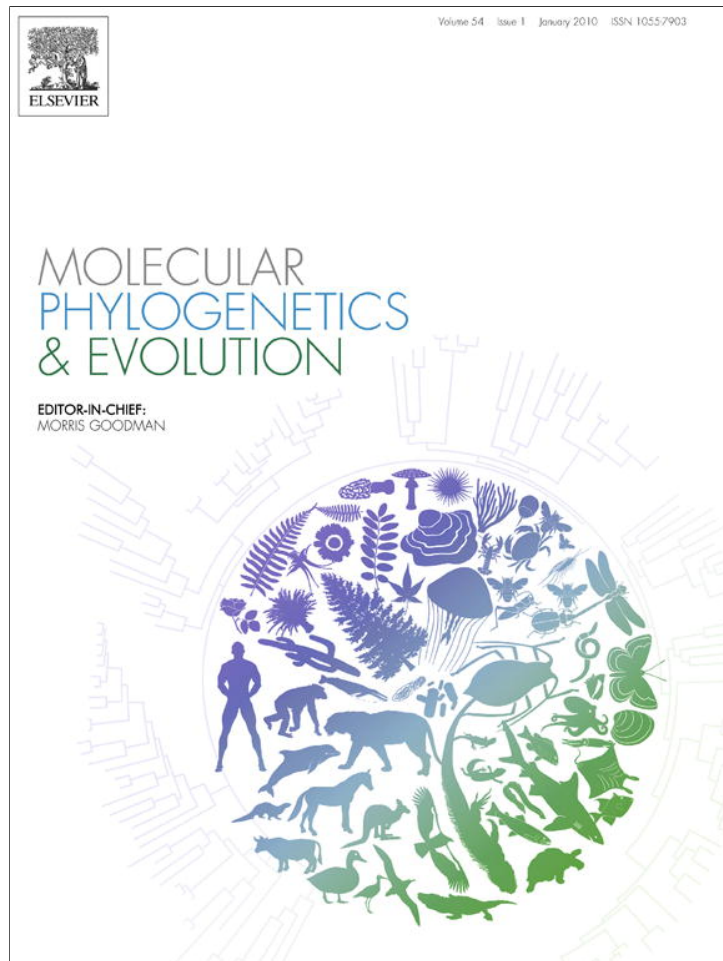


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Short Communication

The systematic position of Opistocystidae (Annelida, Clitellata) revealed by DNA data

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ABSTRACT

Opistocystidae Černosvitov, 1936 is a largely Neotropical oligochaete taxon containing seven species. Its familial status has never been formally challenged, although possible close relationships with Naididae and Phreodrilidae have been noted. Mitochondrial 12S and 16S rDNA, and nuclear 18S rDNA, of a range of aquatic oligochaete taxa, including *Trieminentia corderoi* (Opistocystidae), were analysed by Bayesian inference. This showed that *T. corderoi* is a derived lineage within Naididae, closely related to *Pristina* and its monotypic subfamily Pristininae. Opistocystidae as a whole (with its three genera, *Opistocysta*, *Trieminentia*, and *Crustipellis*) is thus likely to be a group within Naididae.

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1. Introduction

The aquatic representatives of the oligochaetous Clitellata appear to be a paraphyletic assemblage of different “microdrile” families, which to a large extent have been classified on the basis of the segmental position of their genital organs (see Erséus, 2005). The largest family is Naididae Ehrenberg, 1828 (*sensu* Erséus et al., 2008), a taxon occurring world-wide with about a thousand species. Naididae was earlier restricted to about 180 species, excluding the many worms traditionally referred to as Tubificidae Vejdovský, 1876, but DNA data have verified that Naididae in its old sense is phylogenetically nested within the latter (Christensen and Theisen, 1998; Erséus et al., 2002; Sjölin et al., 2005; Envall et al., 2006). This led to the suggestion to consider Naididae as a subfamily (Naidinae) within Tubificidae (Erséus and Gustavsson, 2002; Erséus et al., 2005), but The International Commission on Zoological Nomenclature (2007) ruled that Naididae maintains its precedence over Tubificidae, with the consequence that all former tubificids instead are proposed to be part of an enlarged Naididae (Erséus et al., 2008). Moreover, as Envall et al. (2006) showed that Naididae in its former, restricted, sense is likely to be polyphyletic, Erséus

et al. (2008) advocated that *Pristina* Ehrenberg, 1828, should be referred to a separate subfamily, Pristininae Lastockin, 1921, well separated from Naidinae Ehrenberg, 1828; the latter containing all other former naidid genera. Both Naidinae and Pristininae occur globally in freshwater, but a few naidines are estuarine.

A taxonomically and geographically more restricted aquatic oligochaete family, Opistocystidae Černosvitov, 1936 (authorship recognized by intent; Harman, 1969), is a largely Neotropical taxon with seven nominal species (four inadequately described), known from Argentina, Brazil, Paraguay and Uruguay in the South to the southern United States in the North, and classified into three genera (Harman and Loden, 1978): *Opistocysta* Černosvitov, 1936 (type species, *O. funiculus* Cordero, 1948), *Trieminentia* Harman and Loden, 1978 (type species, *O. corderoi* Harman, 1969), and *Crustipellis* Harman and Loden, 1978 (type species, *O. tribranchiata* Harman, 1969). Brinkhurst (1966) also reported a worm, “most likely to belong to *O. funiculus*”, from Africa, which may reflect a recent introduction of an opistocystid onto this continent. The genera are separated by the location of their genital organs; testes and ovaries are in segments XXI–XXII in *Opistocysta*, XIV(or XV)–XV(or XVI) in *Trieminentia*, and XI–XII in *Crustipellis*. Otherwise, they are morphologically similar, all with a long prostomial protuberance (“proboscis”), three (one median and two lateral) ciliated caudal appendages (gills), hair chaetae in all dorsal bundles (normally together with needle chaetae), atria covered by diffuse prostate glands, and terminating in eversible penial-to-pseudopenial copu-

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latory structures, and spermathecae located in the segment immediately posterior to the one bearing the male pores (Harman, 1969; Brinkhurst and Jamieson, 1971; Harman and Loden, 1978; Brinkhurst and Marchese, 1989). The predominant mode of reproduction seems to be asexual by budding (Harman and Loden, 1978), i.e., paratomic fission (see, e.g., Dehorne, 1916; Bely and Wray, 2001). All these features except the segmental positions of the genitalia make opisthocystids similar to members of Naidinae and Pristininae, a resemblance recognized already by Leidy (1880), who placed the first described opisthocystid, *O. flagellum* (Leidy, 1880) in *Pristina*. However, Harman (1969) and Harman and Loden (1978) regarded *O. flagellum* as a species *inquirenda*.

Brinkhurst and Jamieson (1971) placed Opisthocystidae in an unresolved superfamily (Tubificioidea) together with Phreodrilidae, Dorydrilidae, and Tubificidae/Naididae, but its exact phylogenetic position has only been briefly discussed in the literature. Černosvitov (1936) regarded it as closely related to “Naididae” (i.e., today’s Naidinae and Pristininae together), a view also shared by Čekanovskaja (1962, Fig. 67), and Omodeo (1998). A morphology-based cladistic analysis also supported this position, but this time with Naididae/Opisthocystidae nested within “Tubificidae” (Brinkhurst, 1994). Moreover, the possibility that opisthocystids even are derived “Naididae” was noted by Brinkhurst (1986, p. 14) and Erséus (1990).

On the other hand, Černosvitov (1936) noted that both Opisthocystidae and the southern hemisphere family Phreodrilidae have their spermathecae placed in a segment posterior to (instead of anterior to) that of the atria, an unusual condition among aquatic oligochaetes. Using this line of evidence, Timm (1981) placed Opisthocystidae closest to Phreodrilidae in his intuitive scheme of aquatic oligochaete evolution.

New material of *Trieminentia corderoi* from the Paraná River floodplain in Argentina enabled the first examination of DNA sequences from an opisthocystid. In this study, parts of the mitochondrial (12S and 16S rDNA) and nuclear genome (18S rDNA) were sequenced and compared to the corresponding sequences of other aquatic oligochaete taxa, with the aim to clarify the systematic position of Opisthocystidae.

2. Material and methods

Specimens of the *Trieminentia corderoi* were collected (August 18, 2006) in a floodplain lake connected to the Middle Paraná River (31°39.9'S, 060°35.4'W), NW of Paraná City, Entre Ríos Province, Argentina. The site is a silty-sandy bottom, 0.3 m deep, with a rich cover of macrophytes (pH 7.4; O₂ 8.23 mg/l). Worms were preserved in 96% ethanol, but in most specimens, the body wall then burst in numerous places, forming a rough surface with a multitude of small thorn-like tissue fragments and exuded body fluids all over it. Nevertheless, the complete worms had remnants of the prostomial proboscis and the three caudal appendages typical of the family. One sexually mature individual (Ref. # CE2037) was cut into two parts, and the anterior region including the clitellum and genitalia was stained in paracarmine, dehydrated, and mounted whole in Canada balsam on a microscope slide (as a voucher), while the posterior part was used for DNA extraction. The voucher is located in the Swedish Museum of Natural History, Stockholm (SMNH 104787). Another worm (CE2038, SMNH 104788), not used for sequencing, was prepared in the same way.

The two specimens have dorsal bundles containing finely serrated hair chaetae and minute, hair-like needle chaetae; and everted pseudopenes (=male pores) in segment XVI. Unfortunately, the quality of preservation does not allow observations of internal features, such as morphology of atria and prostate glands, and appearance and location of spermathecae. If compared to the three

opisthocystid species considered as valid and described by Harman and Loden (1978), our material thus resembles the external appearance of *Trieminentia corderoi*, despite the fact that we discovered fine serrations on the sides of the hair chaetae (only visible at highest magnification); a feature not reported in previous descriptions of this species. Most importantly, *T. corderoi* has its male pores in XV or XVI (Harman and Loden, 1978), while *Crustipellis tribranchiata* has these pores in XII, and also possesses long needle chaetae with a nodulus (not seen in our form), and *Opisthocysta funiculus* has male pores as far back as in XXII. The identity of our specimens as *T. corderoi* is further strengthened by the morphological details in other worms of the sampled population (observed by Marchese).

All other taxa analyzed here are those studied by Envall et al. (2006, Table 2), with a total of 51 different naidids (4 Tubificinae, 4 Phallo-drilinae, 4 Limnodriloidinae, 12 Rhyacodrilinae, 23 Naidinae and 4 Pristininae), which together with *Trieminentia corderoi* were regarded as the ingroup, and 5 outgroup species (2 Enchytraeidae, 1 each of Phreodrilidae, Lumbricidae and Lumbriculidae).

DNA of specimen CE2037 was extracted using the DNeasy® Blood and Tissue Kit (Qiagen). PCR of 12S (381 bp), 16S (484 bp) and 18S (1726 bp) was carried out with PuReTaq Ready-To-Go PCR Beads (GE Healthcare). Primers used and thermocycling protocols followed are listed by Envall et al. (2006). PCR products were checked by electrophoresis on an agarose gel containing ethidium-bromide (3%), and then purified using the E.Z.N.A.® Cycle-Pure kit (GE Healthcare). The PCR products were sequenced by Macrogen Inc., using primers listed by Envall et al. (2006).

The sequences of *Trieminentia corderoi* (GenBank Nos.: GU002446, 12s; GU002447, 16s; GU002448, 18s) were added to those used by Envall et al. (2006), and aligned using ClustalW in MegAlign (DNASTar Inc.), with default settings (e.g., pairwise gap opening penalty 15/multiple gap opening penalty 15; see Section 4). The combined dataset was analyzed by Bayesian inference, using MrBayes, version 3.1.2 (Huelsenbeck and Ronquist, 2001). The models used in the analysis were selected under the Akaike information criterion in MrModeltest, version 2.3 (Nylander, 2004), in conjunction with PAUP, version 4.0b10 (Swofford, 2002). The GTR+I+G model was selected for all three genes. Substitution rates, character state frequencies, gamma shape parameters, and proportions of invariable sites were unlinked between the genes (Ronquist and Huelsenbeck, 2003). Four runs times four Markov chains (one cold and three heated) were run simultaneously for 20 million generations; trees were sampled every 100th generation. Each of the chains was started from a random starting tree. The first 20,000 trees sampled during the burn-in phase were discarded. Gaps were treated as missing data.

The dataset was also analysed by parsimony resampling with TNT 1.1 (Goloboff et al., 2008), using jackknife (35% removal probability), and 1000 replicates.

3. Results

The data matrix contains 2727 characters, of which 788 are informative. The Bayesian inference tree (Fig. 1) gives maximum support (posterior probability, pp 1.00) for the naidid ingroup, and although poorly resolved in the basal part, several of its main clades also are strongly supported: one consisting of all Phallo-drilinae except *Bathydrilus* (pp 1.00), and most closely related to the “rhyacodriline” *Heterodrilus* (pp 1.00); one with all Tubificinae (pp 1.00), but with *Branchiura* (earlier regarded as a rhyacodriline) nested within it (pp 0.96); one with all Limnodriloidinae (pp 1.00); and finally, a large clade consisting of all Naidinae, *Pristina* (Pristininae), *Trieminentia* and the “rhyacodriline” genera *Ainudrilus*, *Rhyacodrilus*, *Epirodilus* and *Monopylephorus* (pp 1.00). Within this

large clade, *Pristina* (pp 1.00) and *Trieminentia* are sister groups (pp 1.00), and together form the sister of all remaining taxa (pp 1.00). Among the remaining taxa the two species of *Ainudrilus* (pp. 1.00) are the sister of a less supported clade (pp 0.94), within which Naidinae (pp 1.00) is the sister of a group comprising *Rhyacodrilus*, *Epirodrius* and *Monopylephorus* (pp 1.00). Thus, *Trieminentia* is with maximum support placed as the sister group of *Pristina*.

For all jackknife frequencies >50% (not shown in Fig. 1) except one, the parsimony resampling supports nodes also present in the Bayesian tree. The exception is that the parsimony analysis places *Chaetogaster* (instead of *Dero*) as the sister to all remaining Naidinae (latter with 86% jackknife support). The sister group relationship between *Trieminentia* and *Pristina* is supported by 100% jackknife support, as is the placement of these two taxa as the sister to the clade comprising Naidinae, *Ainudrilus*, *Rhyacodrilus*, *Epirodrius* and *Monopylephorus*.

4. Discussion

The analyses of the combined ribosomal mitochondrial and nuclear data give maximum support for *Trieminentia* being a member of the family Naididae *sensu* Erséus et al. (2008), and more specifically, with a position within the large clade also comprising Naidinae, *Pristina* (Pristininae), and a paraphyletic assemblage of various “Rhyacodrilinae”; and with *Pristina* as its sister group in this taxon sample (Fig. 1). Although *Trieminentia* is not the type genus of Opistocystidae, it seems reasonable to assume that Opistocystidae as defined by Harman and Loden (1978) is monophyletic (morphological evidence for this is discussed below). If this assumption proves correct, the type (*Opistocysta*) of Opistocystidae would be nested within, and Opistocystidae would become a junior synonym of, Naididae Ehrenberg, 1828.

The phylogenetic position of Opistocystidae proposed herein is corroborated by morphological evidence. All “opistocystids” fully described to date have male ducts with diffuse prostate glands, and eversible pseudopenes, two features typical also of the rest of the large naidine–rhyacodriline–pristinine group. At the same time, the opistocystid genera have hairs and needles – or hair-like needles – in all dorsal bundles, as is common for freshwater naidids, including *Pristina* but excluding the naidines; all Naidinae show reductions in the dorsal distribution of chaetae. A possible synapomorphy of Pristininae, *Opistocysta*, *Trieminentia* and *Crustipellis* is the proboscis, but a similar prostomial appendage is present also in the naidine genera *Arceonais*, *Ripistes* and *Stylaria*.

Opistocysta and *Trieminentia* have their sexual organs in an unusually posterior position, whereas *Crustipellis* have them in the position (testes in XI; ovaries and male pores in XII) that has been regarded as the plesiomorphic state for aquatic microdrile groups (Brinkhurst, 1994). However, as the present study suggests that the former opistocystids are terminally nested members of Naididae, which normally have the genitalia in X–XI or even anterior to that, it is likely that they share a derived state of this character, i.e., a general backward shift of genitalia. Regardless of this, however, two other characters support the monophyly of the former opistocystids: the posterior position of the spermathecae vis-à-vis the atria, and the particular arrangement of caudal gills, including two longer ventro-lateral and one shorter dorso-median appendages. Members of the naidine genus *Dero* also possess posterior gills, which are lamelliform or digitiform processes around the anus, but they are always in multiples of two (all paired) and may have evolved convergently to those of *Opistocysta*, *Trieminentia* and *Crustipellis*.

Expanding Pristininae Lastoĉkin, 1921 to include the former Opistocystidae would be in accordance with the tree obtained (Fig. 1). Other alternatives, e.g., lowering the rank of Opistocystidae

Černosvitov (1936) to yet another subfamily within Naididae, are also possible, as the morphological coherence of *Opistocysta*, *Trieminentia*, and *Crustipellis* support their identity as a separate lineage. On the basis of the present molecular study, we are only able to conclude that *Trieminentia* is closely related to *Pristina*; *Opistocysta* (the family type), e.g., was not included in our molecular study. However, as a working hypothesis, and for the time being, we suggest to regard all former opistocystids as members of the Pristininae.

Whether or not the paratomic reproduction is homologous in Naidinae, Pristininae and Opistocystinae, is still an enigma. Functionally, the process appears similar in all three groups, in that the secondary zooid regenerates a new head end in the fission zone of cloning individuals (for *Opistocysta funiculus* (*sensu* Harman and Loden, 1978), see Černosvitov (1936, Fig. 9)), but the number of regenerated segments differs between taxa (Bely and Wray, 2004). The tree obtained in the present study, as well as those from the previous studies by Erséus et al. (2002) and Envall et al. (2006), suggest that, in the Pristininae + *Trieminentia* clade, this mode of reproduction has evolved convergently from that in Naidinae. Bely and Wray (2004), who also found *Pristina* to be a group separate from Naidinae, noted that these groups “display a remarkable diversity of modes of fission” and also found it possible that these modes may have multiple origins.

Envall et al. (2006) used the data of all taxa of the present study, except *Trieminentia corderoi*, in Bayesian analyses of datasets of combined 12S + 16S + 18S datasets under two different combinations of alignment parameters (different settings in Clustal X). Trees obtained using alignments “15/45 penalties” and “15/15 penalties (default setting)”, both placed *Pristina* as sister of *Ainudrilus*, but this had support only in the 15/45 alignment. By and large, the basal resolution of the naidinae–rhyacodriline–pristinine–opistocystine clade was less supported in the 15/15 alignment of the Envall et al. study than in the single alignment (also 15/15) of the present study. The difference may be attributed to the fact that we added an opistocystid to the sampled taxa, and/or that our Bayesian analysis was run for ten times as many generations as in the previous study. Still, however, the somewhat different topologies in the two studies suggest that the estimated phylogeny of this large lineage within Naididae may not have yet arrived to its final, fully corroborated, state. Additions of taxa and molecular as well as morphological data, and the use of other analytical methods may still change the conclusions regarding the detailed relationships of the lower-level taxa. Nevertheless, our study has not refuted the previous conclusions by Envall et al. (2006) that Pristininae is a taxon well separated from Naididae, and that Rhyacodrilinae, as previously defined indeed is an artificial taxon.

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