH079454	
Eysturoy, Faroe Islands	N 62°16'	W 07°00'	2003		<i>Austeruseus faeroensis</i>	TarCPH_4	MH079470	MH079481
Godhavn, Greenland	N 69°14'	W 53°32'	2004		<i>Ramazzottius cataphractus</i>	TarCPH_30		MH079508
Helsingør, Denmark	N 56°02'	E 12°36'	2009		<i>Halobiotus crispae</i>	Tar789		MH079516
Ikka Fjord, South Greenland	N 61°12'	W 48°00'	2007		<i>Bertolanius weglarskae</i>	TarCPH_1	MH079469	
Ivittuut, Greenland	N 61°12'	W 48°10'	2007		<i>Adorybiotus granulatus</i>	Tar720	MH079463	MH079494
			2007		<i>Adorybiotus granulatus</i>	Tar721	MH079464	MH079495
			2007		<i>Adorybiotus granulatus</i>	Tar722	MH079465	MH079496
			2007		<i>Adorybiotus granulatus</i> ^a	Tar723	MH079466	MH079497
			2007		<i>Adorybiotus granulatus</i> ^a	Tar724	MH079467	MH079498
Madrid, Spain	N 40°45'	W 4°01'	2008		<i>Milnesium eurytostomum</i>	Tar745	MH079457	MH079476
			2008		<i>Milnesium eurytostomum</i>	Tar746	MH079459	MH079511
			2008		<i>Milnesium eurytostomum</i>	Tar757	MH079458	MH079512
Milodon Cave, Patagonia, Chile	S 51°34'	W 72°37'	2004		<i>Macrobiotus hufelandi</i>	TarCPH_19		MH079484
			2004		<i>Mesobiotus liviae</i>	TarCPH_24		MH079488
			2003		<i>Macrobiotus furcatus</i>	TarCPH_28	MH079468	MH079492
Nivå, Denmark	N 55°56'	E 12°30'	2004		<i>Isohypsibius prosostomus</i>	TarCPH_14		MH079509
			2004		<i>Macrobiotus hufelandi</i>	TarCPH_21		MH079487
Øland, Sweden	N 56°44'	E 16°40'	2004		<i>Ramazzottius oberhaeuseri</i>	TarCPH_31	MH079474	MH079507
Østerlien, Disko Island, Greenland	N 69°15'	W 53°31'	2009		<i>Milnesium cf. tardigradum</i>	Tar763		MH079479
Røen Sø, Disko Island, Greenland	N 69°15'	W 53°31'	2004		<i>Adropion prorsirostre</i>	TarCPH_10		MH079514
			2004		<i>Astatumen trinacriae</i>	TarCPH_5		MH079515
			2005		<i>Calohypsibius ornatus</i>	Tar778		MH079502
			2005		<i>Calohypsibius ornatus</i>	Tar779		MH079503
			2005		<i>Calohypsibius ornatus</i>	Tar795	MH079472	MH079504
			2005		<i>Calohypsibius ornatus</i>	Tar801	MH079471	MH079505
			2004		<i>Disphascon pingue</i>	TarCPH_9	MH079473	
			2004		<i>Macrobiotus hufelandi</i>	TarCPH_20		MH079485

(Continues)

TABLE 1 (Continued)

Locality	Coordinates		Collection		Code	Genbank accession number	
			Year	Species		18S rRNA	28S rRNA
Svaneke, Denmark	N 55°08'	E 15°08'	2004	<i>Astatumen trinacriae</i>	TarCPH_6		MH079501
Vellerup, Denmark	N 55°14'	E 11°51'	2005	<i>Halobiotus crispae</i>	TarCPH_12		MH079510
Wombeyan caves, Australia	S 34°19'	E 149°59'	1996	<i>Doryphoribius zyxiglobus</i>	Tar787	MH079475	
				<i>Doryphoribius zyxiglobus</i>	Tar788		MH079483
Zackenbergl, Sydkæret,	N 74°30'	W 20°30'	2004	<i>Macrobiotus</i> sp.	TarCPH_22		MH079491
Greenland			2004	<i>Doryphoribius macrodon</i>	TarCPH_11		MH079482

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.

(Table 1) with the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol (including the 10-min incubation at 72°C after adding Buffer AL, currently deleted from manufacturer protocol), and re-suspended in 100 µl of ddH₂O, as described by Guil and Giribet (2009).

A fragment from the nuclear ribosomal 18S rRNA (663–706 bp depending on the species), which showed most of the genetic variation in previous tardigrades analyses, was amplified using the universal primer pair 18S a2.0 (5'-ATG GTT GCA AAG CTG AAA C-3'; Whiting, Carpenter, Wheeler, & Wheeler, 1997) and 18S 9R (5'-GAT CCT TCC GCA GGT TCA CCT AC-3'; Giribet, Carranza, Bagaña, Riutort, & Ribera, 1996). Amplifications were performed in a 22 µl volume of a solution containing 14 µl of ddH₂O, 1 µl of 10× polymerase chain reaction buffer, 2 µl of dNTP's mix (10 mM), 1.0 µl of each primer (100 µM), 0.1 µl of AmpliTaq[®] DNA polymerase (Applied Biosystems) and 3.0 µl of DNA template. The PCR protocol developed to amplify the 18S rRNA fragments consisted of an initial denaturing step at 94°C for 5 min, 35 amplification cycles (94°C for 10 s, 42–45°C—depending on taxon—for 30 s and 72°C for 30 s), a final elongation step of 7 min at 72°C, and a rapid thermal ramp to 4°C. A fragment of the nuclear ribosomal 28S rRNA (1,344–1,446 bp depending on the species) was amplified using the pair of universal primers: 28Sa (5'-GAC CCG TCT TGA AAC ACG GA-3'; Whiting et al., 1997) and 28Srd5b (5'-CCA CAG CGC CAG TTC TGC TTAC-3'; Schwendinger & Giribet, 2005). Amplifications were performed as for 18S rRNA. All PCR products were checked for the presence of amplicons of the expected size on a 1.0% agarose gel electrophoresis. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) using the manufacturer's protocols. Fragments successfully sequenced for each taxon and specimen are shown in Table 1.

Cycle sequencing with AmpliTaq DNA polymerase was as described by Guil and Giribet (2012). Cycle-sequenced products were cleaned using a standard protocol with ethanol, sodium acetate and formamide. The BigDye[®]-labelled

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 SEQUENCHER 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 18S a2.0 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)

Taxa	Species	Genes	
		18S a2.0-9R	28S a-5b
Arthropoda			
Mandibulata			
Pancrustacea			
	<i>Allacma fusca</i>	EU368610	EU376054
Myriapoda			
	<i>Dendrothereua homa</i>	FJ660705	FJ660746
Arthropoda			
Chelicerata			
Xyphosura			
	<i>Limulus polyphemus</i>	M20083	M20084
Priapulida			
	<i>Priapulidus caudatus</i>	AF025927	AY210840

TABLE 2 Genbank accession numbers for outgroups used in analyses

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 (Table 3), making relevant conclusions achieved. Sequenced eutardigrade species represent all eutardigrade superfamilies, 92% of families and 59% genera (Table 3 and Supporting information Table S1). Seven species and one

genus (*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 3 Diversity of genera, subfamilies, families and superfamilies analysed are presented globally and by class (Heterotardigrada and Eutardigrada) in absolute numbers; percentages were included respect total diversity within Tardigrada, following Degma et al. (2018) and more recent taxonomic changes (Cesari et al., 2016; Guidetti et al., 2016; Vecchi et al., 2016)

Taxa level	Total	Heterotardigrada	Eutardigrada
Absolute numbers			
Genera	63	27	36
Subfamilies	13	8	5
Families	17	6	11
Superfamilies	4	0	4
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, Echiniscoididae 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 eurytomum* (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, Macrobiotoida 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

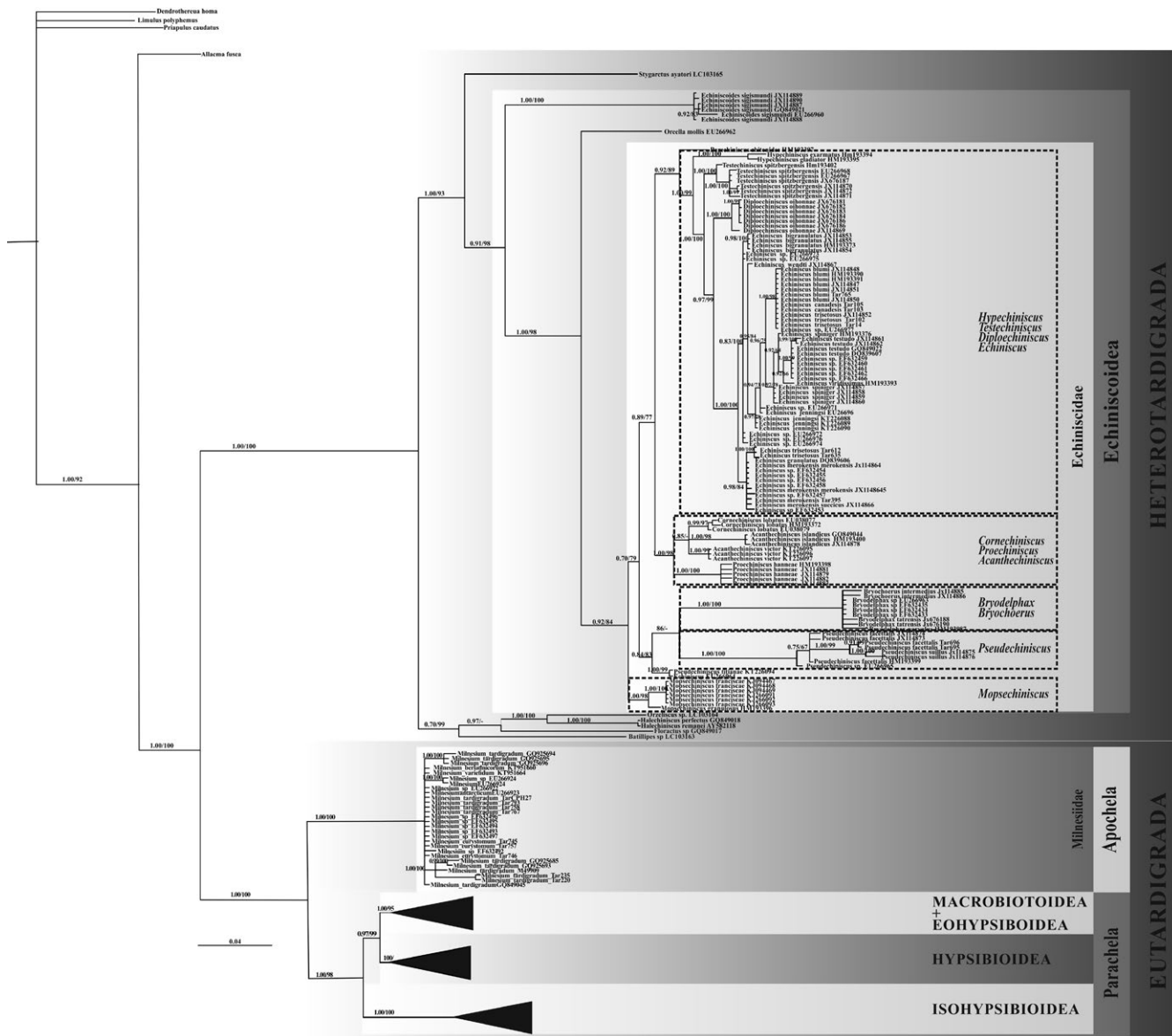


FIGURE 1 Bayesian phylogram obtained with the nuclear 18S a2.0-9R data set (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information Table S1. Parachelan 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

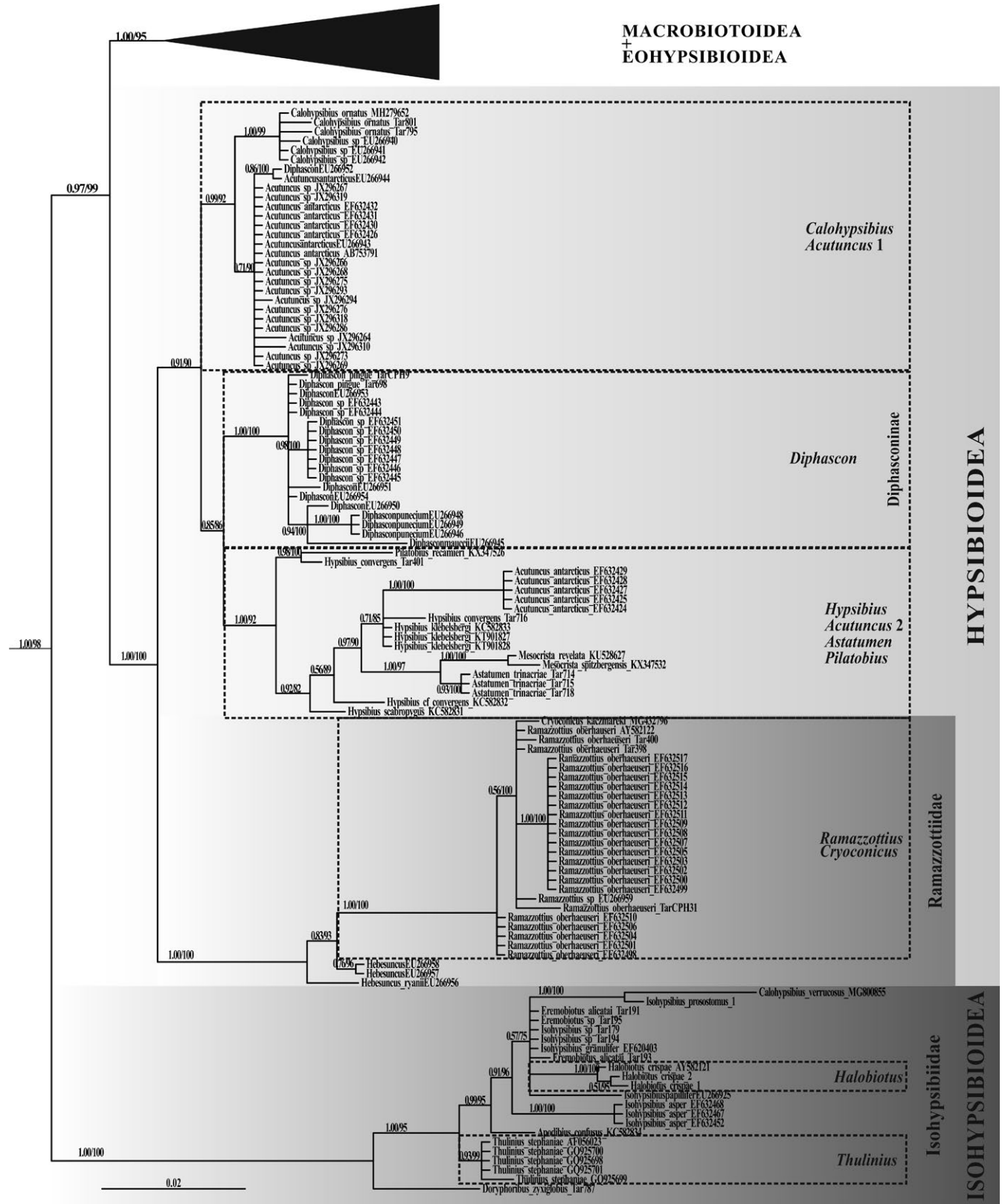


FIGURE 2 Bayesian phylogram obtained with the nuclear 18S a2.0-9R for the superfamilies Hypsibioidea and Isohypsibioidea (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following 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

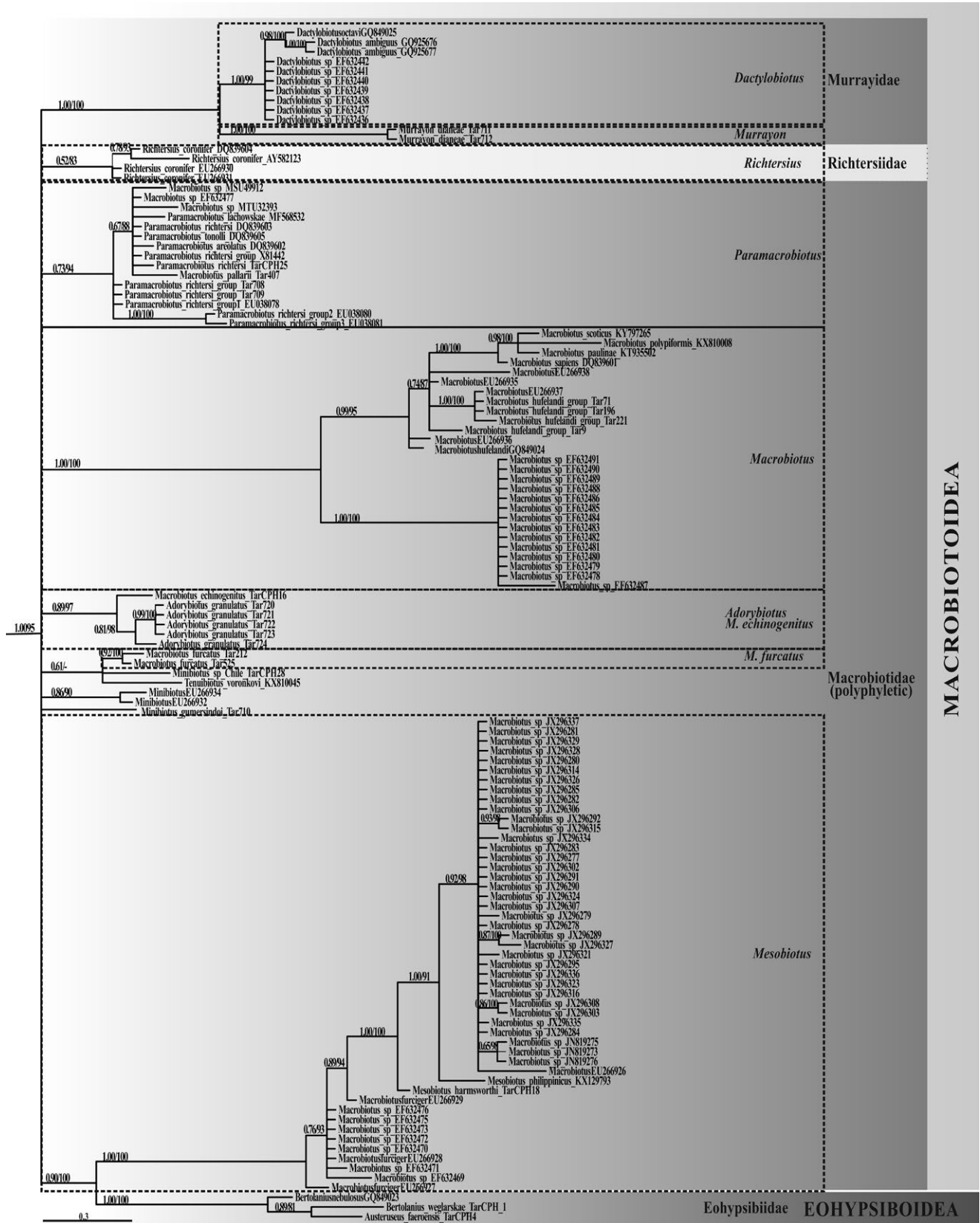


FIGURE 3 Bayesian phylogram obtained with the nuclear 18S a2.0-9R for the superfamilies Macrobiotoidea and Eohypsiboidea (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following 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

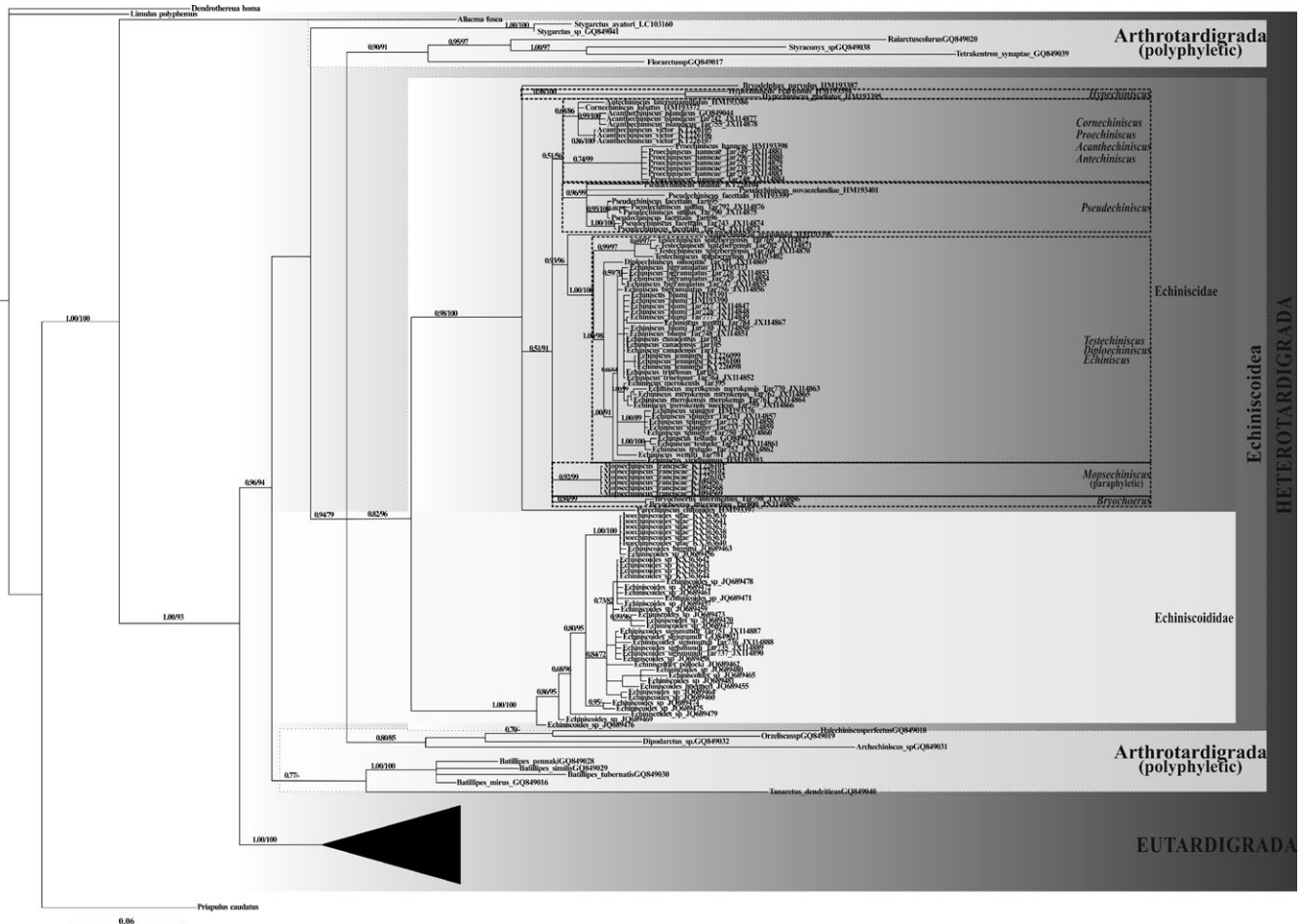


FIGURE 4 Bayesian phylogram obtained with the nuclear 28S a-5b for the class Heterotardigrada (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information 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

Macrobiotoidae, 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 Richtersiidae (*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 Hypsibioidae was divided into five clades with combined analysis (Figure 6): (a) Ramazzottiidae, (b) Diphascioninae, (c) Calohypsibiidae, (d) subfamily

Itaquaconinae with *Hypsibius* and (e) *Acutuncus*. When considering 18S data, similarly four phyletic lines were supported (Figure 2): (a) Ramazzottiidae, (b) Diphascioninae, (c) Calohypsibiidae with *Acutuncus* and (d) subfamily Itaquaconinae with *Hypsibius* and other *Acutuncus* specimens.

The superfamily Isohypsibioidae 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 kathmanae* and *Doryphoribius macrodon*; and (g) *Thulinus*, *Isohypsibius* species (including *I. dastychi*), *Pseudobiotus megalonyx*, *Haplomacrobiotus* and *Hexapodibius*.

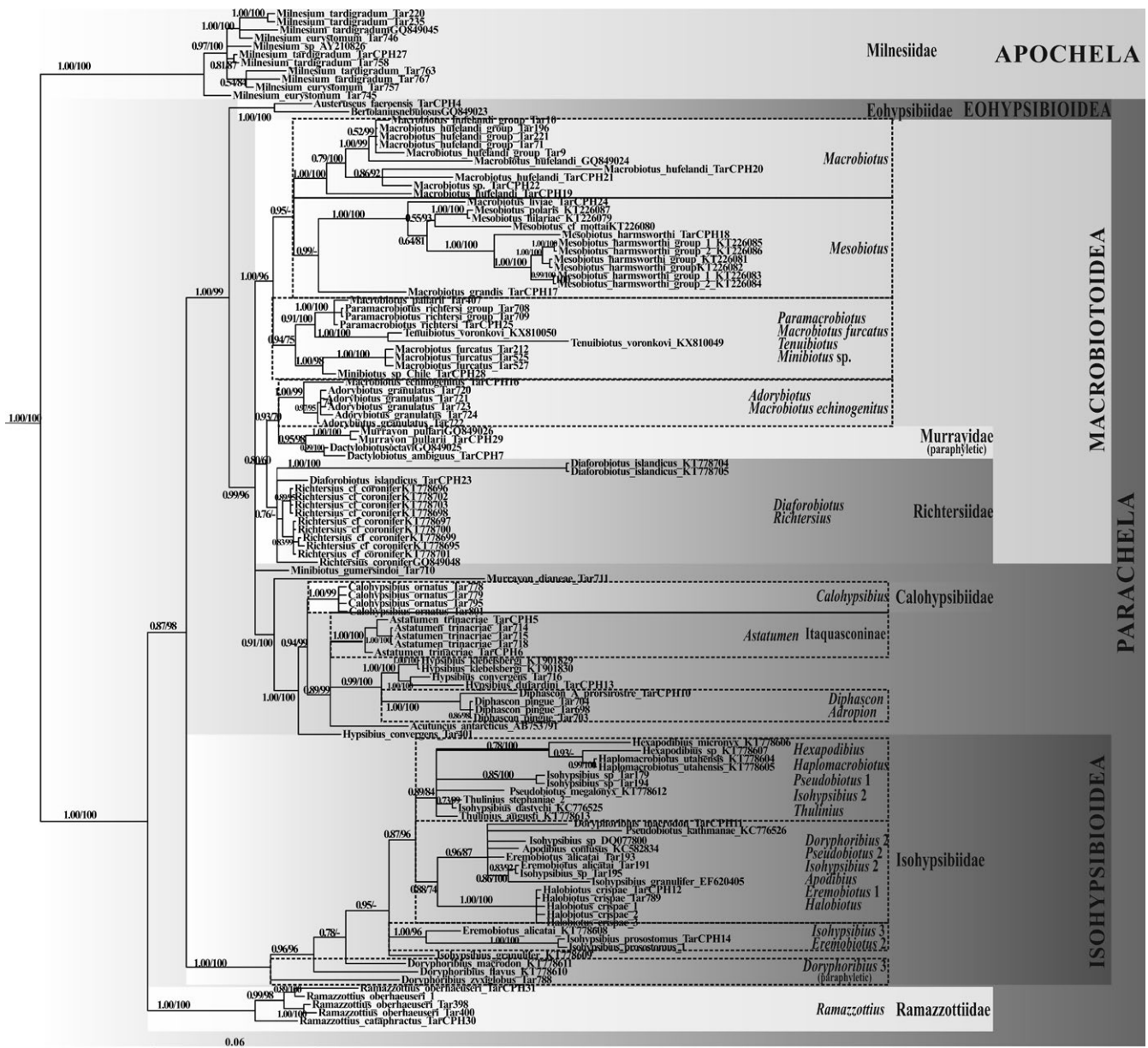


FIGURE 5 Bayesian phylogram obtained with the nuclear 28S a-5b for the class Eutardigrada (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information Table S1. Orders, superfamilies, 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 & Muccii, 1983). Eutardigrada monophyly has also been examined resulting dependent on the selection of outgroups for analyses (Guil & Giribet, 2012). In that study, the order Apochelela was independent of class Eutardigrada.

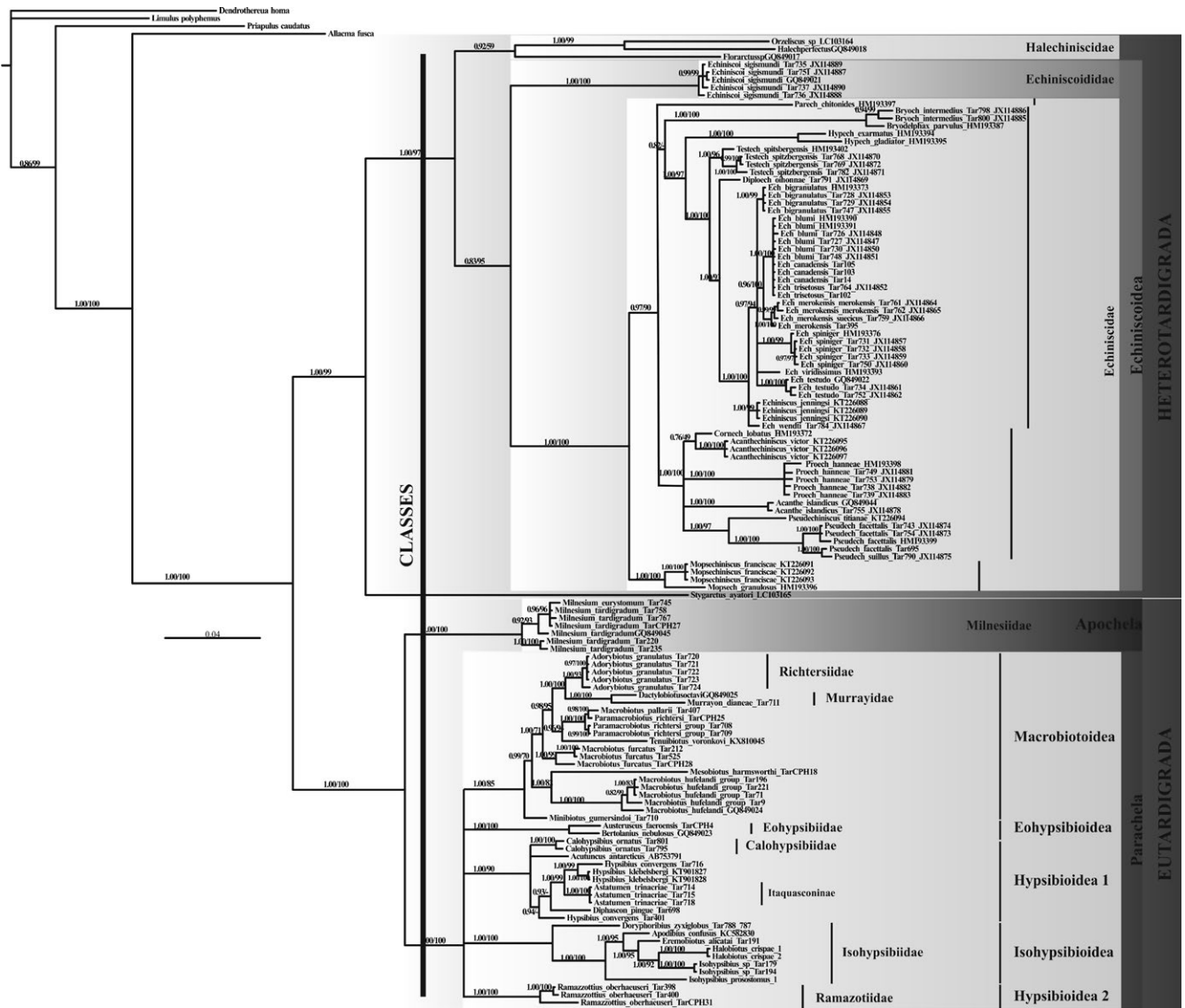


FIGURE 6 Bayesian phylogram obtained combining nuclear genes 18S rRNA and 28S rRNA data set (Supporting information Table S1). First number above branches is posterior probabilities obtained in the BI. Second number is bootstrap support values from ML. Taxa are named following Supporting information 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

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

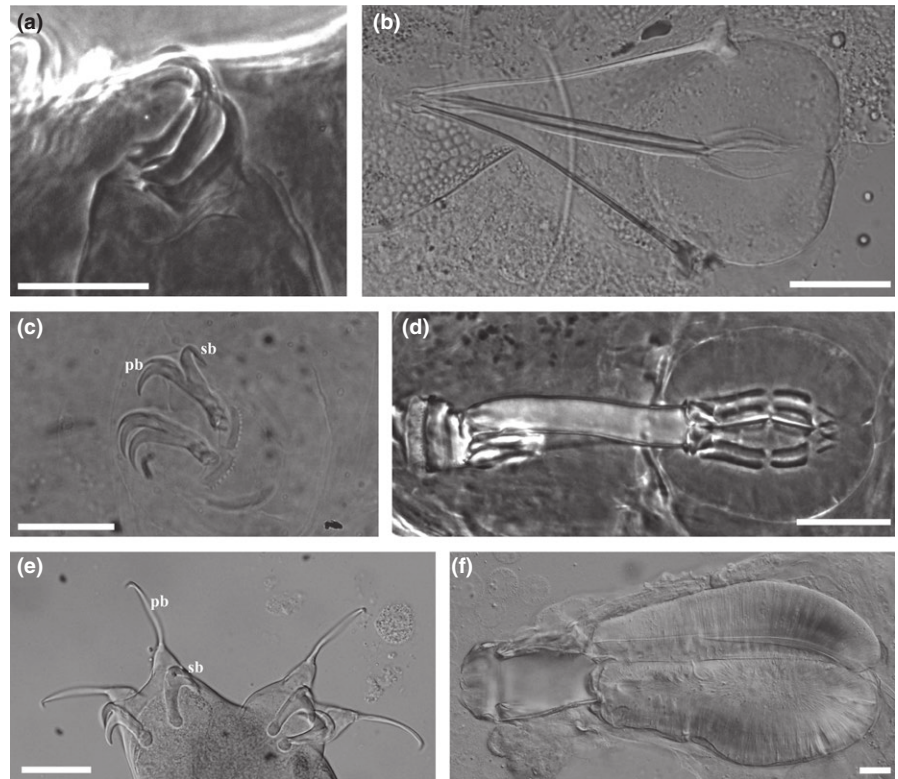


FIGURE 7 Images from optical microscope with phase contrast of: (a, b) claws and buccopharyngeal apparatus, respectively, of *Echiniscus blumi* representing the class Heterotardigrada; (c, d) claws and buccopharyngeal apparatus of *Richtersius coronifer* and *Macrobiotus terminalis*, respectively, representing the order Parachela of the class Eutardigrada; and (e, f) claws and buccopharyngeal apparatus, respectively, of *Milnesium tardigradum*, representing the order Apochela of the class Eutardigrada. Scale bar = 20 μm

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 (Eohypsibioidea, Macrobiotioidea, Hypsibioidea, Isohypsibioidea) as orders within the class and suppression of order Parachela. Detailed taxonomic information is available in the Systematics section. Composition and diagnosis for former superfamilies (now orders) and families are as in Bertolani et al. (2014), Cesari et al. (2016), Guidetti et al. (2016) and Vecchi et al. (2016).

Internal relationships in Parachela confirmed clades as in other studies (e.g., Murrayidae, Macrobiotidae, Richtersiidae, Eohypsibiidae, Isohypsibiidae, 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 Eohypsibioidea (Figure 6),

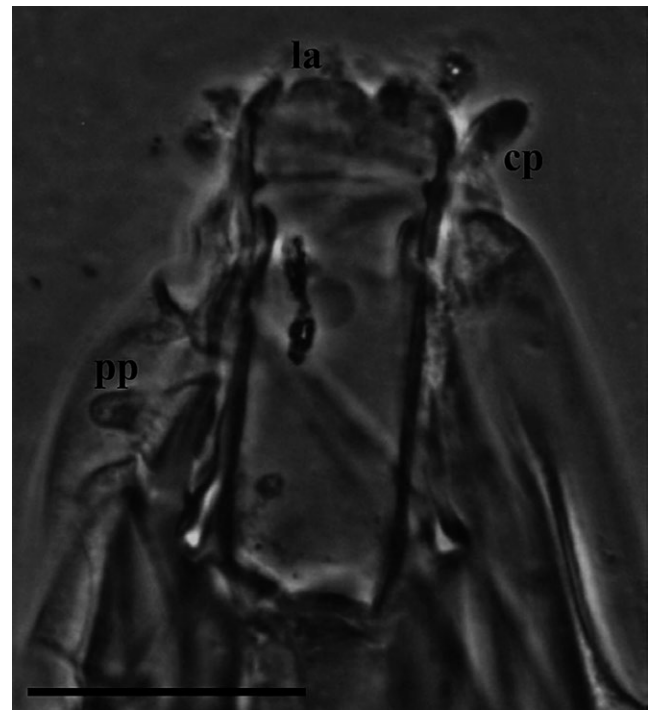


FIGURE 8 Image from optical microscope with phase contrast of *Milnesium tardigradum* head showing: **la**, lamellae; **cp**, cephalic papillae; and **pp**, peribuccal lamellae. Scale bar = 20 μm

being probably caused by differential biodiversity analysed (*Bertolanius* 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 *Hypsibius*, and *Acutuncus*; Figure 6). And finally, two lines can be detected within Isohypsibioidea: 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 *Eremobiotus*, *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, Jørgensen, & Hansen, 2017; Guil & Giribet, 2012; Guil, Machordom, et al., 2013; Jørgensen et al., 2011). Few attempts to organize the heterotardigrade classification have been done (Møbjerg, Kristensen, & Jørgensen, 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) *Hypechiniscus*, *Testechiniscus*, *Diploechiniscus* and *Echiniscus*; (b) *Bryodelphax* and *Bryochoerus*; (c) *Acanthechiniscus*, *Cornechiniscus* and *Proechiniscus*; (d) *Pseudechiniscus* 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., Pseudechiniscinae 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): Echiniscini tribe n. and Bryodelphaxini tribe n. Three tribes organize internally the subfamily Pseudechiniscinae subfam. n. based on specific presence of pseudosegmental plates and phylogenetic support with molecular information (Figure 6): Cornechiniscini tribe n., Pseudechiniscini 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 *Hypsibius*, 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 *Pseudechiniscus*, *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 Arthrotardigrada; (b) heterotardigrades from another more easy-to-find genera, such as the terrestrial *Echiniscus* (order Echiniscoidea); (c) one apotardigrade (newly created class Apotardigrada, formerly order Apochela, e.g., *Milnesium*); and (d) an eutardigrade (e.g., from the new created order Macrobiozoidea, formerly superfamily).

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 homoplastic evolution (Guil & Sanchez-Moreno, 2013; Guil, Jørgensen, 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 & Biserov, 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 *calohypsibius* type of claw with two phyletic lines: one with normally developed claw (*Calohypsibius*) and the other with different grades of claw reduction (*Parhexapodibius*, *Hexapodibius*, *Haplomacrobotus* and *Haplohexapodibius*) (Bertolani & Biserov, 1996; Pilato, 1989; Pilato & Binda, 2010). Phylogenetically, it has been demonstrated that former Calohypsibiidae was polyphyletic, with *Calohypsibius* within Hypsibioidea (Figure 6), and the claw reduced genera within Isohypsibioidea (creating a new family Hexapodibiidae; Cesari et al., 2016). The other lineage, Necopinatidae, was composed by *Necopinatum* and *Apodibius*, 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, *Apodibius* inclusion, a clawless genus, within Isohypsibioidea (Figure 6; Dabert et al., 2014) allows hypothesizing its claw evolution from an original *isohypsibius* claws from an isohypsibioidean ancestor until claw lost in current *Apodibius*. Claws' modification in the soil-dwelling *Apodibius* 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. Hohberg and Lang (2016) related *Apodibius* to *Doryphoribius* and *Hexapodibius* based on ventral lamina presence. However, *Apodibius* shares phylogenetic lineage with genera without ventral lamina, that is, *Pseudobiotus*, *Eremobiotus*, *Isohypsibius* and *Thulinus* within Isohypsibioidea (Figures 2 and 6). Then, ventral lamina presence (*Doryphoribius*, *Hexapodibus*, *Haplomacrobotus*, *Apodibius*) or absence (*Eremobiotus*, *Halobiotus*, *Isohypsibius*, *Pseudobiotus*, *Thulinus*) is homoplastic within the Isohypsibioidea 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)

Diagnosis: Cirri A present. With heterotardigrada-like spines. Heterotardigrade-like claws with no differentiation in main and secondary branches. Pharyngeal bulb with Eutardigrada-like macroplacoids.

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 **Eohypsibioidea** Bertolani & Kristensen, 1987 comb. n.

Order **Hypsibioidea** Pilato, 1969 comb. n.

Order **Macrobotoidea** Thulin, 1928 comb. n.

Order **Isohypsibioidea** 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.

Composition: Taxonomic accounts and classification as in Kristensen, 1987, Degma et al., 2018, and Fontoura, Bartels, Jørgensen, Kristensen, & Hansen, 2017.

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 cirriphores. External and internal buccal cirri with cirriphores.

Composition: *Echiniscus* C.A.S. Schultze, 1840 (type genus), *Diploechiniscus* Vicente et al., 2013, *Testechiniscus* Kristensen, 1987, *Hypechiniscus* Thulin, 1928.

Tribe **Bryodelphaxini** tribe n.

Diagnosis: Cirri A are filaments with cirriphores. External and internal buccal cirri without cirriphores.

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'.

Composition: *Cornechiniscus* Maucci & Ramazzotti, 1981 (type genus), *Acanthechiniscus* Vecchi et al., 2016, *Proechiniscus* Kristensen, 1987.

Tribe **Pseudechiniscini** tribe n.

Diagnosis: Only pseudosegmental plate IV' present.

Composition: *Pseudechiniscus* Thulin, 1911 (type genus), *Mopsechiniscus* 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: *Antechiniscus* 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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