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Morphological and molecular analyses on *Richtersius* (Eutardigrada) diversity reveal its new systematic position and lead to the establishment of a new genus and a new family within Macrobiotoidea

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Important contributions have been made to the systematics of Eutardigrada in recent years, but these have also revealed that several taxa are polyphyletic and that cryptic species are present. To shed light on the taxonomy and systematic position of the genus *Richtersius* (Eutardigrada, Macrobiotoidea), six populations attributed to *Richtersius coronifer* were collected and analysed from morphological (light and scanning electron microscopy) and molecular (*mitochondrial cytochrome oxidase subunit 1*, *18S*, *28S*) points of view. In particular, a new morphometric index (claw common tract: length of the common tract of the claw/total claw length × 100) and a new morphological character (stalk system) were introduced. Our integrative study was able to unveil the 'cryptic' species diversity within *Richtersius*, showing that the genus contains more than one evolutionary lineage. A morphological peculiarity in the animals of all lineages is the dimorphism in the morphology of the cuticle. Cuticular pores are present in the newborns and are lost with the first moult; this morphological change represents a novelty in the life cycle of eutardigrades. The phylogenetic analyses carried out on *Richtersius* populations and other Macrobiotoidea show that *Richtersius* is closely related to *Macrobiotus islandicus*, whereas *Adorybiotus granulatus* is more related to *Richtersius* and *M. islandicus* than to other members of the genus *Macrobiotus* (type genus of Macrobiotidae); therefore, the genus *Macrobiotus* and the family Macrobiotidae are not monophyletic. Based on these results, the new genus *Diaforobiotus* (for *M. islandicus*) and the new family Richtersiidae (composed of *Richtersius*, *Diaforobiotus* gen. nov., and *Adorybiotus*) are established.

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ADDITIONAL KEYWORDS: *Adorybiotus coronifer* – cryptic species – cuticular pores – ***Diaforobiotus* gen. nov.** – Eutardigrada – *Macrobiotus islandicus* – molecular phylogeny – **Richtersiidae fam. nov**.

INTRODUCTION

There have been important contributions to the systematics of Eutardigrada in the last 10 years, allowing the creation of more robust monophyletic taxa (Guidetti & Bertolani, 2005; Guidetti, Bertolani & Degma, 2007a; Sands *et al.*, 2008; Guidetti *et al.*, 2009; Pilato & Lisi, 2011; Marley, McInnes & Sands, 2011; Bertolani *et al.*, 2014; Vecchi *et al.*, 2016). The taxon that has received most attention is the family Macrobiotidae, formerly the largest in the phylum Tardigrada and including many polyphyletic taxa. The genus *Richtersius* is currently placed within the Macrobiotidae (Guidetti & Bertolani, 2005; Degma, Bertolani & Guidetti, 2015) and certainly belongs to the Macrobiotoidea but the phylogenetic relationships within this superfamily are unclear (Guidetti *et al.*, 2005,

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2009; Jørgensen *et al.*, 2010; Guil & Giribet, 2012; Bertolani *et al.*, 2014). *Richtersius coronifer* (Richters, 1904), the only species of the genus, has a troubled taxonomic history. It was described as *Macrobiotus coronifer* from specimens collected at Klaas Billen-Bay (Spitzbergen, Arctic). Then, the genus *Adorybiotus* was erected by Maucci & Ramazzotti (1981) for *Macrobiotus granulatus* (Richters, 1903) and *M. coronifer*, based on the presence in both species of large dorsal and ventral crests on the buccal tube, and lunules with large teeth on their margins. In the same paper, Maucci & Ramazzotti (1981) also described the neotype of *Adorybiotus coronifer* from specimens collected at Bodö (Norway). Subsequently, Pilato & Binda (1987) erected the new genus *Richtersia*, into which they transferred only *Richtersia coronifer* based on the peculiar morphology of the dorsal crests of the buccal tube. After 2 years, the genus name was changed to *Richtersius* owing to synonymy with a nematode genus (Pilato & Binda, 1989). Although *Richtersius* is a monospecific genus, cryptic species have been identified within this taxon but not described as separate species (Rebecchi *et al.*, 2003; Faurby *et al.*, 2008). *Richtersius* is still a monospecific genus because more than 110 years after the description of *R. coronifer* from the Arctic no other similar species have been described.

To shed light on the morphological and molecular diversity within *Richtersius*, six populations attributed to *R. coronifer* were studied with an integrative approach and compared with the neotype and related material from the type locality of the species. Moreover, to identify the phylogenetic position of *Richtersius* within Macrobiotoidea, other taxa in this superfamily were also evaluated from a molecular and morphological point of view. Based on these results further morphological analyses were performed on *Macrobiotus islandicus* Richters, 1904, to clarify its systematic position.

MATERIAL AND METHODS

Specimens attributed to *R. coronifer* were collected (Table 1) at six different sites. Three sites were in Italy (25–250 km from each other) and the others were from widely scattered locations in West Greenland, Sweden, and Mongolia. Specimens of *M. islandicus* were collected at three sites, in Italy, Norway, and Greenland (Table 1).

Morphological analyses

Animals and eggs were extracted from mosses in all collected samples according to Guidetti *et al.* (2014). Animals and eggs were mounted on slides in Faure-Berlese fluid for light microscopy observations, using phase contrast and differential interference contrast, with a Leica DM RB microscope equipped with a Nikon DS-Fi 1 digital camera. Measurements of *R. coronifer* specimens were carried out using a filar micrometer. The buccal tube length was measured from the anterior end of the cuticular mouth lips (see Guidetti *et al.*, 2012) to the posterior portion of the buccal tube. The other morphometric measurements and the *pt* index (length of a structure/buccal tube length × 100) were taken according to Pilato (1981). In addition, we introduce here the claw common tract index, (*cct* index) which is the length of the common tract of the claw (measured from the claw base to the separation point between the first and the second branch)/total claw length × 100.

Specimens of *Richtersius* from Öland (Sweden), Greenland, Mongolia, and Pratignano (northern Italy 1) were prepared for scanning electron microscopy (SEM) according to Guidetti *et al.* (2014), and observed with a Philips SEM XL 40, available at the 'Centro Interdipartimentale Grandi Strumenti' at the University of Modena and Reggio Emilia, Italy.

For comparison, the neotype of *R. coronifer* (slide CT4063), together with specimens of *M. islandicus* from Norway (CT4198), Greenland (14155-6), and Iceland (14592, 14597, 14610, 14615, 14612) from the Maucci collection (Civic Museum of Natural History of Verona, Italy) were observed.

In addition, three slides (BASDk002-004) from the British Antarctic Survey (BAS) collection were observed. The specimens on the slides were collected in Greenland and belong to the same population from which the GenBank sequences EU266930-1 (attributed to *R. coronifer*) were derived.

To detect whether dimorphism exists between newborns and adults in *Richtersius*, two eggs from each Greenlander, Swedish, and Mongolian populations were kept in water at 20 °C until they hatched. The newborns were maintained at 20 °C on a few wet leaves of moss and observed periodically until their first moult.

The reproductive mode in tardigrades is determined by analysing the sex ratio, which is obtained by observing the type of germinal cells found within the gonad (Rebecchi *et al.*, 2003). To define the reproductive mode of *Richtersius* populations, specimens (20 for each population) from Mongolia, Greenland, and central Italy were fixed *in toto* in Carnoy fluid (methanol/acetic acid, 3/1), individually mounted on slides, and stained with a drop of aceticlactic orcein. The type of germinal elements present in the gonad was detected by observing all animals with the Leica DM RB microscope.

Molecular analyses

Genomic DNA was extracted from single adult tardigrades following the protocol described by Cesari

Table 1. The table reports the sampling site, geographic coordinates, metres above sea level, substrate, and code of the sample from which the specimens of *Richtersius coronifer* and *Macrobiotus islandicus* were extracted. GenBank accession numbers of the nucleotide sequences of *R. coronifer* and *M. islandicus* populations analysed (new sequences in bold) are reported.

Population	Sampling site	Geographic coordinates	m.a.s.l.	Substrate	Sample code	GenBank accession number		
						18S	***28S***	***cox1***
Richtersius coronifer								
Greenland	Red River, Disko Island	69°15.248′N, 53°30.145′E	25	Moss on basalt rock	C3585	**KT778712-3**	**KT778702-3**	EU244607 **KT778690-4**
Sweden	Möckelmossen, Öland Island,	56°31.732′N, 16°29.474′E		Moss (*Orthotricum cupulatum*) on rock	C2353	AY582123	GQ849048	EU251385, EU244606
Northern Italy 1	Pratignano, Northern Apennines, Modena	44°09.197′N, 10°48.415′E	1500	Moss (*Leucodon sciuroides*) on beech bark	C2369	HQ604987-8	**KT778695-6**	AY598780-1
Northern Italy 2	Sasso del Corvo, Piandelagotti, Northern Apennines, Modena	44°12.774′N, 10°31.974′E	1280	Moss (*Homalothecium sericeum*) on rock	C3226	**KT778706-7**	**KT778697-8**	EU251383-4
Central Italy	Serra Santa Mountain, Central Apennines, Gualdo Tadino, Perugia		1260	Moss (*Tortula laevipila*) on rock	C1545	**KT778711**		
Mongolia		46°47.311′N, 101°57.848′E	1790	Moss on rock	C2595 C2592	**KT778708-10**	**KT778699-701**	**KT778683-9**
Macrobiotus islandicus								
Norway	Å, Lofoten Islands	67°52.941′N, 12°58.953′E		Moss on rock	C2661	HQ604972	**KT778704-5**	
Italy	Cucco Mountain, Central Apennines, Perugia		1120	Moss (*Homalothecium philippeanum*) on rock	C1553			
Greenland	Arctic station, Disko Island	69°15.248′N, 53°30.145′E	15	Moss on gneiss rock	C3585			

cox1, cytochrome oxidase subunit 1.

et al. (2009). Molecular investigations were carried out using fragments of the nuclear *18S* and *28S* genes, and of the mitochondrial *cytochrome oxidase subunit 1* (*cox1*) gene.

New molecular analyses of one or more of the *18S*, *28S*, and *cox1* genes were carried out on specimens of all populations of *Richtersius*, and on specimens of *M. islandicus* from the population of Norway (Table 1). The primers and cycles utilized for amplification of the *18S* and the *28S* genes were those described in Vecchi *et al.* (2016). Sequences of *cox1* were amplified according to Bertolani *et al.* (2011a). The amplified products were gel purified and sequenced as indicated in Vecchi *et al.* (2016). Nucleotide sequences of the newly analysed specimens were submitted to GenBank (for accession numbers see Table 1). For phylogenetic analyses, *18S* and *28S* nucleotide sequences of other Macrobiotoidea species obtained from GenBank were considered (Table S1). A sequence pertaining to the species *Milnesium* cf. *tardigradum* (Eutardigrada, Apochela) was used as the outgroup (Table S1).

The sequences were aligned with the Muscle algorithm, using default parameters implemented in MEGA5 (Tamura *et al.*, 2011). The resulting alignment was inspected for accuracy by searching for software homology misinterpretations. The GBlocks program (Catresana, 2000) was used for applying relaxed settings and parameters (values are as specified in Bertolani *et al.*, 2014) and for discarding uninformative regions of the alignment. The combined analyses (*18S* + *28S*) were performed by applying Bayesian inference (BI) and maximum likelihood (ML) as described in Bertolani *et al.* (2014).

The new molecular analyses for the *cox1* gene were carried out on seven *Richtersius* specimens from Mongolia and five specimens from West Greenland following the protocol of Cesari *et al.* (2009). For appropriate molecular comparisons, we included in our analysis *cox1* sequences from GenBank attributed to other *Richtersius* specimens from the population of Sweden, northern Italy 1, northern Italy 2, and China (Table S2). Pairwise nucleotide sequence divergences between scored haplotypes were calculated utilizing both the *p*-distance and the Kimura two-parameter model by using MEGA5.

RESULTS

Morphological results

Richtersius coronifer

Morphological differences amongst populations attributed to *Richtersius* were mainly observed in the characters related to the cuticle surface, claws, and buccal–pharyngeal apparatus. The new morphometric index (claw common tract) and the reproductive mode proved to be very useful to differentiate certain populations of *Richtersius*.

Cuticular pores: In all six populations of *Richtersius*, specimens with and without cuticular pores (pearls) were observed. Pores (Fig. 1A–D) were generally present in specimens shorter than 500 μm in length. To clarify this situation, we followed six newborns from their hatching up to their first moult. After the newborns hatched, all their cuticles had pores that were distributed over the entire cuticular surface (Fig. 1A–D). After the first moult the new cuticles were without pores.

Diversity in the morphology of cuticular pores was observed amongst the specimens of the six *Richtersius* populations. Greenland and Mongolian specimens exhibited many small cuticular pores (Ø = 1–2 μm) with regular margins (Fig. 1D), whereas the Italian and Swedish specimens showed rare, scattered, and large pores (Ø = 2–4 μm) with thick, irregular margins (Fig. 1A–C).

Claws: In all populations, the animals did not differ in the relative lengths of the claws, and the ranges (maximum–minimum) of the *pt* indexes of claw lengths on all legs overlapped amongst populations (Table 2). However, clear differences were recorded in the relative lengths of the common tract (tract of the claw in which the main and secondary branch are fused), with populations exhibiting common tracts that were either short or long with respect to claw length (Table 2). These differences allowed us to identify two groups of populations: one made up of the Norwegian neotype and the Greenland and Mongolian populations (short common tracts; Fig. 1E–G), and the other made up of the Swedish and Italian populations (long common tracts; Fig. 1H–J). For each leg, the ranges of the claw common tract index did not overlap or overlapped only slightly for the claws of the first and fourth legs, but if the newborns were not considered, there was no overlap.

In all populations, the animals had claws with an evident 'stalk system' composed of a laminar stalk (connecting the common tract to the lunule) and two posterior lateral expansions, whose distal tips were connected to the stalk where it came in contact with the lunule (Fig. 1F, I, J). Although never described before, a similar 'stalk system' is also present in other Macrobiotoidea (e.g. Guil & Guidetti, 2005: fig. 2C; Kaczmarek *et al.*, 2011: fig. 3).

Buccal–pharyngeal apparatus: The buccal tube wall became thicker from the anterior to the posterior portion in all specimens of all populations (Fig. 2A–

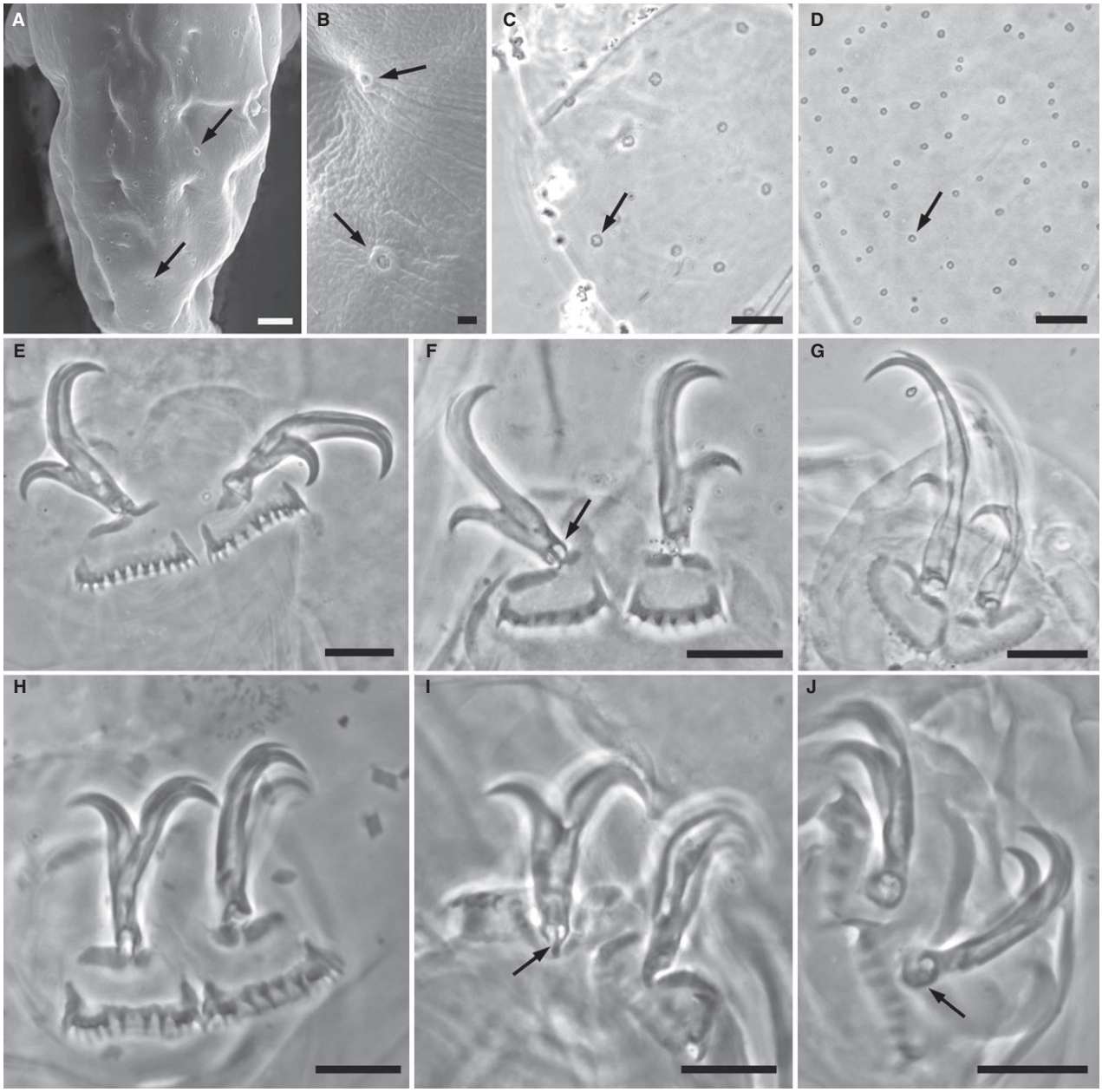

Figure 1. *Richtersius coronifer*. A, dorsal cuticle of a newborn with pores (arrows) (population from Sweden). B, cuticular pores of a newborn (arrows) (Sweden). C, cuticular pores of a newborn with irregular margin (arrow) (northern Italy 1). D, cuticular pores of a newborn with regular margin (arrow) (Greenland). E, claws on the second leg in an adult (neotype) (Norway). F, frontal view of the claws on the hind leg with an evident stalk system in an adult (arrow) (Greenland). G, claws on the hind leg in a newborn (Greenland). H, claws on the second leg in an adult (northern Italy 1). I, frontal view of the claws on the first leg with an evident stalk system in an adult (arrow) (Sweden). J, lateral view of the claws on the second leg with an evident stalk system in an adult (arrow) (central Italy). A, B: scanning electron microscopy; C–J: phase contrast. Scale bars: A, C–J = 10 μm, B = 1 μm.

D). In particular, the buccal tube of the neotype (Fig. 2A), the Greenlander (Fig. 2D), and the Mongolian specimens became extremely thick after the stylet support insertions, but it reduced its thickness before its end within the pharynx.

The wide buccal crown (see Guidetti *et al.*, 2012) was followed dorsally by a huge apophysis with a posterior crest connected to the buccal tube (Fig. 2A–C). This crest was long in the specimens of all populations, with the exception of the Italian ones in

Table 2. Morphometric data of the *Richtersius* populations. Ten animals (seven adults and three newborns) were measured for each population.

Richtersius population	tl (μm)	*pt* ssi	*pt* cl I	*cct* cl I	*pt* cl II–III	*cct* cl II–III	*pt* cl IV	*cct* cl IV
Norway neotype	769.0	72.7	26.1	52.2	29.5	50.0	37.5	45.5
Greenland Mean (SD)	622.8 (135.7)	75.2 (1.6)	27.4 (3.1)	53.7 (2.9)	31.2 (3.1)	49.6 (2.8)	38.8 (4.7)	48.4 (2.4)
Min.–max. total	467.7–875.6	72.2–77.5	23.9–33.8	47.6–56.5	27.2–36.8	44.0–53.8	33.3–47.9	44.1–51.5
Min.–max. without newborns	497.5–875.6	72.2–76.3	23.9–27.8	54.2–56.5	27.2–32.2	44.0–53.8	33.3–42.3	46.9–51.5
Sweden Mean (SD)	533.3 (131.9)	69.3 (1.3)	27.7 (2.1)	60.7 (5.8)	29.0 (1.5)	62.6 (4.3)	36.9 (3.5)	60.3 (7.4)
Min.–max. total	318.4–676.6	67.5–72.0	25.5–31.4	50.0–69.6	27.3–31.4	57.1–69.6	30.5–41.2	52.4–75.5
Min.–max. without newborns	467.7–676.6	67.5–72.0	25.8–31.2	58.8–69.6	28.0–31.2	57.9–69.6	30.5–38.7	58.6–75.9
Northern Italy 1 Mean (SD)	571.3 (147.8)	63.6 (2.5)	27.9 (2.3)	68.4 (4.5)	29.2 (1.9)	67.5 (4.6)	38.1 (4.1)	63.0 (8.6)
Min.–max. total	368.0–806.0	58.4–66.0	24.5–31.1	58.8–73.9	27.3–32.4	60.0–75.0	33.3–44.6	50.0–82.4
Min.–max. without newborns	498.0–806.0	58.4–65.7	24.5–31.1	58.8–73.9	27.3–32.4	63.6–75.0	34.8–44.6	58.1–69.0
Northern Italy 2 Mean (SD)	515.3 (144.8)	69. 9 (1.0)	28.5 (3.9)	59.7 (3.6)	29.5 (3.5)	59.8 (4.5)	37.1 (4.0)	57.4 (4.1)
Min.–max. total	298.5–746.3	68.5–71.9	21.3–36.5	52.6–64.7	23.0–35.1	55.6–70.0	27.9–41.1	50.5–63.6
Min.–max. without newborns	368.2–746.3	68.5–71.9	21.3–31.5	55.0–64.7	23.0–35.1	55.6–70.0	27.9–41.1	57.1–63.6
Central Italy Mean (SD)	490.5 (164.3)	67.7 (1.5)	27.8 (2.6)	65.6 (4.8)	29.0 (2.3)	64.8 (4.1)	39.8 (7.1)	64.9 (5.4)
Min.–max. total	279.0–696.5	65.3–70.1	23.6–30.7	60.0–76.5	23.6–32.1	60.0–71.4	32.7–58.0	50.0–68.4
Min.–max. without newborns	453.0–696.5	66.2–69.3	23.9–30.7	60.9–76.5	27.6–32.1	60.0–71.4	35.5–43.8	64.0–68.2
Mongolia mean (SD)	576.3 (104.2)	73.8 (1.3)	26.3 (2.2)	47.9 (4.4)	28.9 (2.6)	47.2 (4.4)	35.3 (3.4)	47.4 (4.6)
Min.–max. total	398.0–706.5	71.7–75.8	22.5–29.3	41.7–54.5	24.4–34.1	41.2–54.5	30.0–41.5	40.0–54.2
Min.–max. without newborns	507.5–706.5	72.5–75.6	22.5–29.3	41.7–54.5	25.4–34.1	42.9–54.5	30.0–41.5	45.5–54.2

cct, claw common tract index; cl I–IV, claws from the first to the fourth pair of legs; *pt*, *pt* index; ssi, stylet support insertion; tl, animal total length.

which it was shorter (Fig. 2B). Ventrally, the buccal crown was followed by a short ventral lamina with a large anterior hook that was particularly large in the northern Italy population 1 (Fig. 2B).

The *pt* index of the stylet support insertion (*pt* SSI) differed amongst populations. In the neotype, Greenlander, and Mongolian specimens the *pt* SSI was ≥ 72, whereas in the Italian and Swedish populations it was ≤ 72 (Table 2; Fig. 2A–D).

Eggs: Specimens of all samples laid free eggs with ornamented shells. The eggs were large (Ø > 100 μm) and yellowish/brown. The egg shell processes were long and thin cones, with an internal reticulated structure. The surface of the shell between the processes was smooth; the shell surface was generally difficult to see owing to the frequent presence of debris attached to it. No differences have been identified so far in the egg shell morphology amongst populations.

Reproductive biology: The populations from Greenland, Mongolia, and central Italy included females and several males with spermatozoa within the gonad, and therefore they can be considered gonochoric-amphimictic. As reported in a previous study (Rebecchi *et al.*, 2003), the populations from Sweden and northern Italy 2 were unisexual and parthenogenetic, whereas the population from northern Italy 1 was gonochoric-amphimictic.

Macrobiotus islandicus

In all three populations attributed to *M. islandicus* and in the specimens from the Maucci collection, the buccal–pharyngeal apparatus consisted of a buccal ring with buccal lamellae, a buccal tube with an

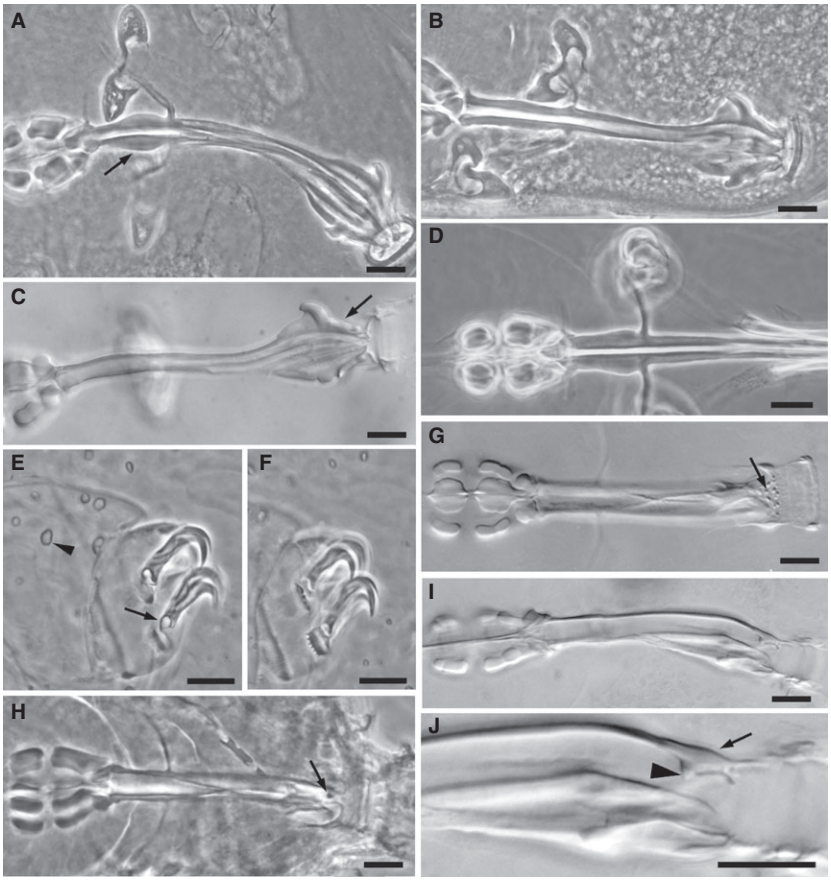

Figure 2. *Richtersius coronifer* (A–D) and *Diaforobiotus islandicus* (E–J). A, buccal–pharyngeal apparatus, with enlarged terminal portion (arrow) of the buccal tube (neotype) (Norway). B, buccal–pharyngeal apparatus (population from northern Italy 1). C, buccal–pharyngeal apparatus, with the dorsal thickening in the anterior portion of the buccal tube (arrow) (Sweden). D, buccal–pharyngeal apparatus (Greenland). E, F, lateral view of the claws on the second leg at different focus levels. The evident stalk system (arrow) and cuticular pores (arrowhead) on the leg are visible (Norway). G, buccal–pharyngeal apparatus (ventral view), with some strong, scattered round teeth in the buccal armature (arrow) (Italy). H, buccal–pharyngeal apparatus (dorsal view), with the large tooth (arrow) on the internal surface of the buccal tube (Norway). I, buccal–pharyngeal apparatus (lateral view) (Greenland). J, anterior portion of the buccal tube (enlargement of I), with the large tooth on internal surface (arrowhead) and the dorsal thickening (arrow) (Greenland). A, B, D–F, H: phase contrast; C, G, I, J: differential interference contrast. Scale bars = 10 μm.

evident anterior bend, and a pharynx with pharyngeal apophyses and two macroplacoids (Fig. 2G–J). The buccal tube was characterized by: a buccal armature formed by a wide posterior band of small teeth, followed by some large, scattered round teeth, and without transverse crests; a ventral lamina; and a dorsal thickening in the anterior portion of the buccal tube, corresponding to a large tooth on the internal surface of the tube (Fig. 2H–J). The entire cuticle had pores on its surface (Fig. 2E). The claws showed an evident stalk system (Fig. 2E) and were characterized by large teeth on the lunules on all pairs of legs (Fig. 2F).

The eutardigrade specimens from Greenland on slides BASDk002-004 of the BAS collection were also attributed to *M. islandicus*.

Molecular results

Genetic distances

Genetic distances amongst specimens of *Richtersius* were based on the partial sequence (582 bp) of the mitochondrial gene *cox1*. A total of 13 haplotypes was found, with none shared amongst any of the *Richtersius* populations. The number of haplotypes found in each population differed depending on the population and number of analysed specimens. Two specimens were analysed for the Swedish, northern Italy 1, and northern Italy 2 populations, revealing one, one, and two haplotypes, respectively. Three haplotypes were found in the six specimens of the Greenland population, whereas the seven specimens of the Mongolian population showed six different haplotypes (Table S2).

Genetic distances showed low diversity within each population (*p*-distance ranging from 0 to 2.9%), whereas high distances were recorded amongst populations, ranging from 9.6 to 22.2%, with the exception of the comparison between the two parthenogenetic populations (northern Italy 2, Sweden), which showed a *p*-distance of ≤ 2.6% (Table S2).

Phylogenetic analyses

Both the BI and ML phylogenetic trees computed from the combined *18S* and *28S* sequences of Macrobiotoidea species showed the same topologies (Fig. 3), albeit with a few differences in the support values of some nodes. The phylogenetic relationships within Macrobiotoidea reflect those found in previous studies (Bertolani *et al.*, 2014; Vecchi *et al.*, 2016). Within the superfamily, three well-supported clusters were found. The first cluster was composed of the genera *Paramacrobiotus* and *Minibiotus*, the second of *Macrobiotus*, *Mesobiotus*, and *Xerobiotus*, and the third of *Adorybiotus*, *Richtersius*, *Murrayon*, *Dactylobiotus*, and the species *M. islandicus*. The relationships within the latter cluster were not completely resolved. *Murrayon* and *Dactylobiotus* were sister groups (they belong to the family Murrayidae), whereas *Adorybiotus* was related to them but with low support values. All sequences pertaining to *Richtersius* specimens (both newly analysed and retrieved from GenBank) clustered together in a sister group of the *M. islandicus* lineage, composed of the sequences of *M. islandicus* from Italy (HQ604972) and those from Greenland, originally attributed to *R. coronifer* (EU266930-1).

Within the *Richtersius* lineage, two well-supported sister groups were present (Fig. 3), one formed by the Greenland and Mongolian populations, the other formed by the Italian and Swedish populations. In this last group, there were two additional clusters: one with the central Italy and northern Italy 1 populations, and the other group containing the parthenogenetic populations from northern Italy 2 and Sweden.

DISCUSSION

Our integrative study also identified the 'cryptic' species diversity previously found amongst some populations of *Richtersius* attributed to the same species (Rebecchi *et al.*, 2003; Faurby *et al.*, 2008), showing that the genus contains more than one evolutionary lineage.

A molecular approach is undoubtedly useful to identify and discriminate tardigrade species. Previous studies on some *Macrobiotus* taxa (Cesari *et al.*, 2009; Bertolani *et al.*, 2011a,b; Cesari *et al.*, 2011, 2013) identified a threshold in genetic difference between *cox1* gene sequences that can help in discriminating two species: a *p*-distance and/or Kimura two-parameter value higher than 3%. The analysed populations of *Richtersius* showed genetic distances often much higher than 3% amongst them (Table S2). These genetic differences, the morphological differences identified amongst populations and between the populations studied here and the neotype specimen of *R. coronifer*, and the presence of different evolutionary lineages associated with each population together lead to the conclusion that there is more than one species in the genus *Richtersius*. Further morphological data on animals and eggs, and statistical analyses of morphometric characters are needed to eventually establish new species from the populations studied here. These potential species can be grouped into two different evolutionary lineages, identified by both the molecular phylogenetic analysis (Fig. 3) and their peculiar morphological characters (Figs 1, 2). One lineage consists of *R. coronifer* from the neotype locality and the gonochoric-amphimictic populations of *Richtersius* from Mongolia and Greenland. This cluster is characterized by small cuticular pores in the newborns, claws with a very long main branch (longer than the common tract), stylet supports inserted on the buccal tube at more than 72% of the tube length, and increased thickness of the buccal tube wall posterior to the stylet support insertion points. The other lineage is characterized by large cuticular pores in the newborns, claws with a long common tract (longer than the main branch), and stylet supports inserted on the buccal tube at less than 72% of the tube length. Within this latter lineage, there are two clusters differentiated by their reproductive modes: one is made up of parthenogenetic populations (northern Italy 2, Sweden), whereas the other is composed of gonochoric-amphimictic populations (northern Italy 1, central Italy).

With regard to intraspecific variability, the presence of cuticular pores only in some specimens of *R. coronifer* had already been reported by some authors (e.g. Rodríguez-Roda, 1952; Maucci & Ramazzotti, 1981; Maucci, 1986; Dastych, 1988), but it remained unclear as to whether the pores were characteristic of the species or a result of intraspecific variability. Our data demonstrate that the presence or absence of pores is a result of intraspecific variability because the pores are present in the cuticle of the newborn and then lost at the first moult. The different sizes and shapes of the pores amongst *Richtersius* populations will be useful characters for species discrimination. The biological significance of these cuticular pores, present only during a short period of the life cycle of the animal, as well as of the pores in all tardigrade species, remains unknown. The peculiar pattern of the presence/absence of pores during the life cycle of *Richtersius*

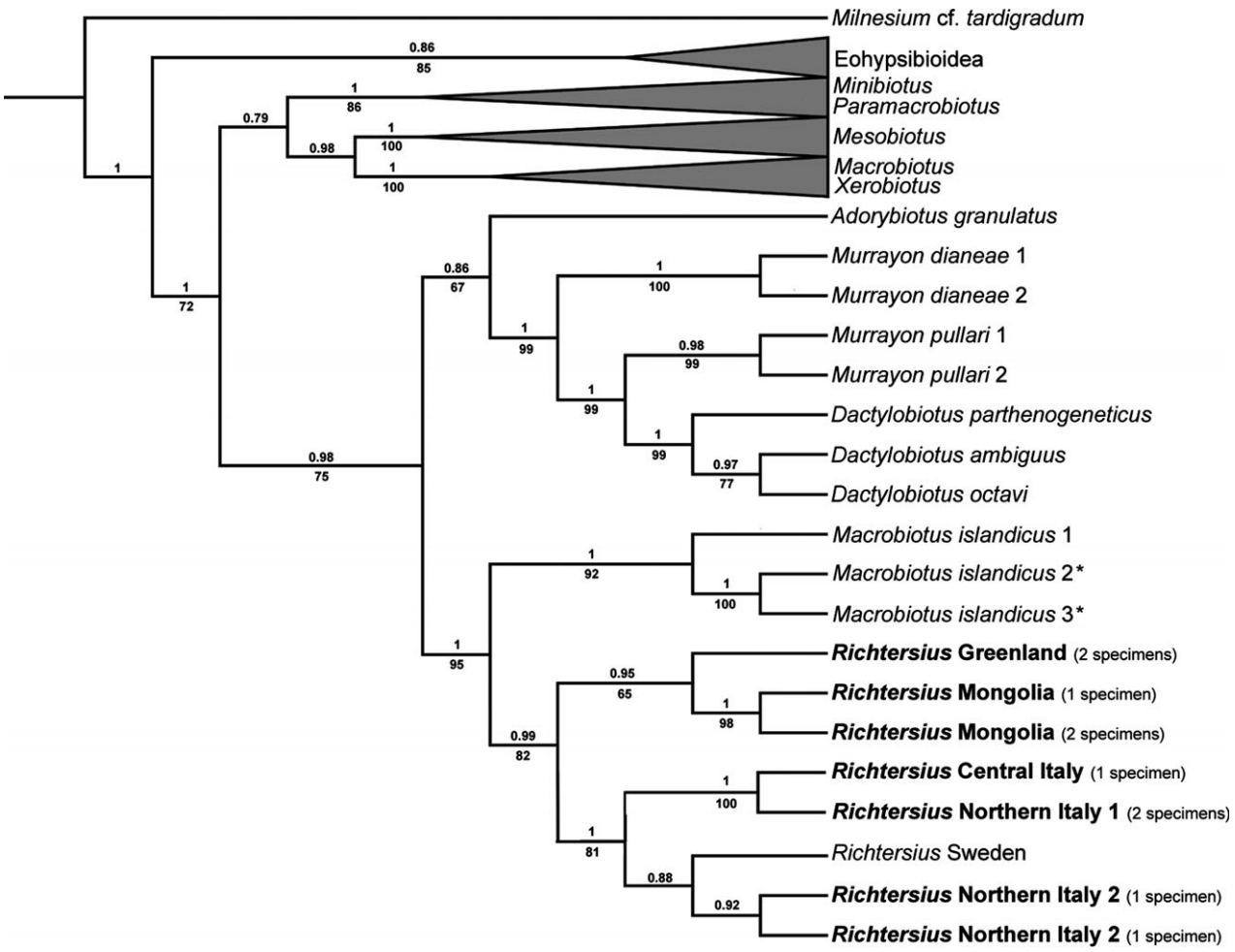

Figure 3. Phylogenetic reconstruction of Macrobiotoidea based on combined data set (*18S* + *28S* rRNA sequences) and obtained by Bayesian inference (BI) and maximum likelihood (ML). Values above branches: BI posterior probability values; values under branches: ML bootstrap values (values below 65 are not reported). In bold, newly generated sequences; in grey, clusters of genera or species. *, GenBank sequences (EU266930-1) wrongly attributed on the basis of morphology to *Richtersius coronifer*.

suggests that in this genus the pores are related to the newborn way of life, or that they are involved during the development of the embryo inside the egg, perhaps in increasing cuticle permeability. The finding of pores in *Richtersius* is a novelty for eutardigrades as no similar type of dimorphism has been previously reported between newborns and adults in eutardigrade species, whereas this kind of dimorphism is well known in heterotardigrade species (Bertolani *et al.*, 1984; Hansen *et al.*, 2016). In eutardigrade species, the morphological changes during the life cycle had previously been linked only to morphometric (e.g. Guidetti *et al.*, 2007b; Bartels, Nelson & Exline, 2011) or to sexual characters (Rebecchi & Nelson, 1998).

A second result of our research is the confirmation and additional evidence that some taxa within Macrobiotoidea are not monophyletic, as indicated by previous studies (Sands *et al.*, 2008; Guidetti *et al.*, 2009; Guil & Giribet, 2012; Bertolani *et al.*, 2014; Vecchi *et al.*, 2016). Our phylogenetic analyses showed that the genus *Macrobiotus* and the family Macrobiotidae are not monophyletic. In fact, *M. islandicus* does not belong to the lineage of the other *Macrobiotus* species but to a phylogenetic lineage related to *Richtersius*. *Macrobiotus islandicus,* and the genera *Adorybiotus* and *Richtersius*, belong to a lineage closer to the Murrayidae family than to *Macrobiotus* (the type genus of Macrobiotidae). For these reasons and based on the presence of peculiar morphological characters, the new genus *Diaforobiotus* gen. nov. is established for *M. islandicus*, and the new family Richtersiidae fam. nov. is established for the genera *Richtersius* and *Diaforobiotus* gen. nov.

together with *Adorybiotus* (see Taxonomic account). Although not completely supported by the molecular data, perhaps because of taxon and/or gene sampling problems, the relationship amongst *Richtersius*, *Diaforobiotus* gen. nov., and *Adorybiotus* is supported by several morphological characters: a cuticular thickening on the anterior dorsal wall of the buccal tube (Fig. 2C, J), large teeth on all lunules (Figs 1E–J, 2F), absence of transverse crests in the buccal armature (Fig. 2A–C, G, H), two macroplacoids in the pharynx (Fig. 2), and presence of cuticular pores, at least in the newborns (Figs 1A–D, 2E, F). Based on molecular data, Richtersiidae fam. nov. and Murrayidae belong to the same lineage, but there is no morphological support, e.g. synapomorphies, to erect a new taxon or to attribute all the genera in the two families to the same family.

TAXONOMIC ACCOUNT

RICHTERSIIDAE FAM. NOV.

Description: Double claws Y-shaped, with the two branches forming an evident common tract of variable length. Large teeth on all lunules. Buccal tube with ventral lamina and a cuticular thick on the anterior, dorsal wall of the buccal tube (which can form a large apophysis). Absence of transverse crests in the buccal armature. Two macroplacoids in the pharynx. Cuticular pores (at least in a phase of the life cycle). Eggs laid freely with cuticular processes on their surface.

Type genus. Richtersius

Composition. Richtersius, *Diaforobiotus* gen. nov., *Adorybiotus* (provisionally, see Remarks).

Remarks. Based on molecular data, *Richtersius*, *Diaforobiotus* gen. nov., and *Adorybiotus* do not belong to Macrobiotidae, but the phylogenetic relationship of *Adorybiotus* with the other genera of the families Richtersiidae fam. nov. and Murrayidae needs to be clarified with further molecular data. Based on several morphological affinities with *Richtersius* and *Diaforobiotus* gen. nov. (i.e. a cuticular thickening on the anterior dorsal wall of the buccal tube, large teeth on all lunules, absence of transverse crests in the buccal armature, two macroplacoids in the pharynx, and presence of cuticular pores), we place *Adorybiotus* in this family.

DIAFOROBIOTUS GEN. NOV.

Description: Peribuccal lamellae (ten) and ventral lamina present. A dorsal thickening present in the anterior portion of the buccal tube, in conjunction with a large tooth on the internal surface of the tube. Some strong, scattered round teeth present posterior to the second band of teeth of the buccal armature present.

Type species. Macrobiotus islandicus Richters, 1904.

Etymology. Diaforobiotus, from *diaforos* (Greek) = different, different from all other macrobiotoid genera.

Composition. Diaforobiotus islandicus (Richters, 1904).

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REFERENCES

Bartels PJ, Nelson DR, Exline RP. 2011. Allometry and the removal of body size effects in the morphometric analysis of tardigrades. *Journal of Zoological Systematics and Evolutionary Research* **49:** 17–25.

Bertolani R, De Zio SG, Gallo MDA, Morone De Lucia MR. 1984. Postembryonic development in heterotardigrades. *Monitore Zoologico Italiano* **18:** 307–320.

Bertolani R, Biserov V, Rebecchi L, Cesari M. 2011a. Taxonomy and biogeography of tardigrades using an integrated approach: new results on species of the *Macrobiotus hufelandi* group. *Invertebrate Zoology* **8:** 23–36.

Bertolani R, Rebecchi L, Giovannini I, Cesari M. 2011b. DNA barcoding and integrative taxonomy of *Macrobiotus hufelandi* CAS Schultze 1834, the first tardigrade species to be described, and some related species. *Zootaxa* **2997:** 19–36.

Bertolani R, Guidetti R, Marchioro T, Altiero T, Rebecchi L, Cesari M. 2014. Phylogeny of Eutardigrada: New molecular data and their morphological support lead to the identification of new evolutionary lineages. *Molecular Phylogenetics and Evolution* **76:** 110–126.

Catresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analyses. *Molecular Biology and Evolution* **17:** 540–552.

Cesari M, Bertolani R, Rebecchi L, Guidetti R. 2009. DNA barcoding in Tardigrada: the first case study on *Macrobiotus macrocalix* Bertolani & Rebecchi, 1993 (Eutardigrada, Macrobiotidae). *Molecular Ecology Resources* **9:** 699–706.

Cesari M, Giovannini I, Bertolani R, Rebecchi L. 2011. An example of problems associated with DNA barcoding in tardigrades: a novel method for obtaining voucher specimens. *Zootaxa* **3104:** 42–51.

Cesari M, Guidetti R, Rebecchi L, Giovannini I, Bertolani R. 2013. A DNA barcoding approach in the study of tardigrades. *Journal of Limnology* **72:** 182–198.

Dastych H. 1988. *The Tardigrada of Poland. Monografie Fauna Polski 16*. Warsaw: Panstwowe wydawnictwo naukowe.

Degma P, Bertolani R, Guidetti R. 2015. Actual checklist of Tardigrada species. http://www.tardigrada.modena.unimo.it/miscellanea/Actual%20checklist%20of%20Tardigrada.pdf, pp. 40. Accessed 15/07/2015.

Faurby S, Jönsson KI, Rebecchi L, Funch P. 2008. Variation in anhydrobiotic survival of two eutardigrade morphospecies: a story of cryptic species and their dispersal. *Journal of Zoology* **275:** 139–145.

Guidetti R, Bertolani R. 2005. Tardigrade taxonomy: an updated check list of the taxa and a list of characters used in their identification. *Zootaxa* **845:** 1–46.

Guidetti R, Gandolfi A, Rossi V, Bertolani R. 2005. Phylogenetic analysis of Macrobiotidae (Eutardigrada, Parachela): a combined morphological and molecular approach. *Zoologica Scripta* **34:** 235–244.

Guidetti R, Bertolani R, Degma P. 2007a. New taxonomic position of several *Macrobiotus* species (Eutardigrada: Macrobiotidae). *Zootaxa* **1471:** 61–68.

Guidetti R, Colavita C, Altiero T, Bertolani R, Rebecchi L. 2007b. Energy allocation in two species of Eutardigrada. *Journal of Limnology* **66:** 111–118.

Guidetti R, Schill RO, Bertolani R, Dandekar T, Wolf M. 2009. New molecular data for tardigrade phylogeny, with the erection of *Paramacrobiotus* gen. n. *Journal of Zoological Systematics and Evolutionary Research* **47:** 315–321.

Guidetti R, Altiero T, Marchioro T, Sarzi Amadè L, Avdonina AM, Bertolani R, Rebecchi L. 2012. Form and function of the feeding apparatus in Eutardigrada (Tardigrada). *Zoomorphology* **131:** 127–148.

Guidetti R, Rebecchi L, Cesari M, McInnes SJ. 2014. *Mopsechiniscus franciscae*, a new species of a rare genus of Tardigrada from continental Antarctica. *Polar Biology* **37:** 1221–1233.

Guil N, Giribet G. 2012. A comprehensive molecular phylogeny of tardigrades—adding genes and taxa to a poorly resolved phylum-level phylogeny. *Cladistics* **28:** 21–49.

Guil N, Guidetti R. 2005. A new species of Tardigrada (Eutardigrada: Macrobiotidae) from Iberian Peninsula and Canary Islands (Spain). *Zootaxa* **889:** 1–11.

Hansen JG, Kristensen RM, Jørgensen A, Accogli G, D'Addabbo M, Gallo M. 2016. Postembryonic development, paedomorphosis, secondary sexual dimorphism and population structure of a new *Florarctus* species (Tardigrada, Heterotardigrada). *Zoological Journal of the Linnean Society* **178:** 871–877.

Jørgensen A, Faurby S, Hansen JG, Møbjerg N, Kristensen RM. 2010. Molecular phylogeny of Arthrotardigrada (Tardigrada). *Molecular Phylogenetics and Evolution* **54:** 1006–1015.

Kaczmarek Ł, Gołdyn B, Prokop ZM, Michalczyk Ł. 2011. New records of Tardigrada from Bulgaria with the description of *Macrobiotus binieki* sp. nov. (Eutardigrada: Macrobiotidae) and a key to the species of the *harmsworthi* group. *Zootaxa* **2781:** 29–39.

Marley NJ, McInnes SJ, Sands CJ. 2011. Phylum Tardigrada: A re-evaluation of the Parachela. *Zootaxa* **2819:** 51–64.

Maucci W. 1986. *Tardigrada. Fauna d'Italia*, vol. **24**. Bologna: Edizioni Calderini.

Maucci W, Ramazzotti G. 1981. *Adorybiotus* gen. nov.: nuova posizione sistematica per *Macrobiotus granulatus* Richters, 1903 e per *Macrobiotus coronifer* Richters, 1903 (Tardigrada, Macrobiotidae). *Memorie dell'Istituto Italiano di Idrobiologia, Pallanza* **39:** 153–159.

Pilato G. 1981. Analisi di nuovi caratteri nello studio degli Eutardigradi. *Animalia* **8:** 51–57.

Pilato G, Binda MG. 1987. *Richtersia*, nuovo genere di Macrobiotidae, e nuova definizione di *Adorybiotus* Maucci e Ramazzotti, 1981 (Eutardigrada). *Animalia* **14:** 147–152.

Pilato G, Binda MG. 1989. *Richtersius*, nuovo nome generico in sostituzione di *Richtersia* Pilato e Binda, 1987 (Eutardigrada). *Animalia* **16:** 147–148.

Pilato G, Lisi O. 2011. *Tenuibiotus*, a new genus of Macrobiotidae (Tardigrada). *Zootaxa* **2761:** 34–40.

Rebecchi L, Nelson DR. 1998. Evaluation of a secondary sex character in eutardigrades. *Invertebrate Biology* **177:** 194–198.

Rebecchi L, Rossi V, Altiero T, Bertolani R, Menozzi P. 2003. Reproductive modes and genetic polymorphism in the tardigrade *Richtersius coronifer* (Eutardigrada, Macrobiotidae). *Invertebrate Biology* **122:** 19–27.

Rodríguez-Roda J. 1952. Tardigrados de la Fauna Española. *Trabajos del Museo de Ciencias Naturales de Barcelona* **1:** 1–86.

Sands CJ, McInnes SJ, Marley NJ, Goodall-Copestake WP, Convey P, Linse K. 2008. Phylum Tardigrada: an "individual" approach. *Cladistics* **24:** 861–871.

Tamura K, Peterson D, Peterson N, Stechler G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary. *Molecular Biology and Evolution* **28:** 2731–2739.

Vecchi M, Cesari M, Bertolani R, Jönsson IK, Rebecchi L, Guidetti R. 2016. Integrative systematic studies on tardigrades from Antarctica identify new genera and new species within Macrobiotoidea and Echiniscoidea. *Invertebrate Systematics*. DOI: 10.1071/IS15033.

SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article:

Table S1. Sequences of Macrobiotoidea and *Milnesium* cf. *tardigradum* retrieved from GenBank.
Table S2. Genetic distances between *Richtersius* specimens in the *cytochrome oxidase subunit 1* gene (582 bp). Below diagonal: Kimura two-parameter values; above diagonal: *p*-distances. In bold: new data.