**Phylogeny of the asexual lineage Murrayidae (Macrobiotoidea, Eutardigrada) with the description of *Paramurrayon* gen. nov. and *Paramurrayon meieri* sp. nov.**

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**Running title**: Phylogeny of the asexual lineage Murrayidae

**Abstract.**

The peculiar family Murrayidae (*Murrayon, Dactylobiotus, Macroversum*) contains relatively rare species living in hydrophilic and freshwater habitats on all continents and contains two of the six exclusively freshwater tardigrade genera. This family probably represents an example of the evolution and persistence of an asexual lineage that differentiated into several taxa without sexual reproduction. Analyses of nuclear and mitochondrial genes (18S, 28S, ITS2, *cox1*) and the increase of five taxa to the phylogenetic analyses of Murrayidae led us to infer that *Murrayon* is polyphyletic, being composed of two “species groups” that also find morphological supports: the “*dianeae* group” characterized by peculiar egg processes (rod-shaped and covered with a cuticular layer), animals with large, evident epicuticular pillars and small claws; the “*pullari* group” characterized by conical egg processes, animals with very small epicuticular pillars and proportionally larger and longer claws. This latter group is a sister group to *Dactylobiotus*. Within the genus only *Murrayon hastatus* has an uncertain position with eggs of “*dianeae* group” and animals of “*pullari* group”. We propose the erection of *Paramurrayon* gen. nov. (for the “*dianeae* group” of species), the emendation of *Murrayon*, and new taxonomic keys for both genera. Possible scenarios of the evolution of the taxa within Murrayidae are hypothesised based on synapomorphic characters. *Paramurrayon meieri* sp. nov. from Norway is described with an integrative approach. Photographs of type material of *Murrayon stellatus, Murrayon nocentiniae, Murrayon ovoglabellus* and *Macroversum mirum* are shown for the first time, together with descriptions of new characters. *Murrayon hibernicus* is considered as *nomen dubium*, and *Murrayon* *hyperoncus* is transferred to *Macrobiotus* pending further analyses*.*

**Key words:** molecular phylogeny, species delimitation, asexual lineages, synapomorphy, *Macroversum*, *Dactylobiotus*, parthenogenesis

**Short summary**

The Murrayidae (*Murrayon, Dactylobiotus, Macroversum*) contains hydrophilic and freshwater species and probably represents an example of the evolution of an asexual lineage. Thanks to the integrative description of a new Norwegian species(*Paramurrayon meieri* sp. nov.), we were able to perform multigene phylogenetic analyses of the family identifying a new evolutionary line. This led to the erection of *Paramurrayon* gen. nov. and to develop possible scenarios of the evolution of the taxa within Murrayidae based on synapomorphic characters. New original pictures of several species of Murrayon together with descriptions of new characters allowed a better definition of the species.

**Introduction**

The family Murrayidae (Macrobiotoidea, Eutardigrada) contains relatively rare species living in terrestrial hydrophilic and freshwater habitats (e.g. mosses, liverworts, leaf-litter, lakes, streams) in all continents (Kaczmarek *et al*. 2014, 2015, 2016; McInnes *et al*. 2017; Nelson *et al*. 2020). The family comprises three genera: the limnic *Dactylobiotus* with 14 species, the limnic and monospecific *Macroversum*, and the limnic/hydrophilic *Murrayon* with eight species (Degma and Guidetti 2022). *Dactylobiotus* and *Macroversum*, two of the six exclusively freshwater genera in Tardigrada, are both in Murrayidae (the others, *Carphania, Thulinius,* *Pseudobiotus* and *Grevenius*, belong to other families).

Murrayidae is an evolutionarily interesting taxon. So far, no males have been found and parthenogenesis is confirmed for several species. Thus, the family likely represents an asexual lineage that has differentiated into at least three genera and 20 species without sexual reproduction (Guidetti *et al*. 2000, 2005; Altiero *et al*. 2018), populating both freshwater and hydrophilic habitats all around the world. The apparent adaptation and preference for limnic habitats, with the reduced danger of dehydration, probably is the cause for the limited anhydrobiotic capabilities of the species in this family tested so far (Guidetti *et al.* 2011; Bertolani *et al.* 2019).

The taxon Murrayinae was erected as subfamily by Guidetti *et al.* (2000) based on the presence of epicuticular pillar-like structures and the two branches of the double claws diverging immediately after a short basal portion. Within tardigrades, this taxon was one of the first to be investigated using molecular analyses and was raised to family level based on phylogenetic evidence by Guidetti *et al*. (2005), who described Murrayidae using an integrative approach. Murrayidae was included in the superfamily Macrobiotoidea by Marley *et al.* (2011) based on phylogenetic analyses using 18S rRNA sequence data and morphological characteristics.

Although *Murrayon pullari* (Murray, 1907) was the first tardigrade species from which the *cox1* sequence was obtained (Guidetti *et al.* 2005), only a few species of Murrayidae have been analysed with molecular techniques (Guidetti *et el.* 2005; Sands *et al.* 2008; Guil and Giribet 2012; Bertolani *et al.* 2014; Guil *et al.* 2019; Kihm *et al.* 2020; Pogwizd and Stec 2020; Stec *et al.* 2020). Previous studies have shown that Murrayidae contains two main phylogenetic lineages (Bertolani *et al.* 2014; Stec *et al.* 2020): one group containing the *Dactylobiotus* species and *M. pullari*; the other containing *Murrayon dianeae* (Kristensen, 1982) as a sister taxon (no molecular data are available for *Macroversum*). Thus, a monophyletic *Dactylobiotus* apparently was included in a paraphyletic *Murrayon*, although the number of taxa used in the analyses was insufficient to produce conclusive results. Morphological cladistic analyses identified a close relationship between *Murrayon* and *Dactylobiotus*, but failed to group *Macroversum* with these two genera, probably due to homoplastic characters (Guil *et al.* 2013).

For a better understanding of the evolution of the family Murrayidae, we present the discovery of a new species from Norway initially placed in the genus *Murrayon*. Careful observations and comparison of type material of species in Murrayidae led us to perform molecular phylogenetic analyses of the family. Based on the results and the morphological characteristics of the analysed species, we propose the erection of a new species and a new genus to ensure monophyletic genera in Murrayidae.

**Material and Methods**

***Specimen extraction, slide mounting and morphological identification***

Several specimens of different species ascribable to *Murrayon* and *Dactylobiotus* were extracted from samples collected in Norway, Italy, Scotland and the USA (Tab. 1). To extract tardigrades from their substrates, fragments of the moss sample were placed in distilled water for about half an hour. After soaking, the samples were sieved (sieve meshes: 500 μm and 38 μm) to separate tardigrades and eggs from the substrate. Animals and eggs were then isolated using a needle and removed with a glass pipette under a stereomicroscope. Specimens were used for molecular analyses or mounted on slides in Hoyer’s fluid for observation and measurements, both carried out under phase contrast [PhC] and differential interference contrast [DIC] with a Leica DM RB microscope equipped with a Nikon DS-Fi 1 or an AmScope MU1803 digital camera, at the Department of Life Sciences, University of Modena and Reggio Emilia (UNIMORE). Morphometric data of animals were recorded according to Kaczmarek and Michalczyk (2017) and handled using the ‘Parachela’ ver. 1.6 template available from the Tardigrada Register (Michalczyk and Kaczmarek 2013), updated with Thorpe’s normalization of the data (as in Massa *et al.* 2021) according to Bartels *et al.* (2011).

For comparative purpose, the type material of the following species was observed: paratypes of *Murrayon nocentiniae* (Ramazzotti, 1961) (slide CT6084 with an animal) and *Murrayon ovoglabellus* (Biserov, 1988) (slide CT14659 with an animal and three eggs) from the Maucci collection (Natural History Museum, Verona, Italy); paratypes of *M. nocentiniae* (slide Tipo115 with an animal and two eggs) from the Ramazzotti collection (Natural History Museum, Verona, Italy); paratypes of *M. ovoglabellus* (slide 968-12 with four eggs) and *Murrayon stellatus* Guidetti, 1998 (slide 5N08B-s28 with an egg) from the Bertolani collection (Department of Life Sciences, UNIMORE, Modena, Italy); the holotype of *Macroversum mirum* Pilato & Catanzaro, 1988 (slide 4084 with an animal) from the Binda and Pilato collection (Department of Biological, Geological and Environmental Sciences "Marcello La Greca", University of Catania, Catania, Italy). Other specimens, not belonging to the type series, were investigated: *Murrayon hibernicus* (Murray, 1911), slide CT9840 from Steinkjer, Norway (Maucci collection); *Murrayon hastatus* (Murray, 1907) slide C1178 from Trento, Italy (animal + egg), *M. dianeae*  slides C881, C991 from Modena, Italy (animals), *M. pullari* slide C2573 from Trento, Italy (animal + egg), slide C2605 from Alpine Stelvio pass, Italy (egg) (Bertolani collection); *M. pullari* slide Tipo143 from Lake Tanganyika, Ruwenzori Mt., Africa (Ramazzotti collection).

***DNA extraction, PCR and sequencing***

Prior to molecular analysis, individuals were observed and identified with LM using the method described in Cesari *et al.* (2011) to obtain photo voucher specimens. Genomic DNA was extracted from 13 animals of 5 species (Tab. 2). The extractions were performed using the kit QuickExtract™ DNA Extraction Solution (Lucigen, WI, USA), following the manufacturer’s protocol. Molecular investigations were carried out using fragments of three nuclear genes (18S, 28S, ITS2) and a mitochondrial gene (*cox1*). The 18S gene was amplified using the primers SSU\_F04 (5’-GCTTGTCTCAAAGATTAAGCC-3’) and SSU\_R26 (5’-CATTCTTGGCAAATGCTTTCG-3’), with PCR protocols described in Bertolani et al. (2014). The 28S gene using primers 28S 4.8aF (5'-ACC TAT TCT CAA ACT TTA AAT GG-3') and 28S 7bR (5'-GAC TTC CCT TAC CTA CAT-3'), with PCR protocols described in Guidetti et al. (2014). The ITS2 gene was amplified utilizing primers ITS3 (5'-GCA TCG ATG AAG AAC GCA G-3) and ITS4 (5'-AGT TTY TTT TCC TCC GCT TA-3'), with PCR protocols described in Wełnicz et al. (2011). The *cox1* gene was amplified using primers LCO 1490 (5’-GGTCAACAAATCATAAAGATATTGG-3’) and HCO 2198 (5’-TAAACTTCAGGGTGACCAAAAAATCA-3’), [Folmer et al., (1994)] and PCR protocols described in Cesari et al. (2009). The amplified products were gel purified using the Wizard Gel and PCR cleaning kit (Promega, Madison, WI, USA). Sequencing reactions were performed using the ABI Prism BigDye Terminator v. 1.1 Sequencing Kit (Applied Biosystems™) on purified amplicons Each sequencing reaction contained 0.2 μM of a single PCR primer to initiate the sequencing reaction, 2 μL of BigDye, 70 ng of purified products, 4 μL of 5x BigDye Terminator v.1.1 Sequencing Buffer and H2O for a final volume of 20 μL. Cycling conditions for sequencing reactions consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Both strands were sequenced using ABI Prism 3100 (Applied Biosystems™). GenBank Accession numbers of the nucleotide sequences of the newly analysed specimens are: for *cox1* OP379716-28 (13 sequences); for ITS2 OP390258-68 (11 sequences); for 18S OP380708-18 (11 sequences); for 28S OP380720-33 (14 sequences) (for more delails see Supplementary material Tab. S1).

***Phylogenetic and species delimitation analyses***

For phylogenetic analyses, the more conservative genes 18S and 28S were used, while for species identification and delimitation analyses the more variable cox1 and ITS2 genes were used. The 18S and 28S nucleotide sequences were aligned with the MAFFT algorithm (Katoh *et al.* 2002) as implemented in the MAFFT online service (Katoh *et al.* 2019) and checked by visual inspection. Sequences of the total genome of *Ramazzottius varieornatus* Bertolani & Kinchin, 1993 (Eutardigrada, Parachela, Hypsibioidea; GenBank A.N.: BDGG01000030 for both genes) were used as outgroup. Other tardigrade sequences from GenBank of Macrobiotoidea were also included in the analysis for comparisons (Table S1 Supporting information). A Bayesian inference (BI) dendrogram was computed with the program MrBayes (Ronquist *et* *al*. 2012) version 3.2.7a on the CIPRES Science Gateway Portal (http://www.phylo.org/sub\_sections/portal/). Best fit model evaluations were performed considering the Akaike Information Criterion and Bayes Information Criterion (jModeltest 2.1.7; Darriba *et al*. 2012) which identified the GTR + Gamma model as the most suitable for both genes. Two independent runs, each of four Metropolis-coupled Markov chains Monte Carlo method, were launched for 70 × 107 generations, trees were sampled every 1000 generations. Convergence of runs was assessed by tracking average standard deviation of split frequencies between runs and by plotting the log likelihood of sampled trees in Tracer v1.5 (Rambaut and Drummond 2007), and the first 7 × 106 sampled generations were discarded as burn-in. A maximum likelihood (ML) analysis was performed with the program RAxML v7.2.4 (Stamatakis 2006) on the CIPRES Science Gateway Portal using the GTR + Gamma model. Bootstrap resampling with 1000 replicates was completed via the rapid bootstrap procedure of Stamatakis *et al*. (2008) to assign support to branches in the ML tree.

For *cox1* sequences, chromatograms were checked for the presence of ambiguous bases, as sequences were translated to amino acids by using the invertebrate mitochondrial code implemented in MEGA X (Kumar *et al*. 2018) to check for the presence of stop codons and therefore of pseudogenes. Nucleotide sequences of *cox1* were aligned with the MAFFT algorithm as described above, the ITS2 sequences were aligned with the ClustalW algorithm implemented in MEGAX and checked by visual inspection. For appropriate molecular comparisons, we included in our analysis *cox1* and ITS2 sequences from GenBank pertaining to other Murrayidae specimens (Table S1 Supporting information). Pairwise nucleotide sequence divergences between scored haplotypes were calculated using p-distance by using MEGA X.

Putative species were inferred by using Assemble Species by Automatic Partitioning method (ASAP; Puillandre *et al*. 2021). In this distance-based method, cox1 sequences were sorted into hypothetical species based on the barcode gap (i.e. whenever the divergence among organisms belonging to the same species is smaller than divergence among organisms from different species). The method first detects the barcode gap as the first significant gap beyond a model-based one-sided confidence limit for intraspecific divergence, and then uses it to produce several partitions of the data. The ASAP then computes an ad hoc ASAP-score for each defining partition, with the lower score indicating the better partition. The analysis was performed on the ASAP website (https://bioinfo.mnhn.fr/abi/public/asap/ ), using p-distances as distance unit.

**Results**

***Molecular characterization of the new species and the newly analysed specimens***

Matrixes with p-distances comparing the new sequences of *cox1* and ITS2 with those available in GenBank belonging to Murrayidae (i.e., *Dactylobiotus* and *Murrayon* specimens) are provided in Tab. S2 Supplementary material*.* For *cox1*, the p-distances between the two populations of the new speciesfrom Norway (samples 282 and 247) was 0.8–1.5%, while the p-distance within each population ranged from 0–0.3% and 0% for samples 282 and 247, respectively. The p-distance between sequences of the new Norwegian species and a Spanish specimen from GenBank (identified as *M*. cf. *dianeae*) was 0.2–2.1%, and the new species and *M*. cf. *pullari* specimens from Italy and Scotland ranged from 17.7–19.3% (Tab. S2). The two specimens of *M*. cf. *pullari* from Glendoebeg and Oban (Scotland) had a p-distance of 16.3%, these two specimens had a p-distance of 12.8–18.0% in comparison to the Italian *M*. cf. *pullari* specimens.

The ASAP analysis (Tab. 2) identified four species within *Murrayon* according to the available *cox1* sequences, corresponding to: i) the new species from Norway (together with the specimen from Spain identified as *M*. cf. *dianeae*); ii) *M*. cf. *pullari* from Italy (from two populations found in Northern Italy in localities 56 km apart along the Apennine ridge; Guidetti *et al*. 2005; Stec *et al*. 2020); iii) *M*. cf. *pullari* from Glendoebeg (Scotland); iv) *M*. cf. *pullari* from Oban (Scotland). Therefore, according to the ASAP analysis, there are at least three species similar to *M. pullari* (species ii, iii, and iv); we call this group of similar species as “*pullari* complex”.

Based on ITS2, all four specimens of the new species from Norway have the same haplotype. The p-distances range from 16.1–23.7% with respect to the *M.* cf. *pullari* specimens from Oban and Glendoebeg (Scotland), which in turn differ from each other by a p-distance of 18.4%.

The two Italian specimens of *D*. *parthenogeneticus* obtained in this study have the same *cox1* haplotype, which is very similar (p-distance 0.2–1.8%) to those available in GenBank corresponding to specimens identified as *D*. *parthenogeneticus* collected in Italy, England, France and Poland (Tab. S2).

The two specimens of *Dactylobiotus grandipes* (Schuster *et al*., 1978), from the *locus typicus* of this species (Lake Tahoe, California, USA), have a p-distance of 0.3% and differ from all the other sequences of *Dactylobiotus* for a p-distance of 10.0–19.3% (Tab. S2).

The ASAP analysis (Tab. 2) identified four species within *Dactylobiotus* according to the available *cox1* sequences, corresponding to: i) *D. grandipes* + *Dactylobiotus selenicus* Bertolani, 1982 from Finland (they are together because, although their p-distance ranged from 10.0–10.2%, ASAP identified a threshold of 11.4%); ii) *Dactylobiotus ovimutans* Kihm *et al*., 2020 (from the *locus typicus* in Antarctica); iii) *D*. *parthenogeneticus* from several European areas.

For ITS2 (Tab. S2), all three newly sequenced specimens of *D. parthenogeneticus* have the same haplotype, which is the same or very similar (p-distance 0–1.0%) to those in GenBank for *D. parthenogeneticus* from Poland, France, and England. The two newly sequenced specimens of *D. grandipes* from the type locality have the same haplotype, which differs from *D. parthenogeneticus* by having a p-distance of 1.1–2.2% and from *D. selenicus* from Finland of 0.9%.

***Phylogenetic analyses***

Phylogenetic molecular analyses of Macrobiotoidea were carried out with Bayesian and Maximum Likelihood inferences on a3668 bp dataset comprising sequences of 18S (1853 bp) and 28S (1815 bp). Both approaches display the same topology for the relationships of the considered taxa but with different support values for some of the branches (Fig. 1). Tree topology of the relationships between the families is essentially similar as those determined by Guidetti *et al*. (2021) and Stec *et al*. (2020, 2021), but differs in not supporting the family Macrobiotidae. At the base of the superfamily, there is an unresolved node with five well supported branches, *Macrobiotus+Xerobiotus, Sisubiotus, Mesobiotus, Minibiotus+Tenuibiotus+Paramacrobiotus,* and a branch leading to a cluster grouping the families Richtersiusidae (*Richtersius+Diaforobiotus*), Adorybiotidae (*Adorybiotus+Crenubiotus*) and Murrayidae (*Murrayon+Dactylobiotus*). In this last cluster, Adorybiotidae is the sister taxon of Murrayidae. Within Murrayidae, two main clusters are present (Fig. 1), one grouping sequences of the new Norwegian species and *M. dianeae*, the other formed by the sequences of *M*. *pullari* (or *M*. cf. *pullari*) in a sister group relationship with the sequences of *Dactylobiotus* species [*D. parthenogeneticus, D. grandipes, D. selenicus, D. ovimutans*, *Dactylobiotus ambiguus* (Murray, 1910), and *Dactylobiotus octavi* Guidetti *et al*., 2006)]. It has to be underlined that from these analyses the genera *Minibiotus* and *Diaforobiotus* resulted as non-monophyletic (Figure S1 Supplementary file) and *D. ovimutans* (from Antarctica) formed a not well supported cluster with the other species of its genus (Fig. 1).

***Taxonomy of* Murrayon** Bertolani & Pilato

A new species from Norway attributable to *Murrayon* was found in this study. Based on the data reported in the Results and Discussion sections, this new species and other species of *Murrayon* are ascribed to a new genus; both the new genus (*Paramurrayon* gen. nov.) and the new species (*Paramurrayon* *meieri* sp. nov.) are described below in the “Diagnoses and descriptions of new taxa” section.

According to the checklist of Tardigrada (Bertolani and Guidetti 2005; Degma *et al*. 2007; Degma &Guidetti 2022), in *Murrayon* there are eight species: *M. pullari* (type species), *M. dianeae*, *M. hastatus*, *M. hibernicus*, *Murrayon hyperoncus* Meyer *et al*., 2014, *M. nocentiniae*, *M. ovoglabellus*, and *M. stellatus*.

Based on the data in literature and the observations of type material, some taxonomic considerations on some species of *Murrayon* are reported below.

***Murrayon hibernicus*** Murray

Murray (1911) described the former *Macrobiotus hibernicus* from Ireland based on a “young squeezed out of the egg”, reporting that cuticular structures of the animal (necessary for taxonomic identification) may not have been fully developed. In the original description (Murray 1911), the characteristics of the cuticle are not mentioned and the presence of three macroplacoids (“nearly equal, about twice as long as broad”) and a “comma” (microplacoid) are reported. However, in the original drawings, the microplacoid is not present, and the third macroplacoid seems larger than the others (fig. 15b in Murray 1911). Twenty-four years later, based on French specimens, Cuénot (1932) reported the presence of dots (very fine grains) on the cuticle surface forming dorsolateral bands. He also reported the presence of three macroplacoids (the third the longest, the second the shortest and in contact with the first), and the absence or presence of a microplacoid (it was drawn as a very small dot; fig. 55 in Cunéot 1932). Based on western Canadian animals (eggs were not found) from Vancouver Is. (British Columbia), Kathman (1990) was the only one to report an extension of the dorsolateral band of larger dots onto the dorsal surface of the legs. Sixty-one years after Murray’s description of the species, Argue (1972), based on eastern Canadian specimens (New Brunswick), reported that the egg processes were “often prolonged with hairs” (a.k.a. with long spines or with thin threadlike appendages; Fig. 2F). One year later, using Italian specimens, the buccal armature of *M. hibernicus* was described by Pilato (1973). Only in 1998, *Macrobiotus hibernicus* was transferred to *Murrayon* (Guidetti 1998).

Almost no images are available of animals and eggs of *M. hibernicus.* There are only three drawings of an animal: those in lateral view in Cunéot (1932) (most subsequent drawings were based on this drawing, e.g. Marcus (1936), Ramazzotti and Maucci (1983), Maucci (1986); Séméria (2003), and Kathman (1990), and in ventral view in Dastych (1988). While there are several drawings of eggs by Murray (1911), Cunéot (1932), Marcus (1936), Maucci (1986) and Dastych (1988), but only Dastych showed the “hairs” on process tips. The monographs on tardigrades by Thulin (1928), Marcus (1929, 1936), Ramazzotti (1972) and Ramazzotti and Maucci (1983) report the presence of a dorsolateral band of dots on the cuticle for this species but not the presence of the “hairs” on the egg processes. The only photographs of *M. hibernicus* are those reported by Argue (1972) of the feeding apparatus, the close-up of the cuticle of an animal, and the eggs. Egg processes of Canadian specimens in the pictures in Argue (1972) look different in shape (in particular in their distal portions) from those found in our Norwegian sample (see below). These two types of eggs led to the hypothesis of the presence of more than one species having the general characters attributed to *M. hibernicus*. Based to the numerous reports from different regions in the Holarctic, Kaczmarek *et al*. (2016) hypothesized the presence of a species complex related to *M. hibernicus*.

Cunéot (1932), Argue (1972), Pilato (1973), and Kathman (1990) added new characters to the first description of *M. hibernicus* by Murray (1911), using specimens collected from different areas of the world (all far from the type locality); therefore, the species described by Murray (1911) is not necessarily the same (although similar) to the species recorded by those authors. Moreover, according to Morgan (1977), no type material of *M. hibernicus* is available in the Natural Museum of Scotland, where most of the type series of James Murray are still available.

For all the above-mentioned reasons (i.e. the partial and incomplete description of the species from an animal artificially extracted from its egg, absence of type material, addition by other authors of characters from specimens collected in different areas, probable presence of a species complex), *M. hibernicus* needs taxonomic revision and, awaiting new data, we propose this species to be considered *nomen dubium.*

***Murrayon hyperoncus*** Meyer, Domingue & Hinton

Meyer *et al*. (2014) reported the presence of cuticular pillars and a hook on the ventral margin of the strengthening bar of the buccal tube in *Murrayon hyperoncus*, but these characters are not visible in any figures in the paper. The claws were defined as *Murrayon* type (or *pullari* type), but there is no picture of the claws in lateral view in which the laminar (quadrangular) stalk is visible. In *M*. *hyperoncus*, there are also characters not present in any other species of the genus (or even of the family), such as a large microplacoid (absent in *Murrayon* and Murrayidae) and a buccal armature very similar to those found in most Macrobiotidae (i.e. an anterior and a posterior bands of small teeth evident, as in e.g. *Macrobiotus* and *Paramacrobiotus*), while in the other species of the genus the armature is absent or very weak. Moreover, the eggs of this species are unknown. For the above-mentioned reasons and since *M.* *hyperoncus* has characters similar to those of *Macrobiotus* species, we propose to transfer *M. hyperoncus* to thegenus *Macrobiotus* (*Macrobiotus* *hyperoncus* comb. n.) pending further analyses*.*

***Murrayon nocentiniae*** Ramazzotti

Observation of paratypes (in lateral view) revealed that the cuticle is smooth, without dots or visible epicuticular pillars. Buccal armature is not visible. Pharyngeal apophyses are large, the two macroplacoids are wide, the first has an evident constriction at about half of its length, and the second has a subterminal constriction (Figs 3A, B). Claws have small smooth lunules (proportionally larger on hind legs) and evident accessory points on the main branches (Figs 3C, D). Eggs have a diameter of about 66 µm without ornamentations (61 µm according to Ramazzotti 1961), egg processes are in shape of small cones, about 3–4 µm height and 2–3 µm in diameter at the base, their tips can be bi- or trifurcated (Fig. 3E). There are about 28–30 processes (35–40 according to Ramazzotti 1961) on the circumference of the eggs (Fig. 3F).

***Murrayon ovoglabellus*** Biserov

Observation of paratypes revealed that in contrast to what was reported in the original description, eggs attributed by Biserov (1988) to this species are not completely smooth (Fig. 3L). The eggs have faint, scattered conical processes of different shapes and size (generally in the shape of long thin cones) that are irregularly spread on the egg surface (Fig. 3K). We confirm that the animals have a smooth cuticle, without dots or visible epicuticular pillars. In the buccal armature, the first band of teeth is not visible, the second band is barely visible, dorsally the central transverse crest is large and round and the lateral crests are in the shape of a small dash, ventrally the central crest is a small dot and the lateral crests are very narrow (Fig. 3G). Pharyngeal apophyses are small; the two macroplacoids are long, straight, and thin; the first macroplacoid has an indentation at about half of its length, the second in its sub-terminal portion (Figs 3G, H). The claws are large with relatively large smooth lunules (proportionally larger on the hind legs), and evident accessory points on the main branches (Figs 3I, J).

**Discussion**

Compared to previous studies, our molecular phylogeny of Murrayidae includes more taxa and confirms the paraphyletic status of the genus *Murrayon* with respect to *Dactylobiotus.* As also reported by Bertolani *et al*. (2014), species assigned to *Murrayon* belong to two different phylogenetic lineages: species of the *pullari*-complex forming a sister group to the species of *Dactylobiotus* and not grouping with the new species from Norway and *M. dianeae*, which are clustered together*.*

The results from the molecular analyses are supported by morphological characteristics of the taxa. In fact, based on egg and animal morphologies, two groups can be identified in *Murrayon*: the *dianeae*-group (*M. dianeae, M. stellatus,* and the new Norwegian species; Figs 2, 4, 5) and the *pullari*-group (*M. pullari,* with the other undescribed species of the *“pullari* complex”, *M. nocentiniae, M. ovoglabellus*; Figs 3, 6). The *dianeae*-group is characterized by egg processes (Figs 2, 6) that are more or less cylindrical with enlarged apices (rod- or chalice-shaped) covered with a cuticular layer that connects the apex of the processes to the egg surface (in the past these processes were considered immersed in a hyaline matrix), the adults have large, evident (with LM) epicuticular pillars (Figs 2, 4), which produce a pattern of dots on the animal surface, small claws (Figs 2, 4), and are commonly found in terrestrial/hydrophilic habitats. The *pullari*-group is characterized by eggs with conical processes (even reduced as in *M. ovoglabellus*; Figs 3, 6), the adults have a smooth cuticle without dots (with LM), although the epicuticular pillars are present (Guidetti *et al*. 2000), proportionally larger and longer claws (Figs 3, 6), and are commonly found in freshwater habitats. *Murrayon hastatus* (Fig. 6G) has an uncertain position: based on adult morphology it could belong to the *pullari*-group, while based on egg morphology it could belong to the *dianeae*-group. Egg processes similar to those found in *M. hastatus* and in the species of the *dianeae*-group are present in taxa belonging to other distantly related lineages: e.g. several *Minibiotus* species (Macrobiotidae; see Claxton 1998), *Eohypsibius nadjae* Kristensen, 1982 (Eohypsibiidae), and *Oreella mollis* Murray, 1910 (Carphaniidae, Heterotardigrada); the eggs of the latter two species are described in Bertolani *et al*. (1996), for *Oreella* see also Dastych *et al*. (1998).

Our molecular phylogeny places the species of the *pullari-*complex as a sister group to *Dactylobiotus* (Fig. 1). The morphology of the egg processes of *M. pullari* (i.e. conical; Fig. 6B) is the same or very similar to that of *Dactylobiotus* species (e.g. see *D*. *parthenogeneticus, D. dispar, Dactylobiotus vulcanus* Kaczmarek *et al*., 2012). The egg processes of *D. octavi* can be considered as cones not completely everted, similar to the egg of *D. ovimutans* (Kihm *et al*. 2020). The cluster (*pullari-*complex + *Dactylobiotus* spp.; Fig. 1) is characterized by egg processes in shape of cones and epicuticular pillars not visible with light microscopy (*Dactylobiotus* species have epicuticular pillars visible only with electron microscopy; Guidetti *et al*. 2000). Moreover, *Dactylobiotus* species (e.g. *D. dispar, D. parthenogeneticus*) and *M. ovoglabellus* and *M. pullari* (Murray 1907; Bertolani 1982; Biserov 1988) usually release free eggs but can release eggs within their old cuticle (exuviae), while *M. hastatus* releases free eggs but sometimes releases eggs into the exuviae of small crustaceans, probably ostracods (Fig. 6C; Murray 1907; Bertolani 1982).

Nevertheless, claw morphology of thespecies in the *pullari-*group(V-shaped) is different from that of *Dactylobiotus* or *Macroversum* species (L-shaped; Figs 7C-G), while it is more similar to that of the *dianeae*-group (V-shaped; see description below in “Diagnoses and descriptions of new taxa”).

For all the above-mentioned reasons, we propose the erection of the new genus *Paramurrayon* for the *dianeae*-group and consequently to emend the diagnosis of *Murrayon* (see below in “Diagnoses and descriptions of new taxa”). By doing so, we retain the natural (monophyletic) genus-level groups that are morphologically defined and present a more resolved classification compared to the alternative scenario of regrouping all *Murrayon* and *Dactylobiotus* species into one genus.

Based on the molecular phylogeny, two possible parsimonious evolutionary scenarios for the family Murrayidae can be hypothesised (Figs 7A, B), differing by the unknown ancestral status of the egg processes and claw sizes.

For Murrayidae, the results of this study partially resolve the taxonomic paradox underlined by Guidetti *et al*. (2006): “there are closely related species, which share a very similar morphology of the animals but clearly differ in their egg morphology. Conversely there are species belonging to different evolutionary lines that have similar eggs, but very different adult morphology”. The paradox is only partially solved because *M. hastatus* has adults similar to *Murrayon* and eggs similar to *Paramurrayon* gen. nov., unfortunately, there are no molecular data for *M. hastatus*. In Macrobiotoidea, there are genera (e.g. *Macrobiotus, Minibiotus*) in which species have very similar adult morphology but different egg morphology, the opposite condition (i.e. very similar egg morphology and different animal morphology) is present only for the genus *Xerobiotus*, whose taxonomic status is debated (Stec *et al*. 2021, 2022; Massa *et al*. 2021). For this reason, the species *M. hastatus* is retained in the genus *Murrayon* awaiting molecular confirmation.

Recent advances in clarifying tardigrade systematics (see Degma & *Guidetti* 2022), obtained due to the integrative taxonomic approach and molecular phylogenetics, have resulted in a more uniform morphological characters in eggs and adults within each taxon. Nevertheless, in spite of these achievements, the “taxonomic paradox” still persists in several taxa (e.g. of Macrobiotidae and Eohypsibiidae). From an evolutionary point of view, this paradox generates further questions related to the speciation processes, monophyly and definition of taxa, as well as the mosaic-like evolution of the adult and egg characters in tardigrades.

**Diagnoses and descriptions of new taxa**

***Murrayon*** Bertolani & Pilato (emended diagnosis)

Claws of *Murrayon* type (= *pullari* type): basal section of the double claw appears as a trapezoidal lamina; primary and secondary branches similar in length and joined to each other for a very short portion, forming an acute angle (V-shaped); lunules present. Two macroplacoids in the pharynx. Epicuticular pillar-like structure not visible with light microscopy.

***Species composition***: *M. pullari* (type species), *M. hastatus, M. nocentiniae*, *M. ovoglabellus.*

***Remarks***: The species have conical egg processes (except *M. hastatus*) and are found in freshwater or hydrophilic habitats. Eggs of *M. hastatus* have rod-shaped processes covered by a cuticular lamina. *Murrayon hastatus* species is retained in this genus pending molecular confirmation.

***Paramurrayon* gen. nov.**

ZooBank: XXXXXXXXXXXXXXXXXXXXXX

Small claws of the *Murrayon* type (= *pullari* type): basal section of the double claw appears as a trapezoidal lamina; primary and secondary branches similar in length and joined to each other for a very short portion, forming an acute angle (V-shaped); lunules present. Three macroplacoids in the pharynx. Epicuticular pillar-like structure visible with light microscopy (dorsally as dots, laterally as pillars). Eggs with chalice-shaped rod processes covered by a thin cuticular lamina (like a blanket), connecting the distal part of the chalice-shaped rods to the egg surface.

***Species composition***: *P. dianeae* comb. n. (type species), *P*. *meieri* sp. nov.*, P. stellatus* comb. n., *P. hibernicus* comb. n. (*nomen dubium*)*.*

***Remarks***: All species are found in terrestrial/hydrophilic habitats. Each egg process has a long, thin threadlike filament on its dorsal margin.

**Etymology**: Para (near or resembling; in Greek) + *Murrayon*.

***Paramurrayon* *meieri* sp. nov.** (Figs 4, 5; morphometric data in Tab. 3)

*ZooBank*: XXXXXXXXXXXXXXXXXXXXXX

*Holotype*: slide 282s36a

*Paratypes*: 31 animals mounted on slides in Hoyer’s medium and 11 eggs mounted in Faure Berlese’s or Hoyer’s medium, in addition to the five specimens used for molecular analysis.

*Type locality*: site 72 of the Geitaknottane Nature Reserve (Kvam, Hordaland, Norway), N 60.11139°, E 5.86491°, 126 m a.s.l., lichen sample 282, composed by a mix of *Lobaria virens* (With.) J.R. Laundon and *Lobaria pulmonaria* (L.) Hoffm., collected on 14th August 2017, on the bark of a hazel tree (*Corylus avellana* L.) in a broad-leaved deciduous forest.

Additional locality: site S Førlandsvatnet of Førland/Sletthei Landscape Protection Area (Lund, Rogaland, Norway), N 58.55935°, E 6.43991°, 273 m a.s.l., bryophyte sample 247 [mix of *Hylocomium splendens* (Hedw.) Schimp.*, Barbilophozia lycopodioides* (Wallr.) Loeske + *Rhytidiadelphus loreus* (Hedw.) Warnst.], collected on 12th August 2017, on a boulder in a *Betula pubescens* Ehrh. Forest.

*Etymology*: The species is dedicated to the Norwegian tardigradologist Terje Meier for his passion for water bears.

*Type repositories:* the holotype (slide 282s36) and 10 paratypes are deposited in the collection of the NTNU University Museum (NTNU-VM), Trondheim, Norway, 10 paratypes deposited in the Bertolani Collection (Department of Life Sciences, University of Modena and Reggio Emilia, Italy), 5 paratypes in the tardigrade slide collections of the Natural History Museum of Verona (Italy).

*Description*: animals from 140 µm to 241 µm in length, colourless, eye-spots visible in eight of the 32 animals mounted on slides. The entire cuticle surface presents a fine punctation, each dot representing the head of an epicuticular pillar (Figs 4A, C). The dorsal dots generally have smaller diameters than those on the ventral region of the body. There is an evident dorsolateral band of large dots (corresponding to larger pillars) that runs continuously along each side of the body (Fig. 4A); the two dorsolateral bands are in contact on the dorsal part of the cephalic region (Fig. 4C) and on the dorsal portion of the last body segment, in alignment with the hind legs. Each band is wider where each leg is located, extending almost to the proximal portion of the legs (Fig. 4A). The central region of the band has the larger pillars that can reach 1 µm in height (visible with LM in lateral view; Fig. 4A).

The buccal ring of the feeding apparatus is characterized by 10 small, barely visible peribuccal lamellae. The buccal tube is relatively large. The buccal armature, identifiable in larger specimens, is formed by three small dorsal transverse crests (two laterals and one central anterior to the other two; Fig. 4F) and two very small, rounded teeth corresponding to the ventral-lateral crests (Fig. 4G); other components of the buccal armature are not visible with LM. In lateral view, the ventral lamina (strengthening bar) has the hook-shaped apophysis (typical for the family) in the anterior region (Fig. 4H). The two stylet supports (about 9 µm long) are inserted on the buccal tube at about 2/3 of its length and have a bifurcated end connected to the stylet furca (Fig. 4D). The stylet furca has two evident additional rounded apophyses on the furca branches. The pharyngeal bulb contains large apophyses and three macroplacoids, the first is the longest and the second the shortest (Fig. 4E). They are rod-shaped in lateral view. In frontal view, the first macroplacoid is posteriorly enlarged, the second is quadrangular (sometime almost rounded) and positioned very close to the first, and the third has a subterminal constriction (Fig. 4E).

The claws are small and of the *pullari* type (V-shaped and with a squared peduncle in lateral view; Fig. 4H-J), with small lunules (proximally thinner and distally thicker; Fig. 4K), and evident accessory points on the main branches. In lateral view, the first three pair of legs display a longitudinal thickening connected to the external claw, this thickening appears located within the cuticular layer of the leg (Fig. 4J).

Eggs are laid freely (Fig. 5). They have a diameter (without processes) of ca. 50-59 µm. The egg shell is covered with lines of chalice-shaped rod processes (4-5 µm in height), which form an irregular pattern on the surface of the egg, not always delimiting closed areas (Figs 5A, C, E, G). Chalice-shaped rod processes have an enlarged distal head with a variable diameter (Figs 5D, H). Each line of rods is covered by a thin cuticular lamina (like a type of blanket), connecting the distal part of the chalice-shaped rods to the egg surface (Figs 5B, C, D, F). Each rod has a thin threadlike filament of variable length (up to 8.9–10 µm long) on its dorsal margin (Figs 5D, H). The surface between the rod lines appears smooth.

*Differential diagnosis*

The new species has a unique mix of characters that identified it as new to science; in particular, *Paramurrayon meieri* sp. nov. differs from *Paramurrayon dianeae* comb. n. (Figs 2A, D) by having the evident dorsolateral band of large dots on the cuticular surface; from *Paramurrayon stellatus* comb. n. (Fig. 2E) by having a less evident band of dorsolateral dots (star-shaped in frontal view in *P. stellatus* comb. n.) on the cuticular surface due to the smaller size of the cuticular pillars that form the bands, and by the egg processes that are larger, longer and with a larger base and head, bearing a longer threadlike filament (Figs 2A, 5A-H).

***Identification keys of* Murrayidae** Guidetti, Rebecchi & Bertolani

The family Murrayidae is characterized by animals with V-shaped or L-shaped double claws, with the two branches diverging immediately after a short common basal section; ventral deep indentation on the ventral lamina; buccal tube completely rigid; epicuticular layer with pillar-like structures, sometimes visible only at ultrastructural level (Bertolani *et al*. 2014).

There are four genera in the family: *Murrayon* (type genus), *Dactylobiotus, Macroversum,* and *Paramurrayon* gen. nov.

1. Primary and secondary branches of claws similar in length (the primary can be slightly longer) and forming an acute angle (V-shaped claw) (Figs 3D, I, 6D, G) …………………………… 2

– Primary branch almost two times (or more) longer than secondary branch (L-shaped claw; Fig. 7G), each pair of claws connected with a cuticular bar (Figs 7E) ……………….……………… 3

2(1). Epicuticular pillar-like structure visible with light microscopy (dorsally as dots, laterally as pillars; Figs 2D, 4A), eggs with chalice-shaped rod processes covered by a thin cuticular lamina (like a blanket), connecting the distal part of the chalice-shaped rods to the egg surface\* (Figs 2E, F, 5A-H.) …………………………………………………………… *Paramurrayon* gen. nov.

– Epicuticular pillar-like structure not visible with light microscopy egg surface with conical (even small) or rod-shaped processes………………………………………………………………… *Murrayon*

3(1). Lunules of the claws absent ……………………………………………………………………. *Dactyolobiotus*

– Lunules of the claws present (Fig. 7E) …………………………………………….………………. *Macroversum*

\* *Murrayon hastatus* has also this kind of egg (Fig. 6F).

***Murrayon*** Bertolani & Pilato

1. Slender macroplacoids, i.e. first macroplacoid at least four (or more) times longer than wide (Fig. 3H); egg surface with small, very thin, sparse conical processes (Fig. 3L) ………………………………………………………………………………………………………………… *M. ovoglabellus*

– Stumpy macroplacoids, i.e. first macroplacoid about two times longer than wide (Figs 3A, 6A); egg with numerous, evident processes ……………………….………………….………………………. 2

2(1). Egg processes large, rod-shaped and covered by a cuticular lamina (Fig. 6F) … *M. hastatus*

– Egg processes conical or spiniform in shape ..……………………………………………………………………. 3

3(2). Eggs with small, conical processes (Fig. 6B) ……………………………………………………… *M. pullari*

– Eggs with long conical processes bifurcated at the extremities (Fig. 3F) ………… *M. nocentiniae*

***Paramurrayon* gen n.**

*Paramurrayon hibernicus* comb. n. sp. dub. is not considered in the key due to the reasons reported above.

1. Cuticle uniformly and finely punctuated with small dots/pillar-heads … *P. dianeae* comb. n.

– Cuticle with an evident dorsolateral band of larger dots/pillar-heads ……………………………… 2

2(1). Pillar-heads in the central portion of the dorsolateral band of dots, large and very evident, star-shaped in dorsal view; egg processes in shape of small rods° ………. *P. stellatus* comb n.

– Pillar-heads in the central portion of the dorsolateral band of dots not star-shaped in dorsal view; egg processes chalice-shaped with wide head bearing a very long filament (generally longer than the process height) ……………………………………………………………… *P. meieri* sp. nov.

° only one egg was found, intraspecific variability unknown.

For an identification key of ***Dactylobiotus*** species see Kaczmarek *et al*. (2012).

***Macroversum*** contains only one species: *Macroversum mirum* (Fig. 7).

**List of the online Supplemental material**

**Table S1**. GenBank accession numbers of the sequences produced in this study and of the Macrobiotoidea sequences used in phylogenetic analyses.

**Table S2**. Uncorrected p-distance of *cox1* and ITS2 computed among Murrayidae (*Murrayon*, *Paramurrayon* gen. nov. and *Dactylobiotus*) species.

**Table S3**. Morphometric data of *Paramurrayon* *meieri* sp. nov.

**Figure S1**. Phylogenetic relatioships (enlargement of Fig. 1) between *Minibiotus+Tenuibiotus+Paramacrobiotus* (Macrobiotidae) and between *Diaforobiotus+Richtersius* (Richtersiusidae).

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**Data Availability Statement**

The data that support this study are available in the article and accompanying online supplementary material. The molecular data obtained in this study are available in GenBank. Other data that support this study will be shared upon reasonable request to the corresponding author.

**Conflicts of Interest**

The authors declare no conflicts of interest

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**Table 1.** Sampled localities for the examined specimens.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Sample** | **Locality** | | **Geographic coordinates** | **Altitude (m)** | **Substrate** | **Species** |
| 282 | | Geitaknottane Natural Reserve (Norway) | N 60°6.6834'; E 5°51.8946' | 126 | Lichens on *Corylus avellana* (*Lobaria virens, Lobaria pulmonaria*) | *Paramurrayon meieri* gen. n.sp. nov. |
| 247 | | Førland, Sletthei Protected Area (Norway) | N 58°33.561'; E 6°26.3946' | 273 | Moss on boulder (*Hylocomium splendens, Barbilophozia lycopodioides*) | *Paramurrayon meieri* gen. n. sp. nov. |
| C3125 | | Glendoebeg, Fort Augustus, Scotland (UK) | N 57°08.721'; W 4°37.175' | 51 | Freshwater sediment in a stream | *Murrayon* cf. *pullari* |
| C3147 | | Oban, Scotland (UK) | N 56°39.668'; W 5°04.147' | 254 | Plant sediment in a peat bog | *Murrayon* cf. *pullari* |
| C2791 | | Nonantola, Modena (Italy) | N 44°41.397'; E 11°03.243' | 24 | Freshwater sediment in a pond with aquatic plants | *Dactylobiotus* *parthenogeneticus* |
| C3558 | | Pope Beach, Lake Tahoe, California (USA)\* | N 38°56.258'; W 120°01.905' | 1901 | Freshwater sediment in a lake | *Dactylobiotus grandipes* |

\* Locus typicus of *Dactylobiotus grandipes*.

**Table 2.** Groupings of individual *cox1* sequences in species of the genus *Murrayon* as indicated by the ASAP program. Interspecific threshold p-distance value: 11.4% (ASAP-score: 1.50). The specimens analysed in this study are in bold. In parentheses are the GenBank accession numbers. In brackets is the name of the voucher specimen.

|  |  |
| --- | --- |
| **Specimen** | **Species** |
| *Macrobiotus macrocalix* Italy (FJ176211) | 1 |
| *Murrayon* cf. *dianeae* Spain (FJ435801) | 2 |
| ***Paramurrayon meieri* sp. nov. sample 282, Norway (OP379722)** | 2 |
| ***Paramurrayon meieri* sp. nov. sample 282, Norway (OP379723)** | 2 |
| ***Paramurrayon meieri* sp. nov. sample 282, Norway (OP379724)** | 2 |
| ***Paramurrayon meieri* sp. nov. sample 282, Norway (OP379725)** | 2 |
| ***Paramurrayon meieri* sp. nov. sample 282, Norway (OP379726)** | 2 |
| ***Paramurrayon meieri* sp. nov. [V2] sample 247, Norway (OP379727)** | 2 |
| ***Paramurrayon meieri* sp. nov. [V7] sample 247, Norway (OP379728)** | 2 |
| *Murrayon pullari* Italy (AY598772) | 3 |
| *Murrayon* cf. *pullari* Italy (MT808080) | 3 |
| ***Murrayon* cf. *pullari* [V1] Glendoebeg, Scotland (OP379720)** | 4 |
| ***Murrayon* cf. *pullari* [US1] Oban, Scotland (OP379721)** | 5 |

|  |  |
| --- | --- |
| **Specimen** | **Species** |
| *Macrobiotus macrocalix* Italy (FJ176211) | 1 |
| *Dactylobiotus selenicus* Finland (MT808076) | 2 |
| ***Dactylobiotus grandipes* [V1] USA (OP379718)** | 2 |
| ***Dactylobiotus grandipes* [V2] USA (OP379719)** | 2 |
| *Dactylobiotus ovimutans* Antarctica (MT132333) | 3 |
| *Dactylobiotus ovimutans* Antarctica (MT132332) | 3 |
| *Dactylobiotus* sp. Antarctica (EF632528) | 3 |
| *Dactylobiotus* sp. Antarctica (EF632526) | 3 |
| *Dactylobiotus* sp. Antarctica (EF632529) | 3 |
| *Dactylobiotus* sp. Antarctica (EF632527) | 3 |
| *Dactylobiotus* sp. Antarctica (EF632524) | 3 |
| *Dactylobiotus* sp. Antarctica (EF632523) | 3 |
| *Dactylobiotus* sp. Antarctica (EF632525) | 3 |
| *Dactylobiotus parthenogeneticus* Italy (AY598771) | 4 |
| *Dactylobiotus parthenogeneticus* France (MT373804) | 4 |
| *Dactylobiotus parthenogeneticus* Poland (MT373805) | 4 |
| *Dactylobiotus parthenogeneticus* Poland (MT373806) | 4 |
| *Dactylobiotus parthenogeneticus* England (MT373803) | 4 |
| ***Dactylobiotus parthenogeneticus* [V3] Italy (OP379716)** | 4 |
| ***Dactylobiotus parthenogeneticus* [V7] Italy (OP379717)** | 4 |

**Figure Legends**

**Figure 1.** Phylogenetic molecular tree of the superfamily Macrobiotoidea (Bayesian and Maximum likelihood analyses) based on combined dataset (18S + 28S sequences) under the GTR + Gamma model. Numbers above the branches show posterior probability values, while numbers in italics below branches show bootstrap values. Asterisks denote highly supported nodes (both posterior probability value = 1 and bootstrap value = 100). GenBank accession numbers of all the Macrobiotoidea species used in the phylogenetic analyses are given in Table S2. Scale bar = number of substitutions per site.

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**Figure 2.** *Murrayon dianeae* (A-D), *Murrayon stellatus* (E), *Murrayon* cf. *hibernicus* (F). A. Feeding apparatus (frontal view). B. Feeding apparatus (lateral view). C. Claws on the first legs and cuticle with dots (pillars). D. Dorsal-posterior cuticle with dots (pillars). E. Egg (arrowhead = threadlike filament). F. Egg (arrowhead = threadlike filament). A-F: PhC. Slide: A, C-D (C881s2); B (991s9); E (5N08B s28); F (Tipo126). Scale bars: A-F =10 µm.

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**Figure 3.** *Murrayon nocentiniae* (A-F), *Murrayon ovoglabellus* (G-L). A. Feeding apparatus. B. Feeding apparatus. C. Claws on the hind legs. D. Claw on the second pair of legs. E. Egg processes. F. Egg. G. Feeding apparatus (white arrow = dorsal crests, black arrow = ventral crests of buccal armature). H. Macroplacoids. I. Claws on the hind legs. J. Claw on the second pair of legs. K. Egg processes (arrowheads). F. Eggs (arrowheads = egg processes). A-F, J, L: PhC. G-I, K: DIC. Slide: A, C-D (CT6084); B, E-F (Tipo115); G-J (CT14699); K-l (Tipo968-12). Scale bars: A-L = 10 µm.

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**Figure 4.** *Paramurrayon meieri* sp. nov. A. Animal (lateral view). B. Animal (ventral view). C. Head region (dorsal view). D. Feeding apparatus. E. Macroplacoids. F. Buccal armature (dorsal view) (arrowhead = transverse crests). G. Buccal armature (ventral view) (arrowhead = transverse crest). H. Head region (lateral view) (white arrowhead = epicuticular pillars; black arrowhead = hook in the ventral lamina). I. Claws on the second and third pair of legs. J. Claws on the second and third pair of legs (arrowheads = longitudinal thickenings). K. Claw on the second pair of legs (arrowhead = lunula). A-C, H, K-J: PhC. D-G, I: DIC. Slide: B, D-G, I (holotype); A, C, H, K-J (paratypes). Scale bars: A-C, I-J = 20 µm, D, H = 10 µm, E-G, K = 5 µm.

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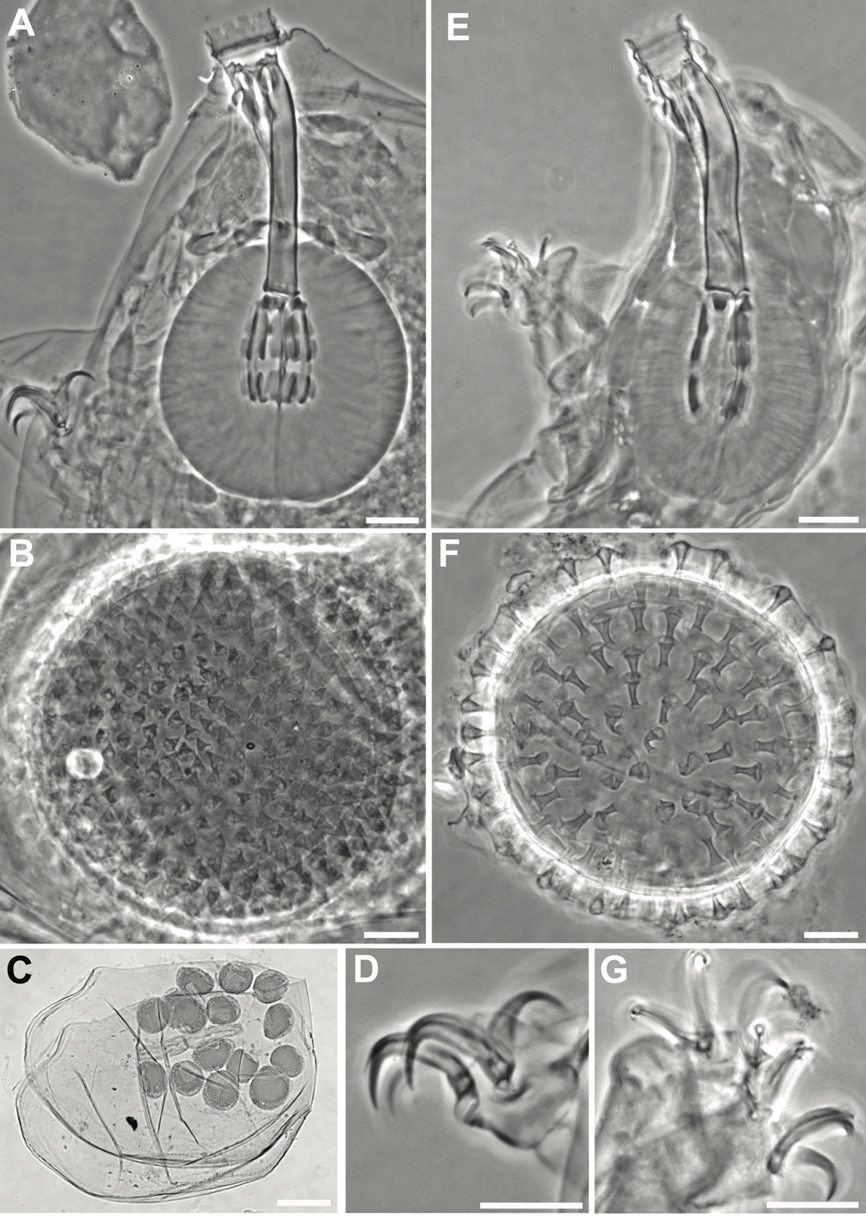
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**Figure 5**. *Paramurrayon* *meieri* sp. nov. A. Egg. B Egg processes in lateral view. C. Surface of the egg. D. Egg processes. E. Egg. F. Surface of the egg. G. Egg. H. Egg processes. White arrowheads = threadlike filaments. Black arrowhead = base of the cuticular lamina covering the processes. Arrows = cuticular lamina covering the processes. A-E, G-H: PhC. F: DIC. Slide: A-E (sample 282 from Geitaknottane Natural Reserve, Norway: type locality); F-H (sample 274 from Førland, Sletthei, Norway). Scale bars: A-H = 10 µm.

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**Figure 6.** *Murrayon pullari* (A-D), *Murrayon hastatus* (E-G). A. Feeding apparatus. B. Egg. C. Eggs within crustacean exoskeleton (probably ostracods). D. Claws on the third pair of legs. E. Feeding apparatus. F. Egg. G. Claws on the hind legs. A-G: PhC. Slide: A, D (C199s3); B-C (Tipo143); E-G (C1178s4). Scale bars: A-B, D-G = 10 µm; C = 100 µm.



**Figure 7.** Evolutionary scenarios for the genera of Murrayidae (A, B), *Macroversum mirum* (C-G; holotype). A, B. Synapomorphies (with [notes]) are represented by numbers. \* = *Murrayon hastatus* is not considered due to its uncertain phylogenetic position. Scenarios A and B differ by the plesiomorphic status of the egg morphology and claw size. 1 = feeding apparatus characterized (in lateral view) by a hook on the margin of the ventral lamina [unknown for *Macroversum*], and two macroplacoids in the pharynx. 2 = pillar-like structures in the inner epicuticle layer [unknown for *Macroversum*]. 3 = symmetrical claws with: the two claw branches diverging immediately after the basal portion (i.e. very short common tract) [with lunules]. 4 = claw V-shaped (i.e. main branch similar in length to secondary branch). 5 = egg of the *Murrayon* type (i.e. conical egg processes) [for *Macroversum* the eggs are unknown]. 6 = egg of the *Paramurrayon* type (i.e. chalice-shaped processes covered by a thin cuticular lamina) [for *Macroversum* the eggs are unknown]. 7 = colonization of freshwater habitats with proportionally larger and longer claws with respect to the ancestor (freshwater tardigrades have longer claws, probably for better adherence to the substrate; Nelson et al. 2015). 8 = reduction of the claws with respect to the ancestor. 9 = claw L-shaped (i.e. main branch longer than the secondary; E-G). 10 = cuticular connection between the lunules. 11 = loss of lunules [presence of cuticular connection between the claws; E-G]. 12 = two, posterior, dorso-lateral papillae. C. Animal in toto. D. Anterior portion of animal with feeding apparatus. E. Second pair of legs. F. Third pair of legs. G. Hind legs. Arrowheads = cuticular bars. Slide: C-G (slide 4084). Scale bars: A = 20 µm, B-E = 10 µm.

