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Phylogenetic Position of the Queer, Backward-bent Entoproct *Loxosoma axisadversum* (Entoprocta: Solitaria: Loxosomatidae)

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The solitary entoproct *Loxosoma axisadversum* Konno, 1972 was found on the body surface of the maldanid polychaete *Nicomache personata* Johnson, 1901 obtained among roots of the seagrass *Phyllospadix iwatensis* Makino collected at a depth of about 1 m in Oshoro Bay, Hokkaido, Japan. The species had previously been known from elsewhere in northern Japan, attached to another maldanid, *Nicomache minor* Arwidsson, 1907. The morphology of the new material is briefly described with photographs taken in life. A preliminary phylogenetic analysis using concatenated partial sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, as well as the nuclear 18S and 28S rRNA genes, placed *L. axisadversum* as the sister taxon to all the rest of Solitaria included in the analysis, indicating non-monophyly of the genus *Loxosoma* Keferstein, 1862.

Key Words: Sea of Japan, marine invertebrates, COI, ribosomal RNA, Kamptozoa, ectosymbiont.

Introduction

The solitary entoproct *Loxosoma axisadversum* Konno, 1972 was originally described based on specimens found attached to the body of the maldanid polychaete *Nicomache minor* Arwidsson, 1907 collected in Fukaura, Aomori Prefecture, Japan (Konno 1972). The species was subsequently reported from Aomori and Hokkaido Prefectures at Akkeshi, Asamushi, Shiretoko, and Shiriyazaki (Fig. 1), on the same species of host polychaete (Konno 1978, 1985). *Loxosoma axisadversum* is morphologically unique in the phylum in that the calyx is recurved so that the mouth is located above the anus within the tentacle crown, contrary to the normal arrangement in other solitary entoprocts, in which the anus is situated above the mouth. Among the 25 species (Nielsen 2010) of *Loxosoma* Keferstein, 1862 as defined by Nielsen (1996), *L. axisadversum* is the smallest in body size, with a maximum body length of just 220 µm, whereas larger species such as *L. pectinaricola* Franzén, 1962 can reach 4 mm (Nielsen 1964). In addition, within the genus, *L. axisadversum* has the lowest number of tentacles, *viz.*, only six, whereas the highest number is found in *L. nung* Nielsen, 1996, which has 34.

Fuchs *et al.* (2010) provided the most comprehensive phylogenetic study of the phylum Entoprocta, although only one species of *Loxosoma* was included, *viz.*, *L. pectinaricola*. Thus, monophyly of this genus was not assessed, but it was shown that *Loxosomella* Mortensen, 1911 is paraphyletic with regard to *Loxocorone* Iseto, 2002, *Loxomitra* Nielsen, 1964, and *Loxosoma*. Our recent new find of *L. axisadversum* prompted us to use molecular methods to investigate the species' phylogenetic position among loxosomatids, with a particular interest in whether it groups with *L. pectinaricola*, thereby supporting the monophyly of *Loxosoma* as currently diagnosed.

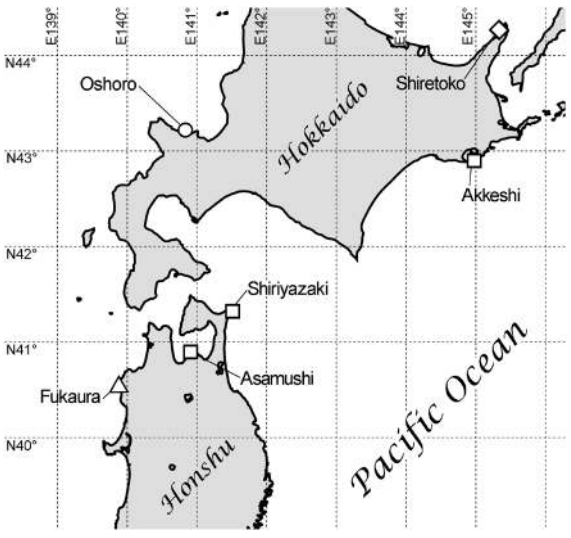


Fig. 1. Map of northern Japan, showing known localities of *Loxosoma axisadversum* Konno, 1972. Sources: triangle, Konno (1972); squares, Konno (1978); diamond, Konno (1985); circle, present study.

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Materials and Methods

The entoprocts were found together with unidentified peritrich ciliates on the body surface of one of two (three, if counting a fragment) specimens of the maldanid polychaete *Nicomache personata* Johnson, 1901 (Fig. 2A, B) collected on 16 August 2014 among roots of the seagrass *Phyllospadix iwatensis* Makino at about 1 m in depth on a rocky shore in Oshoro Bay, Hokkaido, Japan (43°21′50″N, 140°85′54″E; Fig. 1). The entoprocts were photographed alive on the polychaete body surface with a Nikon D5200 digital camera attached to a Nikon SMZ 1500 stereomicroscope by Micronet NYPIXS2-3166, HY1S-FA, and NY1S-1501750 adapters, with external strobe lighting provided by a pair of Morris Hikaru Komachi Di flash units. Ten specimens of the entoprocts were detached from the polychaete under the stereomicroscope, one of which was observed under an Olympus BX51 compound microscope and also photographed with the same digital camera. Five of the detached specimens and a partial tissue sub-sample of the host polychaete were preserved in 99% EtOH for DNA extraction; the rest of the detached entoprocts were fixed in 10% formalin–seawater and preserved in 90% EtOH. The remaining polychaete body with the ectosymbionts (fixed and preserved in 10% formalin–seawater), as well as the other five detached specimens of entoprocts in a glass vial, have been deposited as vouchers in the Hokkaido University Museum, Sapporo, Japan, under the catalogue numbers ZIHU 4935 and 4936, respectively. The other, entoproct-free specimen of *N. personata* (ZIHU 4947) was processed as follows: chaetiger 11 was cut off, fixed in 99% EtOH, and used for DNA extraction; the rest of the body fragments were fixed in 10% formalin–seawater and transferred into 70% EtOH; two fragments of the latter, chaetigers 2–5 and 14–15, were further cut off and mounted on glass slides in Hoyer's medium.

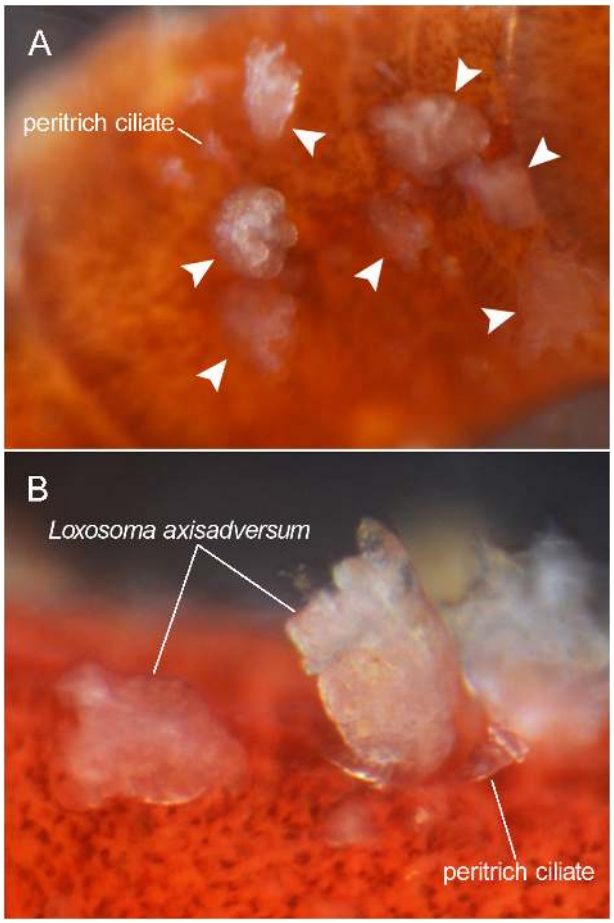


Fig. 2. Photographs of living entoprocts, *Loxosoma axisadversum* Konno, 1972, *in situ* on their host. A, Dorsal surface of the maldanid polychaete *Nicomache personata* Johnson, 1901 (ZIHU 4935) with several entoprocts (indicated by arrowheads) and unidentified peritrich ciliates attached; B, more highly magnified image of *L. axisadversum* (and peritrich) viewed laterally.

Total genomic DNA was extracted from the five entoprocts and two polychaetes using DNeasy Blood & Tissue Kit (Qiagen, USA) following the manufacturer's protocol. PCR amplification was carried out in an iCycler thermal cycler (Bio-Rad Laboratories, USA) using Ex *Taq*® (TaKaRa BIO, Japan), with the primer pairs LCO1490/HCO2198 (Folmer *et al.* 1994) for the mitochondrial cytochrome *c* oxidase subunit I gene (hereafter COI), 18S 1F/9R (Giribet *et al.* 1996) for the 18S rRNA gene (18S), LSU5 (Littlewood 1994)/28S rd4b (Edgecombe and Giribet 2006) for the 28S rRNA gene (28S), and aF/aR (Colgan *et al.* 1998) for histone H3. COI, 18S, and 28S were amplified from *L. axisadversum*, and COI, 18S, and H3 from *N. personata*. Thermal cycling conditions were 1 min at 95°C, 35 cycles of [95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec (for COI and 28S) or 1 min (18S)], followed by 72°C for 7 min. The reaction products were visualized in a 1% agarose gel and purified through enzymatic reaction with exonuclease I and shrimp alkaline phosphatase (TaKaRa BIO, Japan). Sequencing reactions were performed with BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, USA) using the same primer pairs as for amplification. For 18S, the following internal primers were also used: 3F, 4R (Giribet *et al.* 1996); and bi, 2.0 (Whiting *et al.* 1997). Sequencing was done by an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, USA). Base calling was carried out using ATGC ver. 4.0.6 (Genetyx, USA). The resulting sequences have been deposited in DDBJ (accession numbers: LC005490–LC005496 and LC006047–LC006053, Table 1).

A maximum-likelihood (ML) analysis was carried out to assess the phylogenetic position of *L. axisadversum*. Twelve species in Solitaria and three outgroup species in Colonialles were included (Table 2). Sequences were aligned gene by gene by MUSCLE (Edgar 2004a, b) implemented in MEGA ver. 5.2 (Tamura *et al.* 2011), then concatenated with Kakusan4 ver. 4.0 (Tanabe 2011); no significant nucleotide compositional heterogeneity was detected for the combined data set ($p=0.98060$ by chi-square test in Kakusan4). Excluding gap positions, the concatenated matrix comprised 1,371 nt: 488 nt (COI), 581 nt (18S), 302 nt (28S). Optimal substitution parameters in the general time-reversible model (Tavaré 1986) for each partition in the concatenated dataset were estimated also by Kakusan4. The ML analysis was conducted in RAxML ver. 8.0.0 (Stamatakis 2014), with inter-

Table 1. DDBJ/EMBL/GenBank accession numbers for the entoproct *Loxosoma axisadversum* Konno, 1972 and the host polychaete *Nicomache personata* Johnson, 1901 determined in the present study.

Species	Specimen number	Markers			
		COI	18S	28S	H3
Loxosoma axisadversum Konno, 1972 (Entoprocta: Solitaria: Loxosomatidae)	1	LC005494	—	LC005490	—
	2	LC006047	—	—	—
	3	LC006048	LC005492	—	—
	4	LC005495	LC005493	LC005491	—
	5	LC006049	—	—	—
Nicomache personata Johnson, 1901 (Annelida: Maldanidae)	1	LC006052	LC006051	—	LC005496
	2	LC006053	—	—	LC006050

Table 2. List of species included in the phylogenetic analysis, with DDBJ/EMBL/GenBank accession numbers.

Taxa	Markers			Sources
	COI	18S	28S	
Solitaria (ingroup)				
Loxomitra mizugamaensis Iseto, 2002	GU125769	GU125754	GU125739	Fuchs *et al.* (2010)
Loxomitra tetraorganon Iseto, 2002	GU125770	GU125755	GU125740	Fuchs *et al.* (2010)
Loxosoma axisadversum Konno, 1972	LC005495	LC005493	LC005491	Present study
Loxosoma pectinaricola Franzén, 1962	GU125763	GU125733	GU125748	Fuchs *et al.* (2010)
Loxosomella harmeri (Schultz, 1895)	GU125764	GU125749	GU125734	Fuchs *et al.* (2010)
Loxosomella parguerensis Rützler, 1968	GU125761	GU125746	GU125731	Fuchs *et al.* (2010)
Loxosomella plakorticola Iseto and Sugiyama, 2008	GU125767	GU125752	GU125737	Fuchs *et al.* (2010)
Loxosomella stomatophora Iseto, 2003	GU125768	GU125753	GU125738	Fuchs *et al.* (2010)
Loxosomella varians Nielsen, 1964	GU125762	GU125747	GU125732	Fuchs *et al.* (2010)
Loxosomella vivipara Nielsen, 1966	GU125760	GU125745	GU125730	Fuchs *et al.* (2010)
Loxosomella sp. 1	GU125765	GU125750	GU125735	Fuchs *et al.* (2010)
Loxosomella sp. 2	GU125766	GU125751	GU125736	Fuchs *et al.* (2010)
Coloniales (outgroup)				
Barentsia discreta (Busk, 1886)	GU125772	GU125757	GU125742	Fuchs *et al.* (2010)
Barentsia gracilis Sars, 1835	FJ196079	FJ196109	FJ196138	Fuchs *et al.* (2009)
Loxosomatoides sirindhornae Wood, 2005	GU125771	GU125756	GU125741	Fuchs *et al.* (2010)

face data operation assisted by Phylogears2 ver. 2.0 (Tanabe 2008). Nodal support values were obtained by ML analyses of 1,000 bootstrap pseudoreplicates (Felsenstein 1985).

Results and Discussion

Loxosoma axisadversum Konno, 1972
[Japanese name: Ushiromae-rokusosoma]
(Figs 2, 3)

Loxosoma axisadversum Konno, 1972: 23–24, figs 1–8; Konno 1978: 22–23, figs 9–11; Konno 1985: 5.

Material examined. Ten or more specimens alive, of which one was observed under compound microscope: ZIHU 4935 (five specimens fixed in 10% formalin–seawater, preserved in 90% EtOH), ZIHU 4936 (a few individuals attached to the host polychaete, fixed and preserved in 10% formalin–seawater), and five specimens destroyed for DNA extraction.

Description of one specimen. Destroyed for DNA extraction after observation. Body translucent (Fig. 2A, B). Total body length 150 μm. Tentacle crown 140 μm in diameter, with three pairs of very short tentacles (Fig. 3A); those of two larger, upper pairs each with small papilla on distal, abfrontal side near tip (Fig. 3A). Mouth situated above anus within tentacle crown (Fig. 3B). Calyx oval in shape, 90 μm in length and 100 μm in diameter. Peduncle stout, 30 μm long and 25 μm wide (Fig. 3B, C). Pedal disc rounded, 60 μm in length and 40 μm in width.

Distribution on host. Found most densely on dorsal side slightly anterior to middle portion of polychaete's body, with up to about eight individuals on chaetiger 5 (or 6), other individuals mostly found back to chaetiger 10.

Host species. *Nicomache minor* (Konno 1972, 1978, 1985); *N. personata* (this study).

Remarks. The character of the present material agrees well with the morphological account given in the original description by Konno (1972). The body size of the present material falls within the reported range of the species in the original description (Konno 1972). The close geographical proximity of the present sampling site (Oshoro) to the type locality (Fukaura) (*ca.* 300 km; Fig. 1), both under the influence of the Tsushima Current, further justifies our identifi-

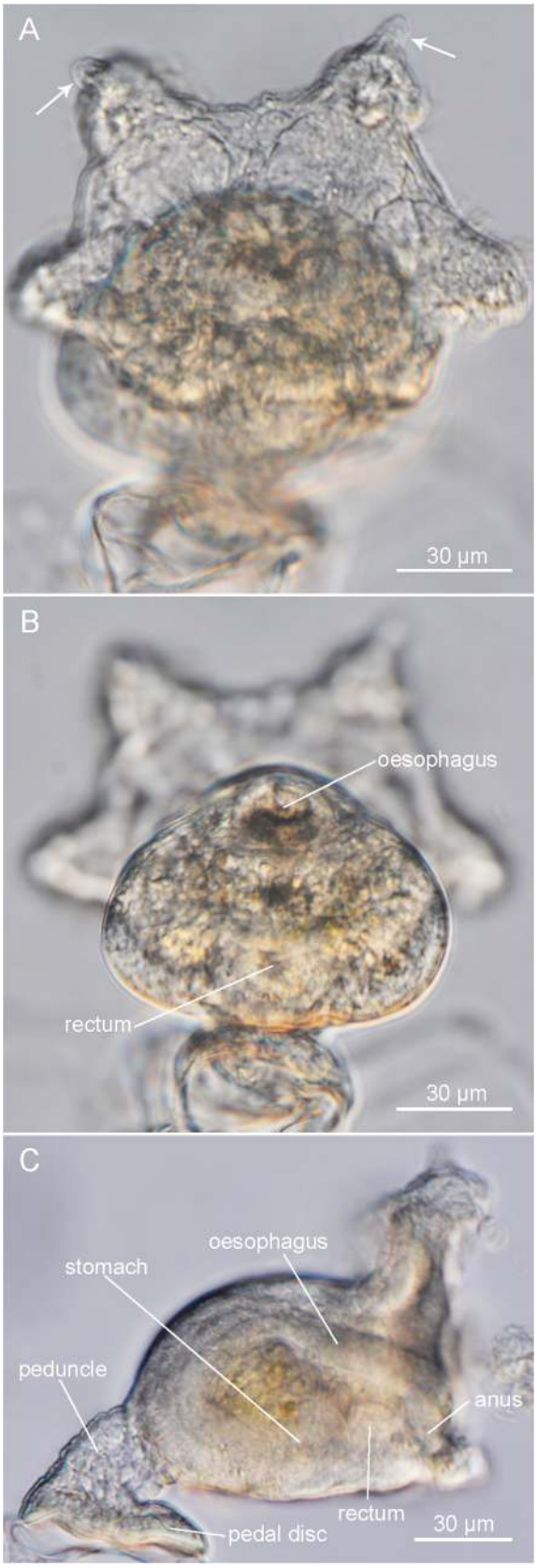


Fig. 3. Photomicrographs of live, slide-mounted *Loxosoma axisadversum* Konno, 1972. A, Frontal view, showing papillae on large tentacles (arrowed); B, same view as A at different focal depth, showing "adverse" position of alimentary canal openings; C, lateral view, showing shape of peduncle and pedal disc.

cation of the material.

Loxosoma axisadversum has previously been known only in northern Japan from one host species, *Nicomache minor* (Annelida: Maldanidae). The latter has been reported to be widely distributed in cold waters in the Northern Hemisphere: Norwegian Sea (Arwidsson 1907), Barents Sea (Arwidsson 1907; Kędra *et al.* 2010), White Sea (Tzetlin and Markelova 1985; Tzetlin *et al.* 1997; Solyanko *et al.* 2011), Sea of Japan (Annenkova 1937; Ushakov 1955; Imajima and Shiraki 1982; Yakovlevich 2013), Sea of Okhotsk (Ushakov 1955; Imajima and Shiraki 1982), Pacific coasts of northern Japan (Imajima and Shiraki 1982), Bering Sea (Ushakov 1955), and Hudson Bay (Berkeley and Berkeley 1943). In the White Sea, Tzetlin and Markelova (1985) found 15 species of symbionts in the tubes of *N. minor*, of which only an unidentified species of "*Loxosomella*" appeared to possess a species-specific relation with the polychaetes. Unfortunately, Tzetlin and Markelova (1985) did not provide any further information, so the identity of their material remains unknown. More recently, however, Borisanova and Krylova (2014) summarized 10 species of *Loxosomella* reported from the White Sea, of which *L. polita* Nielsen, 1964 and *L. similis* Nielsen, 1964 are associated with *N. minor* (Borisanova *in litt.*), but no species of *Loxosoma* has hitherto been reported from this sea area.

We identified the present host maldanid as *N. personata* because it possessed 22 chaetigers and the anal funnel was equipped with 16 papillae (Johnson 1901; Imajima and Shiraki 1982; Imajima 1996; De Assis *et al.* 2007); ours is the first record of *L. axisadversum* from this host species. However, the two maldanids *N. minor* and *N. personata* are quite similar to each other in shape, and the alleged morphological differences between the two (*i.e.*, number of chaetigers: 22 in *N. personata vs* 23 in *N. minor*; Khlebovich 1961; Tarakanova 1974; Imajima and Shiraki 1982) may simply represent intraspecific variation; indeed, they were once regarded as conspecific (Pettibone 1954). A BLAST search (Altschul *et al.* 1997) at the NCBI website (http://blast.ncbi.nlm.nih.gov) with the 331 nt of the histone H3 sequence of what we identified as *N. personata* from Oshoro (DDBJ accession number: LC005496) showed that it was 97% identical (with 93% query coverage) to KF926672, a sequence derived from a polychaete identified as *N. minor* collected in the White Sea (T. Neretina *et al.*, unpublished). The two sequences differ by 1.8% in terms of uncorrected *p*-distance, indicating that the two populations—more than 6,000 km away from each other—are at least mutually closely related, if not the same species. In other annelids, maximum intraspecific difference at the same locality with regard to histone H3 sequence was 1.1% in the opheliid *Ophelina cryptophila* Neave and Glasby, 2013 from Darwin Harbour (between JN182668 and JN182673; Neave and Glasby 2013), while the minimum interspecific difference between *O. cryptophila* and *O. tessellata* Neave and Glasby, 2013 was 3.3% (*e.g.*, between JN182680 and JN182667; Neave and Glasby 2013). *Nicomache minor* and *N. personata* together show a circumpolar distribution but co-occur in Japanese waters (Imajima and Shiraki 1982; De Assis *et al.* 2007), suggesting the possibil-

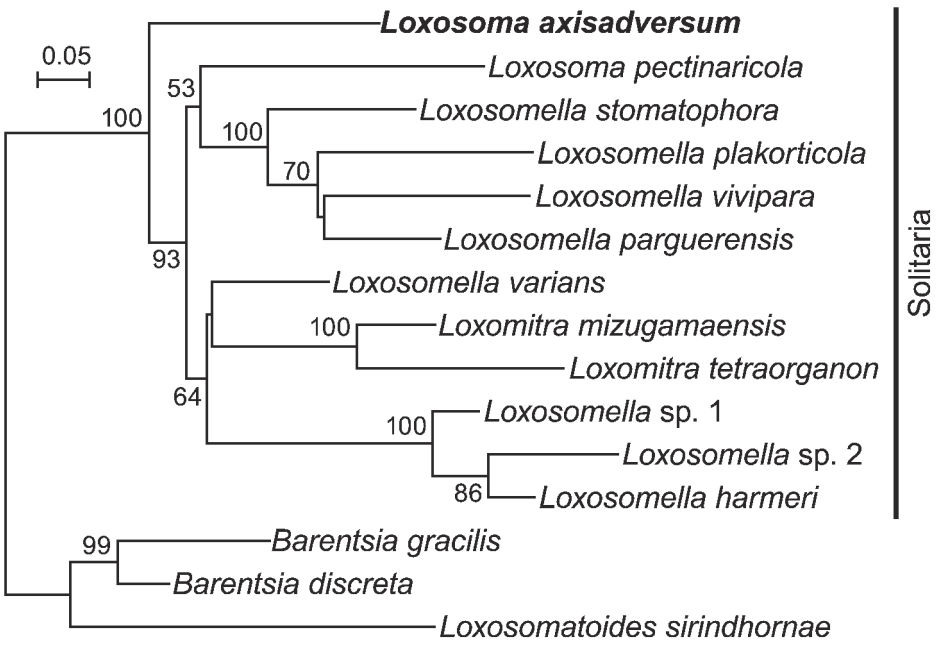


Fig. 4. Maximum likelihood tree based on combined partial COI, 18S, and 28S rRNA gene sequences. Numbers near nodes indicate bootstrap support values ≥50%.

ity of a ring-species complex (Alcaide *et al.* 2014), although resolving this point is beyond the scope of this paper.

Phylogeny. Of the five specimens from which we extracted DNA, PCR amplification of all the three gene markers was successful in only one (specimen number 4, Table 1). The sequences from this specimen were included in the analysis. The other determined sequences from different specimens were identical with respect to each gene marker, except for a single, synonymous substitution in COI. In the resulting ML tree based on the concatenated COI+18S+28S dataset (Fig. 4), *L. axisadversum* appears as sister to all the rest of the solitarians included in the analysis. The same topology was observed in ML trees based only on 18S or 28S, while the COI tree was not well resolved (data not shown). The diagnosis and circumscription of the genus *Loxosoma* based on foot morphology (Mortensen 1911; Nielsen 1996) thus do not appear to reflect the evolutionary relationships among solitary entoprocts.

That *L. axisadversum* and *L. pectinaricola* turned out not to be sister taxa in our analysis may reflect the difference in mode of attachment of their buds to the parent. Based on this feature, Nielsen (1964) proposed a subgeneric discrimination within *Loxosoma*, placing species with buds attached to their parents by the abfrontal margin of the sucking disc in the subgenus *Loxomorpha* Nielsen, 1964 (preoccupied and renamed as *Loxosomina* Nielsen, 1996), while species with buds attached by the central area of the sucking disc were classified into the nominotypical subgenus *Loxosoma*. *Loxosoma axisadversum* and *L. pectinaricola* exhibit bud-attachment modes that are typical of *Loxosoma* (*Loxosomina*) and *Loxosoma* (*Loxosoma*), respectively (Nielsen 1964, 1996; Konno 1972), and this suggests that reassignment of *L. axisadversum* to *Loxosomina* may be in order. Ideally, future analyses with expanded taxon sampling including the type species of all the genus-group names in the family Loxosomatidae should be done to arrive at a better taxonomy of this group with proper application of the genus names reflecting their phylogeny.

Acknowledgments

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