Resolving the systematics of Richtersiidae by multilocus phylogeny and an integrative redescription of the nominal species for the genus *Crenubiotus* (Tardigrada)

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The family Richtersiidae, although established recently with the use of phylogenetic methods, was considered potentially paraphyletic at the time of its erection. Until now, the family comprised four genera, *Richtersius*, *Diaforobiotus*, *Adorybiotus* and a newly erected genus *Crenubiotus*. However, the genetic characterisation for the latter two genera was very limited or absent. To address concerns about the phylogenetic affinity of these two genera, we present a multilocus phylogeny of the families Richtersiidae and Murrayidae based on four molecular markers (18S rRNA, 28S rRNA, ITS-2 and COI). Our results show a distinct evolutionary lineage composed of *Adorybiotus* and *Crenubiotus*, which is sister to Murrayidae. In order to accommodate the phylogenetic and morphological distinctiveness of this lineage, we erect a new family, Adorybiotidae fam. nov. The new taxon differs morphologically from other families in the superfamily Macrobiotoidea by a unique combination of traits: (1) the presence of tubercles/cushions with aggregations of microgranules on their surfaces present on all legs and on the dorso-caudal cuticle, (2) a system of internal septa in claws, and (3) buccal apparatus morphology. Moreover, in order to stabilise the taxonomy and nomenclature in the genus *Crenubiotus*, we redescribe its type species, *Crenubiotus crenulatus*, by means of integrative taxonomy and designate a new neotype based on a population from the original *terra typica*.

Tardigrades are a phylum of microinvertebrates which are found in freshwater, marine and limno-terrestrial environments throughout the world. The first formally described tardigrade was *Macrobiotus hufelandi* C.A.S. Schultze, 1834 and currently over 1300 nominal taxa are recognised within the phylum. Although research on tardigrade systematic started almost two centuries ago, only recently have studies aided by molecular phylogenetics begun to shed more light onto the relationships between taxa within the phylum. Thanks to genetic data for already known, as well as for newly detected species, the discovery and the delimitation of new high rank taxa, such as genera and families, have become more frequent. One example of this trend is the recent erection of the family Richtersiidae Guidetti et al., 2016, which currently comprises four genera: *Adorybiotus* Maucci & Ramazzotti, 1981, *Crenubiotus* Lisi et al., 2020, *Diaforobiotus* Guidetti et al., 2020 and *Richtersius* Filato & Binda, 1989. Guidetti et al. explicitly demonstrated that the genera *Adorybiotus*, *Diaforobiotus* and *Richtersius* do not belong to Macrobiotidae Thulin, 1928 and a family of their own was established. However, at the same time, Guidetti et al. also stressed that the relationships within Richtersiidae need to be clarified with further molecular data as their results indicated a polyphyletic status of the family. More specifically, *Adorybiotus* was shown to be more closely related to Murrayidae than to the other members of Richtersiidae (*Diaforobiotus + Richtersius*). Nevertheless, based on morphological similarities, *Adorybiotus* was provisionally included within the Richtersiidae. The same pattern of relatedness within the family was also recovered by the
In this study, we present an upgraded molecular phylogeny of the families Richtersiidae and Murrayidae based on four genetic markers (18S rRNA, 28S rRNA, ITS-2 and COI). New DNA sequences for six species/populations of Adorybiotus, Crenubiotus and Diaforobiotus, as well as two additional species of the family Murrayidae are added to the dataset available from earlier studies. Our results show that Adorybiotus and Crenubiotus form a clade that is more closely related to the family Murrayidae than to the other two genera of Richtersiidae (Richtersiidae and Diaforobiotus). These results, together with evident differences recovered by morphological analysis, led us to the erection of a new family within Macrobiotoidea. Finally, the analysis of a population of Crenubiotus crenulatus from the original terra typica (Svalbard, Norway) under the integrative taxonomy framework enabled us to redescribe the species and propose a new neotype that will stabilise the taxonomy and will help to uncover the diversity within this recently erected genus.

Results
Phylogenetic analysis. We have obtained good quality sequences of all four markers for all sequenced individuals. The phylogenetic reconstruction performed with BI and ML methods (Fig. 1) showed almost identical topologies, with lower support values for the ML tree. The superfamily Macrobiotoidea was recovered monophyletic and composed of four well supported clades (Fig. 1; the following sentences describe the topology of the tree from the bottom to the top). The first clade represents the family Macrobiotoidea, the second clade comprises a part of the family Richtersiidae (Richtersius and Diaforobiotus but not Adorybiotus and Crenubiotus), the third clade contains Murrayidae (i.e. Murrayon and Dactylobiotus), and the fourth clade is composed of Adorybiotus and Crenubiotus. The last three clades are more closely related to each other than to the family Macrobiotoidea.
Moreover, the family Richtersiidae is a sister group to Murrayidae + (Adorybiotus + Crenubiotus). Given the evident phylogenetic and morphological distinctiveness, the Adorybiotus + Crenubiotus clade is further elevated to the family level (see the next section below for more details). The majority of the genera in the families Richtersiidae, Murrayidae and in the new family Adorybiotidae were retrieved as monophyletic. The only two paraphyletic genera in our reconstruction were Murrayon and Adorybiotus. In the genus Murrayon, M. dianae was found to be sister to the Murrayon cf. pullari + the Dactylobiotus clade. A similar topology was found for Adorybiotus, with Adorybiotus sp. JP008 being sister to Adorybiotus granulatus + Crenubiotus crenulatus.

**Description of the new family.** Systematic and taxonomic account

**Phylum:** Tardigrada Doyère, 1840

**Class:** Eutardigrada Richters, 1926

**Order:** Parachela Schuster et al., 1980 (restored in Morek et al. 2021).

**Superfamily:** Macrobiotoidea Thulin, 1928

**Family:** Adorybiotidae fam. nov. Stec, Vecchi & Michalczyk. 

ZooBank: urn:lsid:zoobank.org:act:CC69D220-D0D8-43CA-86AE-E998FB843D6D. 

(Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12).

**Diagnosis:** Tubercles/cushions with aggregations of microgranules on their surfaces present on all legs and on the dorso-caudal cuticle. Cuticular pores present in all instars. Double Y-shaped claws, with the two branches forming an evident common tract of a variable length. Large, comb-like lunulae under claws on each leg, equipped with long and evenly distributed teeth. Buccal tube with the ventral lamina and a cuticular thickening (which can form a large apophysis) on the antero-dorsal wall of the buccal tube. Two macroplacoids and

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**Figure 2.** Adorybiotus cf. granulatus from Japan: buccal apparatus. (a) Dorsal projection of the entire buccal apparatus seen in NCM; left lower insert: ventral projection of the anterior portion of the buccal apparatus; right upper insert: ventral projection of the placoids. (b) Buccal crown and oral cavity followed by the buccal tube opening, frontal view. (c) Buccal crown, lateral view. (d) Placoids, dorsal view. The filled arrow indicates the cuticular hook on the T-shaped apophysis, empty arrows indicate the lateral triangular apophysis, filled indented arrowheads indicate the bulbous apophysis at the anterior end of the ventral lamina, filled flat arrowheads indicate constrictions in the macroplacoids, empty flat arrowheads indicate dorsal spikes, Roman numerals indicate the bands of teeth in the oral cavity. Scale bars in μm.
microplacoid positioned close to the second macroplacoid in the bulbus. Ornamented eggs without areolation on the surface between the egg processes laid freely to the environment.

Type genus: *Adorybiotus* Maucci & Ramazzotti, 1981\(^2\).

Family composition: *Adorybiotus* (Figs. 2, 3, 4, 5), *Crenubiotus* Lisi et al., 2020\(^1\). (Fig. 6, 7, 8, 9, 10, 11, 12).

Adorybiotidae *fam. nov.*, by the combination of morphological characters of animals and eggs is unique within the superfamily Macrobiotoidea, and it differs specifically from the family:

- **Macrobiotidae** Thulin, 1928\(^1\) by: large comb-like lunulae under claws on each leg equipped with long and evenly distributed teeth (lunulae smaller, often without teeth and when equipped with teeth, they are short and not as regularly arranged as in Adorybiotidae *fam. nov.*); and by the system of internal septa within claws on each leg as described by Lisi et al.\(^1\) (the system of internal septa absent in Macrobiotidae).

- **Richtersiidae** Guidetti et al., 2016\(^1\) by: the presence of tubercles/cushions with aggregations of microgranules on their surfaces on all legs and on the dorso-caudal cuticle (granulation on legs and on the dorso-caudal cuticle absent in Richtersiidae); the presence of the microplacoid in the bulbus (microplacoid absent in Richtersiidae); and by egg process morphology (processes in the shape of cones with wide bases and very narrow elongated apices or processes with concave bases in the shape of cooling towers and apices divided into 2–4 thick horizontal branches in Adorybiotidae *fam. nov.* vs egg process in the shape of elongated, thin, conical spikes in Richtersiidae).

- **Murrayidae** Guidetti et al., 2005\(^2\) by: cuticular pores (absent in Murrayidae); large comb-like lunulae under claws on each leg equipped with long and evenly distributed teeth (lunulae without teeth in Murrayidae); the system of internal septa within claws on each leg as described by Lisi et al.\(^1\) (the system of internal septa absent in Murrayidae); the presence of tubercles/cushions with aggregations of microgranules on their surfaces on all legs and on the dorso-caudal cuticle (only regular granulation present but tubercles/cushions absent in Murrayidae); and by claw morphology (the common tract of the claw longer than the half of the entire claw height in Adorybiotidae *fam. nov.* vs the common tract of the claw shorter than the half of the entire claw height in Murrayidae).

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**Figure 3.** *Adorybiotus cf. granulatus* from Japan: claws. (a) Claws II (PCM). (b) Claws IV (PCM). (c) Claws III (SEM). (d) Claws IV (SEM). The arrowhead indicates paired cuticular swellings/thickenings under the claws. Scale bars in µm.
Genus: **Crenubiotes** Lisi et al., 2020.

*Crenubiotes crenulatus* (Richters, 1904); *Macrobiotus crenulatus* Richters, 1904; *Macrobiotus dentatus* Binda, 1974.

Material examined: 39 animals, and 22 eggs. Specimens mounted on microscope slides in Hoyer's medium (28 animals + 16 eggs), fixed on SEM stubs (10 + 6), processed for DNA sequencing (1 animal).

Neotype locality: 78°12′58.4″N, 15°20′45.1″E; 53 m asl: Norway, Svalbard, Spitsbergen, Lower part of the Bjørndalen (Bjørn valley; Nordenskiöld Land); moss from tundra on the valley slope; coll. 23.07.2016 by Wojciech Maciejowski.

Type depositories: Neotype (slide NO.429.06 with 3 neoparatypes) and remaining 18 neoparatypes (slides: NO.429.*, where the asterisk can be substituted by any of the following numbers 03, 07–09) and 14 eggs (slides: NO.429.*: 01–05) and SEM stub 20.01 are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30–387, Kraków, Poland. Six neoparatypes (slide NO.429.11) and two eggs (slide NO.429.10) are deposited in the Pilato and Binda collection at the University of Catania, Italy.
Redescription of *Crenubiotus crenulatus* (Richters, 1904). *Animals* (measurements and statistics in Table 1): When alive, body almost transparent in juveniles and yellowish in adults; after fixation in Höyer’s medium body transparent (Fig. 6). Eyes present, visible also in specimens mounted in Höyer’s medium. Body cuticle with larger elliptical (0.8–2.0 µm in diameter) and smaller circular (0.3–0.8 µm) pores distributed randomly on the entire body cuticle with the largest elliptical pores being present in the dorso-cephalic and dorso-caudal cuticle (Figs. 7a–c, 8a–f). Patches of dense very visible granulation that comprises of small tubercles/cushions and aggregations of microgranules on their surfaces present on all legs and on the dorso-caudal cuticle (Figs. 7a–c, 8a–d). These tubercles are slightly less developed and evident compared to *Adorybiotus* (Fig. 4a–g) but still obvious (Figs. 7a–c, 8a–d). On legs I–III, the dense granulation comprises of two patches on the external and internal leg surfaces respectively, that are connected with each other by a narrower band of granulation extending from them on the proximal leg surface (Figs. 7a,b, 8a,b). Two pulvini are present on each leg I–III, one on the external and the other—on the internal leg surface (Figs. 7a,b, 8a,b). On legs IV, dense granulation covers evenly the dorsal and lateral leg surfaces (Figs. 7c, 8c,d). This dense granulation is also present as a wide
granulation band on the caudal cuticle that extends across the terminal body segment from the left body side, through dorsal surface, to the right side (Figs. 7c, 8d). Beside these dense granulation patches, very fine granulation is present and evenly distributed on the entire body surface but visible only under SEM (Fig. 8a–f).

**Remarks:**

The large elliptical pores reported by Lisi et al. as located laterally to the mouth in *C. revelator* are also present in *C. crenulatus*, however their exact position and shape could not be determined as the mouth was retracted in almost all analysed specimens.

**Figure 6.** *Crenubiotus crenulatus* s.s. (Richters, 1904) from Spitsbergen: adult habitus, dorso-ventral projection, neotype. Scale bars in μm.

**Figure 7.** *Crenubiotus crenulatus* s.s. (Richters, 1904) from Spitsbergen: cuticular granulation seen in PCM. (a) Granulation on the external surface of leg II. (b) Granulation on the internal surface of leg II. (c) The wide granulation band on the caudal cuticle and granulation on legs IV. The filled flat arrowhead indicates the cuticular bulge/fold on the external leg surface, the empty flat arrowhead indicates the cuticular bulge/fold on the internal leg surface, the arrow indicates the wide granulation band on the caudal cuticle. Scale bars in μm.
Claws slender, of the Richtersiidae type. Primary branches with distinct accessory points, a long, constricted in the middle, common tract with a system of internal septa, and with an evident stalk connecting the claw to the lunula (Fig. 9a–h). The common tract apparently longer than the half of the entire claw height (Fig. 9a,d–g–h). Large, comb-like, triangular lunulae with long and evenly distributed teeth present on all legs (Fig. 9a–h). Under PCM, the lower portion of the lunulae just above the dentation is evidently darker and visible as a dark arc (Fig. 9a–b, d–e). The lunulae are curved and clamped around the cuticular swelling/thickening present under them (Fig. 9g–h) what is well visible in SEM whereas in PCM on the lower focal plane it is visible as a darkening beneath the lunules (Fig. 9c,f). Paired muscle attachments present just below lunulae on legs I–III (Fig. 9a).

Mouth antero-ventral. Bucco-pharyngeal apparatus of modified “Macrobiotus type” (Fig. 10a), i.e. with ten peribuccal lamelae, a rigid buccal tube with the ventral lamina which is provided with an additional ventral thickening in its anterior portion, that appears as an elongated trapezoidal structure pointing towards the mouth.

**Figure 8.** *Crenubiotus crenulatus* s.s. (Richters, 1904) from Spitsbergen: cuticular granulation seen in SEM. (a) Granulation on the external surface of leg III. (b) Granulation on the internal surface of leg III. (c) Granulation on leg IV. (d) The wide granulation band on the caudal cuticle and granulation on legs IV. (e) Sparse cuticular granulation and pores on the dorsal cuticle. (f) Sparse cuticular granulation and pores on the ventral cuticle. The filled flat arrow indicates the cuticular bulge/fold on the external leg surface, the empty flat arrowhead indicates the cuticular bulge/fold on the internal leg surface, the filled arrow indicates the wide granulation band on the caudal cuticle, empty arrows indicate the sparse cuticular granulation. Scale bars in μm.
opening in the ventral view (Fig. 10c) and is visible as a ridge in the lateral view (Fig. 10d). Based on LCM observations, the oral cavity armature is poorly developed and composed only of the third band of teeth (Fig. 10b,c). The first and the second band of teeth are absent or not visible under LCM (Fig. 10b,c). The teeth of the third band are located within the posterior portion of the oral cavity, anteriorly to the buccal tube opening (Fig. 10b,c). The third band of teeth is divided into the dorsal and the ventral portion. Under LCM, both the dorsal and the ventral portions are seen as two distinct transverse ridges and each of them forms a globular thickening at the medial extremity (Fig. 10b,c). Median teeth absent (Fig. 10b,c). Bulbus spherical (Fig. 10a), with triangular apophyses, three anterior cuticular spikes (typically only two are visible in any given plane) and two rod-shaped macroplacoids (2 < 1) and a microplacoid positioned close to the second macroplacoid (Fig. 10a,e). The first macroplacoid is anteriorly narrowed and constricted in the middle whereas the second has a sub-terminal constriction (Fig. 10e).

Eggs (measurements and statistics in Table 2): Laid freely, yellowish, spherical with conical processes with elongated apices and egg surface without areolation (Figs. 11a–d, 12a–f). The elongated process apices are often multifurcated into short flexible filaments (Figs. 11a–d, 12a–f). These elongated distal portions of the processes seem to be sometimes separated from the lower portion of the process by a faint septum (Fig. 11a) but this is
caused a circular thickening on the inner process wall (Fig. 11d). The labyrinthine layer between the process walls is visible as a very faint reticular pattern with circular margins under LCM (Fig. 11a–d). The faint meshes are visible in the lower part of the processes but are not visible in the elongated upper part (Fig. 11a–d). The entire surface of processes is smooth under SEM (Fig. 12a–f). Processes attached to the egg by a ring of short thickenings, seen as dark projections visible only under LCM, which gives the process bases a jagged appearance (Fig. 11b–c). Only rarely some these projections might be elongated making the impression of connection between two neighbouring processes (Fig. 11b), however this character should be treated with a dose of caution as all the eggs were covered with debris, thus these thin connectors may be an artefact. Besides these structures, egg surface between processes appears as smooth under LCM (Fig. 11a–d), whereas it is slightly wrinkled in SEM (Fig. 12a–e).

Reproductive mode. The examination of 28 adults freshly mounted in Hoyer’s medium revealed no testes or spermathecae filled with spermatozoa, which suggests that the species is (at least facultatively) parthenogenetic.

DNA sequences. We obtained sequences for all four of the above-mentioned molecular markers from one of the two individuals destined for DNA extraction and sequencing, which are as follow: 18S rRNA (GenBank: MT812474), 994 bp long; 28S rRNA (MT812468), 735 bp long; ITS-2 (MT812606), 398 bp long; COI (MT808079), 629 bp long.

Phenotypic differential diagnosis. To date, the genus Crenubiotus comprises only two species: the nominal C. crenulatus and an extremely similar species, C. revelator, recently described from Colombia. Despite the overall similarity, C. crenulatus differs from C. revelator by: the absence of the circular median tooth in the ventral portion of the third band of teeth in the oral cavity (the median tooth present in C. revelator), the presence of only two lateral teeth in the ventral portion of the third band of teeth, which have thickenings in their medial

Figure 10. Crenubiotus crenulatus s.s. (Richters, 1904) from Spitsbergen: buccal apparatus seen in PCM. (a) Dorsal projection of the entire buccal apparatus. (b) Dorsal view of the oral cavity armature. (c) Ventral view of the oral cavity armature. (d) Lateral view of the anterior portion of the buccal apparatus. (e) Ventral view of placoids. Empty flat arrowheads indicate dorsal spikes, filled indented arrowheads indicate the ventral thickening/additional ridge on the ventral lamina, empty indented arrowheads indicate the third band of teeth in the oral cavity armature, the arrow indicates the putative dorsal residual apophysis, filled flat arrowheads indicate constrictions in the macroplacoids. Scale bars in μm.
extremity that resemble circular teeth (the teeth without thickenings in *C. revelator*), a shorter ventral lamina (19.8–25.3 µm [53.4–61.7] in *C. crenulatus* vs. 12.1–18.5 µm [44.5–50.7] in *C. revelator*), larger eggs (ranges of full and bare diameter for four *C. crenulatus* eggs: 122.4–126.5 µm and 88.5–93.9 µm vs. 97.8 and 78.1 µm of the sole known egg of *C. revelator*).

**Discussion**

By the analysis of both morphological and molecular data, we explicitly demonstrated the presence of a previously unrecognised phyletic lineage within the superfamily Macrobiotoidea which comprises two genera, *Adorybiotus* and *Crenubiotus*, for which genetic data were extremely limited or absent. To accommodate the phylogenetic and morphological distinctiveness of this group from the remaining three families within the Macrobiotoidea, we erected the new family *Adorybiotidae* fam. nov. Furthermore, in order to enhance taxonomic studies on the recently erected genus *Crenubiotus* and stabilise its nomenclature, we provided an integrative redescription of *C. crenulatus* based on the population from original terra typica and replaced the existing, inadequate neotype with the new one that is associated with DNA barcodes.

Two genera analysed in this study, *Murrayon* in the family Murrayidae and *Adorybiotus* in *Adorybiotidae* fam. nov., appear to be paraphyletic. As already shown by Bertolani et al.⁶, *Murrayon cf. pullari* IT.338 is more closely related to *Dactylobiotus* than to *Murrayon dianae*. However, the paraphyly should be treated with great caution because the *M. dianae* branch is exceptionally long, thus it could lead to topological artefacts. The unbalanced sequencing may also be the cause behind the paraphyly of *Adorybiotus*, as only the 18S rRNA was sequenced for *Adorybiotus granulatus* in Bertolani et al.⁶, in contrast to the other analysed populations of the *Adorybiotidae* fam. nov., for which a complete set of four markers was available. Thus, a better sampling within *Murrayon* and *Adorybiotus*, both in terms of the number of species and sequenced markers, is needed to verify the phyletic relationships within the two genera.

As was pointed out in the Introduction, thanks to the easier acquisition of genetic data and their use in phylogenetic studies, the relationships between major evolutionary lineages within the phylum Tardigrada are

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**Figure 11.** *Crenubiotus crenulatus s.s.* (Richters, 1904) from Spitsbergen: eggs seen in PCM. (a–c) Details of egg processes and surface under a ×1000 magnification, upper inserts shows details of the ‘reticulation’ present in egg processes of a given egg. (d) Midsections of egg processes under a ×1000 magnification. Filled flat arrowheads indicate divided tips of the processes, filled indented arrowheads indicate faint septae, empty flat arrowheads indicate short thickenings/projections around processes bases, empty indented arrowheads indicate thickenings within the processes walls between main and distal parts of the processes, the arrow indicates elongated thickenings/projections around processes the bases. Scale bars in µm, scale bar for inserts in figures a–c are twice as long as those in the main photos.
being gradually resolved. The increasing popularity of integrative taxonomy also contributed to the recognition of considerable and yet undescribed species diversity within this animal group, e.g. 10,11,24–26. The recent years showed several times that old, outdated and inadequate tardigrade species descriptions or redescription are often the major obstacle in resolving the systematics within genera and species complexes24,25,27–31. In the

**Figure 12.** *Crenabiotus crenulatus* (Richters, 1904) from Spitsbergen: eggs seen in SEM. (a) Entire egg. (b) Details of egg surface. (c,d) Details of egg processes. (e,f) Details of egg process apices. Scale bars in μm.
| Character                      | N  | Range         | Mean   | SD   | Neotype |
|-------------------------------|----|---------------|--------|------|---------|
|                              |    | µm            | pt     | µm  | pt     | µm    | pt    | µm    | pt    |
| Body length                   | 18 | 380–584 897–1361 | 441 1099 | 54 104 | 515 1223 |
| Buccal tube                   |    |               |        |      |        |       |      |       |       |
| Buccal tube length            | 18 | 33.6–42.9     | 40.1   | 2.4  | 42.1   |
| Styllet support insertion point | 18 | 24.4–31.7 71.9–74.6 | 29.6 73.7 | 1.9 0.7 | 31.0 73.6 |
| Buccal tube external width    | 18 | 3.6–5.0 9.2–12.1 | 4.3 10.8 | 0.4 0.8 | 4.5 10.7 |
| Buccal tube internal width    | 18 | 1.8–2.8 5.0–6.6 | 2.3 5.7 | 0.3 0.5 | 2.5 5.9 |
| Ventral lamina length         | 16 | 19.8–25.3 53.4–61.7 | 23.1 57.5 | 1.6 2.3 | 24.6 38.4 |
| Placoid lengths               |    |               |        |      |        |       |      |       |       |
| Macroplocoid 1                | 18 | 7.5–11.0 21.8–27.2 | 9.5 23.7 | 1.0 1.7 | 9.5 22.6 |
| Macroplocoid 2                | 18 | 4.9–7.4 13.0–17.6 | 6.3 15.7 | 0.6 1.1 | 6.6 15.7 |
| Microplacoid                  | 18 | 2.0–3.0 5.3–7.0 | 2.5 6.2 | 0.3 0.5 | 2.7 6.4 |
| Macroplocoid row              | 18 | 13.8–20.3 39.0–48.2 | 17.5 43.7 | 1.7 2.6 | 18.5 43.9 |
| Placoid row                   | 18 | 16.5–23.2 45.8–55.1 | 20.5 51.1 | 1.7 2.6 | 22.2 52.7 |
| Claw 1 heights                |    |               |        |      |        |       |      |       |       |
| External base                 | 8  | 5.7–9.5 14.5–22.1 | 7.7 18.8 | 1.2 2.5 |        |
| External primary branch       | 16 | 8.6–14.0 21.9–32.6 | 11.5 28.4 | 1.5 2.6 | 12.0 28.5 |
| External secondary branch     | 12 | 6.0–11.0 15.3–25.6 | 8.8 21.7 | 1.4 2.7 | 9.4 22.3 |
| External base/primary branch (cc) | 8  | 64.8–70.4 | 67.5 | 2.1 |        |
| Internal base                 | 8  | 5.4–8.6 13.7–20.7 | 7.6 18.4 | 1.0 2.1 | 8.0 19.0 |
| Internal primary branch       | 16 | 8.4–13.3 21.4–31.3 | 11.1 27.6 | 1.4 2.5 | 11.9 28.3 |
| Internal secondary branch     | 14 | 6.0–9.8 15.3–23.2 | 8.3 20.8 | 1.2 2.2 | 9.2 21.9 |
| Internal base/primary branch (cc) | 8  | 63.9–70.4 | 66.7 | 2.4 | 67.2 |
| Claw 2 heights                |    |               |        |      |        |       |      |       |       |
| External base                 | 15 | 6.3–10.1 16.0–24.9 | 8.4 20.9 | 1.2 2.3 | 8.6 20.4 |
| External primary branch       | 17 | 9.6–15.8 24.7–36.8 | 12.5 31.0 | 1.7 3.1 | 13.3 31.6 |
| External secondary branch     | 15 | 6.4–11.7 16.3–27.7 | 9.4 23.5 | 1.5 3.0 |        |
| External base/primary branch (cc) | 15 | 63.9–70.1 | 67.1 | 2.2 | 64.7 |
| Internal base                 | 13 | 6.0–9.8 15.5–22.8 | 8.1 20.2 | 1.2 2.2 |        |
| Internal primary branch       | 16 | 8.7–15.2 22.4–35.4 | 11.8 29.5 | 1.7 3.1 | 13.1 31.1 |
| Internal secondary branch     | 16 | 6.5–11.5 16.5–26.8 | 9.4 23.4 | 1.5 2.8 | 10.4 24.7 |
| Internal base/primary branch (cc) | 13 | 64.5–70.2 | 68.5 | 1.7 |        |
| Claw 3 heights                |    |               |        |      |        |       |      |       |       |
| External base                 | 14 | 6.4–10.5 16.3–25.9 | 8.7 21.6 | 1.2 2.4 | 9.4 22.3 |
| External primary branch       | 18 | 9.4–16.1 23.9–38.0 | 12.8 31.9 | 1.8 3.5 | 14.2 33.7 |
| External secondary branch     | 17 | 8.3–12.5 22.4–30.9 | 10.1 25.2 | 1.2 2.4 | 9.9 23.5 |
| External base/primary branch (cc) | 14 | 65.4–69.4 | 67.9 | 1.2 | 66.2 |
| Internal base                 | 13 | 6.0–10.0 15.3–24.4 | 8.5 21.0 | 1.2 2.4 | 9.1 21.6 |
| Internal primary branch       | 18 | 9.0–15.5 22.9–36.1 | 12.2 30.4 | 1.7 3.3 | 13.5 32.1 |
| Internal secondary branch     | 18 | 7.2–12.0 18.3–28.2 | 9.6 23.8 | 1.3 2.5 | 9.9 23.5 |
| Internal base/primary branch (cc) | 13 | 62.6–70.7 | 67.6 | 2.2 | 67.4 |
| Claw 4 heights                |    |               |        |      |        |       |      |       |       |
| Anterior base                 | 15 | 6.7–10.5 17.0–25.9 | 9.0 22.3 | 1.2 2.3 | 9.3 22.1 |
| Anterior primary branch       | 18 | 9.8–16.3 24.9–38.3 | 13.5 33.7 | 1.8 3.5 | 14.2 33.7 |
| Anterior secondary branch     | 16 | 7.1–12.0 18.1–29.4 | 10.3 25.6 | 1.4 2.8 | 10.8 25.7 |
| Anterior base/primary branch (cc) | 15 | 64.3–70.8 | 66.4 | 2.0 | 65.5 |
| Posterior base                | 15 | 6.7–11.1 17.0–27.4 | 9.7 23.7 | 1.2 2.7 | 10.5 24.9 |
| Posterior primary branch      | 18 | 9.9–16.9 25.2–39.4 | 14.1 35.1 | 1.9 3.6 | 16.1 38.2 |
| Posterior secondary branch    | 12 | 7.7–12.7 19.6–31.4 | 11.1 27.2 | 1.5 3.4 | 11.9 28.3 |
| Posterior base/primary branch (cc) | 15 | 63.3–70.7 | 66.6 | 2.2 | 65.2 |

Table 1. Measurements [in µm] of selected morphological structures of individuals from the neotype population of _C. crenulatus_ s.s. (Richters, 1904) mounted in Hoyer's medium (N—number of specimens/structures measured; RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation).
families Richtersiidae and Adorybiotidae fam. nov., type species for all genera were described in the beginning of the twentieth century and are insufficient in details under modern standards of tardigrade taxonomy, while the original type series do not exist. However, *Richtersius coronifer* (Richters, 1903)15, the nominal species for the genus *Richtersius*, was recently redescribed by Stec et al.31 under the integrative taxonomy framework. Although a neotype for this species was previously established by Maucci and Ramazzotti12, its designation and redescriptions have been questioned and considered as an impediment for *Richtersius* taxonomy and nomenclature14,15. Thus, Stec and Michalczyk33 have formally designated a new neotype based on a population from the original *locus typicus* integratively examined in Stec et al.31. Importantly, *Crenubiotus crenulatus* (Richters, 1904)32, the type species of its genus, suffers from a similar problem. The species was originally described from the Svalbard Archipelago (specifically from Smeerenburg on Spitsbergen), where it seems to be a common element of the tardigrade fauna, e.g.34–38. Nonetheless, the diagnosis of this species has been questioned for many years, specifically concerning its possible synonymy with *Macrobiotus echinogenitus* Richters, 1904, also originally described from the Svalbard Archipelago32,35,36. The issue has been clarified to some extent by Binda49, who communicated that the types of *C. crenulatus* and *M. echinogenitus* are lost and therefore she redescribed and established neotypes for both those species. Recently, Lisi et al.13 provided further morphological details of the *C. crenulatus* neotype established by Binda49 in order to differentiate it from a new species from Colombia. Nevertheless, there are some more issues regarding the 1988 neotype designation that have to be stressed and appropriate actions should be undertaken. First of all, the neotype of *C. crenulatus* established by Binda comes from Italy (Valtellina) which is almost 3800 km away from the original *locus typicus*. This neotype locality designation does not comply with the International Code of Zoological Nomenclature (Article 75.3.6) that states that the neotype should came as nearly as practicable from the original type locality and, where relevant, from the same geological horizon or host species as the original name-bearing type41. This requirement was in force already when Binda49 made her designation42. Second, the neotype series is in a bad condition, which prevents a detailed morphological characterisation of the species13. Finally, taking into consideration the morphological similarity between *C. crenulatus* and *C. revelator*, which has been highlighted in this study and in Lisi et al.13, the future taxonomic studies on the genus *Crenubiotus* will be challenging without the use of DNA barcodes. Therefore, in order to stabilise the taxonomy and nomenclature within *Crenubiotus*, in agreement with to the International Code of Zoological Nomenclature, we established a new neotype from a population found at 180 km from the original *locus typicus*, on the same archipelago. With an integrative redescription that comprises detailed morphological data and associated DNA barcodes, future species identification will be much less problematic that it would have been with Binda types from northern Italy. Furthermore, as the same rule of ICZN that was mentioned above was violated when establishing the neotype of *M. echinogenitus* based on the population from Algeria, which is ca. 5900 km away from the Svalbard Archipelago, we propose to consider this designation as invalid too. As the original description of *M. echinogenitus* from Richters (1903)32 is vague and there has been confusion around its identity49, we suggest caution in assigning an individual to this species until it is redescribed with material from the original *locus typicus*.

As obstacles in the form of incomplete and outdated descriptions of the type species for *Richtersius* and *Crenubiotus* (Richtersiidae and Adorybiotidae fam. nov., respectively) have been now removed by Stec et al.31 and this study, respectively, attention should be paid to similar issues in the remaining two genera, *Adorybiotus* and *Diaforobiotus*. In this study, we presented some details of *Adorybiotus* morphology and genetics, but they were all based on an undetermined species from Japan that cannot be confidently identified until *Adorybiotus granulatus* (Richters, 1903)32, the type and the only species of the genus, is redescribed by means of integrative taxonomy. The species was described originally from Norway (Merok), and later redescribed by Maucci and Ramazzotti12 based also on a population from Norway (Steinkjer). Importantly, however, this designation can be questioned as Maucci and Ramazzotti12 did not fulfill properly the condition given in Article 75.3.4 of the Code41, which requires mentioning the “reasons for believing the name-bearing type specimen(s) (i.e. holotype, or lectotype, or all syntypes, or prior neotype) to be lost or destroyed, and the steps that had been taken to trace it or them” (this requirement was also in force already when Maucci and Ramazzotti12 made their designation42). This opens up the possibility of an extensive taxonomic study on *A. granulatus* that would result in an integrative redescription and a designation of a new neotype, when a suitable population from central Norway is found. The genus *Diaforobiotus* comprises currently only two subspecies *D. islandicus islandicus* (Richters, 1904)43 (the

| Character                              | N  | Range          | Mean | SD  |
|----------------------------------------|----|----------------|------|-----|
| Egg bare diameter                      | 4  | 88.5–93.9      | 92.2 | 2.5 |
| Egg full diameter                      | 4  | 122.4–126.5    | 125.2| 1.9 |
| Process height                         | 42 | 12.2–21.0      | 16.4 | 2.1 |
| Process base width                     | 42 | 9.4–17.1       | 12.8 | 1.9 |
| Process base/height ratio              | 42 | 56%–98%        | 78%  | 10% |
| Inter-process distance                 | 30 | 2.1–4.1        | 2.9  | 0.5 |
| Number of processes on the egg circumference | 4  | 15–17         | 16.3 | 1.0 |

Table 2. Measurements [in µm] of selected morphological structures of the eggs from the neotype population of *C. crenulatus* s.s. (Richters, 1904) mounted in Hoyer’s medium (N—number of eggs/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation).
Table 3. Information on moss samples with the species/populations sequenced in the present study. a Neotype population. b Candidate neotype population.

| Sample/population code | Species | Coordinates and altitude | Locality | Collector |
|-------------------------|---------|--------------------------|----------|-----------|
| JP008                   | Adorybiotus cf. granulatus | 36°03'31" N 138°20'43" E 2127 m asl | Japan, Northern Mt. Yatagatake, Mugikusa Pass, | Atsushi Suzuki |
| GB.108                  | Crenubiotus sp. | 58°54'37.77" N 3°22'44.01" W 383 m asl | Scotland, Hoy | Brian Blagden |
| NO.429^b                | Crenubiotus cremus | 78°12'58.4" N 15°20'45.1" E 33 m asl | Norway, Svalbard, Spitsbergen, Lower part of the Bjornsdalen valley (Nordenskiöld Land) | Wojciech Maciejowski |
| FL073                   | Dactylobiotus selenicus sp. | 62°13'50.23" N 25°44'29.53" E 82 m asl | Finland, Jyväskylä, Jyväjärvi Lake, | Matteo Vecchi |
| ID.517                  | Diaforobiotus sp. | 1°51'20" S 120°19'25" E 1331 m asl | Indonesia, Lore Lindu, Bada Lembah | Artur Oczkowski & Piotr Gasiorek |
| IS.042^b                | Diaforobiotus islandicus islandicus | 62°52'53.7" N 22°27'21" W 44 m asl | Island, Grindavik, Blue Lagoon | Wojciech Witalinski |
| NO.386                  | Diaforobiotus sp. | 78°44'02" N 10°36'12" E 47 m asl | Norway, Svalbard, Ragnardalen | Michala Bryndová |
| IT.338                  | Murrayon cf. pullari | 44°23'54.26" N 10°0'23.08" E 1594 m asl | Italy, Parma, Corniglio | Matteo Vecchi & Claudio Ferrari |

nominal subspecies) and D. islandicus nicaraguensis (Séméria, 1985)44. For years, numerous records of D. islandicus islandicus accumulated in the literature from localities all over the world45–48 but it has been demonstrated by Guidetti et al.11 as well as by this study that the genus definitely comprises more than one species. Nonetheless, since the types of D. islandicus islandicus do not exist and morphological details of the species are unknown, we consider descriptions of new taxa highly hazardous until the type species is redescribed by means of integrative taxonomy. The Diaforobiotus population from Iceland analysed in this study is a suitable candidate for the neotype population, which together with two other populations from Norway and Indonesia will be revised by us in the near future and published as an integrative revision of the genus. Such integrative redescriptions of the type taxa have opened and will continue to open the windows for describing species diversity within genera or species groups/complexes, further contributing to our understanding of evolution of microscopic invertebrates, including tardigrades.

Material and methods

Samples and specimens. To reconstruct the phylogeny of Richtersiidae and Murrayidae, along with already published data, we analysed eight new populations representing eight species isolated from moss or pond sediment samples collected from eight distinct localities (see Table 3 for details). In our study, by a population we mean a group of conspecific individuals found in a single sample. All samples were processed following a protocol described in detail in Stec et al.49.

DNA sequencing. Genomic DNA was extracted from individual animals following a Chelex 100 resin (Bio-Rad) extraction method by Casquet et al.50 with modifications described in detail in Stec et al.51. Each specimen was mounted in water on a temporary microscope slide and examined under light microscope prior to DNA extraction. We sequenced four DNA fragments, three nuclear (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial (COI) from 2–4 individuals per each of the six newly analysed populations. All fragments were amplified and sequenced according to the protocols described in Stec et al.51; primers with their original references are listed in Table 4. Sequencing products were read with the ABI 3130x1 sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of the Jagiellonian University, Kraków, Poland. Sequences were processed in BioEdit ver. 7.2.552 and submitted to NCBI GenBank.

Phylogenetic analysis. The phylogenetic analyses were conducted using concatenated 18S rRNA + 28S rRNA + ITS-2 + COI sequences for Macrobiotoidea with Bertolanites violibilis (Durante Pasa & Maucci, 1975)55, Eohypsius madjac Krishensen, 198254, Hypsius exemplaris Gasiorek et al. 201858 and Ramazzottius subanomalous (Biserov, 1985)58 as outgroups. We choose isolates from the families Richtersiidae and Murrayidae with all four sequenced markers (18S rRNA, 28S rRNA, ITS-2 and COI) and isolates that overlap with the sequences produced in this study in the cases of 18S rRNA and 28S rRNA. However, there were four exceptions to this: two Adorybiotus granulatus (Richters, 1903)52 isolates, with only 18S rRNA sequences (HQ604961 and HQ604962), that were included as they are the only available sequences for the nominal species of the genus Adorybiotus (although the species identification is uncertain; see the Discussion for details). The other two exceptions were Murrayon dianae (Kristensen, 1982)54 and Dactylobiotus ovimutans Kimh et al., 202056 that were included to...
have a better representation of the Murrayidiae. For the family Macrobiotidae, one to two species of each genus, for which sequences are available in GenBank, were included in the analysis (see Table 5 for GenBank accession numbers). In the analysed dataset, 82% (31/38) terminals had sequences for all four markers.

The 18S rRNA, 28S rRNA and ITS-2 sequences were aligned using MAFFT ver. 757,58 with the G-INS-I method (thread = 4, threadtb = 5, threadid = 0, reorder, adjustdirection, anysymbol, maxiterate = 1000, retree 1, Adorybiotus files). DNA sequences are deposited and available in GenBank. All data generated and analysed during this study are included in the article (and its Supplementary Information).

### Data availability

All data generated and analysed during this study are included in the article (and its Supplementary Information files). DNA sequences are deposited and available in GenBank.

### Table 4. Primers with their original references used for amplification of the four DNA fragments sequenced in the study. COI sequences for all population were amplified with primer set LCO1490-JJ+HCO2198-JJ except Adorybiotus population (JP:008) for which LCO1490+HCOoutset was used.

| DNA marker | Primer name       | Primer direction | Primer sequence (5′–3′) | Primer source |
|------------|------------------|------------------|-------------------------|---------------|
| 18S rRNA   | 18S_Tar_FH       | Forward          | AGGCGAAACCGCGGATGGCTC   | 77            |
|            | 18S_Tar_Rr       | Reverse          | GCCGGAGGCTCCACTCCFGG    |               |
| 28S rRNA   | 28S_Eutar_F      | Forward          | ACCGCTGAACTTAAAGCATAT  | 78,79         |
|            | 28SR0990         | Reverse          | CTTGTGCCGCTTTAAGACG    |               |
| ITS-2      | ITS2_Eutar_F     | Forward          | CTAACGTTAGTGAGGAC       | 80            |
|            | ITS2_Eutar_R     | Reverse          | TCTCCTCGTTATGATGFGC    |               |
| COI        | LCO1490-JJ       | Forward          | CHACAAAYCTAAAGATATYGG   | 81            |
|            | HCO2198-JJ       | Reverse          | AWACTTCCUGRTVCAAAAAATCA|               |
|            | LCO1490         | Forward          | GGTCAACAAACATATAAGATYGG| 82            |
|            | HCOoutset       | Reverse          | GTAATAATATGRTGDCCTC    |               |

### Microscopy and imaging.

Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyser’s medium and secured with a cover slip, following the protocol by Morek et al.69. Slides were examined under an Olympus BX53 light microscope with phase and Nomarski differential interference contrast (PCM and NCM, respectively; named collectively as light contrast microscopy, LCM), associated with an Olympus DP74 digital camera. In order to obtain clean and extended specimens for SEM, tardigrades were processed according to the protocol by Stec et al.49. Specimens were examined under an Olympus BX53 light microscope with phase and Nomarski differential interference contrast (PCM and NCM, respectively; named collectively as light contrast microscopy, LCM), associated with an Olympus DP74 digital camera. In order to obtain clean and extended specimens for SEM, tardigrades were processed according to the protocol by Stec et al.49. Specimens were examined under high vacuum in a Versa 3D DualBeam Scanning Electron Microscope (SEM) at the ATOMIN facility of the Jagiellonian University, Kraków, Poland. All figures were assembled in Corel Photo-Paint X6, ver. 16.4.1.1281. For structures that could not be satisfactorily focused in a single LCM photograph, a vertical stack of 2–6 images were taken with an equidistance of ca. 0.2 μm and assembled manually into a single deep-focus image in Corel Photo-Paint.

### Morphometrics and nomenclature.

All measurements are given in micrometres (μm). Sample size was adjusted following recommendations by Stec et al.70. Structures were measured only if undamaged and their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. The terminology used to describe oral cavity armature and egg shell morphology follows Michalczyk and Kaczmarek71 and Kaczmarek and Michalczyk72 respectively. Macroplacoid length sequence is given according to Kaczmarek et al.73. Buccal tube length and the level of the stylet support insertion point were measured according to Pilato74. The pt index is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage75. Measurements of buccal tube widths, heights of claws and eggs follow Kaczmarek and Michalczyk76. Morphometric data were handled using the “Parachela” ver. 1.7 template available from the Tardigrada Register77 and are given in Supplementary Data S4. Tardigrade taxonomy follows Bertolani et al.6 with updates from Guidetti et al.31, Vecchi et al.32 and Morek et al.5.
Table 5. GenBank accession numbers of the DNA sequences used for phylogeny reconstruction. a Type or neotype population. b Candidate neotype population.

| Species | 18S rRNA | 28S rRNA | COI | ITS-2 | Source |
|---------|----------|----------|-----|-------|--------|
| Hypsibius exemplaris | MG800327 | MG800337 | MG818724 | MG800336 | 20 |
| Ramazzottius subnubimulus | MF001997 | MF001998 | MF001999 | MG432819 | 27 |
| Bertoliani volubilis | HQ604918 | – | AY598769 | – | 822 |
| Elypybius nadiace | HQ604921 | – | – | – | 8 |
| Minibiota loculator | MT023998 | MT024041 | MT023412 | MT024000 | 13 |
| Minibiota pentanulatus | MT023999 | MT024042 | MT023413 | MT024001 | 13 |
| Tenuibiotus voronkovi | KX810045 KX810049 KX810042 KX810046 | – | – | – | 38 |
| Tenuibiotus zandrae | MN443040 MN443035 MN443027 MN443038 | – | – | – | 46 |
| Paramacrobiotus arcoletus | MH646931 MH664948 MH675998 MH666080 | – | – | – | 37 |
| Paramacrobiotus faribanksi | MH664941 MH664950 MH676011 MH666090 | – | – | – | 37 |
| Macrobiotus shonaicus | MG757132 MG757133 MG757136 MG757134 | – | – | – | 35 |
| Macrobiotus caelestis | MK770793 MK770791 MK770790 MK770789 | – | – | – | 60 |
| Xerobiotus pseudohufelandi | HQ604989 | – | AY598767 | – | 822 |
| Melobiotus harmsworthi | MH197146 MH197264 MH195150 MH197154 | – | – | – | 87 |
| Melobiotus dilimanensis | MN257048 MN257049 MN257047 MN257050 | – | – | – | 36 |
| Richtersius coronifer NO.385 | MH681760 MH685175 MH676053 MH681763 | – | – | – | 33 |
| Richtersius aff. coronifer GR.008 | MK211386 MK211384 MK214323 MK211380 MK211381 | – | – | – | 33 |
| Richtersius aff. coronifer IT.120 | MH681761 MH681758 MH676054 MH681764 | – | – | – | 34 |
| Richtersius aff. coronifer IT.317 | MK211387 MK211385 MK214326 MK211382-3 | – | – | – | 34 |
| Richtersius aff. coronifer PL.247 | MH681762 MH681759 MH676055 MH681765 | – | – | – | 31 |
| Diaforobiotus islandicus IS.042 | MT812470 MT812461 MT800807 MT812597 | This study |
| Diaforobiotus sp. NO.386 | MT812471 MT812463 MT800807 MT812598 | This study |
| Diaforobiotus sp. ID.517 | MT812472 MT812462 MT800807 MT812599 | This study |
| Murrayania dianae | FI435737 FI435762 FI435801 | – | – | – | 99 |
| Murrayania cf. pullari IT.338 | MT812477 MT812465 MT800800 MT812603 | This study |
| Dactylobiotus parthenogeneticus FR.149 | MT373694 MT373700 MT373804 MT374191 | – | – | – | 41 |
| Dactylobiotus parthenogeneticus GB.003 | MT373693 MT373699 MT373803 MT374190 | – | – | – | 41 |
| Dactylobiotus selenicus | MT812476 MT812466 MT800807 MT812602 | This study |
| Dactylobiotus ovianus | MT136805 MT132333 | – | – | – | 56 |
| Crenubiotus sp. GR.108 | MT812473 MT812467 MT800807 MT812604-5 | This study |
| Crenubiotus cremulus NO.429 | MT812474 MT812463 MT800807 MT812606 | This study |
| Adorybiotus granulatus | HQ604961-2 – – – | – | – | 8 |
| Adorybiotus cf. granulatus IP.08 | MT812475 MT812464 MT800807 MT812600-1 | This study |

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Author contributions
D.S.: conceived the study, analysed the samples, performed the molecular and morphological analyses, assembled morphological figures, drafted the first version of the manuscript and partially funded the research; M.V.: conceived the study, performed phylogenetic analysis, prepared the phylogenetic figure, drafted the first version of the manuscript; W.M.: collected samples and drafted the first version of the manuscript; Ł.M.: conceived the study, supervised the research, prepared the phylogenetic figure, drafted the first version of the manuscript and partially funded the research. All authors contributed to the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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