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Congruence between molecular phylogeny and cuticular design in Echiniscoidea (Tardigrada, Heterotardigrada)

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Although morphological characters distinguishing echiniscid genera and species are well understood, the phylogenetic relationships of these taxa are not well established. We thus investigated the phylogeny of Echiniscidae, assessed the monophyly of *Echiniscus*, and explored the value of cuticular ornamentation as a phylogenetic character within *Echiniscus*. To do this, DNA was extracted from single individuals for multiple *Echiniscus* species, and 18S and 28S rRNA gene fragments were sequenced. Each specimen was photographed, and published in an open database prior to DNA extraction, to make morphological evidence available for future inquiries. An updated phylogeny of the class Heterotardigrada is provided, and conflict between the obtained molecular trees and the distribution of dorsal plates among echiniscid genera is highlighted. The monophyly of *Echiniscus* was corroborated by the data, with the recent genus *Diploechiniscus* inferred as its sister group, and *Testechiniscus* as the sister group of this assemblage. Three groups that closely correspond to specific types of cuticular design in *Echiniscus* have been found with a parsimony network constructed with 18S rRNA data.

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ADDITIONAL KEYWORDS: 18S rRNA – 28S rRNA – Bayesian – cladistics – DNA taxonomy – Echiniscidae – morphology – parsimony network.

INTRODUCTION

Tardigrades are among the smallest metazoans and one of the least understood animal phyla – from a phylogenetic perspective. However, few studies have compared explicitly results from morphology-based phylogenies with those of molecular-based trees (Møbjerg *et al*., 2007; Cesari *et al*., 2009, 2011a, b; Guil & Giribet, 2009; Bertolani *et al*., 2010, 2011; Jørgensen, Møbjerg & Kristensen, 2011; Guil, Machordom & Guidetti, 2013). In addition, and due to their diminutive size, molecular phylogenies of tardigrades have often been based on DNA extractions from pooled individuals (e.g. Garey *et al*., 1996, 1999; Møbjerg *et al*., 2007; Jørgensen *et al*., 2010, 2011).

Classifications based solely on morphological characters have been corroborated by molecular analyses in many groups of organisms, but disagreement also exists (see Funk & Omland, 2003; Rheindt *et al*., 2011). DNA-based taxonomy can complement traditional (morphological) taxonomy, aiding in the

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discovery and characterization of cryptic species as well as in identifying phenotypic plasticity. Morphological and molecular conflict in phylogenies can be explained by uninformative molecular markers, homogeneous morphologies, and/or homoplasy (Funk & Omland, 2003). Even in well-studied animal groups such as vertebrates, lack of congruence between morphological and molecular data is not uncommon (e.g. Near, 2009; Losos, Hillis & Greene, 2012), and such conflict can be rampant in lesser-known organisms. Approaches attempting to reconcile the conflict between molecular and morphological characters in tardigrades are scarce, to put it mildly.

Echiniscus (Heterotardigrada, Echiniscoidea, Echiniscidae) is the second most diverse genus of tardigrades, after *Macrobiotus* (Eutardigrada, Macrobiotidae), including almost 15% of the total tardigrade species diversity (Guidetti & Bertolani, 2005; Degma & Guidetti, 2007; Degma, Bertolani & Guidetti, 2013). *Echiniscus* species are recognized morphologically on the basis of differentiation in cuticle design and shape and distribution of cuticular 'appendages' (Ramazzotti & Maucci, 1983; Kristensen, 1987). However, the pattern and number of lateral body appendages, as traditionally used for species differentiation within the *Echiniscus blumi–canadensis* complex, have been shown to conflict with molecular hypotheses (Guil & Giribet, 2009). Conflict between molecules and morphology has also been reported at higher taxonomic levels when comparing genera within Echiniscidae (Jørgensen *et al*., 2011).

The objectives of this study are thus: (1) to provide an updated phylogeny of Heterotardigrada to have a well-established framework for the study of the genus *Echiniscus*; (2) to test the monophyly of *Echiniscus*; and (3) to evaluate internal relationships within *Echiniscus* and the validity of the traditional species groups based on cuticular characters.

MATERIAL AND METHODS

Sampling

Specimens for this study were obtained from the Reinhardt M. Kristensen collection of mosses housed in the Natural History Museum of Denmark (University of Copenhagen). 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.

To date, few studies focusing on tardigrades have generated molecular and morphological data for the same specimens (but see Cesari *et al*., 2011b, 2013). In all cases, no parts from the extracted specimen remain, as the small size of tardigrades makes it necessary to use the whole animal for DNA extraction, although in some cases the egg cases are left as vouchers (Cesari *et al*., 2011a). Photographing the specimens prior to DNA extraction becomes the only feasible solution to link genetic and anatomical data, as done by Cesari *et al*. (2011b, 2013). While many authors provide identifications of each individual used for DNA extraction, especially when multiple species coexist in a sample (Cesari *et al*., 2009; Guil & Giribet, 2009, 2012; Bertolani *et al*., 2010, 2011), photographs of specimens preceding DNA extraction become the only unequivocal link between DNA sequences and morphology. For this study each specimen was mounted in a temporary slide in distilled water and identified by light microscopy at the highest possible magnification (100× objective) using phase contrast and following current taxonomic standards (Guidetti & Bertolani, 2005; Marley, McInnes & Sands, 2011). In addition, taxonomically relevant structures (cuticle, claws, buccopharyngeal apparatus, etc.; Ramazzotti & Maucci, 1983; Guidetti & Bertolani, 2005) from each specimen were photographed, recorded, and stored for future morphological queries (to avoid misidentification problems, as previously reported by Guil & Giribet, 2009). MorphoBank (http://www.morphobank.org/) (O'Leary & Kaufman, 2012) is a public database that stores images related to taxonomy and phylogeny, where each image receives an accession number that can then be linked to publications and to the specific sequence data stored in GenBank. Photographic data for each sequenced *Echiniscus* specimen (as well as for other echiniscoidean specimens) in the present study have been deposited in MorphoBank with accession numbers M148490–M148655 (Project number 785).

The temporary slide mounts were dismantled under a stereoscope in clean conditions after identification, and individuals – usually broken due to slide cover pressure – as well as free disaggregated cells were recovered with a clean glass pipette, and transferred into a sterile tube for subsequent DNA extraction. Whenever possible, more than one individual (Tables 1 and 2) was extracted and sequenced per species, on different days, to avoid cross-contamination. If available, multiple specimens were sequenced for species not previously studied molecularly, and from multiple localities, to reflect some of the variability of the species.

The *Echiniscus* sequences studied included up to 11 species (newly sequenced and from GenBank; Table 1), collected in localities around the world, and including five types of cuticle traditionally used to cluster *Echiniscus* species (Ramazzotti & Maucci, 1983; Peluffo, Moly de Peluffo & Rocha, 2002;

Table 1. Species (newly sequenced specimens in bold) of the genus *Echiniscus* and their localities, when available, analysed in the present study

	Species	Code	Locality	Date	Coordinates		18S rRNA	28S rRNA	REFs.
1	***Echiniscus bigranulatus* Richters, 1907**	**Tar728**	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	**JX114897**	**JX114853**	**New**
		Tar729	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	**JX114898**	**JX114854**	**New**
		Tar747	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	**JX114899**	**JX114855**	**New**
		Tar756	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	–	**JX114856**	**New**
		–	Milodon Cave, Patagonia, Chile	–	–	–	HM193373	HM193389	*1 +
2	***Echiniscus blumi* Richters, 1903**	**Tar726**	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	**JX114891**	**JX114848**	**New**
		Tar727	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	**JX114892**	**JX114847**	**New**
		Tar730	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	**JX114893**	**JX114850**	**New**
		Tar748	**Milodon Cave, Patagonia, Chile**	**Nov. 2004**	**S51°34′**	**W72°37′**	**JX114894**	**JX114851**	**New**
		Tar765	**Disko Island, Greenland**	**April 2009**	**N69°19′**	**W54°04′**	**JX114895**	–	**New**
		Tar777	**Røen Sø, Greenland**	**April 2005**	**N69°15′**	**W53°31′**	–	**JX114849**	**New**
		–	Milodon Cave, Patagonia, Chile	–	–	–	HM193374	HM193390	*1 +
		–	Godhavn, Greenland	–	–	–	HM193375	HM193391	*1 +
	***Echiniscus canadensis* Murray, 1910**	Tar103	Madrid, Spain	–	–	–	FJ435715	FJ435786	*2
		Tar105	Madrid, Spain	–	–	–	FJ435714	FJ435784	*2
		Tar14	Madrid, Spain	–	–	–	FJ435713	FJ435785	*2
	***Echiniscus granulatus* (Doyére, 1840)**	–	Germany	–	–	–	DQ839606	–	*3
	***Echiniscus trisetosus* Cuénot, 1932**	Tar102	Madrid, Spain	–	–	–	FJ435716	FJ435781	*2
		Tar612	Madrid, Spain	–	–	–	FJ435717	FJ435782	*2
		Tar635	Madrid, Spain	–	–	–	FJ435718	FJ435783	*2
		Tar764	**Arctic Station, Greenland**	**April 2009**	**N69°19′**	**W54°04′**	**JX114896**	**JX114852**	**New**
3	***Echiniscus merokensis merokensis* Richters, 1904**	Tar395	Madrid, Spain	–	–	–	FJ435719	FJ435787	*2
		Tar761	**Disko Island, Greenland**	**April 2009**	**N69°19′**	**W54°04′**	**JX114907**	**JX114864**	**New**
		Tar762	**Disko Island, Greenland**	**April 2009**	**N69°19′**	**W54°04′**	**JX114908**	**JX114865**	**New**
		Tar770	**Disko Island, Greenland**	**April 2005**	**N69°19′**	**W54°04′**	–	**JX114863**	**New**
	***Echiniscus merokensis suecicus* Thulin, 1911**	**Tar759**	**Disko Island, Greenland**	**April 2005**	**N69°19′**	**W54°04′**	**JX114906**	**JX114866**	**New**
	***Echiniscus spiniger* Richters, 1904**	**Tar731**	**Resmo, Øland, Sweden**	**July 2007**	**N56°39′**	**E16°38′**	**JX114900**	**JX114857**	**New**
		Tar732	**Resmo, Øland, Sweden**	**July 2007**	**N56°39′**	**E16°38′**	**JX114901**	**JX114858**	**New**
		Tar733	**Resmo, Øland, Sweden**	**July 2007**	**N56°39′**	**E16°38′**	**JX114902**	**JX114859**	**New**
		Tar750	**Resmo, Øland, Sweden**	**July 2007**	**N56°39′**	**E16°38′**	**JX114903**	**JX114860**	**New**
		–	Øland, Sweden	–	–	–	HM193376	HM193392	*1 +
	***Echiniscus testudo* (Doyére, 1840)**	–	Nivå, Denmark	–	–	–	GQ849022	GQ849043	*4
		–	France	–	–	–	DQ839607	–	*3
		–	France	–	–	–	EF632459	–	*5
		–	France	–	–	–	EF632460	–	*5
		–	France	–	–	–	EF632461	–	*5
		–	France	–	–	–	EF632462	–	*5
		–	France	–	–	–	EF632464	–	*5
		–	France	–	–	–	EF632466	–	*5
		Tar734	**Samaria Gorge, Crete, Greece**	**Oct. 2004**	**N35°17′**	**E23°57′**	**JX114905**	**JX114861**	**New**
		Tar752	**Samaria Gorge, Crete, Greece**	**Oct. 2004**	**N35°17′**	**E23°57′**	**JX114904**	**JX114862**	**New**

Table 1. *Continued*

	Species	Code	Locality	Date	Coordinates		18S rRNA	28S rRNA	REFs.
4	***Echiniscus jenningsi*** Dastych, 1984	–	–	–	–	–	EU26696	–	*6
	***Echiniscus wendti* Richters, 1903**	**Tar781**	**Røen Sø, Greenland**	**April 2005**	**N69°15′**	**W53°31′**	–	**JX114867**	**New**
		Tar784	**Røen Sø, Greenland**	**April 2005**	**N69°15′**	**W53°31′**	**JX114909**	**JX114868**	**New**
5	***Echiniscus viridissimus*** Péterfi, 1956	–	–	–	–	–	AF056024	HM193393	*7, *1 +
	***Echiniscus* sp.**	–	Antarctic islands	–	–	–	EF632453	–	*5
	***Echiniscus* sp.**	–	Antarctic islands	–	–	–	EF632454	–	*5
	***Echiniscus* sp.**	–	Antarctic islands	–	–	–	EF632455	–	*5
	***Echiniscus* sp.**	–	Antarctic islands	–	–	–	EF632456	–	*5
	***Echiniscus* sp.**	–	Antarctic islands	–	–	–	EF632457	–	*5
	***Echiniscus* sp.**	–	Antarctic islands	–	–	–	EF632458	–	*5
	***Echiniscus* sp.**	–	–	–	–	–	EU266964	–	*6
	***Echiniscus* sp.**	–	–	–	–	–	EU266971	–	*6
	***Echiniscus* sp.**	–	–	–	–	–	EU266972	–	*6
	***Echiniscus* sp.**	–	–	–	–	–	EU266973	–	*6
	***Echiniscus* sp.**	–	–	–	–	–	EU266974	–	*6
	***Echiniscus* sp.**	–	–	–	–	–	EU266975	–	*6
	***Echiniscus* sp.**	–	–	–	–	–	EU266976	–	*6
	***Echiniscus* sp.**	–	–	–	–	–	EU266977	–	*6

Date, collection date. 18S rRNA and 28S rRNA, GenBank accession numbers for each genetic marker and specimen. Refs., references where the GenBank sequences were published, and geographical and ecological information obtained. 1, *bigranulatus* cuticle group. 2, *blumi* cuticle group. 3, *merokensis* cuticle group. 4, *arctomys* cuticle group. 5, *viridis* cuticle group. New, new sequence. –, information not available, not sequenced. +, DNA extraction from more than one individual. References for GenBank sequences: *1, Jørgensen *et al*. (2011); *2, Guil & Giribet (2012); *3, Schill & Steinbrück (2007); *4, Jørgensen *et al*. (2010); *5, Sands *et al*. (2008a); *6, Sands *et al*. (2008b); *7, Garey *et al*. (1999).

Table 2. Heterotardigrades from the order Echiniscoidea used in the analyses

Species	Code	Locality	Date	Coordinates		18S rRNA	28S rRNA	REFs.
Echiniscoides sigismundi	–	–	–	–	–	GQ849021	GQ849042	*1 +
(M. Schultze, 1865)	–	–	–	–	–	EU266960	–	*2
	Tar735	**Lynæs, Seeland, Denmark**	**Sept. 2005**	**N55°56′**	**E11°51′**	**JX114926**	**JX114889**	**New**
	Tar736	**Lynæs, Seeland, Denmark**	**Sept. 2005**	**N55°56′**	**E11°51′**	**JX114927**	**JX114888**	**New**
	Tar737	**Lynæs, Seeland, Denmark**	**Sept. 2005**	**N55°56′**	**E11°51′**	**JX114928**	**JX114890**	**New**
	Tar751	**Lynæs, Seeland, Denmark**	**Sept. 2005**	**N55°56′**	**E11°51′**	**JX114929**	**JX114887**	**New**
Oreella mollis Murray, 1910	–	Antarctica	–	–	–	EU266962	–	*2
Antechiniscus lateromamillatus (Ramazzotti, 1964)	–	Angol, Chile	–	–	–	HM193370	HM193386	*3 +
Bryochoerus intermedius	**Tar798**	**Røen Sø, Greenland**	**April 2005**	**N69°15′**	**W53°31′**	**JX114920**	**JX114886**	**New**
(Murray, 1910)	**Tar800**	**Røen Sø, Greenland**	**April 2005**	**N69°15′**	**W53°31′**	**JX114921**	**JX114888**	**New**
Bryodelphax parvulus Thulin, 1928	–	Øland, Sweden	–	–	–	HM193371	HM193387	*3 +
***Bryodelphax* sp.**	–	Antarctic islands	–	–	–	EF632435	–	*4
	–	Antarctic islands	–	–	–	EF632434	–	*4
	–	Antarctic islands	–	–	–	EF632433	–	*4
	–	Antarctic islands	–	–	–	EU266963	–	*2
Cornechiniscus lobatus	–	–	–	–	–	EU038077	–	*5
(Ramazzotti, 1943)	–	–	–	–	–	EU038079	–	*5
	–	Sinai, Egypt	–	–	–	HM193372	HM193388	*3 +
Diploechiniscus oihonnae (Richters, 1903)	Tar791	Bergen, Norway	Aug. 2009	N60°23′	E5°19′	JX114910	JX114869	New
Hypechiniscus exarmatus (Murray, 1907)	–	Mt. Amigasa, Japan	–	–	–	HM193377	HM193394	*3 +
Hypechiniscus gladiator (Murray, 1905)	–	Mt. Amigasa, Japan	–	–	–	HM193378	HM193395	*3 +
Mopsechiniscus granulosus Mihelčič, 1967	–	Angol, Chile	–	–	–	HM193379	HM193396	*3 +
Parechinicscus chitonides Cuénot, 1926	–	Øland, Sweden	–	–	–	HM193380	HM193397	*3 +
Proechiniscus hanneae	**Tar738**	**Disko Island, Greenland**	**April, 2005**	**N69°15′**	**W53°34′**	**JX114922**	**JX114882**	**New**
(Petersen, 1951)	**Tar739**	**Disko Island, Greenland**	**April, 2005**	**N69°15′**	**W53°34′**	**JX114924**	**JX114883**	**New**
	Tar740	**Disko Island, Greenland**	**April, 2005**	**N69°15′**	**W53°34′**	–	**JX114884**	**New**
	Tar749	**Disko Island, Greenland**	**April, 2005**	**N69°15′**	**W53°34′**	**JX114923**	**JX114881**	**New**
	Tar753	**Disko Island, Greenland**	**April, 2005**	**N69°15′**	**W53°34′**	**JX114925**	**JX114879**	**New**
	Tar796	**Røen Sø, Greenland**	**April, 2005**	**N69°15′**	**W53°31′**	–	**JX114880**	**New**
	–	Godhavn, Greenland	–	–	–	HM193381	HM193398	*3 +
Pseudechinicus facettalis	–	Zackenberg, Greenland	–	–	–	HM193382	HM193399	*3 +
Petersen, 1951	Tar695	Madrid, Spain	–	–	–	FJ435720	FJ435788	*6
	Tar696	Madrid, Spain	–	–	–	FJ435721	FJ435789	*6
	Tar743	**Zackenberg, Sydkæret, Greenland**	**June 2004**	**N74°30′**	**W20°30′**	**JX114914**	**JX114874**	**New**
	Tar744	**Zackenberg, Sydkæret, Greenland**	**June 2004**	**N74°30′**	**W20°30′**	**JX114915**	–	**New**
	Tar754	**Zackenberg, Sydkæret, Greenland**	**June 2004**	**N74°30′**	**W20°30′**	**JX114916**	**JX114873**	**New**
Pseudechiniscus islandicus	**Tar742**	**Vadhorn, Eyturoy, Faroe Islands**	**Nov. 2003**	**N62°01′**	**W6°49′**	–	**JX114877**	**New**
(Richters, 1904)	**Tar755**	**Vadhorn, Eyturoy, Faroe Islands**	**Nov. 2003**	**N62°01′**	**W6°49′**	**JX114919**	**JX114878**	**New**
	–	Tingvala, Iceland	–	–	–	HM193383	HM193400	*3 +
	–	Eyturoy, Faroe islands	–	–	–	AY582119	GQ849044–1	*7, *1
Pseudechiniscus novaezeelandiae (Richters, 1908)	–	Chillan, Chile	–	–	–	HM193384	HM193401	*3 +
***Pseudechiniscus* sp.**	–	–	–	–	–	EU266965	–	*2
Pseudechiniscus suillus	**Tar790**	**Bergen, Norway**	**August 2009**	**N60°23′**	**E5°19′**	**JX114917**	**JX114875**	**New**
(Ehrenberg, 1853)	**Tar792**	**Bergen, Norway**	**August 2009**	**N60°23′**	**E5°19′**	**JX114918**	**JX114876**	**New**
Testechiniscus spitzbergensis	–	–	–	–	–	EU266967	–	*2
(Scourfield, 1897)	–	–	–	–	–	EU266968	–	*2
	–	Godhavn, Greenland	–	–	–	HM193385	HM1933402	*3 +
	Tar782	**Østerlien, Disko; Greenland**	**March 2004**	**N69°15′**	**W53°31′**	**JX114913**	**JX114871**	**New**
	Tar768	**Østerlien, Disko; Greenland**	**March 2004**	**N69°15′**	**W53°31′**	**JX114911**	**JX114870**	**New**
	Tar769	**Østerlien, Disko; Greenland**	**March 2004**	**N69°15′**	**W53°31′**	**JX114912**	**JX114872**	**New**

New sequences are in bold. 18S rRNA and 28S rRNA, GenBank accession numbers. Refs., references where GenBank sequences were published, and geographical and ecological information obtained. New, new sequence. –, information not available, not sequenced. +, DNA extraction from more than one individual. References for GenBank sequences: *1, Jørgensen *et al.* (2010); *2, Sands *et al.* (2008a); *3, Jørgensen *et al.* (2011); *4, Sands *et al.* (2008b); *5, Guidetti *et al.* (2009); *6, Guil & Giribet (2012); *7, Jørgensen & Kristensen (2004).

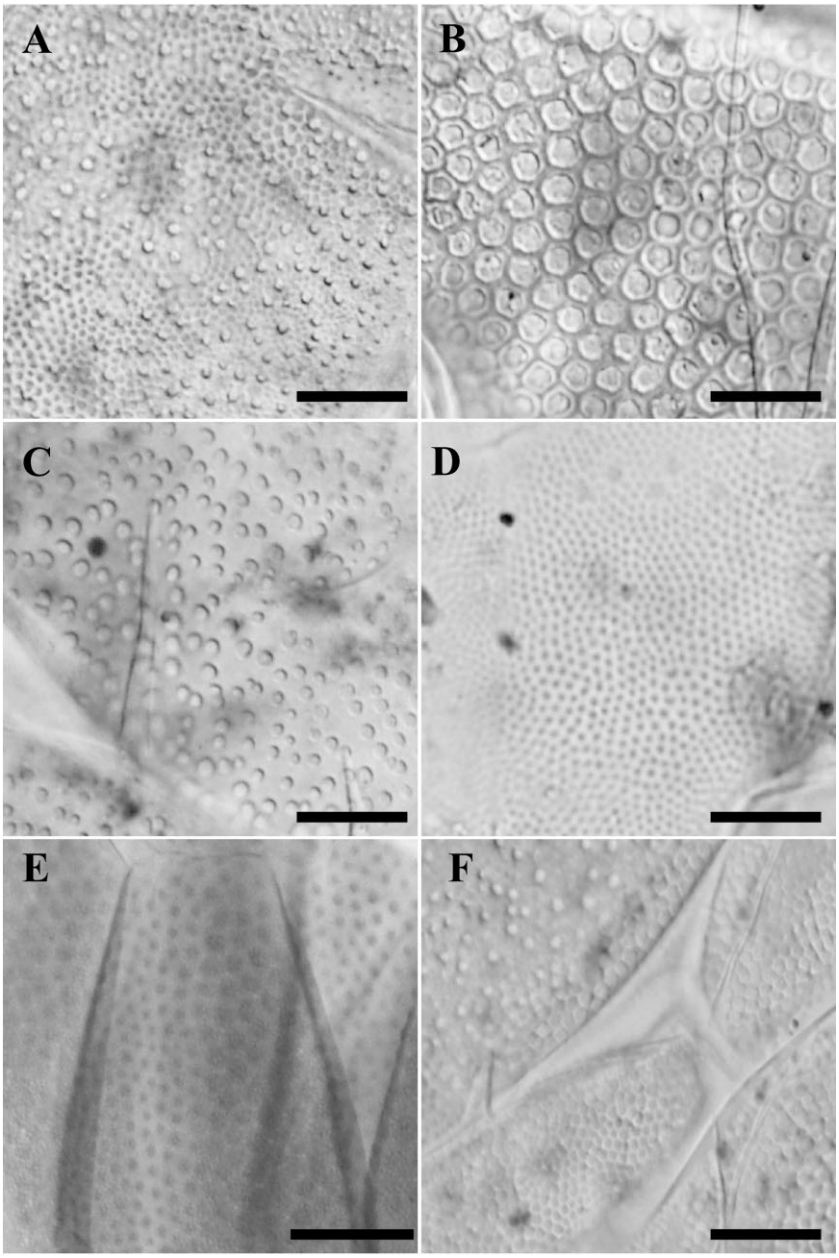


Figure 1. The five types of cuticular designs traditionally used to group *Echiniscus* species and the recently described genus *Diploechiniscus* (Ramazzotti & Maucci, 1983; Peluffo *et al*., 2002; Pilato *et al*., 2007, 2008): A, *bigranulatus*; B, *blumi-canadensis*; C, *merokensis*; D, *arctomys*; E, *viridis*; F, *D. oihonnae*. A to D and F were made using phase contrast. E was made using differential interference contrast. Scale bars: 10 μm.

Pilato, Fontoura & Lisi, 2007; Pilato *et al*., 2008) (Table 1; Fig. 1): *bigranulatus* (Fig. 1A) (*Echiniscus bigranulatus* Richters, 1907 – supporting Fig. S1), *blumi-canadensis* (Fig. 1B) (*E. blumi* Richters, 1903 – Fig. S2 – *E. trisetosus* Cuénot, 1932, *E. canadensis* Murrray, 1910, *E. granulatus* (Doyére, 1840)), *merokensis* (Fig. 1C) (*E. merokensis merokensis* Richters, 1904 – Fig. S3 – *E. merokensis suecicus* Thulin, 1911, *E. testudo* (Doyére, 1840) – Fig. S4 – *E. spiniger* Richters, 1904 – Fig. S5), *arctomys* (Fig. 1D) (*E. wendti* Richters, 1903 – Fig. S6 – *E. jenningsi* Dastych, 1984) and *viridis* (Fig. 1E) (*E. viridissimus* Péterfi, 1956). *Diploechiniscus oihonnae* (Richters, 1903) (Fig. S7) has been recently transferred from *Echiniscus* to a newly described genus, *Diploechiniscus* (Vicente *et al*., 2013). *Diploechiniscus oihonnae* was not formerly placed within any of these groups, as it had a particular kind

'of sculpture design on the cuticle surface (Fig. 1F) only shared with *E. multispinosus* da Cunha, 1944 (Ramazzotti & Maucci, 1983: p. 424; now a synonymy of *D. oihonnae*; Vicente *et al*., 2013). In addition, we analysed other echiniscoids (based on GenBank and newly sequenced specimens), including *Echiniscoides*, *Bryochoerus*, *Proechiniscus*, *Pseudechiniscus*, and *Testechiniscus* (Table 2), as well as some arthrotardigrades (Table 3). Eutardigrades (Table 3) were included to test the monophyly of Heterotardigrada. Non-tardigrade outgroups are those previously employed by Guil & Giribet (2012).

Sequences

The two nuclear ribosomal genes 18S rRNA and 28S rRNA were chosen because they have proven informative for tardigrade phylogeny in previous analyses (Sands *et al*., 2008a; Jørgensen *et al*., 2011; Marley *et al*., 2011; Guil & Giribet, 2012). DNA was extracted from 46 individuals (Tables 1 and 2) with the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol (including 10 min of incubation at 72 °C after adding buffer AL), and re-suspended in 100 μL double distilled H_2O (ddH_2O), as described by Guil & 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 tardigrade analyses, was amplified using the universal primer pair 18S a2.0 (5′-ATGGTTGCAAAGCTGAAAC-3′; Whiting *et al*., 1997) and 18S 9R (5′-GATCCTTCC GCAGGTTCACCTAC-3′; Giribet *et al*., 1996). Amplifications were performed in a 22-μL volume of a solution containing 14 μL ddH_2O, 1 μL of 10× PCR buffer, 2 μL of dNTP 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 consists 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 (1344–1446 bp depending on the species) was amplified using two pairs of universal primers: 28Sa (5′-GACCCGTCTTGAAACA CGGA-3′; Whiting *et al*., 1997) and 28Srd5b (5′-C CACAGCGCCAGTTCTGCTTAC-3′; Schwendinger & Giribet, 2005), and 28Srd4.8a (5′-ACCTATTCT CAAACTTTAAATGG-3′; Schwendinger & Giribet, 2005) and 28Srd7b1 (5′-GACTTCCCTTACCTACAT-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.

Cycle sequencing with AmpliTaq DNA polymerase was as described by Guil & 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 Corp.). 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 JX114891–JX114929 for 18S rRNA, and JX114847–JX114890 for 28S rRNA (Tables 1 and 2).

Analyses

Three sets of analyses were conducted to determine the variability of each genetic marker at different taxonomic levels: (1) 18S rRNA only, (2) 28S rRNA only, and (3) combined analyses of 18S rRNA and 28S rRNA.

A direct optimization approach, which facilitates the analysis of sequences of unequal length without prior alignment (Wheeler, 1996), using parsimony as an optimality criterion, was conducted with the program POY 4.1 (Varón, Sy Vinh & Wheeler, 2010). The 18S rRNA amplicon was divided into three fragments, and the 28S rRNA sequence into ten fragments (Guil & Giribet, 2012), according to internal primers and to accommodate the length heterogeneity of the sequence fragments generated by different authors utilizing different sets of primers. The definition of predefined fragments in this fashion allowed us to treat entire missing fragments as missing data without the need of using random numbers of *N*'s as placeholders (see Wheeler *et al*., 2005: 111). Homology assignment and tree generation were performed simultaneously ('dynamic homology'; Wheeler *et al*., 2005) under a parameter set with indel opening cost of 3, base transformations cost of 2, and indel extensions cost of 1 (parameter set 3221; De Laet, 2005). Different data sets (described above as 1–3) were analysed using the 'max_time' command (24 h), which implements a default search strategy that combines Wagner addition with tree bisection–and–reconnection (TBR) branch swapping, parsimony ratchet (Nixon, 1999), and tree fusing (Goloboff, 1999). The best trees were used for subsequent analyses with 'max_time' command (24 h), changing costs parameters with an indel opening cost of 3, elongation

Table 3. Eutardigrade and arthrotardigrade outgroups used in the analyses

Class, order, superfamily/family	Species	Code	18S	28S	Refs.
Outgroups					
ARTHROPODA,Chelicerata, Xyphosura	***Limulus polyphemus*** Linnaeus, 1758	–	U91490	AF212167	–
ARTHROPODA, Mandibulata, Myriapoda	***Dendrothereua homa*** (Chanmberlin, 1942)	–	FJ660705	FJ660746	–
ARTHROPODA, Mandibulata, Pancrustacea	***Allacma fusca*** (Linnaeus, 1758)	–	EU368610	EU376054	–
PRIAPULIDA	***Priapulus caudatus*** Lamarck, 1816	–	AF025927	AY210840	–
Tardigrada					
EUTARDIGRADA, Parachela, Isohypsibioidea	***Eremobiotus alicatai*** (Binda, 1969)	Tar191	FJ435722	FJ435766	*1
	Halobiotus crispae Kristensen, 1982	–	EF620402	EF620409	*2
EUTARDIGRADA, Parachela, Hypsibioidea	***Astatumen trinacriae*** (Arcidiacono, 1962)	Tar718	FJ435731	FJ435773	*1
	Diphascon (Diphascon) pingue (Marcus, 1936)	Tar698	FJ435736	FJ435776	*1
	Ramazzottius oberhaeuseri (Doyére, 1840)	Tar398	FJ435728	FJ435768	*1
EUTARDIGRADA, Parachela, Eohypsibioidea	***Bertolanius nebulosus*** (Dastych, 1983)	–	GQ849023–5	GQ849046–5	*3
EUTARDIGRADA, Parachela, Macrobiotidea	***Dactylobiotus octavi*** Guidetti, *et al*., 2006	–	GQ849025–5	GQ849049–5	*3
	Macrobiotus hufelandi group	Tar71	FJ435740	FJ435751	*1
	Macrobiotus hufelandi C.A.S. Schultze, 1834	–	GQ849024–5	GQ849047–5	*3
	Minibiotus gumersindoi Guil & Guidetti, 2005	Tar710	FJ435748	FJ435761	*1
	Murrayon pullari (Murray, 1907)	–	–	GQ849050–5	*3
	Murrayon dianeae (Kristensen, 1982)	Tar711	FJ435737	FJ435762	*1
	***Paramacrobiotus richtersi* group**	Tar708	FJ435743	FJ435757	*1
EUTARDIGRADA, Apochela, Milnesiidae	***Milnesium* cf. *tardigradum***	Tar235	FJ435749	FJ435779	*1
		–	AY582120	–	*4
		Tar220	FJ435750	FJ435780	*1
HETEROTARDIGRADA, Arthrotardigrada, Batillipedidae	***Batillipes mirus*** Richters, 1909	–	GQ849016	GQ849027	*3
	Batillipes pennaki Marcus, 1946	–	–	GQ849028	*3 +
	Batillipes similis Schulz, 1955	–	–	GQ849029	*3 +
	Batillipes tubernatis Pollock, 1971	–	–	GQ849030	*3 +
HETEROTARDIGRADA, Arthrotardigrada, Halechiniscidae	***Archechiniscus* sp.**	–	–	GQ849031	*3
	***Dipodarctus* sp.**	–	–	GQ849032	*3 +
	***Florarctus* sp.**	–	GQ849017	GQ849034	*3 +
	***Florarctus* sp2.**	–	–	GQ849033	*4
	Halechiniscus perfectus Schulz, 1955	–	GQ849018	GQ849035	*3 +
	Halechiniscus remanei Schulz, 1955	–	AY582118	–	*4
	***Orzeliscus* sp.**	–	–	GQ849036	*3 +
	Raiarctus colurus Renaud-Mornant, 1981	–	–	GQ849037	*3 +
	***Styraconyx* sp.**	–	–	GQ849038	*3 +
	Tanarctus dendriticus Renaud-Mornant, 1980	–	–	GQ849040	*3 +
HETEROTARDIGRADA, Arthrotardigrada, Stygarctidae	***Stygarctus* sp.**	–	–	GQ849041	*3 +

Refs., references where the GenBank sequences were published. –, Information not available. +, DNA extraction from more than one individual. References for GenBank sequences: *1, Guil & Giribet (2012); *2, Møbjerg *et al*. (2007); *3, Jørgensen *et al*. (2010); *4, Jørgensen & Kristensen (2004).

cost of 1, and a transversion/transition ratio of 2:1 (parameter set 3211). A subsequent round of analyses, using the previous trees as input, was performed to check the stability of tree length. Nodal support was assessed via 100 bootstrap replicates (dynamic homology; and hence the 13 fragments were used for resampling), based on Wagner tree search with random sequence addition of terminals, and local searches using TBR.

The implied alignments (Wheeler, 2003; Giribet, 2005) obtained under the direct optimization parsimony analyses were used to conduct a Bayesian inference (BI) analysis (Huelsenbeck *et al*., 2001). Prior to Bayesian analysis, MrModeltest version 3.7 (Posada & Crandall, 1998) was executed to choose the best-fit model of nucleotide substitution for each of the 18S rRNA, 28S rRNA, and combined matrices under the Akaike information criterion (AIC). In all cases, a General Time Reversible (GTR) model with corrections for invariants and a gamma distribution of site heterogeneity (GTR + Γ + I) was selected as the best-fit model. Bayesian analyses were performed with MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). 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 to the complete log likelihood graphs of all analyses after 10 000 000 generations; around 25 000 trees were discarded as burn-in after analyses of likelihoods of the samples using Tracer version 1.5. Support for nodes is expressed as posterior probabilities, calculated as a 50% majority rule consensus and reported on a maximum clade credibility tree of the post-burn-in sample.

The implied alignments were also used for a maximum-likelihood (ML) search, as in the Bayesian analysis. Models obtained with Modeltest version 3.7 (Posada & Crandall, 1998) for likelihood analyses coincided with those models obtained with MrModeltest for Bayesian approaches for the different data sets studied. ML analyses were conducted in RAxML 7.2.6 (Stamatakis, 2006). The closest substitution model available in RAxML, GTR + Γ + I (Yang, 1996), was thus selected, using a common model to all partitions (as both genes are part of the same locus, and the best-fit model obtained with Modeltest both for the individual and for the combined partitions was GTR + Γ + I). A tree search with 20 replicates was conducted, nodal support consisting of 100 bootstrap replicates. The same analyses (likelihood and Bayesian) were run with the data aligned using MUSCLE (Edgar, 2004) and with the divergent regions trimmed with GBlocks (Castresana, 2000), to compare the effect of different homology statements (implied alignment vs. multiple sequence alignment) on the results.

Relationships among *Echiniscus* 18S and 28S rRNA haplotypes were analysed using a statistical parsimony network estimated with TCS version 1.21 (Clement, Posada & Crandall, 2000). This method estimates the unrooted tree and provides a 95% plausible set of all sequence type linkages within the unrooted network. An analysis of molecular variance (AMOVA) was conducted with ARLEQUIN 3.11 to examine hierarchical population structure by pooling *Echiniscus* species based on their cuticular design. A total of 16 000 permutations were run to guarantee having less than 1% difference with the exact probability in 99% of cases (Guo & Thompson, 1992). Following Srivathsan & Meier (2012), we differentiated species using uncorrected *p*-values generated by PAUP* (Swofford, 1998) instead of Kimura's two-parameter model.

RESULTS

We sequenced 46 heterotardigrade specimens (Tables 1 and 2), 23 of which belong to *Echiniscus*. Genera from six of the 11 heterotardigrade families, and 14 of 17 genera of Echiniscoidea (Table 4) were analysed. Fragments from 663 to 706 bp (depending on the species) for 18S rRNA and from 1344 to 1446 bp for 28S rRNA were sequenced per specimen. Images for all the specimens newly sequenced are provided (Figs S1–S8; and photographs deposited in MorphoBank: project number 785, accession numbers M148490–M148655).

Updating the systematic knowledge of heterotardigrades

Phylogenetic analyses performed with BI and ML yielded similar topologies and support values, but differed from the parsimony result (Fig. 2). This is not unexpected, as the two probabilistic approaches were based on similar evolutionary models, even though all analyses use the same homology scheme. Results from analyses performed with the two homology schemes (POY, Fig. 2, and MUSCLE+GBlocks, Fig 3; additional MUSCLE+GBlocks results are available in Figs S9 and S10) were largely congruent, and thus throughout the paper we refer to the results obtained with the POY analyses and the probabilistic analyses obtained from the implied alignments, except when otherwise indicated. Monophyly of both tardigrade classes, Heterotardigrada and Eutardigrada, was supported in all analyses. Monophyly of the heterotardigrade orders (Figs 2, 3, 6) was not supported, however. The families and subfamilies of Arthrotardigrada were polyphyletic due to the position of certain genera (e.g. *Tanarctus*, *Styraconyx*). Monophyly of the order Echiniscoidea

Table 4. Current accepted classification of Heterotardigrada (orders, families, subfamilies and genera) following Guidetti & Bertolani (2005), and Vicente *et al*., (2013).

Order	Family	Subfamily	Genera
ARTHROTARDIGRADA	Batillipedidae		***Batillipes***
	Coronarctidae		*Coronarctus*
			Trogloarctus
	Halechiniscidae	Archechiniscinae	***Archechiniscus***
		Dipodarctinae	***Dipodarctus***
		Euclavarctinae	*Clavarctus*
			Euclavarctus
			Exoclavarctus
			Moebjergarctus
			Parmursa
			Proclavarctus
		Florarctinae	***Florarctus***
			Ligiarctus
			Wingstrandarctus
		Halechiniscinae	*Chrysoarctus*
			Halechiniscus
			Paradoxipus
		Orzeliscinae	***Orzeliscus***
			Opydorscus
		Styraconyxinae	*Angursa*
			Bathyechiniscus
			Lepoarctus
			Paratanarctus
			Pleocola
			Raiarctus
			Rhomboarctus
			Styraconyx
			Tetrakentron
			Tholoarctus
		Tanarctinae	*Actinarctus*
			Tanarctus
			Zioella
	Neoarctidae		*Neoarctus*
	Neostygarctidae		*Neostygarctus*
	Renaudarctidae		*Renaudarctus*
	Stygarctidae	Megastygarctidinae	*Megastygarctides*
		Stygarctinae	*Faroestygarctus*
			Parastygarctus
			Prostygarctus
			Pseudostygarctus
			Stygarctus
ECHINISCOIDEA	Echiniscoididae		*Anisonyches*
			Echiniscoides
	Carphaniidae		*Carphania*
	Oreellidae		***Oreella***
	Echiniscidae		***Antechiniscus***
			Bryochoerus
			Bryodelphax
			Cornechiniscus
			Diploechiniscus
			Echiniscus
			Hypechiniscus
			Mopsechiniscus
			Novechiniscus
			Parechiniscus
			Proechiniscus
			Pseudechiniscus
			Testechiniscus

Genera included in the present study are in bold type.

was confirmed, with *Echiniscoides* in a basal position. Monophyly of Echiniscidae was rejected due to the inclusion of *Oreella mollis* (Oreellidae is currently included within the order Echiniscoidea; Table 4) (Fig. 2). Relationships among arthrotardigrades received low support in general.

All genera of Echiniscidae were monophyletic with the exception of *Pseudechiniscus*, as *P. islandicus* (Richters, 1904) (from Iceland and the Faroe islands; Table 2) did not group with the other *Pseudechiniscus* species analysed (*P. facettalis* Petersen, 1951, *P. suillus* (Ehrenberg, 1853) and *P. novaezeelandiae* (Richters, 1908)). Three major groups can be found within the clade including the Echiniscidae genera plus *Oreella*. *Parechiniscus*, and the clade including *Bryodelphax–Bryochoerus* appear as two unresolved lineages, the rest of the species clustering together in a poorly supported clade (Fig. 2). *Oreella* (only with 18S rRNA data) and *Mopsechiniscus* were related in the Bayesian and ML analyses but support is negligible (Fig. 2), and they appeared in a more basal position in the parsimony analysis (tree not shown). A clade of *Proechiniscus*, *Antechiniscus*, *Cornechiniscus*, and *Pseudechiniscus islandicus* was obtained in all analyses and received high support. A clade consisting of the other *Pseudechiniscus* species (*P. facettalis*, *P. novaezeelandiae*, and *P. suillus*) together with an *Echiniscus* sp. from GenBank labelled as EU266964 is moderately supported. *Pseudechiniscus facettalis* was not monophyletic, although the Greenlandic *P. facettalis* individuals formed a clade, while the Spanish specimens clustered with a Norwegian *P. suillus* (Table 2). Lastly, *Hypechiniscus* formed a clade with *Testechiniscus*, *Diploechiniscus,* and *Echiniscus*, with *Echiniscus* being monophyletic in all analyses, and the sister group of *Diploechiniscus*. The latter two together constituted the sister group of *Testechiniscus* (Fig. 2).

Relationships within *Echiniscus*

Deep phylogenetic structure within *Echiniscus* is missing for the most part, but a sister group relationship to *D. oihonnae* was well supported in all analyses. The majority of species or complexes of species (all but *E. bigranulatus*) were supported but with different data sets: the *blumi-canadensis* complex, *E. granulatus*, *E. spiniger*, and *E. testudo* with 18S rRNA information (Fig. 4A), and *E. merokensis*, *E. wendti*, *E. spiniger*, and *E. testudo* with 28S rRNA data (Fig. 4B). Instead, the parsimony network constructed with the 18S rRNA data revealed three groups based on cuticular design that had statistical support (AMOVA: $F_{ST} = 0.944$, $P < 0.001$, genetic variation explained: 52.3% among cuticular design groups, 42.2% among populations within cuticular design groups, and 5.5% within populations). These three groups (Fig. 5) are composed of: (1) *blumi-canadensis*; (2) *viridissimus*, *spiniger*, *testudo*, *bigranulatus*, *jenningsi*, and *wendti*; and (3) *trisetosus* Tar612 and 635, *granulatus* and *merokensis*. By contrast, other classical groups based also on cuticle designs (presented in Table 1 and Fig. 1: *bigranulatus*, *blumi-canadensis*, *merokensis*, *arctomys*, and *viridis*; Ramazzotti & Maucci, 1983; Peluffo *et al*., 2002; Pilato *et al*., 2007, 2008) did not find statistical or phylogenetic support from any of the genetic markers used, whether analysed individually or in combination. The parsimony network constructed with the 28S rRNA data or combining 18S and 28S rRNA sequences (networks not shown) did not yield any interpretable groups as specimens appeared mixed, and with no statistical (AMOVA) support ($P > 0.05$) in any case.

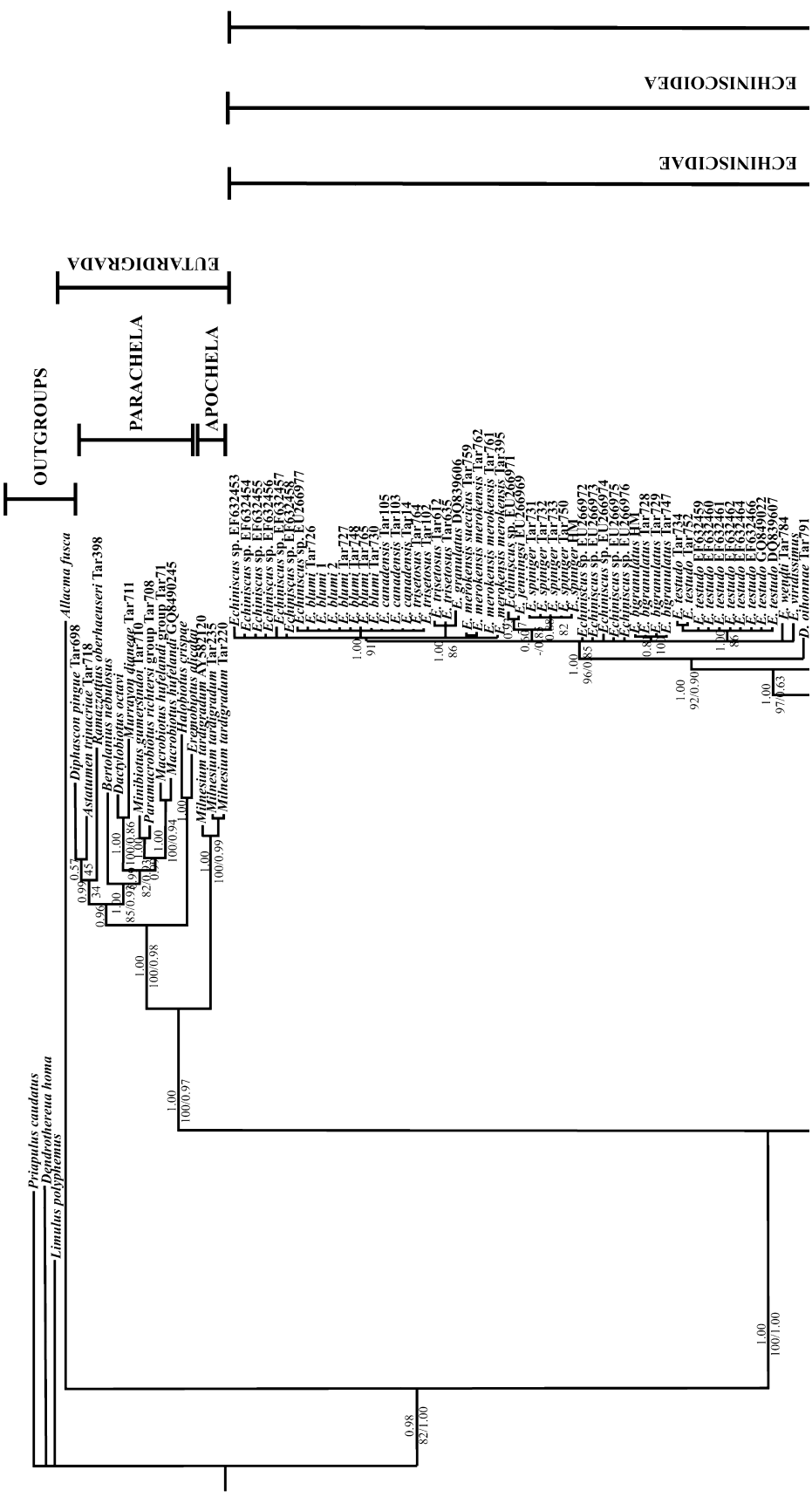
When considering uncorrected pairwise *p*-distances (Table 5), differences within each *Echiniscus* species were up to 0.3% for 18S rRNA and up to 0.8% for 28S rRNA. Comparing uncorrected *P*-values among *Echiniscus* species, differences for 18S rRNA were 0.5–1.7% and for 28S rRNA 0.5–4.3%. *Diploechiniscus oihonnae* (sister group of *Echinsicus*) had differences between 2.6 and 3.5% for 18S rRNA with respect to the *Echiniscus* species, and for 28S rRNA were 1.7–5.0%.

The sequence fragment delimited by primer pair 28Sa to 28S7b1 presented problems because the majority of the sequences were incomplete. A shorter fragment between primers 28Sa and 28S5b was complete in the majority of taxa and had enough differences to discriminate among species (a gap was present between within- and among-species differences; Table 5): within *Echiniscus* species genetic differences were 0.0–0.8%, and among *Echiniscus* species, 1.0–3.7%, this range being 2.4–4.1% for the sister group of *Echiniscus*, *D. oihonnae*, with respect to the *Echiniscus* species. In comparison, differences between *Echiniscus* species and *Testechiniscus* species were 1.8–4.1% for 18S rRNA and 2.8–4.5% for 28S rRNA fragment a-5b. Differences among other Heterotardigrada genera (from the same order) were 4.4–8.7% for 18S rRNA and 4.3–10.4% for 28S (a-5b) rRNA, and in Eutardigrada genera were 3.1–8.1% for 18S rRNA and 4.6–7.8% for 28S (a-5b) rRNA.

DISCUSSION

Heterotardigrade relationships

From the two heterotardigrade groups, arthrotardigrade relationships and their branching with respect to the echiniscoidean genera remain poorly resolved (Fig. 2), as also concluded in previous studies (Jørgensen *et al*., 2010, 2011). Following Jørgensen


OUTGROUPS
PARACHELA
APOCHELA
EUTARDIGRADA
ECHINISCIDAE
ECHINISCOIDEA
Priapulus caudatus
Dendrotherea homa
Limulus polyphemus
Diphascon pingue Tar698
Astatumen trijugum Tar718
Ramazzottius oberhaeuseri Tar398
Bertolanius nebulosus
Dactylobiotus octavi
Minibiotus Sumerstadt Tar710
Paramacrobiotus richtersi group Tar708
Macrobiotus hufelandi group Tar71
Macrobiotus hufelandi FGQ8490245
Halobiotus crispae
Eremobiotus alleganyi
Milnesium tardigradum AY582120
Milnesium tardigradum
Milnesium tardigradum Tar220
Allacma fusca
Echiniscus sp. EF632453
Echiniscus sp. EF632454
Echiniscus sp. EF632455
Echiniscus sp. EF632456
Echiniscus sp. EF632457
Echiniscus sp. EF632458
Echiniscus sp. EU266977
E. blumi Tar726
E. blumi 1
E. blumi 2
E. blumi Tar727
E. blumi Tar728
E. blumi Tar765
E. blumi Tar730
E. canadensis Tar105
E. canadensis Tar103
E. canadensis Tar104
E. canadensis Tar102
E. trisetosus Tar612
E. trisetosus Tar635
E. granulatus DQ839606
E. merokensis sueciae Tar759
E. merokensis merokensis Tar762
E. merokensis merokensis Tar761
E. merokensis merokensis Tar395
Echiniscus sp. EU266971
E. jennings EU266969
E. spiniger Tar731
E. spiniger Tar733
E. spiniger Tar750
E. spiniger HV
Echiniscus sp. EU266972
Echiniscus sp. EU266973
Echiniscus sp. EU266974
Echiniscus sp. EU266975
Echiniscus sp. EU266976
E. bigranulatus HV
E. bigranulatus Tar728
E. bigranulatus Tar729
E. bigranulatus Tar747
E. testudo Tar734
E. testudo Tar735
E. testudo EF632459
E. testudo EF632460
E. testudo EF632461
E. testudo EF632462
E. testudo EF632464
E. testudo EF632466
E. testudo GQ849022
E. testudo DQ839607
E. wendti Tar784
E. virilissimus
D. oihonnae Tar791

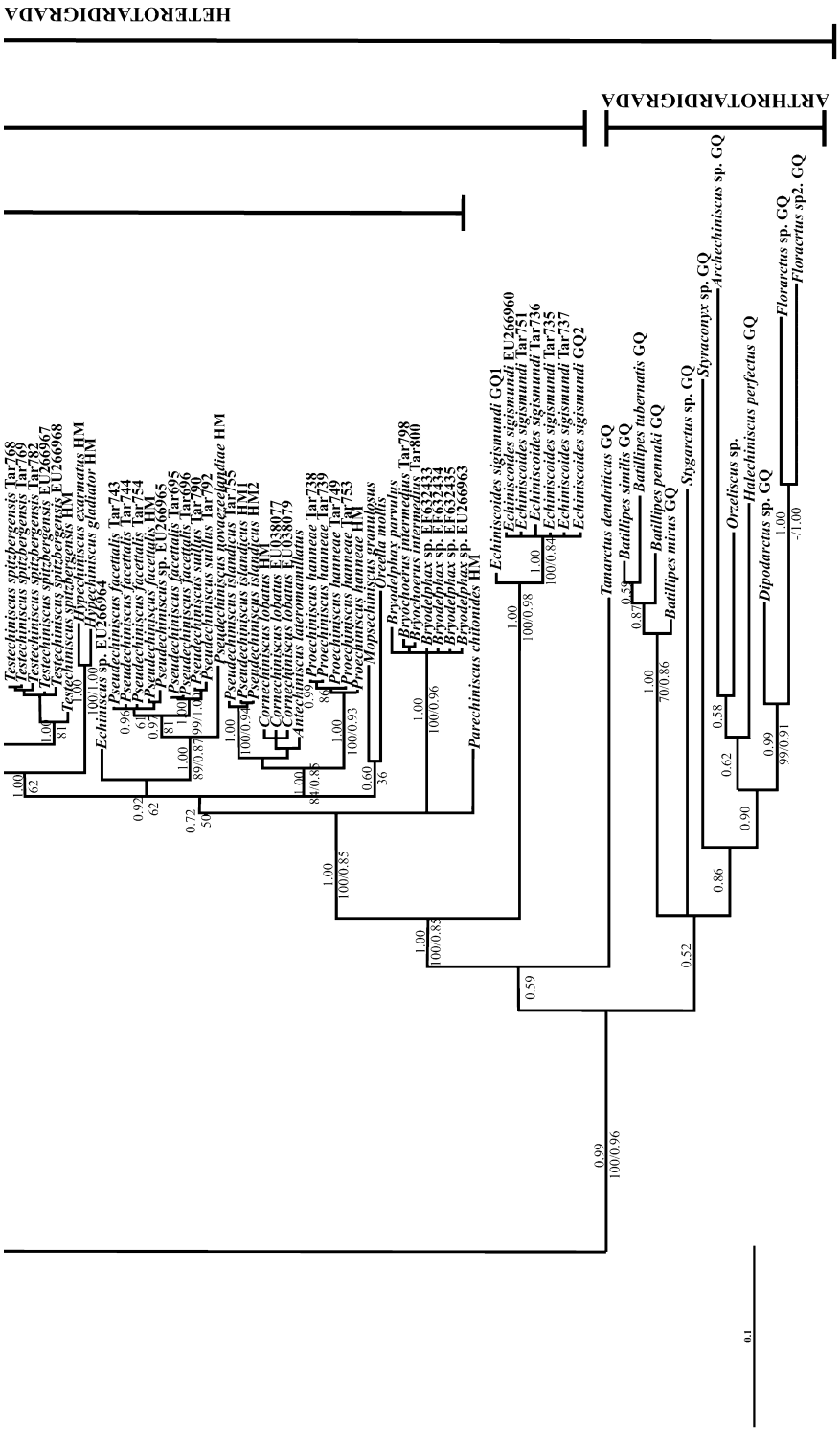
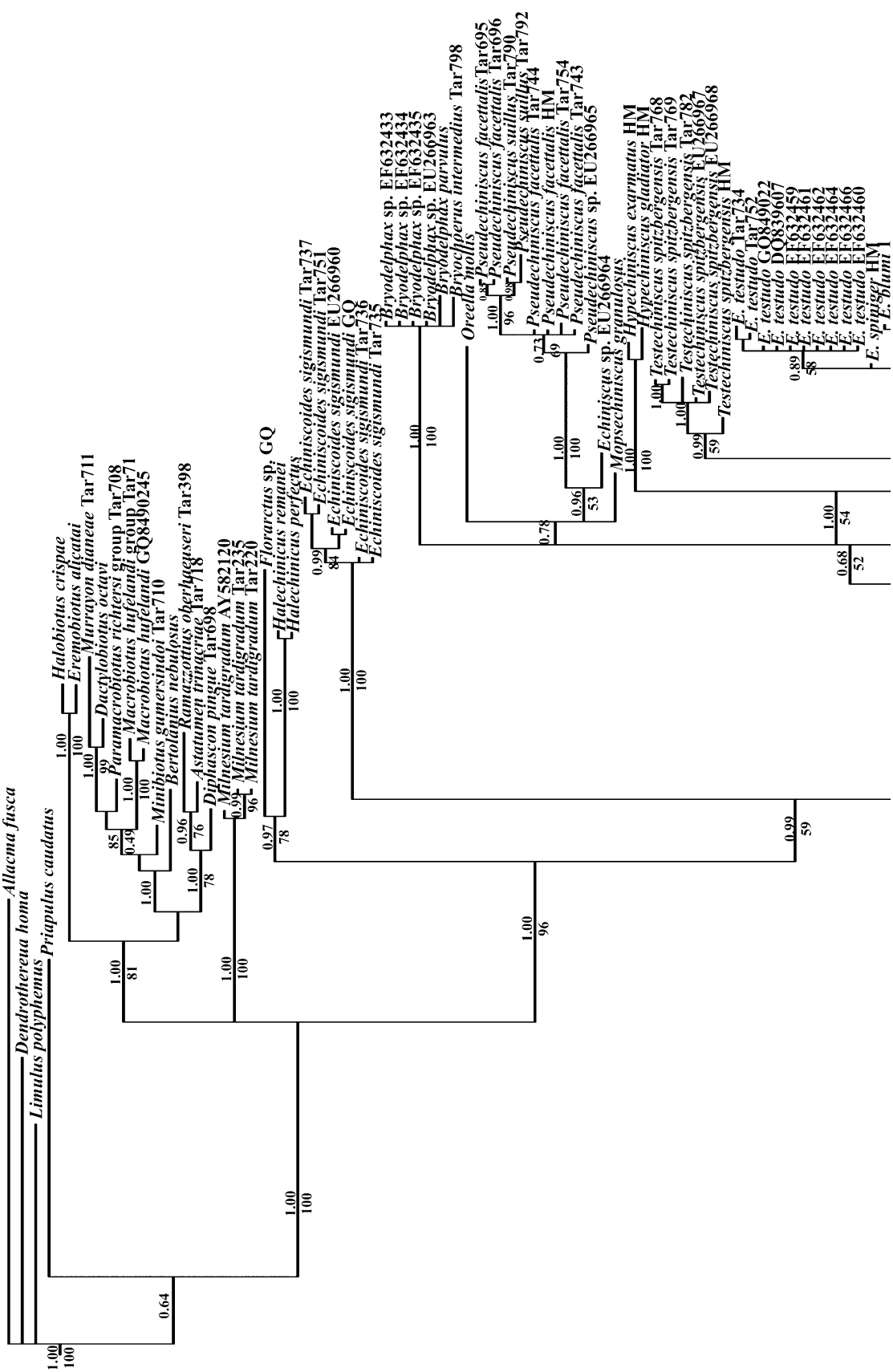


Figure 2. Bayesian phylogram obtained with 18S and 28S rRNA information combined, using all taxa considered in the present study (i.e. outgroups and eutardigrades from Table 3, heterotardigrades from Table 2, and *Echiniscus* species from Table 1). Above branches are posterior probabilities obtained in the Bayesian analysis. Below branches two values are provided: bootstrap support values from the ML analysis, and bootstrap support values from the parsimony analysis. A dash indicates absence of data for a given branch and analysis that had support in other analyses. Tardigrade classes (Heterotardigrada, Eutardigrada), orders (Apochela, Parachela, Arthrotardigrada, Echiniscoidea), and the family Echiniscidae are indicated.


Allacma fusca
Dendrothereua homa
Limulus polyphemus
Priapulus caudatus
Halobiotus crispae
Eremobiotus alıçatai
Murrayon dianeae Tar711
Dactylobiotus octavi
Paramacrobiotus richtersi group Tar708
Macrobiotus hufelandi group Tar71
Macrobiotus hufelandi GQ8490245
Minibiotus gumersindoi Tar710
Bertolanius nebulosus
Ramazzottius oberhauseri Tar398
Astatumen trinacriae Tar718
Diphascon pingue Tar698
Milnesium tardigradum AY582120
Milnesium tardigradum Tar235
Milnesium tardigradum Tar220
Florarctus sp. GQ
Halechinicus remanei
Halechinicus perfectus
Echiniscoides sigismundi Tar737
Echiniscoides sigismundi Tar751
Echiniscoides sigismundi EU266960
Echiniscoides sigismundi GQ
Echiniscoides sigismundi Tar736
Echiniscoides sigismundi Tar735
Bryodelphax sp. EF632433
Bryodelphax sp. EF632434
Bryodelphax sp. EF632435
Bryodelphax sp. EU266963
Bryodelphax parvulus
Bryochoerus intermedius Tar798
Oreella mollis
Pseudechiniscus facettalis Tar695
Pseudechiniscus facettalis Tar696
Pseudechiniscus suillus Tar790
Pseudechiniscus suillus Tar792
Pseudechiniscus facettalis Tar744
Pseudechiniscus facettalis HM
Pseudechiniscus facettalis Tar754
Pseudechiniscus facettalis Tar743
Pseudechiniscus sp. EU266965
Echiniscus sp. EU266964
Mopsechiniscus granulosus
Hypechiniscus exarmatus HM
Hypechiniscus gladiator HM
Testechiniscus spitzbergensis Tar768
Testechiniscus spitzbergensis Tar769
Testechiniscus spitzbergensis Tar782
Testechiniscus spitzbergensis EU266967
Testechiniscus spitzbergensis EU266968
Testechiniscus spitzbergensis HM
E. testudo Tar734
E. testudo Tar752
E. testudo GQ849022
E. testudo DQ839607
E. testudo EF632459
E. testudo EF632461
E. testudo EF632462
E. testudo EF632464
E. testudo EF632466
E. testudo EF632460
E. spinifer HM
E. blumi 1

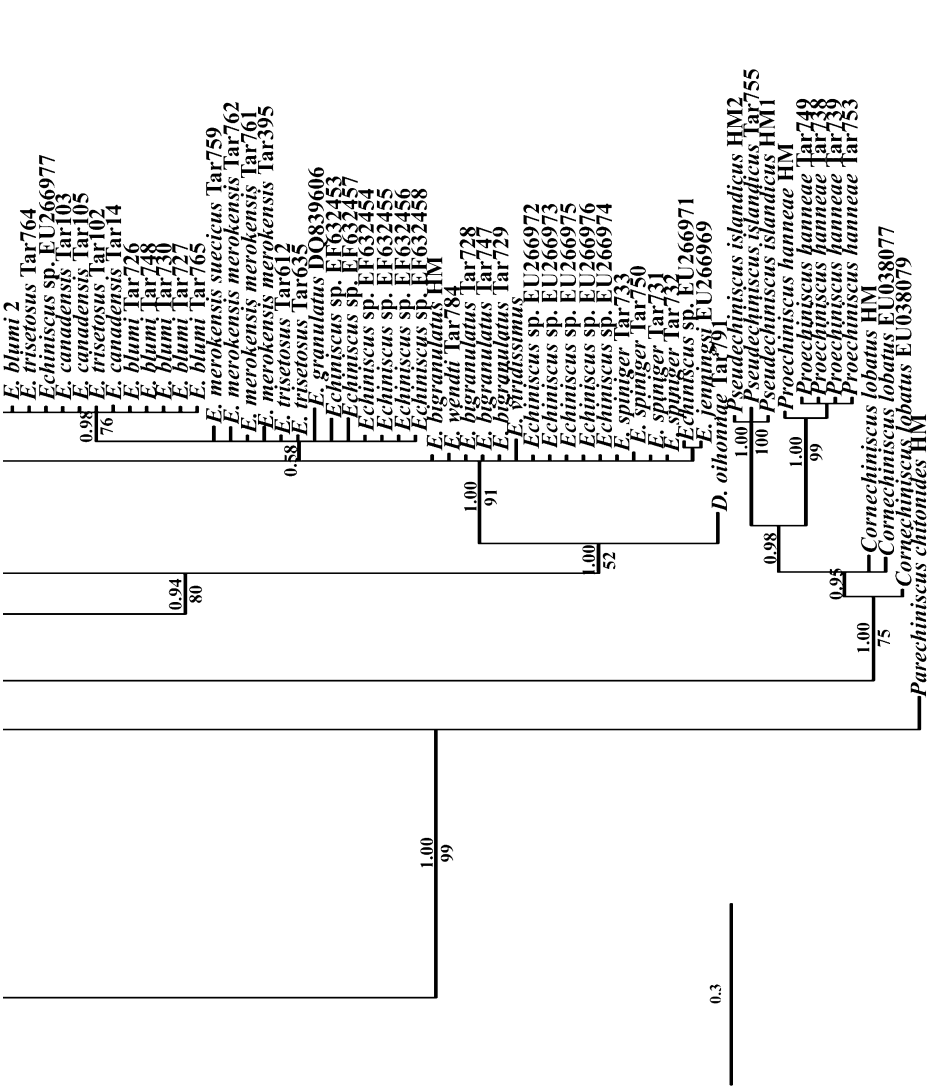


Figure 3. Bayesian phylogram obtained with combined 18S and 28S rRNA, aligned with MUSCLE and trimmed with GBlocks, using all taxa considered in the present study (i.e. outgroups and eutardigrades from Table 3, heterotardigrades from Table 2, and *Echiniscus* species from Table 1). Above branches are posterior probabilities obtained in the Bayesian analysis are provided. Below branches are bootstrap support values from the ML analysis.

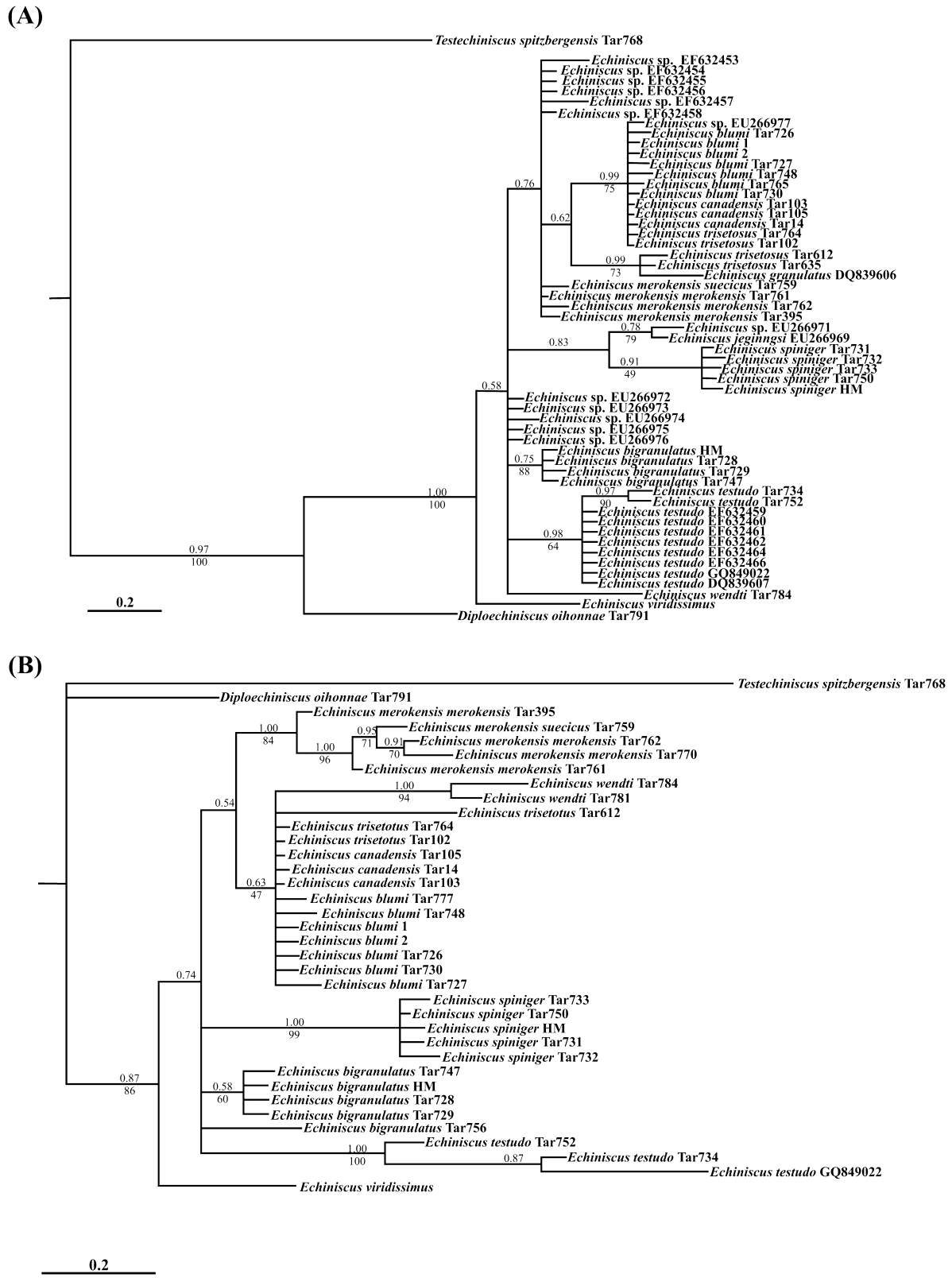


Figure 4. Bayesian phylogram obtained from the analysis of 18S rRNA (A) and 28S rRNA (B) information for *Echiniscus* species and *Diploechiniscus oihonnae*, using *Testechiniscus spitzbergensis* as outgroup. Values above branches indicate posterior probabilities obtained with the Bayesian analysis. Bootstrap support values from ML analysis are provided below branches.

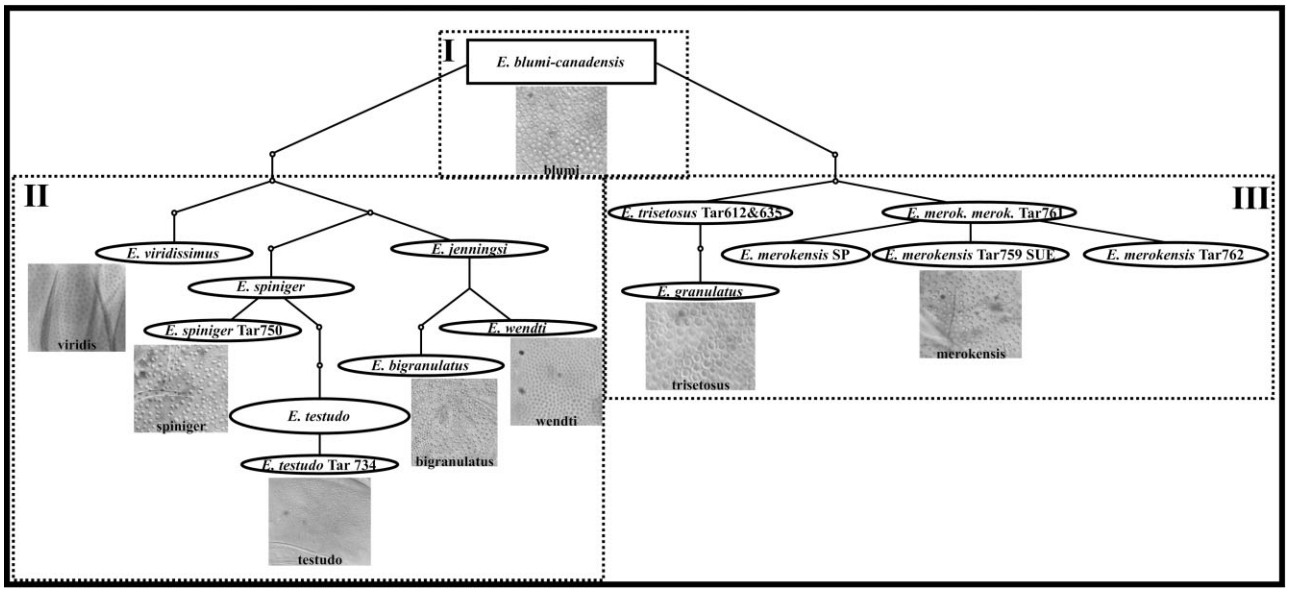


Figure 5. Parsimony network obtained with TCS for the 18S rRNA information from *Echiniscus* species. A photo with the cuticular design for each species is provided. *E. merokensis* SP, Spanish *Echiniscus merokensis merokensis*. *E. merokensis* Tar759 SUE, subspecies *Echiniscus merokensis suecicus*. The three supported groups found among the *Echiniscus* species, based on cuticle design, are identified with dotted squares, and named as I, II, and III.

et al. (2010), the position of *Tanarctus* at the base of Echiniscoidea is questioned due to the short and atypical 28S rRNA sequence, which was conserved in the other heterotardigrades as well as in eutardigrades. Likewise, the positions of *Archechiniscus* and *Orzeliscus* could be questionable based on their 28S rRNA sequences.

Echiniscoidean relationships, the focus of this paper, were better resolved. The sister group of Echiniscidae (including *Oreella*) was *Echiniscoides*, as shown by Jørgensen *et al*. (2010). *Oreella* appears within Echiniscidae (Figs 2, 6) while traditionally it has been given its own monogeneric family, Oreellidae, separated from *Carphania* (Kristensen, 1987). The morphology of *Oreella* clearly distinguishes it from the rest of the echiniscid genera, because it has a series of 'cuticular folds' (and lacks dorsal plates) that divide the body in segments, as dorsal plates do for the echiniscid genera (Kristensen, 1987). Moreover, this genus has been traditionally considered the sister group of Echiniscidae (Kristensen, 1987; Binda & Kristensen, 1986; see also the molecular analysis of Jørgensen *et al*., 2011).

Phenotypic differentiation of Echiniscidae based on the distribution of the dorsal plates (Kristensen, 1987) is used in current classifications, but this system conflicts with molecular phylogenies based on 18S and 28S rRNA (Figs 2, 6), as shown by other authors (Jørgensen, 2000; Jørgensen *et al*., 2011). The phylogenetic lineages proposed based on the presence or absence of the pseudosegmental plate IV′ (Kristensen, 1987) – the *Pseudechinisus*-line (with *Pseudechiniscus*, *Mopsechiniscus*, *Proechiniscus*, *Cornechiniscus*, and *Antechiniscus*) and the *Echiniscus*-line (with *Echiniscus*, *Bryodelphax*, *Bryochoerus*, *Testechiniscus*, and *Hypechiniscus*) – were not corroborated by the present analyses. As in morphological and molecular phylogenetic analyses (Kristensen, 1987; Jørgensen, 2000; Jørgensen *et al*., 2011), *Parechiniscus*, with its weakly sclerotized dorsal plates, appears at the base of Echiniscidae (Figs 2, 6). However, *Bryodelphax* and *Bryochoerus*, as in the morphological phylogeny of Jørgensen (2000), appear in a basal polytomy, together with *Oreella* and *Mopsechiniscus* (Fig. 6). While *Parechiniscus*, *Oreella*, and *Mopsechiniscus* have morphological features that clearly distinguish them from other genera (weakly sclerotized dorsal plates in *Parechiniscus*; 'cuticular folds' in *Oreella*; absence of cirri, long spine shape of cirri A, and thorn-shaped scapular plate in *Mopsechiniscus*), *Bryodelphax* and *Bryochoerus* share with other echiniscid genera the presence of dorsal plates and a similar distribution of sensory organs. The taxonomic validity of *Bryochoerus* has thus been questioned (Kristensen, 1987), as it only differs from *Bryodelphax* in having divided third intersegmental median plates (m3), and not having ventral plates, both morphological characteristics being homoplastic (Kristensen, 1987; Jørgensen, 2000; Jørgensen *et al*., 2011). This may

Table 5. Percentage uncorrected *p*-distances obtained with PAUP* for 18S and 28S rRNA gene sequences within *Echiniscus* species (bold type), between species (bold type) and genera within Tardigrada

Taxonomic level		18S rRNA (%)	28S rRNA (%)	
			a-7b1	a-5b
Within species				
Within *Echiniscus* species		**0.0–0.3**	0.1–0.6	**0.0–0.8**
Within *Testechiniscus* species		0.0–1.2	–	0.0–0.2
Within *Bryodelphax* species		0.9	–	–
Within *Pseudechiniscus* species		0.0–1.3	1.9	0.0–1.9
Within *Milnesium* cf. *tardigradum*		0.0–0.6	0.5	0.5
Among species				
Among *Echiniscus* species		**0.5–1.7**	0.5–3.0/4.3*	**1.0–3.7**
Among *Hypechiniscus* species		1.3	3.7	3.9
P.facettalis Spain	vs. *P.facettalis* Greenland	1.3–1.6	–	1.5
	vs. *P.suillus* Norway	0.7–1.4	0.4	0.0
	vs. *P.islandicus*	8.9–9.9	4.6	6.3
P.facettalis Greenland	vs. *P.suillus* Norway	1.7–3.3	–	1.5
	vs. *P.islandicus*	8.6–9.2	–	5.7
P.novaezeelandiae	vs. *P.facettalis*	0.5–0.6	4.1–6.3	4.6–5.0
	vs. *P.islandicus*	–	6.3	7.4
P.suillus Norway	vs. *P.novaezeelandiae*	–	4.1	4.6
	vs. *P.islandicus*	9.7–13.6	4.9	6.3
Among genera within the same order				
Bryodelphax	vs. *Bryochoerus*	0.8–1.0	–	–
Echiniscus	vs. *Testechiniscus*	**1.8–4.1**	2.9–5.3	**2.8–4.5**
	vs. *Diploechiniscus*	**2.6–3.5**	1.7–3.3/5.0*	**2.4–4.1**
	vs. *Pseudechiniscus*	5.7–8.7	6.0–9.7	5.1–10.4
	vs. *Cornechiniscus*	4.7–6.5	3.8–6.3	4.5–5.1
	vs. *Parechinicus*	5.0–6.1	5.4–7.8	6.3–8.3
	vs. *Mopsechiniscus*	5.4–6.8	6.0–9.2	6.8–9.0
	vs. *Echiniscoides*	11.0–13.5	12.6–14.7	12.6–15.2
Testechiniscus	vs. *Pseudechiniscus*	5.7–8.7	6.3–8.3	4.6–9.6
	vs. *Cornechiniscus*	4.4–6.1	3.6–4.3	4.3–4.5
	vs. *Parechinicus*	4.5–5.3	5.4–6.5	6.4–6.6
	vs. *Mopsechiniscus*	5.2–5.5	5.9–6.8	6.8–7.1
	vs. *Echiniscoides*	12.0–13.7	12.4–13.1	12.9–13.6
Macrobiotus	vs. *Paramacrobiotus*	3.1–3.3	4.8–5.5	4.7–5.6
	vs. *Minibiotus*	3.1–3.2	5.9	4.5–5.9
	vs. *Halobiotus*	7.8–8.1	11.6	11.5
	vs. *Diphascon*	5.4–5.6	7.7	6.9–7.8
Ramazzottius	vs. *Diphascon*	4.5	7.4	5.0
	vs. *Astatumen*	3.7	7.1	4.6
Halobiotus	vs. *Eremobiotus*	1.2	6.5	7.5
Among genera from different orders				
Florarctus	vs. *Echiniscus*	12.8–14.6	12.0–19.4	11.1–20.1
Milnesium	vs. *Macrobiotus*	8.0–8.2	12.6–12.8	11.6–13.0
	vs. *Paramacrobiotus*	6.7–7.0	11.9	11.9
	vs. *Bertolanius*	7.1	12.6	12.7
	vs. *Eremobiotus*	9.0–9.5	13.3	12.7–12.9

*With respect to *E.testudo* GQ849043, comparisons were not possible because no complete sequences were available in any of the specimens sequenced.

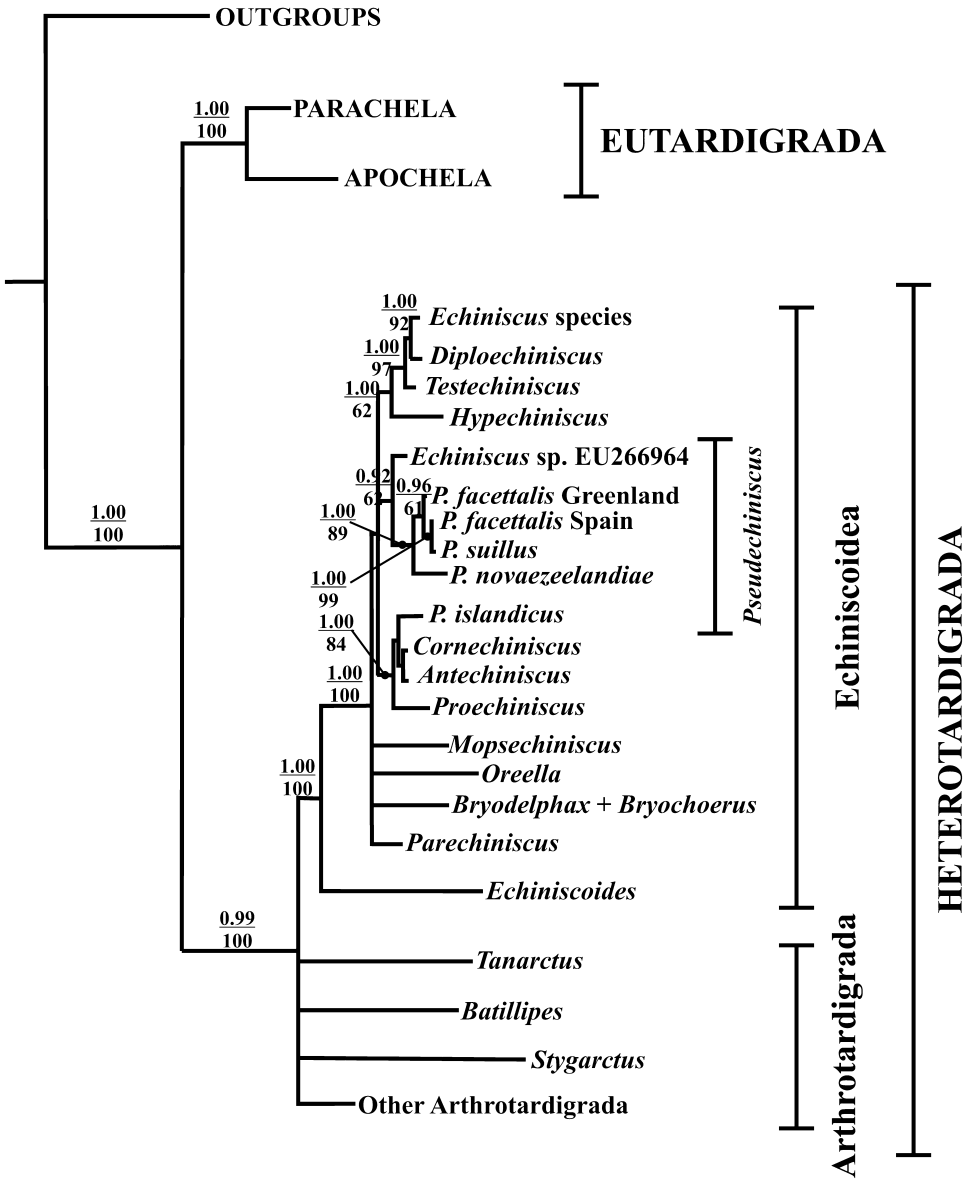


Figure 6. Summary of phylogenetic relationships of heterotardigrade genera obtained in the present study. Values above branches indicate posterior probabilities obtained with Bayesian analysis. Bootstrap support from ML analysis is provided below branches. Tardigrade classes (Heterotardigrada, Eutardigrada), heterotardigrade orders (Arthrotardigrada, Echiniscoidea), and the polyphyletic genus *Pseudechiniscus* are indicated.

thus be the reason why *Bryochoerus* and *Bryodelphax* appear in the same clade, but testing the monophyly of each genus is required before taxonomic changes are proposed.

Another lineage obtained within Echiniscidae comprises all *Pseudechiniscus* species except for *P. islandicus* (Fig. 6 and Fig. S8). The polyphyly of *Pseudechiniscus* is hard to explain morphologically following the current classification system for *Pseudechiniscus* (Ramazzotti & Maucci, 1983). Within the clade including the other *Pseudechiniscus* species, four lineages were found: one for the misidentified *Echiniscus* sp. EU266964 (see Sands *et al*., 2008b; Guil & Giribet, 2012), and three lineages for *Pseudechiniscus* belonging to the *suillus* group. *Pseudechiniscus novaezeelandiae* can be distinguished morphologically by the transversally divided intersegmental median plates 1 and 2 (m1, m2). A notched terminal plate (IV) in *P. facettalis* differentiates it from *P. suillus* (Ramazzotti & Maucci, 1983), so no morphological explanation can be provided for the *P. facettalis* differentiation. Further investigation

is needed to clarify if the Spanish *P. facettalis* specimens were misidentified, as no morphological voucher is available for these GenBank sequences. This is precisely why we emphasize the necessity to keep a photographic record of the extracted animals for public access, as proposed here. An alternative could be that 18S and 28S rRNAs are not suitable markers to solve phylogenetic relationships among these closely related species – although it works well in many other groups of panarthropods (see, for example, Bisset *et al*., 2005; Okamoto, Urushima & Hasegawa, 2009; Zhi-Huan *et al*., 2011), or these constitute cryptic species.

Another clade within Echiniscidae comprises *Proechiniscus*, *Pseudechinicus islandicus*, *Cornechiniscus*, and *Antechiniscus* (Fig. 6) as in Jørgensen *et al*. (2011). This clade resembles Kristensen's (1987) *Pseudechiniscus*-line, composed of *Pseudechiniscus*, *Mopsechiniscus*, *Cornechiniscus*, and *Antechiniscus*. As discussed above, the morphology of *Mopsechiniscus*, especially for sensory organs, is quite different from that of other Echiniscidae, which could explain its basal position within the family (Fig. 6). *Proechiniscus*, *Cornechiniscus*, and *Antechiniscus* share the presence of pseudosegmental plates II′ and III′ and paired segmental plates II and III (Kristensen, 1987). However, the well-supported inclusion of *P. islandicus* and exclusion of other *Pseudechiniscus* species (Figs 2, 6) remain morphologically challenging. Finally, a clade including *Hypechiniscus*, *Testechiniscus*, *Diploechiniscus*, and *Echiniscus* (Fig. 6) resembles Kristensen's (1987) *Echiniscus*-line, but excludes *Bryodelphax* and *Bryochoerus*. These four genera (*Hypechiniscus*, *Testechiniscus*, *Diploechiniscus*, and *Echiniscus*) share: subdivided (in *Diploechiniscus*) or undivided (*Hypechiniscus*, *Testechiniscus*, and *Echiniscus*) intersegmental median plates m1 and m2, an undivided m3 for the four genera, and the absence of pseudosegmental plates (I′, II′, III′, and IV′). *Hypechiniscus* branches early in this clade, with *Testechiniscus* as sister group to the clade comprising *Diploechiniscus* and *Echiniscus* (as in Jørgensen, 2000; Jørgensen *et al*., 2011; Vicente *et al*., 2013), contrary to the hypotheses in which *Pseudechiniscus* was the sister group of *Echiniscus* (Jørgensen *et al*., 2010), or formed part of a clade including also *Bryodelphax* and *Bryochoerus* (Kristensen, 1987). The monophyly of *Echiniscus* is thus corroborated (as in Jørgensen *et al*., 2011; Vicente *et al*., 2013).

Evolutionary importance of cuticular design in *Echiniscus*

Contrary to the conflict found among the dorsal plate configuration in echiniscid genera, and molecular phylogenetic data (using 18S rRNA and 28S rRNA), cuticular design seems to contain evolutionary signal within *Echiniscus*. However, the traditional cuticular design groups (see Ramazzotti & Maucci, 1983; Peluffo *et al*., 2002; Pilato *et al*., 2007, 2008), i.e. *bigranulatus*, *blumi-canadensis*, *merokensis*, *arctomys*, and *viridis* (Fig. 1 and Figs S1–7), did not coincide with the groups found in our parsimony network (Fig. 5). The three groups of *Echiniscus*, supported by the AMOVA (named I, II, and III; Fig. 5), show different types of cuticular design. One group (group I, Fig. 5) is for the *E. blumi-canadensis* group, with the typical polygonal sculpture of *blumi-canadensis*. Another group (group II) includes species with granulation in their cuticles, from the large, roundish, densely distributed granulation of *E. viridissimus* to *E. spiniger* and *E. testudo* with small roundish pores of different sizes, regularly but not densely distributed, *E. jenningsi* and *E. wendti* with similar cuticular designs of very minute granulation regularly and densely distributed (Dastych, 1984), and finally *E. bigranulatus*, with mixed large and fine granulation evenly distributed. In the third group (group III), we include two *E. blumi-canadensis*, which could be a case of misidentification (Guil & Giribet, 2009), *E. granulatus* (pores structured in polygonal areas; Ramazzotti & Maucci, 1983) and *E. merokensis*, which shows a cuticle with pores of various sizes and shapes and smooth cuticle between pores (Fig. 5). In contrast to the evolutionary signal in cuticular design, we found little biogeographical signal in our data even at broad geographical scale: for example, *E. blumi* from Chile and Greenland share 18S rRNA and/or 28S rRNA haplotypes; this is also the case of *E. merokensis* from Spain and Greenland, and of *E. testudo* from Greece, France, and Denmark. This may support the idea that microscopic animals can achieve broad distributions mediated by long-distance passive dispersal (Fenchel & Finlay, 2004).

Monophyly of the different *Echiniscus* species analysed is phylogenetically supported (Fig. 4), but with different data sets, depending on the species: 28S rRNA supports monophyly of four *Echiniscus* species (*E. merokensis*, *E. wendti*, *E. spiniger*, and *E. testudo*) while 18S rRNA confirms monophyly of three species (*E. granulatus*, *E. spiniger*, and *E. testudo*), and one complex of species (*Echiniscus blumi-canadensis*). Two species or complexes of species remain problematic. *Echiniscus bigranulatus* is monophyletic except for specimen Tar756 (GenBank accession number: JX114856; Fig. 3 and Fig. S1). The *Echiniscus blumi-canadensis* complex (comprising *E. blumi*, *E. canadensis*, *E. mediantus* Marcus, 1930, *E. trisetosus*, *E. dearmatus* Bartoš, 1935, and probably *E. marleyi* Li, 2007) has not been supported by 28S rRNA data, as opposed to the rest of the

Echiniscus species complexes studied (Fig. 4B). In contrast, the *blumi-canadensis* complex finds 18S rRNA support (Fig. 4A). However, this complex of species has been problematic for a long time, due to high morphological variability (Guil, 2008) not reflected in the molecular information (at least for COI data; Guil & Giribet, 2009), and apparently supported by the present study (morphospecies of the complex, *E. blumi*, *E. canadensis*, and *E. trisetosus* are not phylogenetically differentiated either by 18S rRNA data or 28S rRNA). Two specimens of the *blumi-canadensis* complex had different sequences (coded as Tar612 and Tar635; GenBank accession numbers: FJ435717, FJ435782, FJ435718, and FJ435783; Table 1) when compared with the rest of the *blumi-canadensis* individuals. These two specimens (Tar612 and Tar635) were closely related to *E. granulatus*, indicating a possible misidentification (for these GenBank sequences there is no morphological voucher).

With the current sampling two clear trends are noted: (1) the distribution of plates within the family Echiniscidae is in conflict with the phylogenetic information derived from 18S and 28S rRNA sequence data; and (2) the cuticular design contains evolutionary signal congruent with the 18S rRNA information within *Echiniscus*. Together with morphological and any other source of information, this would contribute towards a more integrative taxonomic approach within this group of minute animals. We also emphasize the importance of generating and making available morphological information for the study of these tiny animals, as argued previously by Pleijel *et al*. (2008).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Light micrographs of *Echiniscus bigranulatus* (coded as Tar756; accession number: JX114856): A, habitus; B, buccal tube; C, cuticle in head and sensory organs; D, cuticle in posterior side; E, dental collar and claws in PIV.

Figure S2. Light micrographs of *Echiniscus blumi* (coded as Tar730; accession numbers: JX114893 & JX114850): A, habitus; B, buccal tube; C, dental collar and claws in PIV; D, cuticle in posterior side.

Figure S3. Light micrographs of *Echiniscus merokensis merokensis* (coded as Tar761; accession numbers: JX114907 & JX114864): A, habitus; B, buccal tube; C, cuticle in head; D, dental collar and claws in PIV.

Figure S4. Light micrographs of *Echiniscus testudo* (coded as Tar752; accession numbers: JX114904 & JX114862): A, habitus; B, cuticle in head; C, cuticle in posterior side.

Figure S5. Light micrographs of *Echiniscus spiniger*: A, habitus (coded as Tar750; accession numbers: JX114903 & JX114860); B, cuticle in head (coded as Tar750; JX114903 & JX114860); C, cuticle in posterior side (coded as Tar750; JX114903 & JX114860); D, cuticle in posterior side (coded as Tar733; JX114902 & JX114859).

Figure S6. Light micrographs of *Echiniscus wendti* (coded as Tar781; accession number: JX114867): A, habitus; B, cuticle posterior side; C, cuticle in head; D, dental collar and claws in PIV.

Figure S7. Light micrographs of *Diploechiniscus oihonnae* (coded as Tar791; accession numbers: JX114910 & JX114869): A, habitus; B, buccal tube; C, cuticle in posterior side; D, cuticle in head.

Figure S8. Light micrographs of *Pseudechiniscus islandicus* (coded as Tar755; accession numbers: JX114919 & JX114878): A, habitus; B, cuticle in head and sensory organs; C, cuticle in mid-body; D, posterior side, segmental plate IV.

Figure S9. Bayesian phylogram obtained with 18S rRNA information combined and aligned with MUSCLE and trimmed with GBlocks, using all taxa considered in the present study (i.e. outgroups and eutardigrades from Table 3, heterotardigrades from Table 2, and *Echiniscus* species from Table 1). Above branches are posterior probabilities obtained in the Bayesian analysis. Below branches are bootstrap support values from the ML analysis.

Figure S10. Bayesian phylogram obtained with 28S rRNA (between primers 28Sa and 28Srd5b) information combined and aligned with MUSCLE and trimmed with GBlocks, using all taxa considered in the present study (i.e. outgroups and eutardigrades from Table 3, heterotardigrades from Table 2, and *Echiniscus* species from Table 1). Above branches are posterior probabilities obtained in the Bayesian analysis. Below branches are bootstrap support values from the ML analysis.