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Actinarctus doryphorus (Tanarctidae) DNA barcodes and phylogenetic reinvestigation of Arthrotardigrada with new *A. doryphorus* and Echiniscoididae sequences‡

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Abstract

Little is still known about the diversity and evolution of marine arthrotardigrades, as they are generally difficult to sample, resulting in a limited amount of molecular data for barcoding and phylogenetic studies. With the current study, we provide the first investigation into COI haplotype diversity in a marine tanarctid and at the same time readdress arthrotardigrade phylogeny. Specifically, we provide COI mtDNA, 18S and 28S rDNA sequences from a population of *Actinarctus doryphorus* (Tanarctidae) sampled off the coast of Roscoff, France and further provide new 18S sequences from two marine echiniscoidids. *A. doryphorus* COI sequences confirmed the presence of a single species and further revealed five haplotypes shared among nine sequenced individuals. Our 18S and 28S rDNA datasets were individually and combined analysed with Bayesian inference and Maximum Likelihood. *Actinarctus doryphorus* was placed together with *Tanarctus* sequences within a maximally supported Tanarctidae, confirming previous interpretations that the clade is distinct from Halechiniscidae. Although several studies in recent decades have concluded that the marine arthrotardigrades are paraphyletic, recent studies have argued that the clade may not be paraphyletic. Our phylogenetic analyses consistently inferred Arthrotardigrada as paraphyletic, as the clade includes the monophyletic Echiniscoidea. Accordingly, we propose that it is time to suppress the order Arthrotardigrada as it clearly does not reflect tardigrade phylogeny.

Key words: 18S rRNA, 28S rRNA, Bayesian inference, COI, Maximum Likelihood, phylogenetics, Tardigrada

Introduction

Microscopic representatives of Ecdysozoa include Tardigrada, which divide into two major evolutionary lineages Heterotardigrada and Eutardigrada (Giribet & Edgecombe 2017; Jørgensen *et al.* 2018). Numerous recent studies have focused on the evolution and diversity of eutardigrades and echiniscid heterotardigrades living in semi-terrestrial and freshwater habitats (*e.g.* Jørgensen *et al.* 2007; Bertolani *et al.* 2014; Gąsiorek & Michalczyk 2020; Stec *et al.* 2020; Morek *et al.* 2021), whereas marine echiniscoideans (Gąsiorek & Kristensen 2022; Møbjerg *et al.* 2016, Møbjerg *et al.* 2020) and the predominantly marine arthrotardigrades have received much less attention. The arthrotardigrades are known for their large morphological variation, small size and widely scattered distribution (Hansen *et al.* 2012; Jørgensen *et al.* 2014; Møbjerg *et al.* 2018). In addition to difficulties in sampling, it is also difficult to amplify sequences from arthrotardigrade taxa. Hence, phylogenetic analyses of these marine heterotardigrades are challenged by a limited number of sequences. Consequently, Arthrotardigrada has been inferred as paraphyletic (Jørgensen *et al.* 2010; Fujimoto *et al.* 2017), polyphyletic (Guil *et al.* 2013, 2019) and more recently, monophyletic (Fleming & Arakawa 2021).

Universal primers, including the widely used "Folmer primers" (Folmer *et al.* 1994), generally bind to arthrotardigrade sequences with low affinity impairing amplification or amplifying non-tardigrade DNA (Jørgensen *et al.* 2010). Notably, only four arthrotardigrade sequences of the mitochondrial cytochrome c oxidase subunit I (COI) are available to date: *Batillipes pennaki* (see Ryu *et al.* 2007), *Styraconyx takeshii* (see Fujimoto *et al.* 2020),

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Florarctus sp. (GenBank Accession number MT999946) and *Batillipes longispinosus* (KF938943), with *Batillipes* species represented by partial mitochondrial genome sequences. The small quantity of COI sequences has prevented DNA barcoding approaches, such as metabarcoding studies and analyses of haplotypes or patterns of speciation and distribution, from being applied to this group of tardigrades.

Here, we present the first comprehensive approach analysing average nucleotide composition, haplotype diversity and sequence variation (uncorrected *p*-distances) of nine COI sequences from the tanarctid *Actinarctus doryphorus*. In addition, we readdress arthrotardigrade phylogeny using newly obtained 18S and 28S rDNA sequences of *A. doryphorus* and we further include new sequences from the marine echiniscoideans *Isoechiniscoides sifae* and *Neoechiniscoides aski* in our analyses.

Material and methods

Collection of specimens, DNA extraction and PCR amplification. Specimens of *Actinarctus doryphorus ocellatus* Renaud-Mornant, 1971 (Tanarctidae) were obtained from shell gravel collected off the coast of Roscoff, France in 2020 at the location known as Trezen ar Skoden (see Persson *et al.* 2019; Neves *et al.* 2021). Tardigrades and other meiofauna were extracted from the shell gravel using freshwater shocking, followed by sieving through a 30 μm mesh net, and finally, retransfer to filtered seawater from the locality. Living specimens were observed and photographed (using up to 40× objectives) with a DP27 camera mounted on an Olympus BX53 compound microscope. The specimens were subsequently used for DNA extractions. Specifically, DNA was extracted from sixteen *A. doryphorus* specimens using the Qiagen DNeasy Blood & Tissue kit.

Sequences of the nuclear ribosomal subunit 18S and 28S genes were amplified using the primer pairs: 1F (Giribet *et al.* 1996) and 23R (Blaxter *et al.* 1998), and 28Srda (Whiting *et al.* 1997) and 28Srd5b (Schwendinger & Giribet 2005)/28S rd7b1 (Whiting, 2002), respectively. The mitochondrial COI barcode gene fragment was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.* 1994)/HCOout (Schwendiger & Giribet 2005). PCRs were run with the DreamTaq™ DNA Polymerase (Thermo Fisher Scientific) under the following conditions: 95 °C for 3 min, 35 cycles of 95 °C for 1 min, 43 °C for 1 min and 72 °C for 1.3 min, followed by elongation at 72 °C for 7 min. The products were evaluated on 1% agarose gels, purified using NucleoSpin™ Gel and PCR Clean-up Kit (Macherey-Nagel) and subsequently sequenced at Eurofins Genomics. Ribosomal subunit and COI sequences were obtained from five and nine specimens, respectively (Fig. 1). Contigs were trimmed and assembled with ChromasPro 2.1.5 (Technelysium Pty Ltd) using default settings. Alignment of the sequences was carried out using Clustal Omega at EMBL-EBI (Madeira *et al.* 2019).

In addition to the new *A. doryphorus* sequences, we also provide new 18S sequences from two specimens of *Neoechiniscoides aski* (specimens R6 and R8, Møbjerg *et al.* 2020) and two specimens of *Isoechiniscoides sifae* (specimens R1 and R2, Møbjerg *et al.* 2016).

Phylogenetic Analyses. 18S and 28S sequences from arthrotardigrades, echiniscoideans and eutardigrades as well as outgroup taxa were downloaded from GenBank and analysed together with the newly obtained *A. doryphorus* and echiniscoidid sequences (Table S1, Supporting Information). Combined 18S and 28S datasets were created from taxa represented by both 18S and 28S sequences, as well as from selected arthrotardigrades only represented by a 28S sequence in which case the missing 18S sequence was substituted with N's.

Genetic pairwise distances were calculated using the *p*-distance model in MEGA7 version 7.0 (Kumar *et al.* 2016) under default settings (Table 1). All datasets were aligned with MUSCLE (Edgar 2004) in MEGA7, and low-quality ends were manually cut from the alignments prior to trimming under a gap threshold of 0.1 in trimAl v.1.3 (Capella-Gutiérrez *et al.* 2009). Substitution models were detected by ModelFinder (Kalyaanamoorthy *et al.* 2017), which was set to operate like JModelTest using the Bayesian Information Criterion (BIC) for the Bayesian inference (BI) and the Akaike Information Criterion (AIC) for the Maximum Likelihood (ML) analyses. The GTR+F+I+G4 model was inferred for both 18S and 28S datasets, according to AIC. According to BIC, TIM3e+I+G4 was the inferred model for the 18S dataset and TIM3+F+I+G4 for the 28S dataset. ML analyses were conducted in IQ-Tree (Nguyen *et al.* 2015) with nodal support evaluated by 1000 Bootstrap (BS) replicates. Combined 18S and 28S datasets ran with the same number of BS replicates in IQ-Tree using the partition model (Chernomor *et al.* 2016). BI analyses were performed in MrBayes version 3.2.7 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) with nodal support estimated by posterior probabilities (PP). Since the TIM3 substitution

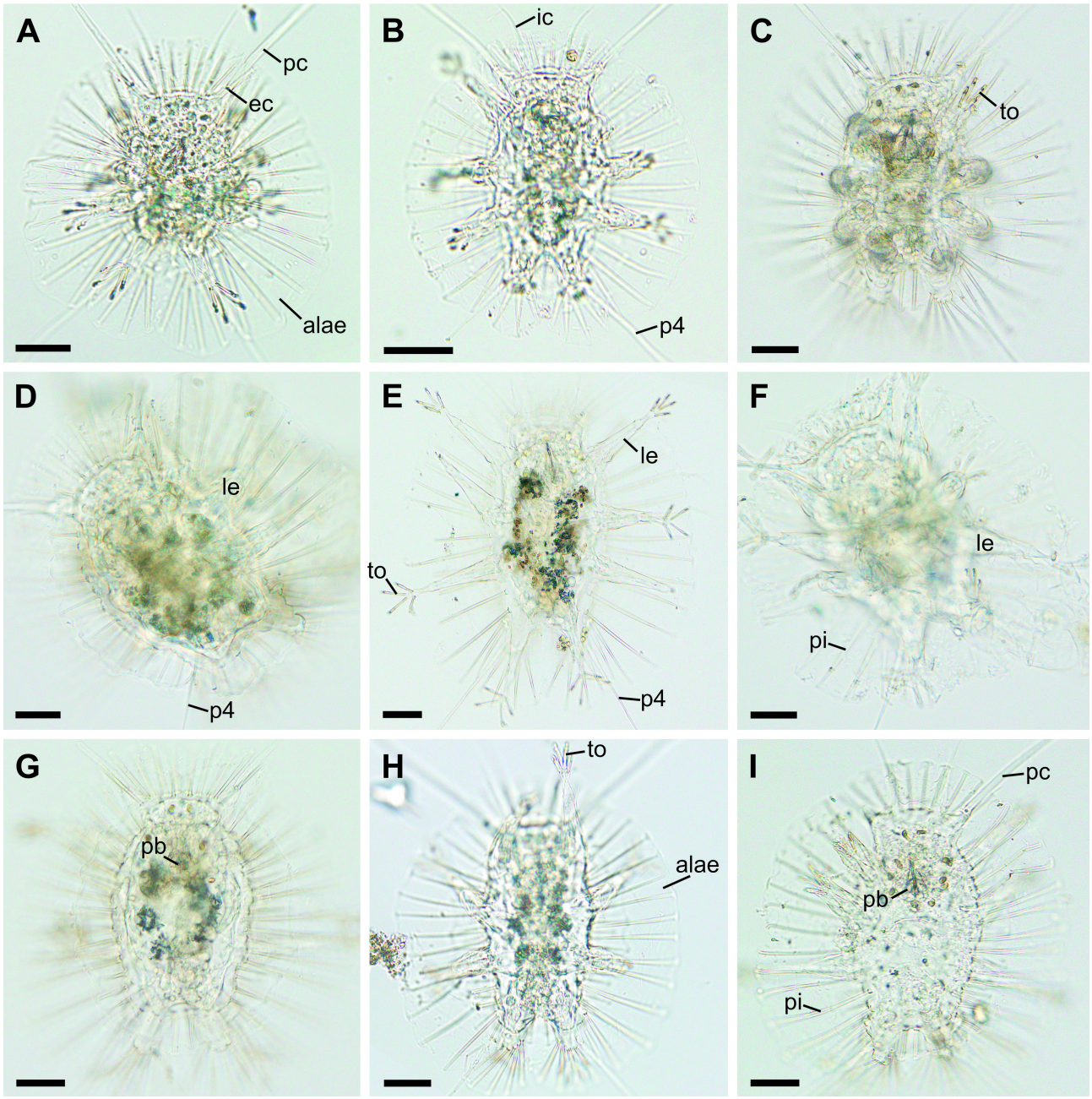

FIGURE 1. *Actinarctus doryphorus* specimens sampled at Roscoff, France, of which the cytochrome c oxidase subunit I (COI), 18S and 28S rDNA fragments were amplified. Light microscopic images of live animals. Characteristics of the species include *e.g.* telescopic legs (le) with four-terminal toes (to), the cuticular wing-like structure (alae), which contains epicuticular pillars (pi) and a set of head appendages, including external cirri (ec), internal cirri (ic) and primary clavae (pc) (see Persson *et al.* 2019 for more details). **A.** Specimen 06, presumably female. **B.** Specimen 07, presumably juvenile. **C.** Specimen 08, presumably male. **D.** Specimen 10, presumably juvenile. **E.** Specimen 12, female. **F.** Specimen 17, presumably juvenile. **G.** Specimen 18, presumably female. **H.** Specimen 22, presumably male. **I.** Specimen 23, presumably male. COI was obtained from all specimens, 18S from specimen no. 08, 10, 12, 17 and 18, and 28S from specimen no. 08 and 10. Specimens in A, C, E, F, H and I are seen ventrally, whereas B, D and G are seen dorsally. Anterior is up (and left in D, F). Pharyngeal bulb (pb), sensory organ of the fourth leg (p4). Scale bar = 20 µm in D, F otherwise 30 µm.

model is not supported in MrBayes, it was replaced by the GTR model as the closest over-parameterized model (Chapman *et al.* 2016; Huelsenbeck & Rannala 2004). Therefore, the model settings in MrBayes, according to BIC, were GTR+I+G4 for the 18S and GTR+F+I+G4 for the 28S datasets, respectively. From the default settings in

MrBayes, Diagnfreq was set to 1000 and the sample frequency to 100. The suitability of the default burn-in setting was verified by the ESS values and graphing of the Bayesian log-likelihoods of respective shorter analyses in Tracer version 1.7.2 (Rambaut *et al.* 2018). All analyses were performed with 4 million replicates. The phylogenetic tree visualization was performed in CorelDRAW. Nodes were considered significantly supported with BS values ≥95 and PP ≥0.95 (Fujimoto *et al.* 2017; Nguyen *et al.* 2015), moderately supported at BS 70–95 and PP 0.8–0.95, and poorly supported at BS <70 and PP <0.8.

TABLE 1. GenBank Accession numbers and average pairwise genetic distance (uncorrected *p*-distance) for the *Actinarctus doryphorus* COI sequences with haplotypes Ad1–5. The average pairwise distance across all sequences is 0.005. Specimens were sampled from Roscoff, France in 2020.

Specimen no.	Sequence length (bp)	Haplotype	Average *p*-distance	GenBank No.
06	642	Ad1		OQ411297
07	635	Ad1		OQ411298
17	633	Ad1	0.002	OQ411302
22	633	Ad1		OQ411303
23	634	Ad1		OQ418991
18	632	Ad2	0.006	OQ260192
12	660	Ad3	0.004	OQ411301
08	640	Ad4	0.006	OQ411299
10	648	Ad5	0.009	OQ411300

The average nucleotide composition and pairwise genetic distances across the COI data matrix were calculated in MEGA7. Intraspecific diversity was estimated as the number of haplotypes, the number of polymorphic sites, haplotype diversity, and nucleotide diversity using the software DnaSP v.6 (Rozas *et al.* 2017). The percentage identity between COI sequences from *Florarctus* sp. (GenBank Accession number MT999946), *Styraconyx takeshii* (LC488166), *Echiniscus testudo* (EU244601), *Echiniscoides sigismundi* (HM193403), the presumed COI-region of *Batillipes longispinosus* (KF938943), *Batillipes pennaki* (DQ099433) and *A. doryphorus* no. 12 were calculated in NCBI Multiple Sequence Alignment Viewer 1.22.1 after alignment with MUSCLE in MEGA7 following manually trimming of ends.

Results

We succeeded in amplifying 632–660 bp fragments of COI from nine *Actinarctus doryphorus* specimens, 689–1399 bp fragments of 18S from four specimens and 619–1398 bp fragments of 28S from two specimens. All sequences have been uploaded to GenBank (Tables 1 & 2). The average nucleotide composition of COI was: A (26.2%), C (22.9%), G (15.3%) and T (35.6%), showing an AT bias (AT: 61.8%, CG: 38.2%). The sequence variation was distributed with nine point mutations, all transition substitutions. With five recognized haplotypes of COI (Ad1–5), the estimated nucleotide diversity was 0.00435±0.00135 and the haplotype diversity was 0.722±0.159. The genetic *p*-distances between haplotypes ranged from 0.002 to 0.009 (Table 1). The sequence from specimen 10 (see Fig. 1) showed the highest genetic distance compared to the sequences from the other specimens.

The obtained *Actinarctus* COIs had an identity of ~ 68% with *E. testudo* and the other arthrotardigrade COIs, except for *S. takeshii* which had an identity of 57.4%. The identity between *Actinarctus* and *E. sigismundi* was slightly higher at 62.7%. As a comparison, the identity between the presumed COI regions of the two *Batillipes* sequences was 80.8% and 75.3% when compared to *E. testudo* and *Florarctus* sp., respectively, and 67.8% when compared to *E. testudo* and *E. sigismundi.*

Overall, the phylogenetic topologies inferred by the BI and ML analyses were congruent for each of the respective datasets. Our analyses consistently inferred Arthrotardigrada as paraphyletic, as the clade includes the monophyletic Echiniscoidea (Figs. 2–4). Pairwise distances for 28S revealed the highest range within the Arthrotardigrada (0.032–0.377) (Table 3). Based on the 18S sequences, the largest range of variation was calculated between Arthrotardigrada and Echiniscidae (0.000–0.259) (Table 3). According to all phylogenetic analyses, Echiniscoididae was inferred to be a sister-group to Echiniscidae. Most relationships within the monophyletic Echiniscoidea received weak support (Figs. 2–4).

TABLE 2. Sequence length and GenBank Accession numbers for the 18S and 28S rDNA sequences obtained from *Actinarctus doryphorus* specimens. The 18S rDNA sequence from specimen 08 and the 28S rDNA sequence from specimen 10 were included in the phylogenetic analyses. In addition, new 18S sequences from *Isoechiniscoides sifae* and *Neoechiniscoides aski* are listed together with previously published 28S sequences from selected specimens (see Møbjerg *et al.* 2016; 2022).

Specimen no.	18S length (bp)	GenBank No.	28S length (bp)	GenBank No.
Actinarctus doryphorus				
08	1420	OP901695	619	OP882620
10	1404	OP901696	1393	OP882621
12	1382	OP901697	–	–
17	1028	OP901698	–	–
18	689	OP901699	–	–
Isoechiniscoides sifae				
R1	1828	OP908041	1415	KX363636
R2	1828	OP908042	1415	KX363637
Neoechiniscoides aski				
R6	1813	OP908043	1417	KX363642
R8	1813	OP908048	1417	KX363643

TABLE 3. Pairwise genetic distances (uncorrected *p*-distances) among various heterotardigrade taxa and *Actinarctus*.

Taxon comparison	18S	28S
Arthrotardigrada – Echiniscidae	0.000–0.259	0.010–0.344
Arthrotardigrada – Echiniscoididae	0.048–0.2	0.188–0.200
Actinarctus – Arthrotardigrada	0.033–0.242	0.058–0.192
Actinarctus – Tanarctidae	–	0.058–0.069
Actinarctus – Halechiniscidae	0.033–0.139	0.148–0.209
Actinarctus – Stygarctidae	0.120	0.136–0.185
Actinarctus – Styraconyxidae	0.184	0.127–0.190
Actinarctus – Batillipedidae	0.195–0.242	0.175–0.182
Actinarctus – Coronarctidae	–	0.192
Within Arthrotardigrada	0.003–0.242	0.032–0.377

In the 28S tree (Fig. 2), the parachelan Hypsibioidea was inferred as polyphyletic as *Ramazzottius* sp. grouped with Macrobiotoidea (*Macrobiotus* + *Richtersius*) and Eohypsibioidea (*Bertolanius*). However, the current taxon sampling does not adequately cover eutardigrade sequence diversity and we, therefore, refrain from discussing eutardigrade relationships any further. The monophyly of Heterotardigrada was confirmed with *Coronarctus* sp. in a basal position. *Actinarctus doryphorus* was placed as a sister-group to *Tanarctus*, which was supported by the lowest range in genetic pairwise distances for 28S among all arthrotardigrade taxa (Fig. 2, Table 3). Within the maximum supported Tanarctidae (PP 1.0, BS 100), the relationship between *A. doryphorus* and *Tanarctus diplocerus* Fujimoto *et al.* 2013 was largely unsupported (PP 0.65, BS –). The arthrotardigrades seemed to group into five clades (besides the basally placed Coronarctidae, represented by *Coronarctus* sp.) mainly supported by the BI analysis. Specifically, Renaudarctidae (*Nodarctus* + *Renaudarctus*) and Stygarctidae (*Stygarctus* + *Parastygarctus*) were well supported and inferred as sister-group with moderate support (PP 1.00, BS 53). Tanarctidae (*Tanarctus* + *Actinarctus*) and the well-supported Styraconyxidae (*Raiarctus* + *Styraconyx* + *Cyaegharctus* + *Tetrakentron*) (PP 1.0, BI 98) were placed in a trichotomy with the remaining taxa. Since *Styraconyx takeshii* Fujimoto *et al.* 2020 and *Styraconyx* sp. formed sister relationships with *Raiarctus colurus* Renaud-Mornant, 1981 and *Tetrakentron synaptae* Cuénot, 1892, respectively, instead of being grouped, *Styraconyx* was inferred as polyphyletic within Styraconyxidae. With strong BI, but insignificant ML support (PP 0.98, BS 53), a clade consisting of Halechiniscidae (*Halechiniscus* + *Dipodarctus* + *Orzeliscus* + *Florarctus*) + Archechiniscidae (only presented by one 28S rDNA from *Archechiniscus* sp.) was inferred as sister-

group to Echiniscoidea and thus gives rise to a paraphyletic Arthrotardigrada. As *Halechiniscus perfectus* Schulz, 1955 and *Halechiniscus churakaagii* Fujimoto, 2015 were more closely related to *Archechiniscus* sp., Halechiniscidae was also inferred to be paraphyletic. However, this relationship was poorly supported (PP 0.62, BS –) and the sequence diversity of Archechiniscidae was not adequately covered (Fig. 2).

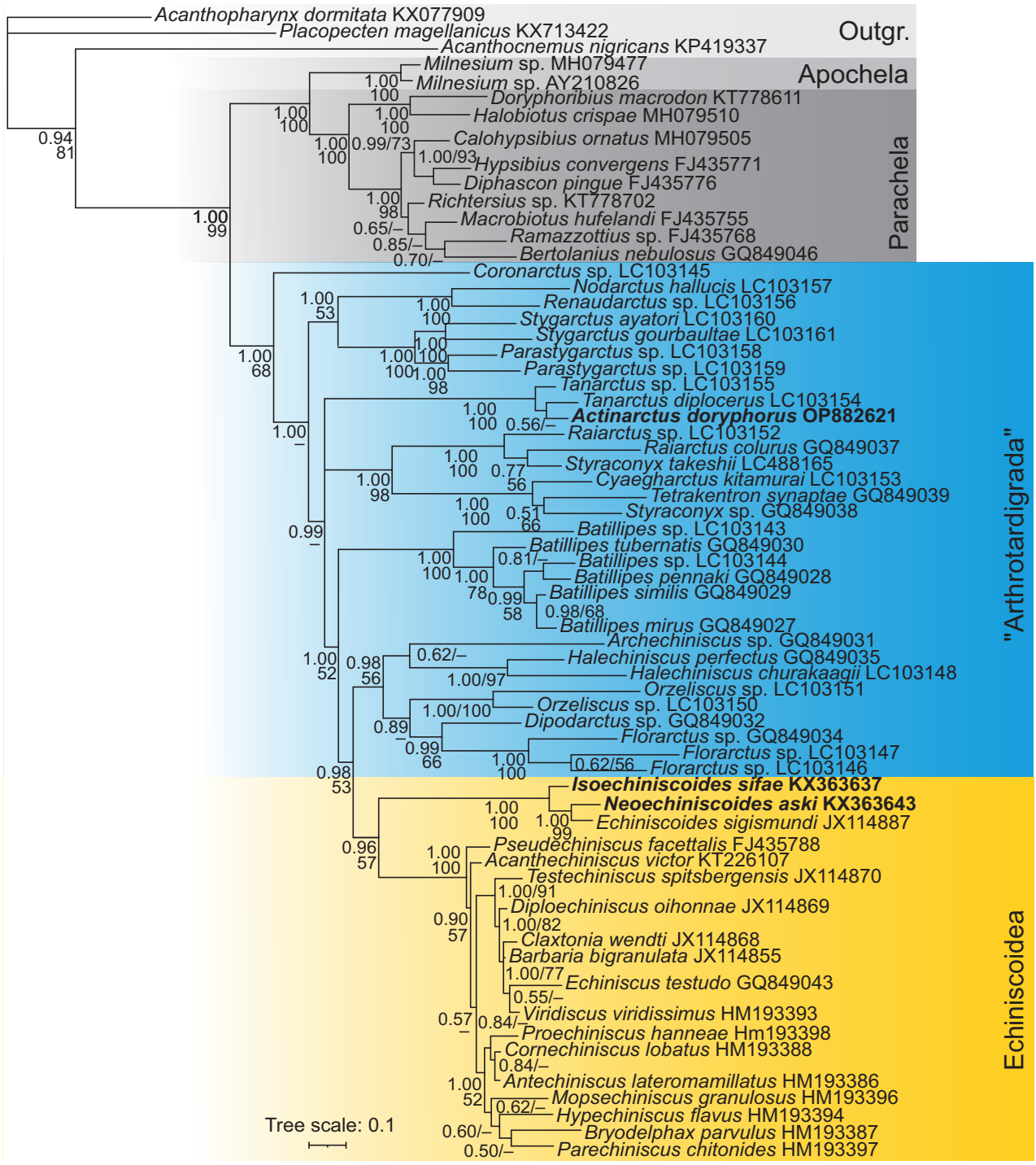

FIGURE 2. Phylogenetic tree inferred from BI analyses of the 28S rDNA dataset. *Actinarctus doryphorus* is placed together with *Tanarctus* sequences within a maximum supported Tanarctidae. Arthrotardigrada is inferred to be paraphyletic. Posterior probabilities (upper value) relating to BI analyses and bootstrap values (lower) from ML analyses are shown at the nodes. (–) indicates bootstrap values <50.

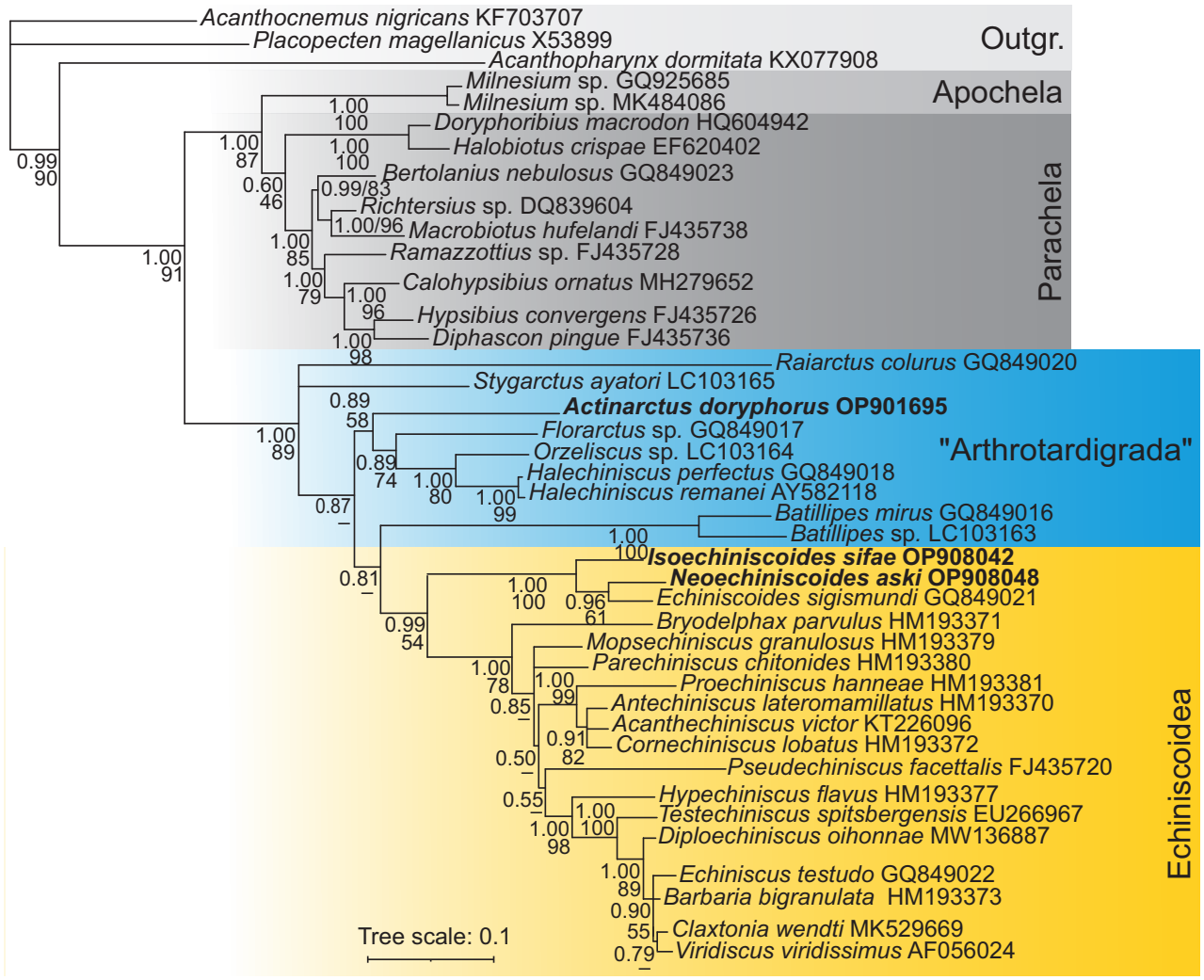

FIGURE 3. Phylogenetic tree inferred from BI analyses of the 18S rDNA dataset. Arthrotardigrada is inferred to be paraphyletic. Posterior probabilities (upper value) relating to BI analyses and bootstrap values (lower) from ML analyses are shown at the nodes. (–) indicates bootstrap values <50.

Analyses of both 18S and 28S datasets strongly supported the monophyly of Batillipedidae, but interspecific relationships revealed moderate to low support within the 28S tree (Fig. 2). In the 18S tree, Batillipedidae formed a sister-group to Echiniscoidea and thus inferred Arthrotardigrada as paraphyletic (Fig. 3). Moreover, as no other tanarctid 18S sequences were available, *A. doryphorus* was inferred as a sister-group to the halechiniscid clade with moderate to low support (PP 0.89, BS 58). The low pairwise distance between 18S *Actinarctus* and Halechiniscidae supported this position (Table 3). With no available coronarctid 18S sequence, *R. colurus* and *Stygarctus ayatori* Fujimoto, 2014 were inferred at a basal position within Heterotardigrada and formed an unresolved trichotomy with the rest of the clade. As compared to other species, the branch of *R. colurus* was remarkably long (Fig. 3). Interestingly, the alignment of the 18S sequences revealed a 74 bp long unique region in *A. doryphorus* not present in other tardigrade sequences. Specifically, this insert seems to present a secondary structure loop.

The analysis of the combined 18S/28S dataset (Fig. 4) revealed many polytomies and thus failed to resolve the relationships for many Heterotardigrada taxa. Also in this dataset, Arthrotardigrada was inferred to be paraphyletic as a clade formed by Coronarctidae + Batillipedidae + Tanarctidae + Halechiniscidae + Archechiniscidae formed the sister-group to Echiniscoidea (Fig. 4).

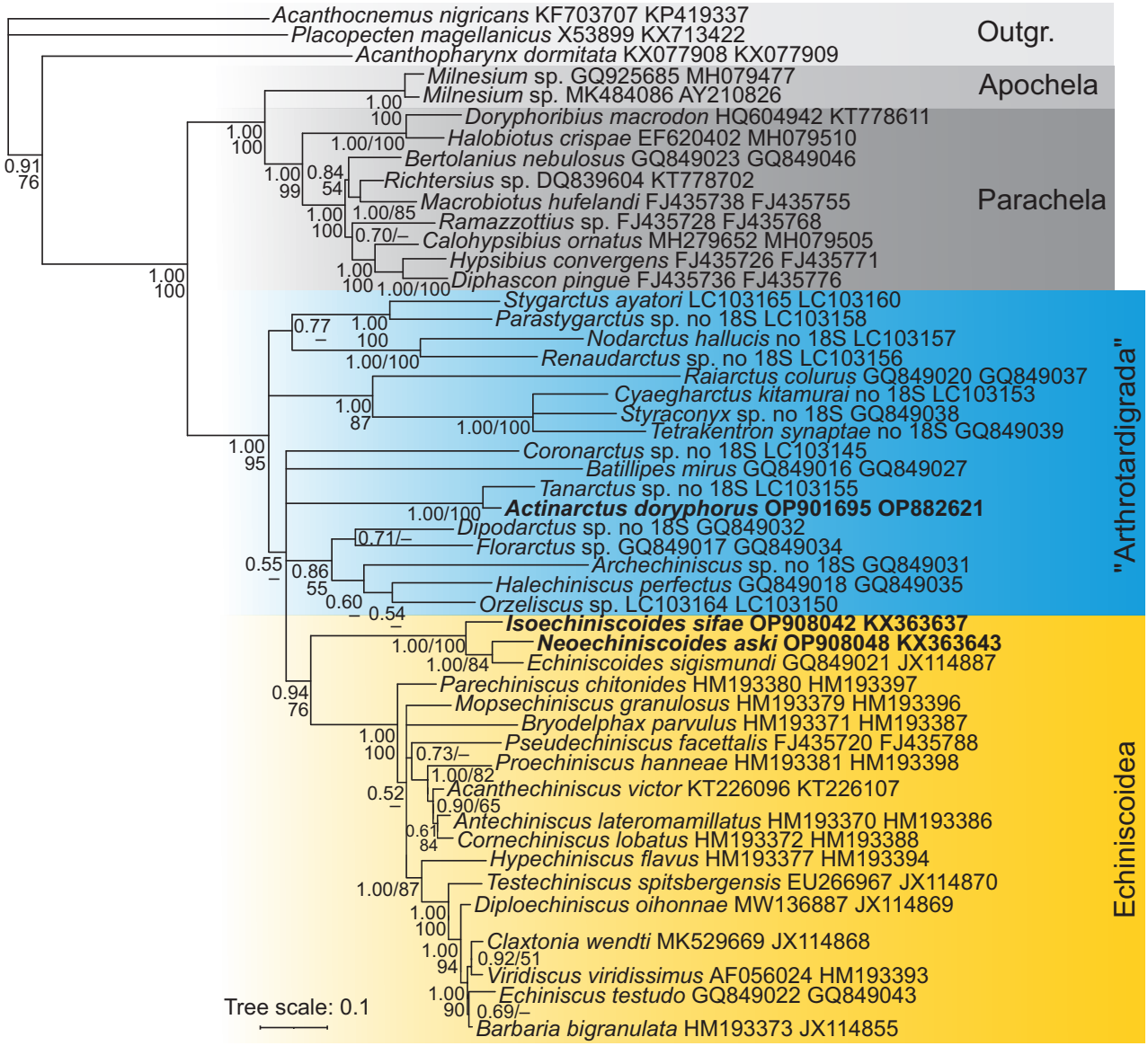

FIGURE 4. Phylogenetic tree inferred from BI analyses of the combined 18S/28S rDNA dataset. Unavailable 18S sequences were substituted with N's (indicated by "no 18S"). *Actinarctus doryphorus* is placed together with *Tanarctus* and Arthrotardigrada is inferred to be paraphyletic. Posterior probabilities (upper value) relating to BI analyses and bootstrap values (lower) from ML analyses are shown at the nodes. (–) indicates bootstrap values <50.

Discussion

This study provides the first investigation into COI haplotype diversity in a marine tanarctid, *i.e.*, *Actinarctus doryphorus,* and at the same time readdresses arthrotardigrade phylogeny using new 18S and 28S sequence data. Specifically, we succeeded in obtaining COI, 18S and 28S rDNA sequences from nine, five and two *A. doryphorus* specimens, respectively.

The average haplotype diversity of COI within *A. doryphorus* is similar to the haplotype diversity of COI within *E. testudo* from European, Asian and Northern African populations (Jørgensen *et al.* 2007). With a sample size of only nine sequences, we refrain from comparing the genetic distances with calculations for *Echiniscoides* (see Møbjerg *et al.* 2016) or *Echiniscus* (see Jørgensen *et al.* 2013), as these studies used a sample size of 242 and 185 sequences, respectively. COI is a highly variable DNA marker (Guil & Giribet 2009) and as such used in barcoding studies. The genetic distance of 0.002–0.009 between the obtained *A. doryphorus* COI fragments demonstrated a high similarity and thus confirms the presence of a single species at Roscoff. To further improve our understanding

of marine tardigrade diversity, investigate phylogeographic structures, and detect isolation and speciation, more COI sequences from marine specimens at different localities are needed. Additionally, more 18S sequences from other *Actinarctus* species are needed in order to confirm whether the observed 74 bp insert within the 18S *A. doryphorus* sequence is unique for this genus or species.

Echiniscoididae was inferred as monophyletic and placed as a sister-group to Echiniscidae forming a monophyletic Echiniscoidea in all our analyses. However, our phylogenetic analyses reconfirmed the paraphyly of Arthrotardigrada, as the clade includes the monophyletic Echiniscoidea. It is thus clear that Arthrotardigrada are not monophyletic. The latter is emphasized by the apparent lack of defining apomorphic characters, with *e.g.* the median cirrus clearly representing a plesiomorphic character as its rudiments are found even among eutardigrades (Persson *et al.* 2012; Gross *et al.* 2021). Accordingly, we propose to suppress the order Arthrotardigrada as it clearly does not reflect tardigrade phylogeny. We suggest retaining the monophyletic Echiniscoidea as a superfamily, and further suggest that future discussions on the phylogeny of marine heterotardigrades focus on establishing sister-group relationships between the currently recognised families within the former Arthrotardigrada (Degma & Guidetti 2023).

Notably, our analyses provide the first molecular support for the position of *A. doryphorus* within Tanarctidae. Specifically, the tree inference of the 28S dataset grouped *Actinarctus* + *Tanarctus* separately from Halechiniscidae. The close relationship between *Actinarctus* and *Tanarctus* is additionally supported by the lowest calculated pairwise genetic distance among all arthrotardigrade taxa. Both findings support the elevation of Tanarctidae by Fujimoto *et al.* (2017). Tanarctidae and Styraconyxidae were well-supported distinct clades. However, the relationship between these clades and the remaining taxa was presented as a trichotomy. An unsupported bifurcation of Tanarctidae and Styraconyxidae was also inferred in the Fujimoto *et al.* (2017) phylogenetic tree based on the combined 18S and 28S rDNA dataset. The current phylogenetic inferences again raise the question of the position of *Archechiniscus* as it is inferred to be the sister-group to *Halechiniscus* or *Halechiniscus* + *Orzeliscus*. However, these relationships have almost no branch support. Jørgensen *et al.* (2010) suggested that *Archechiniscus* should retain its family status, as it had been included in Halechiniscidae based on arguments that it showed "obvious affinities" with *Styraconyx*, which at the time was regarded as a Halechiniscidae member and not as belonging to its own family (Styraconyxidae), which is the current consensus. We suggest keeping Archechiniscidae until further evidence has been gathered and emphasise that it is apparent that a close relationship between *Archechiniscus* and Halechiniscidae exists. This is also evident from the many inferences of a sister-group relationship between *Archechiniscus* and *Halechiniscus* (Jørgensen *et al.* 2010; Fujimoto *et al.* 2017). Besides the Tanarctidae, Styraconyxidae, Halechiniscidae and Archechiniscidae, the remaining arthrotardigrade taxa grouped into the families Coronarctidae, Renaudarctidae, Stygarctidae and Batillipedidae.

We note that many nodes were only well supported by the BI, and not the ML, analyses. The latter may be related to the alignment of sequences amplified with different primers covering different regions of the 18S or 28S rRNA genes, thereby weakening the alignment due to only partial sequence overlaps. Primers, which are more appropriate for these genes in the different species, should be designed to retrieve more sequences and optimize alignments. As a template region of DNA, the *A. doryphorus* sequences of this study could be used. Moreover, inadequate taxon sampling or too large phylogenetic distances between outgroup and ingroup taxa may lead to insignificant support values (De la Torre-Bárcena *et al.* 2009). Notably, the inferred trees of each dataset were identical and in agreement with the findings of Jørgensen *et al.* (2010), Bertolani *et al.* (2014) and Fujimoto *et al.* (2017).

The highest genetic distance for the 28S sequence was calculated within the clades forming the paraphyletic Arthrotardigrada, suggesting a high substitution rate (Jørgensen *et al.* 2010) and/or an old lineage age (Cádiz *et al.* 2018). An old lineage age of these marine heterotardigrades supports the assumption that tardigrades originated from the sea (Kristensen, 1981; Renaud-Mornant, 1982; Jørgensen *et al.* 2010). The marine origin is further supported by the basal position of the marine Echiniscoididae within Echiniscoidea, with the remaining echiniscoidean taxa being limno-terrestrial.

Conclusion

The obtained *A. doryphorus* COI gene fragments provide molecular confirmation that the specimens examined in this study are the same species. The tanarctids, *Actinarctus* and *Tanarctus* were grouped in a distinct clade from Halechiniscidae supporting that the tanarctids constitute their own family, Tanarctidae. Our analyses consistently

infer Arthrotardigrada as paraphyletic and we accordingly propose to suppress the order, as it clearly does not reflect tardigrade phylogeny. The high genetic distances between marine heterotardigrade species suggest high heterogeneity and an old lineage age. More molecular data are needed to increase the understanding of the phylogenetic relationships among the marine heterotardigrades. Furthermore, morphological characters could advantageously be included in future investigations, in combination with the DNA sequences, to increase our understanding of heterotardigrade phylogeny.

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