



# Molecular phylogeny of naidid worms (Annelida: Clitellata) based on cytochrome oxidase I

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## Abstract

Naidids are tiny, primarily freshwater oligochaete annelids which reproduce asexually by fission. We investigated the phylogenetic relationships within this group by sequencing 1224 bp of the mitochondrial gene cytochrome oxidase I (COI) from 26 species of naidids (representing 13 of the 23 genera currently recognized), as well as from four tubificids, their closest allies. Although not completely concordant, maximum parsimony and Bayesian inference analyses agreed in several important respects, with no well-supported conflicts. Our study, the first detailed molecular investigation of naidid relationships, suggests that naidids fall into two groups, one comprised of the genus *Pristina*, and another comprised of all other genera sampled. The clear division of naidids into these two groups best matches an early, simple classification of the group by Lastoĉkin (1924); the more recent classifications proposed by Sperber (1948) and Nemeĉ and Brinkhurst (1987) are not as consistent with our results. We note that our study suggests the genus *Stylaria* is comprised of two distinct species, *Stylaria lacustris* and *Stylaria fossularis*, rather than merely two morphotypes of a single species. Based on our phylogenetic results, we suggest that pigmented eyes evolved only once among naidids but must have been lost multiple times, and that the elongation of the prostomium into a proboscis evolved at least twice independently. The simplest form of fission, architomy (fragmentation), occurs in two of the most basally branching naidid genera, and may represent the plesiomorphic condition for naidids.

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## 1. Introduction

Asexual reproduction by fission has evolved independently in many different annelid groups (Lasserre, 1975; Schroeder and Hermans, 1975). Most commonly, fission occurs in only one or a few closely related species within larger groups of strictly sexual species, suggesting that fission is a derived trait that evolved relatively recently in these groups. One group of oligochaetes, the naidids, stands out for being the largest fissiparous group of annelids. Naidids comprise well over one hundred

species, all of which are known (or thought) to be capable of fission (Brinkhurst and Jamieson, 1971). Most naidids reproduce by a type of fission called paratomy, a remarkable process in which a new head and tail are intercalated in the middle of a worm's body, forming a transiently linked chain of individuals (Bely and Wray, 2001; Dehorne, 1916). Although fission is the primary mode of reproduction throughout this group, most, if not all, naidids also periodically reproduce sexually.

Naidids are small, delicate aquatic worms found worldwide in many bodies of fresh and brackish water (Brinkhurst and Jamieson, 1971). Most species are detritivores living in sediments or among aquatic vegetation, but a few species are carnivorous or parasitic. In part because of their fissiparous mode of reproduction, which may allow a worm to produce a new offspring every few days, naidids can reach extremely high densities under favorable conditions (Cheng et al., 1993; McElhone, 1978), and thus are important members of

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many aquatic ecosystems. As a group, naidids display a great diversity of morphologies, behaviors, modes of fission, and regeneration potentials, among other features (Bely, 1999a; Brinkhurst and Jamieson, 1971; Dehorne, 1916; Sperber, 1948; Stephenson, 1930). Because it is relatively easy to collect a wide variety of naidids from the field, and because many species are easy and inexpensive to rear in the laboratory, naidids make ideal subjects for comparative evolutionary studies. Despite the prevalence, diversity, and tractability of the naidids, however, the evolution of this group has remained poorly studied.

A close relationship between naidids and the Tubificidae has long been recognized (Sperber, 1948; Stephenson, 1930; Vaillant, 1890). Naidids have traditionally been placed in their own family, the Naididae, united primarily by the presence of fission and by the more anterior placement of gonadal segments as compared to that in tubificids. However, recent morphological (Brinkhurst, 1994; Erséus, 1987, 1990) and molecular (Christensen and Theisen, 1998; Erséus et al., 2000, 2002; Martin et al., 2000) analyses suggest that naidids stem from within the Tubificidae, leading to the recent proposal by Erséus et al. (2002) to dismiss the family Naididae and formally absorb it into the Tubificidae. Additional studies are still required to fully resolve the relationships among the major tubificid subgroups, and to test the monophyly of some of these subgroups, including the naidids. Because of these phylogenetic uncertainties and the current lack of a revised classification, in this paper we use the terms “naidid” and “tubificid” in their common usage, referring to species traditionally recognized as being part of the Naididae and Tubificidae, respectively. We recognize that the classification of these oligochaetes will likely be revised in the near future.

In contrast to the attention focused, especially recently, on the relationship between naidids and tubificids, relationships among naidids have received scant attention. In the last one hundred years, only three formal hypotheses on naidid relationships, all based on morphology, have been entertained: Lastoĉkin (1924) devised an early classification of the group, Sperber (1948) provided a taxonomic monograph of the naidids and a modified classification, and, more recently, Nemeĉ and Brinkhurst (1987) analyzed a small morphological dataset which led them to propose yet another classification. The intergeneric relationships suggested by these three hypotheses are incongruent in several important respects, including what the major subdivisions are within the naidids (Fig. 1). The lack of consensus between these three morphology-based classification schemes appears to reflect, at least in part, the limited number of independent morphological characters available for study in these worms, as well as the considerable homoplasy in some of these characters.

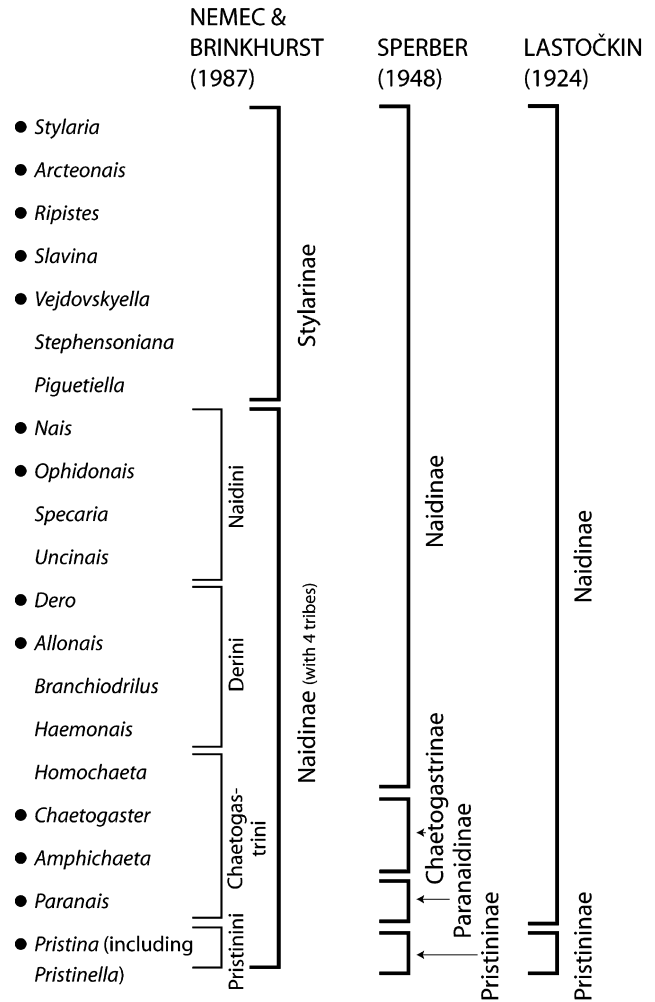


Fig. 1. Naidid classifications (subfamilies and tribes) proposed by Lastoĉkin (1924), Sperber (1948), and Nemeĉ and Brinkhurst (1987). Genera included in our study are marked by bullets. The list of 20 naidid genera presented here (Brinkhurst, 1985; Brinkhurst and Coates, 1985; Brinkhurst and Jamieson, 1971; Collado and Schmelz, 2000) does not include three naidid genera, *Bratislavia* (Kosel, 1976), *Neonais* (which remains inadequately described) (Brinkhurst and Jamieson, 1971; Sokolskaya, 1962), and *Rhopalonais* (Dzwilllo and Grimm, 1974), as these genera had not yet been described at the time Lastoĉkin and Sperber devised their classifications. Nemeĉ and Brinkhurst (1987) tentatively placed these three genera in the Naidinae tribes Pristinini, Naidini, and Derini, respectively. Nemeĉ and Brinkhurst (1987) considered the placement of *Stephensoniana* and *Piguetiella* in the Stylarinae only tentative. The genus *Pristina* was split into *Pristina* and *Pristinella* by Brinkhurst (1985), but *Pristinella* was subsequently dismissed on the grounds that species with a mix of “*Pristina*” and “*Pristinella*” characters have now been described (Collado and Schmelz, 2000). Of the four *Pristina* species we sampled, one (*P. osborni*) was previously assigned to *Pristinella*.

Molecular data have recently been used, with great success, to elucidate the relationships within and among a growing number of clitellate groups (e.g., Apakupakul et al., 1999; Beauchamp et al., 2001; Christensen and Theisen, 1998; Erséus et al., 2000, 2002; Martin, 2001; Siddall and Bureson, 1998; Siddall et al., 2001). However, thus far, the molecular studies that include naidid

taxa have focused mainly on elucidating the relationship between naidids and tubificids, and have included at most seven naidid species (Christensen and Theisen, 1998; Erséus et al., 2000, 2002); no molecular studies have yet focused specifically on the relationships among naidid taxa.

We initiated a molecular phylogenetic study of naidids based on the nucleotide sequence of most of the mitochondrial gene cytochrome oxidase I (COI). The present study represents the first detailed molecular phylogenetic investigation of the naidid oligochaetes, and includes representatives from each of the major naidid subgroups (subfamilies or tribes) recognized in the three naidid classifications (Lastočkin, 1924; Nemeč and Brinkhurst, 1987; Sperber, 1948). Our results allow us to evaluate these differing naidid classification schemes and to provide a phylogenetic framework to begin investigating the evolution of asexual reproduction and morphology within this group.

## 2. Methods

### 2.1. Taxonomic sampling

We collected sequence information from 26 species of naidids (Table 1), representing 13 of the 23 genera currently recognized (Fig. 1). Our sampling included representatives of all naidid subfamilies or tribes as defined by Lastočkin (1924), Sperber (1948), and Nemeč and Brinkhurst (1987). For some species, we also collected data on multiple individuals originating from different geographic localities. Collections or acquisitions were made between 1995 and 2001. Collection localities are listed in Table 1.

In addition to the naidid taxa, this study included four tubificid species, for which we collected sequence data, and one species from the family Lumbricidae, for which we obtained sequence data from Boore and Brown (1995) via GenBank (Table 1). We expected our tubificid taxa to fall outside the naidids. However, to avoid having to make any assumptions about the relationship between our naidid and tubificid taxa, the lumbricid *Lumbricus terrestris* was included as a more distant outgroup.

Voucher specimens were either fixed in formaldehyde and transferred to 70–95% ethanol, or placed directly into ethanol. Taxonomic identifications were made using Kathman and Brinkhurst (1998) or Brinkhurst (1986), and typically cross-checked with Hiltunen and Klemm (1985). Because naidid worms are so small, the entire worm, and preferably several worms, is used for DNA extractions. For this reason, for most naidid taxa asexually reproducing laboratory cultures (see Bely, 1999b for culture conditions) were established from

single individuals, so that we could both produce additional tissue for molecular analyses and, more importantly, preserve voucher specimens of the exact taxa sampled. For the few taxa for which laboratory cultures could not be established, wild-collected individuals from the same sampling effort were preserved as voucher specimens.

### 2.2. DNA extraction

DNA was extracted from live or ethanol-preserved worms using the DNeasy Tissue Extraction Kit (Qiagen), or by proteinase K digestion in a CTAB buffer followed by a phenol/chloroform extraction and DNA precipitation. In nearly all cases, extractions were made from either a single individual or multiple individuals from an isogenic culture (i.e., started from a single individual). However, in a few cases, extractions were made from several wild-caught specimens (from the same sampling effort) because preparations from single worms did not yield PCR products and we were unable to establish isogenic cultures. Christer Erséus generously provided the DNA sample of *Vejdovskyella comata*.

### 2.3. Gene amplification and sequencing

A 1305 bp region (excluding primers) of cytochrome oxidase I (COI) spanning most of the coding sequence was amplified by PCR in two pieces, a 709 bp 5' fragment and a 730 bp 3' fragment, which overlapped slightly. For most taxa, the primers used to amplify the first half of the gene were the published primers LCO1490 and HCO2198 (Folmer et al., 1994); we designed two additional primers to amplify the second half of the gene, COI-A<sup>+</sup> (5'-cctgtctctgctggtctattaciat-3' corresponding to the amino acids PVLGAIMT) and COI-B<sup>-</sup> (5'-tagtcagaatatcgccgaggtaticc-3' corresponding to GMPRRYSYD). For a few taxa, these two primer pairs did not work well and were thus modified slightly. Primer HCO2198 was replaced by primer COI-E<sup>-</sup> (5'-tacttctgggtgtccgaagaatca-3') and COI-A<sup>+</sup> was replaced by either COI-C<sup>+</sup> (5'-ccggtactagcaggagcgattaciat-3') or COI-D<sup>+</sup> (5'-ccagtattgcaggagcaattaciat-3'). Thermal cycling parameters were as follows: 94 °C for 1 min; 35 cycles of 94 °C for 30–60 s (depending on template), 40–50 °C for 30–60 s (depending on template), 72 °C for 30–90 s (depending on template); and 72 °C for 5 min. PCR products were purified by spin column-based PCR purification kits (Qiagen or Gibco-BRL), using either direct PCR product or bands excised from agarose gels. Both strands of all PCR products were sequenced on an automated sequencer. Sequence fragments were assembled and edited using Sequencher (version 3.1.1, Gene Codes). Of the 1305 bp amplified from COI, 1224 bp were included in analyses.

Table 1  
Taxa used in this study, GenBank accession numbers (COI sequence), and collection localities

Species	GenBank Accession Nos.	Collection site
Annelida: Clitellata: Tubificidae?: "naidids"		
<i>Allonais paraguayensis</i> (Michaelsen, 1905)	AF534828	Purchased from Wards (sold as <i>Stylaria</i> )
<i>Amphichaeta raptisae</i> (Chapman, 1981)	AF534829	Wildcat Creek, Richmond, California
<i>Arcteonais lomondi</i> (Martin, 1907)	AF534830	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Chaetogaster diaphanus</i> (Gruithuisen, 1828) (IA)	AF534831	Lake Okoboji, Iowa Lakeside Laboratory, Okoboji, Iowa
<i>Chaetogaster diaphanus</i> (Gruithuisen, 1828) (ONT)	AF534832	Opeongo Lake, Algonquin Park, Ontario
<i>Chaetogaster diastrophus</i> (Gruithuisen, 1828)	AF534833	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Chaetogaster linnaei</i> (von Baer, 1827)	AF534834	Chicken Ranch Slough, Sacramento, California
<i>Dero</i> ( <i>Dero</i> ) <i>digitata</i> (Müller, 1773) (IA)	AF534835	Fort Defiance State Park, Iowa
<i>Dero</i> ( <i>Dero</i> ) <i>digitata</i> (Müller, 1773) (NY)	AF534836	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Dero</i> ( <i>Aulophorus</i> ) <i>furcata</i> (Müller, 1773)	AF534837	Purchased from Connecticut Valley Biological Supply (sold as <i>Dero</i> )
<i>Dero</i> ( <i>Dero</i> ) <i>obtusa</i> (d'Udekem, 1855)	AF534838	Fort Defiance State Park, Iowa
<i>Dero</i> ( <i>Aulophorus</i> ) <i>vaga</i> (Leidy, 1880)	AF534839	Tuckahoe Lake, Tuckahoe State Park, Maryland
<i>Dero</i> ( <i>Dero</i> ) sp.	AF534840	Jemmerson Slough, Okoboji, Iowa
<i>Nais bretscheri</i> (Michaelsen, 1899)	AF534843	Rock Creek, Washington, D.C.
<i>Nais communis</i> (Piguet, 1906)	AF534845	Fort Defiance State Park, Iowa
<i>Nais variabilis</i> (Piguet, 1906) (IA)	AF534844	Lake Okoboji, Iowa Lakeside Laboratory, Okoboji, Iowa
<i>Nais variabilis</i> (Piguet, 1906) (MD)	AF534841	Wye Oak State Park, Maryland
<i>Nais variabilis</i> (Piguet, 1906) (NY)	AF534842	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Ophidonais serpentina</i> (Müller, 1773)	AF534846	Wildcat Creek, Richmond, California
<i>Paranais frici</i> (Hrabě, 1941) (CA)	AF534847	Napa River, Kennedy Park, Napa, California
<i>Paranais frici</i> (Hrabě, 1941) (NY)	AF534848	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Paranais litoralis</i> (Müller, 1784) (CA)	AF534849	Napa River, Kennedy Park, Napa, California
<i>Paranais litoralis</i> (Müller, 1784) (NY)	AF534850	Flax Pond, Setauket, New York
<i>Pristina aequisetata</i> (Bourne, 1891) (IA)	AF534851	Fort Defiance State Park, Iowa
<i>Pristina aequisetata</i> (Bourne, 1891) (NY)	AF534852	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Pristina leidy</i> (Smith, 1896)	AF534853	Purchased from Carolina Biological Supply (sold as <i>Stylaria</i> )
<i>Pristina longiseta</i> (Ehrenberg, 1828)	AF534854	Osnabruck, Germany (collected by R. Hessling)
<i>Pristina osborni</i> <sup>a</sup> (Walton, 1906)	AF534855	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Ripistes parasita</i> (Schmidt, 1874)	AF534856	Opeongo Lake, Algonquin Park, Ontario
<i>Slavina appendiculata</i> (d'Udekem, 1855) (IA)	AF534857	Jemmerson Slough, Okoboji, Iowa
<i>Slavina appendiculata</i> (d'Udekem, 1855) (ONT)	AF534858	Opeongo Lake, Algonquin Park, Ontario
<i>Stylaria fossularis</i> (Leidy, 1852) (ONT)	AF534859	Opeongo Lake, Algonquin Park, Ontario
<i>Stylaria lacustris</i> (Linnaeus, 1767) (IA)	AF534860	Jemmerson Slough, Okoboji, Iowa
<i>Stylaria lacustris</i> (Linnaeus, 1767) (ONT)	AF534861	Opeongo Lake, Algonquin Park, Ontario
<i>Stylaria lacustris</i> (Linnaeus, 1767) (GE)	AF534862	Osnabrück, Germany (collected by R. Hessling)
<i>Vejdovskyella comata</i> (Vejdovský, 1883)	AF534863	Lake Längen, Alingsås, Sweden (collected by C. Erséus)
Annelida: Clitellata: Tubificidae: Rhyacodrilinae		
<i>Branchiura sowerbyi</i> (Beddard, 1892)	AF534864	Constitution Marsh Sanctuary, Cold Spring, New York
Annelida: Clitellata: Tubificidae: Tubificinae		
<i>Limnodrilus hoffmeisteri</i> (Claparède, 1862)	AF534865	Constitution Marsh Sanctuary, Cold Spring, New York
<i>Tubifex tubifex</i> (Müller, 1774)	AF534866	Western Fisheries Research Center, USGS, Sand Point, Lake Washington, Washington (supplied by C. Rasmussen)
<i>Tubifex</i> sp.	AF534867	Wildcat Creek, Richmond, California
Annelida: Clitellata: Lumbricidae		
<i>Lumbricus terrestris</i> (Linnaeus, 1758)	NC_001673	Boore and Brown (1995)

Note. Where multiple representatives of a species were sampled, the geographic location is indicated in parentheses after the species name. Subgenera of *Dero* are shown in parentheses.

<sup>a</sup> *Pristina osborni* was until recently placed in the now dismissed genus *Pristinella* (Collado and Schmelz, 2000).

#### 2.4. Phylogenetic analyses

Sequence alignments were unambiguous, since there was no length variation in the amplified region of the gene. Pairwise (uncorrected "p") sequence distances were calculated using PAUP\* (version 4.0b10, Swofford,

2002). Phylogenetic trees were reconstructed by maximum parsimony using PAUP\* and by Bayesian inference (Huelsenbeck et al., 2001b) using MrBayes (version 2.01, Huelsenbeck and Ronquist, 2001) on a Power Macintosh G4 (OS 9.0.4). Only *L. terrestris* was defined as an outgroup.

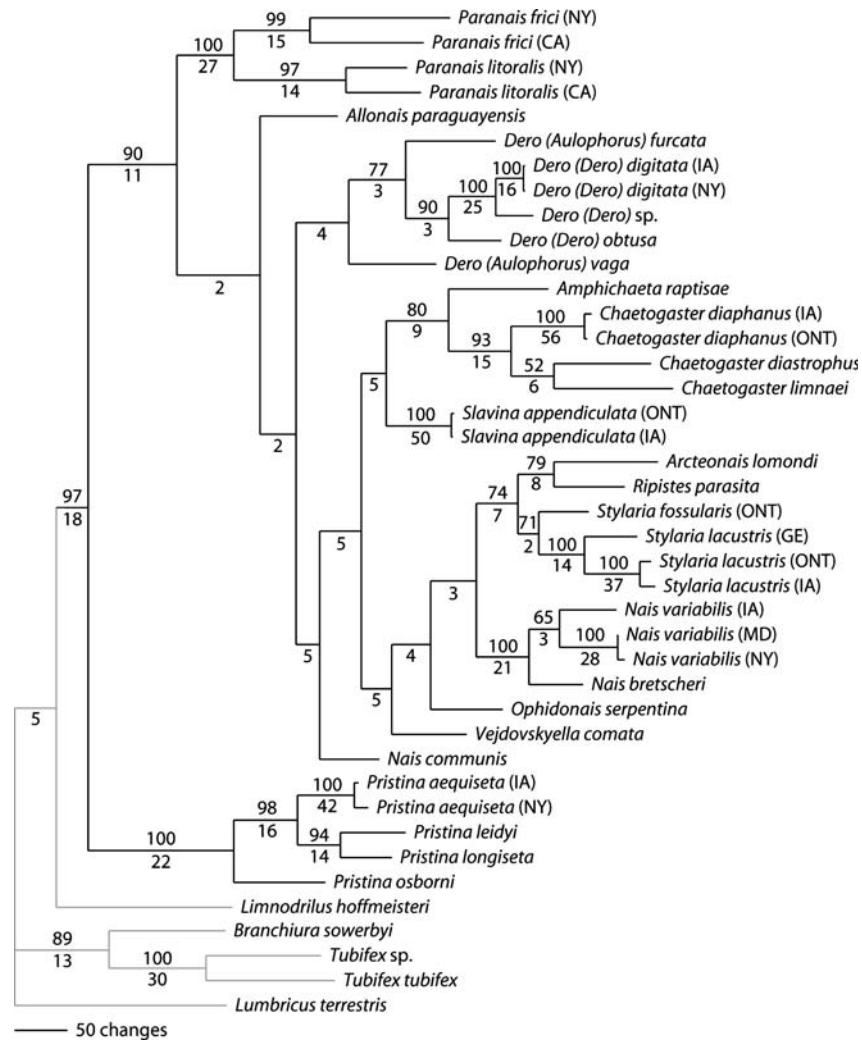


Fig. 2. Single shortest tree from maximum parsimony analysis based on nucleotides plus amino acids (tree length = 4195; consistency index = 0.299, retention index = 0.495, rescaled consistency index = 0.148). Bootstrap values (1000 replicates) greater than 50% are indicated above branches. Bremer support indices are indicated below branches. Naidid branches are black; outgroup branches are gray.

Maximum parsimony analyses were performed using all 1224 nucleotides (“DNA analysis”), as well as with the 1224 nucleotides plus their 408 amino acid translation, for a total of 1632 characters which were equally weighted (“DNA + AA analysis”). Appending the amino acid translation to a nucleotide dataset is a method used to give additional weight specifically to non-synonymous nucleotide substitutions (e.g., Agosti et al., 1996; Birstein and DeSalle, 1998; Flores-Villela et al., 2000; Leys et al., 2000; Schubert et al., 2000). Parsimony trees were obtained using the heuristic search option, constructed using 30 random additions of the sequences, TBR branch swapping, and remaining parameters set to PAUP\* defaults. The level of support for each node was evaluated by non-parametric bootstrapping (Felsenstein, 1985) using 1000 bootstrap replicates (each based on 30 random additions of the sequences) in PAUP\*, as well as by generating Bremer decay indices (Bremer, 1988) using TreeRot v.2 (Sorenson, 1999). Parsimony

analyses constrained to recover specific clades of interest (see Sections 3 and 4) were performed in PAUP\* on the DNA + AA dataset, using all other settings identical to unconstrained analyses.

Bayesian inference trees were based on all 1224 nucleotides. The analyses we report were carried out using a six-parameter nucleotide substitution model (general time reversible, or GTR, Yang et al., 1994) with parameters estimated independently for each of the three codon positions (Huelsenbeck et al., 2001a). Analyses performed using a simpler model of sequence evolution (two-parameter HKY model (Hasegawa et al., 1985), with parameters estimated independently for each codon position) or a more complex model (GTR + I +  $\Gamma$ , which includes a parameter for the proportion of invariant sites and a parameter describing a  $\Gamma$  distribution of rates of change, e.g., Gu et al., 1995) produced nearly identical topologies (differing only in a slightly altered position of *Allonais paraguayensis*) and (with few

exceptions) very similar posterior probability values (data not shown). Each of four replicate runs was initiated from a random starting tree, proceeded with four chains (one heated and three cold), and was allowed to run for one million generations with trees sampled every 100 generations (10,001 trees retained). Based on plots of likelihood scores versus generation (“burn-in” plots) during each run, we determined that stationarity had clearly been reached by generation 300,000 (i.e., after generation 300,000, changes in tree topology and parameter estimates no longer continued to improve the tree likelihood scores). Therefore, the first 3001 trees (from the first 300,000 generations) were discarded, and a majority rule consensus of the remaining 7000 trees was generated in PAUP\* (Huelsenbeck et al., 2001a).

### 3. Results

#### 3.1. Sequence data

There was no length variation among our COI sequences, and their amino acid translations had no stop codons, giving us confidence that our sequences are indeed mitochondrial in origin and not contaminated by nuclear copies. The 1224 bp COI sequence for each taxon has been deposited in GenBank (see Table 1 for accession numbers).

Using all 1224 nucleotides, uncorrected pairwise distances ranged from 0.2 to 11.0% within naidid species, 5–17% between naidid species within a genus, 12–21% between naidid genera, 19–24% between naidids and the tubificid outgroup taxa, and 21–25% between naidid taxa and the lumbricid outgroup. Although most of these values are unsurprising, the midwest and east coast (IA and MD/NY) *Nais variabilis*, the east and west coast (NY and CA) *Paranais frici*, the east and west coast (NY and CA) *Paranais litoralis*, and the North American and European (ONT/IA and GE) *Stylaria lacustris* samples showed remarkably high (9–11%) intraspecific sequence divergences, possibly indicating significant population structure or cryptic species.

#### 3.2. Parsimony analysis

The nucleotide dataset contained 508 (out of 1224) parsimony informative sites, and the combined nucleotide plus amino acid dataset contained 598 parsimony informative characters (508/1224 nucleotides plus 90/408 amino acids). The parsimony analysis based on nucleotides only and the parsimony analysis based on nucleotides plus amino acids each identified a single shortest tree (DNA analysis: tree length = 3758; consistency index = 0.267; retention index = 0.467; rescaled consistency index = 0.125; DNA + AA analysis: tree length = 4195; consistency index = 0.299; retention in-

dex = 0.495; rescaled consistency index = 0.148; Fig. 2). These trees were identical in topology. However, as might be expected, the nucleotide + amino acid tree had higher bootstrap and Bremer support values for most nodes (results from DNA + AA analysis are shown in Fig. 2). Increased support, especially for deeper nodes, has also been found by other authors using this method of weighting (e.g., Birstein and DeSalle, 1998; Leys et al., 2000). References to parsimony results in the remainder of this paper refer specifically to the combined DNA + AA analysis.

The naidids included in this study are united by a strongly supported stem branch, and themselves fall into two well-supported clades: one comprised of species in the genus *Pristina* and another comprised of all remaining naidid taxa (Fig. 2). Within the larger, non-*Pristina* clade, the most basally branching genus is *Paranais*, followed by *Allonais*, *Dero*, and one species of *Nais* (*Nais communis*), but none of these nodes are well supported. The remaining genera (including the other two species of *Nais*) form a clade, within which an (*Amphichaeta* + *Chaetogaster*) clade and an ((*Arcteonais* + *Ripistes*) + *Stylaria*) clade are well supported.

Intragenetic and intraspecific groupings are generally very well supported. All species and genera for which we have multiple representatives are recovered as monophyletic groups, with the exception of the genus *Nais*. Two of the three *Nais* species form a clade, but *N. communis* branches far from this clade. (In light of these findings, the DNA extraction, amplification, and sequencing for *N. communis* were each performed a second time in order to confirm the validity of this sequence. In addition, multiple specimens from our isogenic culture were identified, all of which clearly keyed out as *N. communis*.) However, the shortest tree which recovered a monophyletic *Nais* clade was only five steps longer (tree length 4200 versus 4195).

#### 3.3. Bayesian inference analysis

The four replicate MrBayes runs recovered identical consensus tree topologies with nearly identical posterior probabilities for each clade. These analyses recovered with high probability all of the moderately to well-supported nodes from the parsimony analysis (Fig. 3): naidids form a clade; naidids are divided into a *Pristina* clade and a clade including all remaining naidids; and among the non-*Pristina* group, (*Amphichaeta* + *Chaetogaster*) and ((*Arcteonais* + *Ripistes*) + *Stylaria*) form clades. All species and genera are recovered as monophyletic, with just two exceptions, both involving *Nais*. In the Bayesian analyses (as in the parsimony analysis) *N. communis* does not group with the other two *Nais* species. Furthermore, in the Bayesian analyses (but not the parsimony analysis) *N. variabilis* is paraphyletic (*Nais bretscheri* is placed within this species). In both the

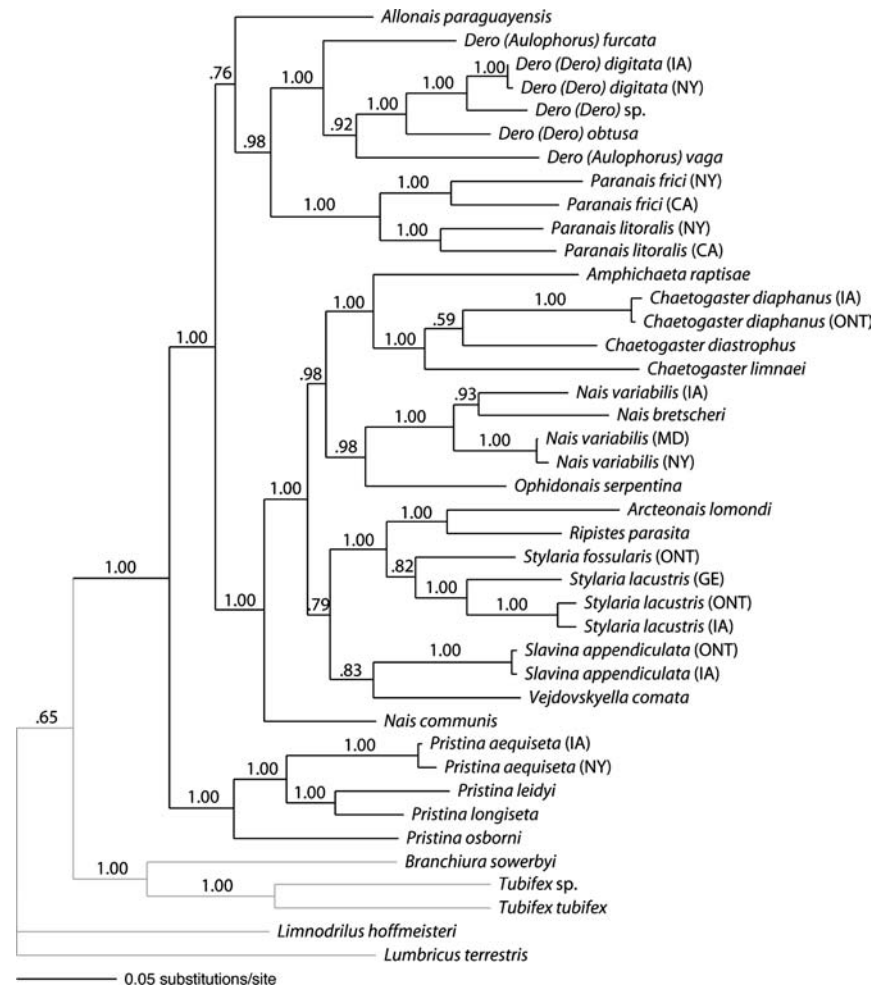


Fig. 3. Consensus tree from one of four replicate Bayesian inference runs. This tree is a consensus phylogram based on mean branch lengths. Posterior probabilities (the frequency of each clade among the 7000 trees retained from the run) are indicated above each branch. Naidid branches are black; outgroup branches are gray. The three other runs produced consensus trees with identical topologies and nearly identical posterior probabilities to those from the run shown here.

Bayesian and parsimony analyses, *Paranaeis*, *Allonais*, *Dero*, and *N. communis* are the most basally branching lineages within the non-*Pristina* clade, although in the Bayesian tree the first three of these form a clade rather than branch sequentially. However, these results are not strongly supported in either analysis.

In addition to the above relationships identified in the parsimony and Bayesian analyses, several additional nodes were recovered with high ( $\geq 0.95$ ) posterior probabilities in the Bayesian analyses (Fig. 3): *Dero* groups with *Paranaeis*, *Nais* (excluding *N. communis*) groups with *Ophidonais*, and (*Nais* + *Ophidonais*) groups with (*Amphichaeta* + *Chaetogaster*). (*Slavina* + *Vejvodskyella*) group with moderate (0.83) probability, and this clade is united with the ((*Arcteonais* + *Ripistes*) + *Stylaria*) clade, again with moderate (0.79) probability. These five genera are united with the ((*Amphichaeta* + *Chaetogaster*) + (*Nais* + *Ophidonais*)) clade with high probability.

#### 4. Discussion

We sequenced most of the mitochondrial gene COI from 26 species of naids representing 13 genera, and analyzed these sequences using maximum parsimony (using both all nucleotides equally weighted and all nucleotides plus their amino acid translation) and Bayesian inference (using all nucleotides). The two parsimony analyses produced identical tree topologies, with the DNA + AA analysis showing higher support values for most nodes. The parsimony tree and the Bayesian tree were congruent in many respects, and produced no well-supported conflicts (Figs. 2 and 3). As expected based on simulation studies showing that Bayesian inference posterior probabilities provide support for correct nodes with fewer characters than non-parametric bootstrapping (Alfaro et al., 2003), Bayesian inference produced a better supported tree: 30/38 nodes had high ( $\geq 0.95$ ) posterior probabilities in our

Bayesian tree, while only 26/38 nodes had high ( $\geq 70\%$ ) bootstrap values in our (DNA + AA) parsimony tree. In addition, Bayesian inference recovered more nodes that are in agreement with hypothesized naidid relationships based on morphology (see next section).

#### 4.1. Intergeneric relationships and naidid classification

Both the parsimony and Bayesian analyses recovered a well-supported clade comprised of all naidids included in our study. The naidids we sampled are thus clearly closely related; however, because our sampling within the Tubificidae was limited, this finding cannot be used as evidence supporting the monophyly of the naidids. Indeed, a recent molecular analysis of the Tubificidae, which included many tubificids and several naidids, recovered the naidids as paraphyletic (two genera of rhyacodriline tubificids fell within the naidids), although this topology was not well supported (Erséus et al., 2002). The question of naidid monophyly must await more detailed analyses, which should include extensive sampling of both naidids and tubificids (especially the Rhyacodrilinae).

Although both morphological and molecular data have identified rhyacodriline tubificids as the closest relatives of the naidids (Christensen and Theisen, 1998; Erséus, 1990; Erséus et al., 2000, 2002), the single rhyacodriline in our dataset, *Branchiura sowerbyi*, did not form a monophyletic group with the naidids in either of our analyses. The subfamily Rhyacodrilinae is likely to be paraphyletic or polyphyletic (Erséus et al., 2002), and *B. sowerbyi* itself is an unusual rhyacodriline, only recently placed in the Rhyacodrilinae (Baker and Brinkhurst, 1981), which may explain the placement of *B. sowerbyi* in our trees.

All naidid genera and species were consistently recovered as monophyletic groups, with two exceptions: the genus *Nais* and the species *N. variabilis*. In both analyses, *N. communis* was separated from the other two species of *Nais*, making this genus polyphyletic based on our results. The genus *Nais* is comprised of over a dozen described species (Brinkhurst and Jamieson, 1971), so we are hopeful that additional intrageneric sampling might help resolve the monophyly, or lack thereof, of this genus. In the Bayesian analyses (but not the parsimony analysis), *Nais variabilis* was recovered as paraphyletic, with *N. bretscheri* nested within this species. The possibility that *N. bretscheri* (and *N. pardalis*, which we did not sample) may be part of the *N. variabilis* complex has previously been suggested based on morphology (Hiltunen and Klemm, 1980; Kathman and Brinkhurst, 1998). The genus *Pristinella*, which was split from *Pristina* only in 1985 (Brinkhurst, 1985), has now been synonymized with it once again (Collado and Schmelz, 2000). *Pristina osborni*, the one species in our dataset that was formerly placed in *Pristinella*, does in-

deed cluster with the other *Pristina* species we included, and the genetic divergence within the whole *Pristina* clade (including *P. osborni*) is comparable to that within other naidid genera.

With respect to relationships among the naidid genera, our analyses confidently place *Pristina* at the base of our naidid clade. *Paranais*, *Allonais*, and *Dero* are the next most basal lineages; these genera form a clade in our Bayesian tree, but branch sequentially in our parsimony tree. The molecular analyses of Erséus et al. (2002), based on the nuclear 18S ribosomal gene, found similar positions for *Pristina* and *Dero*, lending additional support to these placements. (*Paranais* branched elsewhere in their tree, and *Allonais* was not included in their dataset.) Among the remaining naidids, our analyses recovered with high support a clade of (*Amphichaeta* + *Chaetogaster*) and a clade of (*Arcteonais* + *Ripistes*) + *Stylaria*. Close affinities between the genera within each of these clades has previously been suggested on morphological grounds (Nemec and Brinkhurst, 1987; Sperber, 1948). In addition, both Sperber (1948) and Nemec and Brinkhurst (1987) argued based on morphology that *Slavina* and *Vejdovskyella* are closely allied, that these two genera are in turn closely related to *Arcteonais*, *Ripistes*, and *Stylaria*, and that *Nais* is closely allied to *Ophidonais*; these relationships were indeed recovered in our Bayesian analysis, although not our parsimony analyses.

Three different classification schemes have been proposed for naidids in the last one hundred years: those of Lastoĉkin (1924), Sperber (1948), and Nemec and Brinkhurst (1987) (Fig. 1). Because our study includes representatives from each of the naidid subfamilies or tribes recognized by these authors, we are able to explore which aspects, if any, of these classifications might reflect the phylogenetic history of this group as inferred from our molecular data. Our phylogenetic analyses recovered a clear division of the naidids into two clades: one comprised of species in the genus *Pristina*, and another comprised of all other naidid taxa sampled. This division is most consistent with the earliest, and simplest, naidid classification (Fig. 4), proposed by Lastoĉkin (1924), which recognizes two subfamilies, the Pristininae (*Pristina*) and the Naidinae (all other naidid genera).

Morphologically, *Pristina* differs considerably from the rest of the naidids (Brinkhurst and Jamieson, 1971). Whereas most naidids form only 3–4 true segments (plus the prostomium/peristomium) anteriorly during each round of fission and possess testes and ovaries in segments IV and V or V and VI, *Pristina* is unique in forming 6 true segments anteriorly during fission and in possessing testes and ovaries more posteriorly, in segments VII and VIII. *Pristina* is also unusual, although not unique, among naidids in having dorsal chaetae (chitinous bristles) beginning in segment II, the

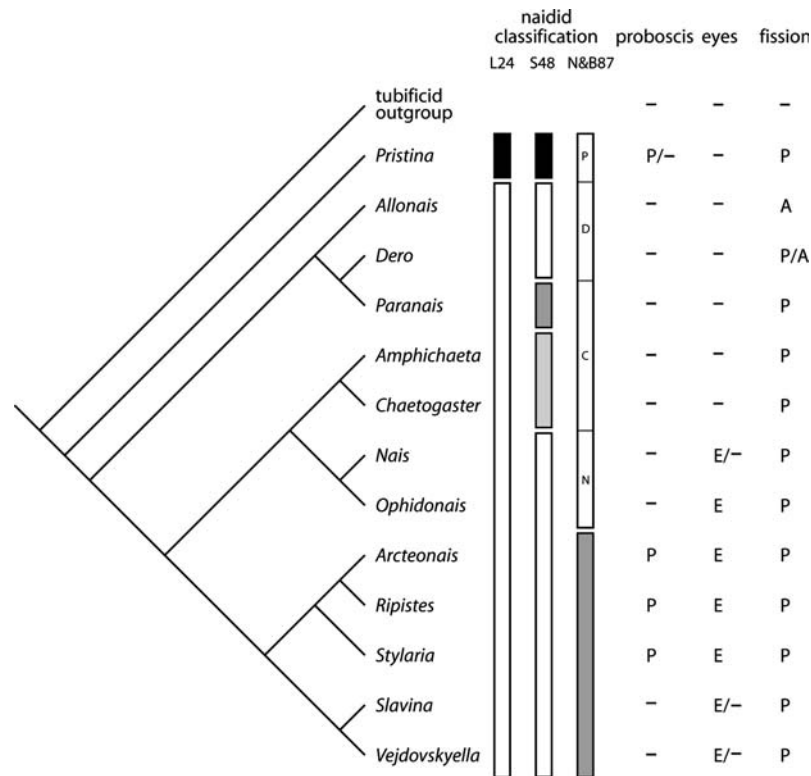


Fig. 4. Naidid classifications and distribution of proboscises, eyes, and type of fission among naidids. The tree topology is from the Bayesian analysis; using the parsimony tree does not significantly alter our conclusions. *N. communis* is omitted from this diagram as it did not group with its congeners. The subfamilial classifications of Lastoĉkin (1924) (L24), Sperber (1948) (S48), and Nemeĉ and Brinkhurst (1987) (N&B87) are coded as follows: Pristininae (black bars); Naidinae (white bars); Chaetogastrinae (light gray bar); Paranaidinae (dark gray bar in S48); Stylarinae (dark gray bar in N&B87). The tribes of N&B87 are Pristinini (P), Derini (D), Chaetogastrini (C), and Naidini (N). Character distributions are marked as follows: proboscis absent (–) or present (P); eyes absent (–) or present (E); fission by architomy (A), paratomy (P), or absent (–). Character information is from Dehorne (1916), Sperber (1948), Brinkhurst and Jamieson (1971), and personal observations.

condition found throughout the tubificids; other naidids typically have several anterior segments devoid of dorsal chaetae. Whether these morphological differences and the division of naidids into the two subgroups that we recovered with our molecular data actually reflect naidid polyphyly, with *Pristina* perhaps having evolved fission independently of the rest of the naidids, is an interesting possibility that we cannot presently address but that clearly warrants future study.

Although some aspects of the Sperber (1948) and Nemeĉ and Brinkhurst (1987) classifications appear in the naidid topologies we recovered, neither classification as a whole satisfyingly matches the results from our molecular analyses (Fig. 4). Sperber (1948), elaborating Lastoĉkin's classification, recognized four subfamilies: the Pristininae (*Pristina*), the Paranaidinae (*Paranais*), the Chaetogastrinae (*Amphichaeta* and *Chaetogaster*), and the Naidinae (all other naidids). (She strongly believed the Chaetogastrinae represented the most basal lineage within the family, and even considered placing them in their own family.) In our analyses, *Chaetogaster* and *Amphichaeta* do indeed form a clade. However, the Naidinae *sensu* Sperber was never recovered as a

monophyletic group: in both of our analyses, the Chaetogastrinae nest well within her Naidinae, and in our Bayesian analysis the Paranaidinae also nest within her Naidinae. In a parsimony analysis constrained to recover a monophyletic Naidinae *sensu* Sperber, the (two) shortest trees are considerably longer (19 steps) than the shortest unconstrained tree (4214 versus 4195 steps). (This constrained tree still retains the Chaetogastrinae as a clade.)

The major division of the naidids that Nemeĉ and Brinkhurst (1987) recognize is between the subfamily Stylarinae (represented in our dataset by *Stylaria*, *Arcteonais*, *Ripistes*, *Vejdovskyella*, and *Slavina*) and the subfamily Naidinae *sensu* Nemeĉ and Brinkhurst (i.e., all other naidids), the latter being further subdivided into four tribes. Again, this classification clearly does not reflect our estimates of naidid relationships, since the Stylarinae always nest well within the Naidinae *sensu* Nemeĉ and Brinkhurst. The shortest parsimony tree constrained to recover a monophyletic Naidinae and Stylarinae *sensu* Nemeĉ and Brinkhurst is much longer (38 steps) than the shortest unconstrained tree (4233 versus 4195 steps). The classification of Nemeĉ

and Brinkhurst was largely influenced by results of parsimony and cluster analyses performed on a limited (24 character) morphological character set and using a subjectively reconstructed ancestor, which could affect the rooting of the tree. Indeed, differences between our and Nemeč and Brinkhurst's estimates of what the major divisions are within the naidids are partly explained by differences in where the trees are rooted. Although the subfamilial designations of Nemeč and Brinkhurst are at odds with our estimates of naidid phylogeny, our Bayesian analysis did recover some of their proposed groupings, namely a clade including *Stylaria*, *Arcteonais*, *Ripistes*, *Vejdovskyella*, and *Slavina* (of their subfamily Stylarinae), and a clade including *Nais* (excluding *N. communis*) and *Ophidonais* (of their tribe Naidini) (Figs. 1 and 3). In addition, *Chaetogaster* and *Amphichaeta* (part of their tribe Chaetogastrini) grouped together, and *Dero* and *Allonais* (of their tribe Derini) were closely allied, although our placement of *Paranais* with *Dero* and *Allonais* rather than with *Chaetogaster* and *Amphichaeta* made these two tribes paraphyletic.

Clearly, additional generic sampling, greater phylogenetic resolution (achieved through analyses of additional genes, for example), and investigations into the question of naidid monophyly are needed prior to a complete revision of naidid classification. However, it seems apparent that the classifications of Sperber (1948) and Nemeč and Brinkhurst (1987) do not reflect the evolutionary relationships among naidids. Only Lastoćkin's (1924) simple division of the naidids into two subfamilies, the Pristininae and Naidinae, is consistent with our molecular results.

We note here that at the time of this writing, COI sequences are available for four naidid species in GenBank, and two of these sequences differ substantially from those we obtained for the same species. When we combine these four nucleotide sequences, which all come from Christensen and Theisen (1998), with ours and run a parsimony analysis, two of the four sequences (*Stylaria lacustris* and *Nais barbata*) fall into the expected positions on the tree. However, their *Chaetogaster diastrophus* falls well within our *Dero* clade, and their *Dero digitata* groups at the base of our *Nais* clade. We therefore suggest that the GenBank submissions for "*C. diastrophus*" (Accession No. AF054196) and "*D. digitata*" (Accession No. AF054195) should be used with caution.

#### 4.2. Molecular evidence supporting the resurrection of *Stylaria fossularis*

Although our study was not intended to explore species boundaries within the naidids, a well-supported result with respect to *Stylaria* is worthy of note. Two discrete morphotypes are found in the genus *Stylaria*, which differ primarily in the shape of the base of the

prostomium (Kathman and Brinkhurst, 1998). There has been some confusion about whether these represent two morphs of the same species (*S. lacustris*), making *Stylaria* a monotypic genus, or two distinct species (*S. lacustris* and *S. fossularis*). One report suggests that the distinguishing feature of the prostomium is phenotypically plastic, since its morphology is variable in culture (Di Persia, 1975). However, in sympatric populations of the two types, Timm (1997) found that the two morphs differed not only in prostomium morphology but also in chaetal morphology, and therefore concluded that the two were indeed distinct species. Our study included specimens of both *Stylaria* morphs collected from a single locality (Opeongo Lake, Ontario), and these were shown to be highly divergent in COI sequence (11%). Perhaps even more revealing, the individual of the *lacustris* morph from Ontario is clearly more closely related to other individuals of the *lacustris* morph from Iowa and even Europe than it is to the *fossularis* morph from the same Ontario locality (Figs. 2 and 3). This strongly suggests that the *lacustris* and *fossularis* morphs represent distinct species, although more extensive sampling of the two morphs will be necessary for a thorough investigation of species boundaries within *Stylaria*.

#### 4.3. Morphological evolution among the naidids

Naidids display a wide range of morphologies, which no doubt has contributed to the disparity of views on naidid relationships. Features such as the presence/absence of an elongated proboscis, of pigmented eye spots, and of a stomach dilation, as well as the shape and axial distribution of chaetae and the position and detailed anatomy of the reproductive system, all vary across the naidids in such a way as to suggest considerable homoplasy of these morphological features (Nemeč and Brinkhurst, 1987). Using the results of our molecular analyses, it is possible to begin to explore how the diverse morphologies among naidids have evolved.

One feature that varies dramatically among naidids is the shape of the prostomium, the asegmental cap of tissue at the anterior end of the animal. In most naidids (and, indeed, in most annelids), the prostomium is inconspicuous or at most takes the form of a small nub-like projection. However, in many species of *Pristina*, as well as in *Arcteonais*, *Ripistes*, and *Stylaria*, part of the prostomium is greatly elongated into a proboscis (Brinkhurst and Jamieson, 1971), the function of which is still obscure despite the fact that the structure can represent up to one fifth of the animal's body length in some species. Based on our phylogenetic results, it seems clear that a proboscis has evolved at least twice independently, once in the genus *Pristina* and a second time in the ancestor of *Arcteonais*, *Ripistes*, and *Stylaria* (Fig. 4). An elongated proboscis has evolved

independently in several other groups of oligochaetes (Brinkhurst and Jamieson, 1971). It should therefore be possible to investigate the function and evolutionary forces driving the evolution of this character through comparative studies.

Pigmented eye spots appear to be entirely absent among adult tubificids, the closest relatives of naidids, but are found on the lateral surface of the peristomium in a number of naidids (Brinkhurst and Jamieson, 1971). Among the genera in our dataset, eye spots are present in all or most species of *Arcteonais*, *Ripistes*, *Stylaria*, *Slavina*, *Vejdovskyella*, *Ophidonais*, and *Nais* (Fig. 4). Based on our results, and our expectation that it is easier to lose pigmented eyes than to gain them, it seems likely that pigmented eye spots evolved only once, in the common ancestor of the above genera, and were subsequently lost in the lineage leading to *Amphichaeta* and *Chaetogaster*, two genera which are highly derived in many respects (Sperber, 1948).

Although the presence/absence of pigmented eye spots is usually fixed within genera and species, *Nais*, *Slavina*, and *Vejdovskyella* include both eyed and eyeless species, and several species of *Nais* as well as the sole species of *Arcteonais* even vary intraspecifically with respect to this character (Brinkhurst and Jamieson, 1971). With two exceptions (the MD and NY representatives of *N. variabilis*), the individuals we sequenced from these four genera all possessed pigmented eyes; including eyeless taxa in future analyses is likely to reveal additional losses of eyes, which would require revision of our view of the evolution of eyes within this group. In the future, it will be important to determine whether eyelessness is genetically based or can be environmentally induced in intraspecifically variable species, and to investigate the possibility that some species or individuals possess eye spots which escape notice because they are not associated with obvious pigment, as was recently suggested, based on gene expression data, for a species of *Pristina* (Bely and Wray, 2001).

Among naidids, ciliated finger-like projections at the posterior end of the worm are unique to the genus *Dero*. These projections, referred to as gills (which are relatively short) and palps (which are greatly elongated), apparently function as accessory respiratory organs (Stephenson, 1930) and can take a variety of forms, which are important for taxonomic identification (Brinkhurst and Jamieson, 1971). Our sampling included multiple representatives from two of the three *Dero* subgenera (Table 1): *Dero (Dero)*, which possess anal gills but no palps, and *Dero (Aulophorus)*, which possess both anal gills and palps. (The third subgenus, *Allodero*, has no palps but some species possess gills; it is distinguished from *Dero (Dero)* based on other characteristics, such as the mode of fission.) In both our analyses, *Dero (Dero)* was recovered as a monophyletic

clade with high support, but *Dero (Aulophorus)* was paraphyletic with respect to *Dero (Dero)*, suggesting that the elongated palps characteristic of the *Aulophorus* subgenus may be plesiomorphic for the genus, or may have evolved independently multiple times. *Dero* is one of the most speciose naidid genera, with over 30 described species (Brinkhurst and Jamieson, 1971). Therefore a more detailed phylogeny of this genus, including representatives from the third subgenus, *Allodero*, is possible and should yield insights into the evolution of these accessory respiratory organs.

#### 4.4. Evolution of fission among naidids

In annelids, most occurrences of fission involve only one or a few closely related species nested within larger, strictly sexual groups. Naidids are unusual in being a very large fissiparous group: they include well over one hundred species, all apparently capable of fission (Brinkhurst and Jamieson, 1971). As a group, naidids display a remarkable diversity of modes of fission: species differ with respect to where along the body axis fission occurs, the relative sequence of morphogenesis of new tissue and physical separation of daughter zooids (“architomy” versus “paratomy”, see below), whether or not multiple fission zones can develop simultaneously (“fast” versus “slow” fission), and the relative placement of subsequently initiated fission events (i.e., “naidian” versus “stylarian” fission) (Dehorne, 1916). Naidids thus provide an exceptional opportunity to investigate how fission evolves and diversifies.

At the most basic level, reproduction by fission takes one of two forms: architomy (or fragmentation) in which an individual breaks into two (or more) pieces and each piece subsequently replaces the missing regions (i.e., a new head or tail), and paratomy (or budding), in which a new head and tail are intercalated in the middle of a worm’s body before it physically separates into two individual worms. Paratomy produces the unusual phenomenon of worm chains, in which multiple individuals are temporarily linked end to end, all the while being physiologically and behaviorally coordinated (e.g., Drewes and Fournier, 1993).

The developmental processes of naidid fission are very similar to those of regeneration (Bely and Wray, 2001; Dehorne, 1916; Stephenson, 1930), supporting the hypothesis that fission evolved from regeneration. But which form of fission, architomy or paratomy, evolved first? It has been suggested that within a lineage, architomic fission (i.e., fragmentation) evolves first, and that paratomy is derived from architomy (Von Kennel, in Morgan, 1901). This hypothesis is intuitively appealing since architomic fission requires little more than regenerative capabilities, while paratomic fission would seem to require the evolution of a number of additional

developmental and physiological specializations, such as the capability to intercalate new tissue in the middle of a differentiated body, and to have this new tissue develop while it is traversed by a functional nerve cord, gut, and blood vascular system. Despite its intuitive appeal, this “architomy first” hypothesis has remained completely untested.

Among naidids, architomy is far less prevalent than paratomy (Brinkhurst and Jamieson, 1971; Stephenson, 1930). In our dataset, it is found only in *Allonais* (in which all species are known or thought to undergo architomy) and *Dero* (in which most species, including all those we sampled, undergo paratomy, but a few species, such as all species in the subgenus *Allodero*, undergo architomy). (Although some species placed in the genus *Slavina* reproduce by architomy, these may not actually be closely related to *Slavina appendicula* (Nemec and Brinkhurst, 1987; Sperber, 1948), the paratomically reproducing species included in this study.) Interestingly, *Dero* and *Allonais* fall near the base of the naidid tree, and represent two of the most basally branching genera of the Naidinae sensu Lastoĉkin (i.e., non-*Pristina*) clade in our analyses (Fig. 4). Especially if *Pristina* is found to represent an independent origin of fission from the rest of the naidids (a possibility suggested by the preliminary findings of Erséus et al. (2002)), then the architomy seen in *Allonais* and/or *Dero* could be plesiomorphic for the Naidinae, providing support for the “architomy first” hypothesis. In order to confirm this, it will be necessary to better resolve the basal branches within the naidids, and to reconstruct the ancestral type of fission for the genus *Dero*, in which both architomy and paratomy are found. Finally, it will be critical to determine whether the naidids are polyphyletic, since if they do represent multiple origins of fission, the “architomy first” hypothesis would predict that architomy is the ancestral condition for *each* clade that evolved fission.

As for the particular form of paratomy, and the exact location along the body axis at which fission takes place, these features differ widely among naidids. For example, in some species the fission zone always forms in the same segment number within a worm (“naidian fission”) whereas in others new fission zones are formed at progressively more anterior locations (“stylarian fission”), causing the parental worm to become progressively shorter after each round of fission, until it eventually stops fissioning temporarily to replace lost segments through growth (Dehorne, 1916). However, because paratomy has been studied in detail in only a handful of species, and even basic information on fission is lacking for many species, and even entire genera, it is premature to attempt to reconstruct the evolution of these features among the naidids. Once fission is investigated in more detail in a greater number of species, placing such information in a phylogenetic context promises to greatly

expand our understanding of how asexual reproduction by fission has evolved and diversified.

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