Zoological Journal of the Linnean Society, 2008, **154**, 1–26. With 7 figures

Combined-data phylogenetics and character evolution of Clitellata (Annelida) using 18S rDNA and morphology

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Received 30 May 2007; accepted for publication 17 September 2007

This is the first phylogenetic analysis of Clitellata using 18S rDNA in combination with morphological data of a selection of species representing Hirudinida, Acanthobdellida, Branchiobdellida, and eight oligochaetous families. The morphological data set includes 48 somatic (light-microscopical) and 34 spermatozoal (ultrastructural) characters. Eight new sperm models belonging to Lumbriculidae (two), Enchytraeidae (two), Phreodrilidae (one), and Tubificidae (three) are compared with the spermatozoal pattern already described among Clitellata. Somatic characters for each species are extracted from both general literature and the original species description. One new 18S sequence of Lumbriculidae and two of Tubificidae are reported, and are aligned together with corresponding sequences of 36 previously studied clitellate taxa. Two polychaete species are used as outgroups. The phylogenetic trees recovered using parsimony and Bayesian inference as optimization criteria of both individual and combined data sets yield largely consistent results. Our combined-data phylogenetic analysis is congruent with recent molecular studies. Somatic and spermatozoal characters contribute to the 18S rDNA phylogeny under both optimization criteria: in resolving the 18S topology, in adding new nodes, and in increasing the support for many groups. Morphological characters in combination with 18S rDNA suggest the following sister-group relationships: (1) between *Acanthobdella* and Hirudinida, with Branchiobdellida as their plesiomorphic sister group, and (2) between enchytraeids and *Propappus*, with both taxa grouping at the base of a large assemblage containing Lumbricidae, Lumbriculidae, Branchiobdellida, *Acanthobdella*, and Hirudinida. Maximum parsimony and maximum likelihood ancestral character state reconstructions on the combined-data tree indicate a new set of somatic and spermatozoal autapomorphies, and propose new evolutionary trends of somatic and spermatological characters. The observed complexity of the spermatozoal characters patterns among oligochaetous clitellates is discussed. This analysis supports a trend from primarily aquatic forms, with bifid chaetae indefinite in number, towards a more terrestrial mode of life leading to a simplification of the chaetae, thus supporting the hypothesis that the first clitellate was an aquatic form.

ADDITIONAL KEYWORDS: ancestral character state reconstruction – Bayesian inference – parsimony – phylogeny – spermatozoon – systematic – ultrastructure.

INTRODUCTION

Clitellata is a group of annelids that are characterized by their hermaphroditic modality of reproduction, and comprise around 5000 of the over 15 000 species of segmented worms described (Erséus, 2005). The monophyly of clitellates and of several of their constituent taxa is supported by a wide set of data, including general morphology (Purschke *et al.*, 1993; Nielsen, 1995), sperm ultrastructure (Ferraguti, 2000), and DNA sequences (McHugh, 1997; Siddall *et al.*, 2001; Erséus & Källersjö, 2004).

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Within Clitellata the ultrastructure of spermatozoa has proved useful for phylogenetic assessment, not only at high taxonomic levels (Jamieson, Erséus & Ferraguti, 1987; Ferraguti & Erséus, 1999), but also at lower taxonomic levels, among species of branchiobdellids (Cardini & Ferraguti, 2004), enchytraeids (Westheide, Purschke & Middendorf, 1991), and tubificids (Marotta, Ferraguti & Erséus, 2003). Nevertheless, in a study of tubificid relationships based on sperm morphology, Erséus & Ferraguti (1995) pointed out that at low taxonomic levels 'the spermatozoal character patterns are complex and contain elements of convergence and probably also reversal, and therefore they should be used . . . [in tubificids] . . . only in combination with other information'.

In recent years, molecular studies have played a double role with respect to the traditional view of the relationships among clitellates (Jamieson, 2006). On one hand, molecular data have corroborated previous hypotheses based on morphology, at different hierarchical levels. For instance, separately or in combination, several genes have suggested the inclusion of the former aquatic family Naididae within Tubificidae, as a subfamily renamed Naidinae (Christensen & Theisen, 1998; Erséus & Gustavsson, 2002; Sjölin, Erséus & Källersjö, 2005; Envall, Källersjö & Erséus, 2006), as was first hypothesized on morphological grounds by Stephenson (1930), who suggested a tubificid-like ancestor for naidines. Furthermore, combined 18S rDNA and cytochrome oxidase I (COI) sequences corroborated the hypothesis that hirudineans, acanthobdellids, and branchiobdellids comprise a monophyletic group within the oligochaete clade (Martin, 2001; Siddall *et al.*, 2001), as was previously indicated by phylogenetic analyses based on morphological data (Purschke *et al.*, 1993; Brinkhurst, 1994).

On the other hand, molecular data are sometimes in conflict with the hypotheses suggested by traditional morphology (Jamieson, 1988; Jamieson, 2006), mainly concerning the basal diversification of Clitellata. Enchytraeidae, one of the largest oligochaetous clitellate families, abundant in terrestrial as well as freshwater and marine habitats, has been considered to be at the base of Tubificidae and other oligochaetes based on general morphology (Jamieson, 1988) and sperm ultrastructure (Ferraguti & Erséus, 1999; Jamieson, 1983). However, analyses using nuclear 18S rDNA and mitochondrial COI data have suggested that Enchytraeidae is a more derived group, with an unexpected suggestion that this family is closely related to earthworms (Siddall *et al.*, 2001; Erséus & Källersjö, 2004). Moreover, *Propappus*, traditionally grouped together with Enchytraeidae (Michaelsen, 1916; Timm, 1981), appears well separated from enchytraeids in the 18S analysis of Erséus & Källersjö (2004), as was also suggested on morphological grounds by Coates (1986). Finally, *Acanthobdella peledina* was considered to be the sister species of the Hirudinida, in the light of morphology (Purschke *et al.*, 1993), but was placed as the sister group of a clade containing both Hirudinida and Branchiobdella by 18S rDNA and COI studies (Siddall *et al.*, 2001; Erséus & Källersjö, 2004).

The present paper is the first phylogenetic study of broadly sampled clitellates using a total evidence approach. The use of morphological data in combination with molecular data has proved successful in resolving the phylogenies of other animal groups (Wahlberg *et al.*, 2005) because, as pointed out by Chippindale & Wiens (1994), 'unique phylogenetic relationships can emerge when data from different sources are combined into a single analysis'. In this paper, we explore 18S rDNA gene sequences, general somatic characters, and sperm ultrastructure of 39 species belonging to 13 different clitellate families. Our taxon sampling constitutes a representation of the total variability among clitellates, with a clear emphasis on 'microdriles', i.e. the largely aquatic non-earthworm groups. Our aim is to generate a well-corroborated phylogenetic hypothesis, which can be used as an explanation model for the evolution of individual morphological characters in clitellates.

MATERIAL AND METHODS

Specimen collection

The eight species for which the sperm ultrastructure is newly described in this study (Table 1) come from several sources. *Eremidrilus coyote* and *Eremidrilus frigidus* were collected in Coyote Creek (above Gilrog Hot Spring), California, by Steven V. Fend (US Geological Survey, Menlo Park, CA, USA) in 2001; *Fridericia discifera* and *Fridericia montafonensis* were collected by Rüdiger Schmelz in Vorarlberg, Schruns, and Tschagguns village meadow, Austria, in 1995, and in Montafon, Austria, in 1997, respectively; *Insulodrilus bifidus* and *Ainudrilus nharna* were collected from Bow River, Western Australia, by Adrian Pinder (Department of Conservation and Land Management, Wanneroo, Western Australia) in 2000; *Uncinais uncinata* was collected by Christer Erséus in the Säveån river, at Grevasågen sawmill (Västergötland, southwestern Sweden) in 1997. The animals were fixed in a picric acid, paraformaldehyde, glutaraldehyde mixture (Ermak & Eakin, 1976), postfixed in 2% osmium tetroxide, stained *en bloc* in uranyl acetate in 70% ethanol, and embedded in an Epon–Araldite mixture. Ultrathin sections were observed under a Jeol 100SX transmission electron microscope and a Leo 2012 AB transmission electron microscope after staining in lead citrate.

Table 1. List of ingroup and outgroup taxa included in this study, with references to morphological descriptions and GeneBank Accession Numbers for 18S rDNA sequences

Taxon Ingroup taxa	18S rDNA	Sperm ultrastructure	Morphology
Lumbricidae			
Dendrobaena sp.	AJ272527 (*D. clujensis*)	Jamieson *et al.* (1983) (*D. octaedra & D. subrubicunda*)	Jamieson (1988)
Lumbricus castaneus	AF209458	Jamieson *et al.* (1983)	Jamieson (1988)
Lumbricus terrestris	AJ272183		Jamieson (1988)
Lumbricus rubellus	Z83753	Jamieson *et al.* (1983)	Jamieson (1988)
Lumbriculidae			
Eremidrilus coyote	EU126844 (new sequence)	Present study	Fend & Rodriguez (2003)
Eclipidrilus frigidus	AY040692	Present study	Cook (1968), Brinkhurst (1998)
Rhynchelmis alyone	AJ252317	Martin Ferraguti & Kaygorodova (1998)	Martin *et al.* (1998)
Branchiobdellidae			
Branchiobdella sp.	AF310690 (*B. parasita*)	Cardini & Ferraguti (2000) (*B. orientalis*)	Holt (1986)
Xironogiton victoriensis	XVZ83756	Cardini & Ferraguti (2000)	Gelder & Hall (1990)
Cirrodrilus sp.	AF310698 (*C. sapporensis*)	Cardini & Ferraguti (2000) (*C. kawamurai*)	Gelder (1987)
Acanthobdellida			
Acanthobdella peledina	AF099948	Franzén (1991), Westheide & Purschke (1996)	Purschke *et al.* (1993)
Hirudinida			
Piscicola geometra	AF099946	Wissocq & Malécha (1975), Malécha (1975)	Siddall & Burreson (1995)
Hirudo medicinalis	HMZ83752	Wissocq & Malécha (1975)	Siddall & Burreson (1995)
Erpobdella octoculata	AF099949	Wissocq & Malécha (1975)	Siddall & Burreson (1995)
Enchytraeidae			
Enchytraeus albidus	AY040683	Westheide *et al.* (1991)	Nielsen & Christensen (1959), Stephenson (1930)
Fridericia sp.	AF209453 (*Fridericia tuberosa.*)	Present study (*F. discifera & F. montafonensis*)	Healy (1975), Schmelz (1998)
Phreodrilidae			
Insulodrilus bifidus	AF411906	Present study	Pinder & Brinkhurst (1997)
Haplotaxidae			
Haplotaxis. sp.	AY365456 (*H. gordioides*)	Jamieson (1982) (*H. ornamentus*)	Brinkhurst & Fulton (1980)
Tubificidae			
Rhyacodrilinae			
Ainudrilus sp.	AF411871 (*A. paucisetis*)	Present study (*A. nharna*)	Pinder & Brinkhurst (2000)
Heterodrilus minisetosus	AF411885	Erséus & Ferraguti (1995)	Erséus (1981a)
Phallodrilinae			
Olavius sp.	AF411892 (*O. vacuus*)	Ferraguti *et al.* (1994) *Olavius planus*	Erséus (1979, 1984)
Inanidrilus leukodermatus	AF209456	Ferraguti *et al.* (1994)	Erséus (1984)
Pectinodrilus molestus	AF209462	Erséus & Ferraguti (1995)	Erséus (1988, 1992)
Bathydrilus formosus	AF411889	Ferraguti *et al.* (1989)	Erséus (1988)
Limnodriloidinae			
Limnodriloides monothecus	AF411896	Marotta *et al.* (2003)	Erséus (1982a)
Limnodriloides barnardi	AF411894	Marotta *et al.* (2003)	Erséus (1982a)
Smithsonidrilus westoni	AF411902	Marotta *et al.* (2003)	Erséus (1982b)
Smithsonidrilus hummelincki	AF209465	Erséus & Ferraguti (1995), Marotta *et al.* (2003)	Erséus (1990b)
Thalassodrilides ineri	AF411905	Ferraguti *et al.* (1989)	Righi & Kanner (1979)
Thalassodrilides gurwitschi	AF209466	Marotta *et al.* (2003)	Erséus (1981b)
Thalassodrilides bruneti	AF411904	Marotta *et al.* (2003)	Erséus (1990b)
Doliodrilus chinensis	EU126845 (new sequence)	Marotta *et al.* (2003)	Wang & Erséus (2004)
Tubificinae			
Tubifex tubifex	EU126846 (new sequence)	Braidotti & Ferraguti (1982)	Holmquist (1983)
Clitellio arenarius	AF411863	Ferraguti & Ruprecht (1992)	Gustavsson (1995)
Naidinae			
Uncinais uncinata	AY040700	Present study	Sperber (1948)
Paranais frici	DQ459981	Ferraguti *et al.* (1999)	Sperber (1948)
Stylaria lacustris	DQ459973	Ferraguti *et al.* (1999)	Sperber (1948)
Propappidae			
Propappus volki	AY365457	Gustavsson *et al.* (2008)	Coates (1986)
Capilloventridae			
Capilloventer australis	AY365455	Ferraguti *et al.* (1996)	Erséus (1993)
Outgroup taxa			
Arenicolidae			
Arenicola marina	AJ310502	Rouse (1999)	Rouse & Pleijel (2001)
Amphinomidae			
Eurythoe complanata	AY364851	Rouse (1999)	Rouse & Pleijel (2001)

The nearly complete sequences of the 18S rDNA gene of three species were newly obtained for this paper (Table 1). *Eremidrilus coyote* was collected in Coyote Creek below Madrone Soda Springs, Santa Clara Co., California, USA by S. V. Fend in 2003; *Doliodrilus chinensis* was collected in a brackish-water fish pond on the road to Teng Hai, east of Sanya City, Hainan, China by Hongzhu Wang and C. Erséus in 2000, and *Tubifex tubifex* was obtained from a worm culture ('Kultuur B178', originally from Frunze, Kyrgyzstan Republic, Central Asia) maintained in Vörtsjärv Limnological Station, Estonia by Tarmo Timm (collection 2000). DNA was extracted from single specimens, or specimen parts, with the Qiagen Tissue kit according to the manufacturer's instructions. Both strands of DNA were sequenced for all taxa. A nested PCR and sequencing were performed as described previously (Erséus *et al.*, 2002).

Taxa studied

The ingroup and outgroup taxa used for the phylogenetic analyses are listed in Table 1. As Clitellata is probably nested inside Polychaeta, and there is no strongly supported sister group to the Clitellata among the polychaete families (McHugh, 2000; Rota, Martin & Erséus, 2001; Struck, Westheide & Purschke, 2002; Erséus & Källersjö, 2004; Rousset *et al.*, 2007), we selected one 'errant' (*Eurythoe complanata*, Amphinomidae) and one 'sedentary' (*Arenicola marina*, Arenicolidae) polychaetes as outgroups to root the trees, with the considered 18S rDNA, somatic, and ultrastructural data being available for both considered species (see Table 1).

Character sets

Three different character sets were used in this study, and are referred to as the '18S rDNA', 'somatic', and 'spermatozoal' characters. In addition to the three new sequences, GenBank data on the almost complete sequence of the 18S rDNA gene for 39 representatives of Clitellata, as well as two polychaete species, were included in this analysis (Table 1). Forty-eight somatic characters, all treated as unordered, were considered (Appendix 1). Of the eighteen characters concerning external morphology, eight refer to the morphology of chaetae. The other thirty characters deal with internal morphology; twenty of them concern the morphology of the reproductive system. Thirty-four spermatozoal characters, all treated as unordered, were considered (Appendix 2 and Fig. 1): they concern different regions of the sperm cells. Of these, 30 refer to euspermatozoa (i.e. fertilizing sperm, characters 1–30), two to paraspermatozoa (i.e. unfertilizing sperm, characters 33–34), and two other characters deal with the presence/absence of a double sperm line (character 31), and with the various ways in which spermatozoa aggregate to form spermatozeugmata (character 32).

As far as possible, DNA data were collected from the same species for which we have morphological and ultrastructural data. In seven cases, however, we were forced to combine the 18S data of one species with the somatic and spermatological data of another species belonging to the same genus. In these cases, the sperm ultrastructure is homogeneous among different species belonging to the same genus. For instance, we combined the 18S of *Dendrobaena clujensis* with the sperm ultrastructure of *Dendrobaena octaedra* and *Dendrobaena subrubicunda*. As both species have very similar sperm, we assumed that the sperm of *D. clujensis* is similar too, and thus we combined the data from the three taxa.

We used the 18S of *Haplotaxis* cf. *gordioides*, a North American form, and the sperm ultrastructure of *Haplotaxis ornamentus*, a Tasmanian (Australia) haplotaxid species for which such data are known. To be able to analyze the controversial position of the family Haplotaxidae, we included *Haplotaxis* in our data set, although Haplotaxidae is possibly a paraphyletic assemblage of several old lineages (Erséus & Källersjö, 2004), and the two haplotaxid species considered here may even belong to completely different lineages.

Morphological characters for each species were extracted from both general literature (Stephenson, 1930; Brinkhurst & Jamieson, 1971) and, when possible, original species descriptions (see list of taxa and references in Table 1).

Alignment and phylogenetic analysis

Sequences were aligned using ClustalX, v1.8 (Thompson *et al.*, 1997) with default settings; in a previous study of clitellate relationships based on 18S rDNA analyses (Erséus, Prestegaard & Källersjö, 2000), well-supported groups proved to withstand a rather wide range of different alignment parameters.

Each data set and a combination of all data sets were analyzed by parsimony, using PAUP, v4.0b10 (Swofford, 2002), with the following heuristic search settings: random-addition sequence with 1000 replicates, tree-bisection-reconnection (TBR) branch swapping, MulTrees option on, swap on the best tree only, and collapse branches if minimum length is zero (amb –). The complete data set is also analyzed by Bayesian inference, using Mr Bayes 3.1 (Ronquist & Huelsenbeck, 2003). The DNA substitution models for the 18S rDNA analysis was selected with the likelihood-ratio test, as implemented in Modeltest 3.06 (Posada & Crandall, 1998). The general-time

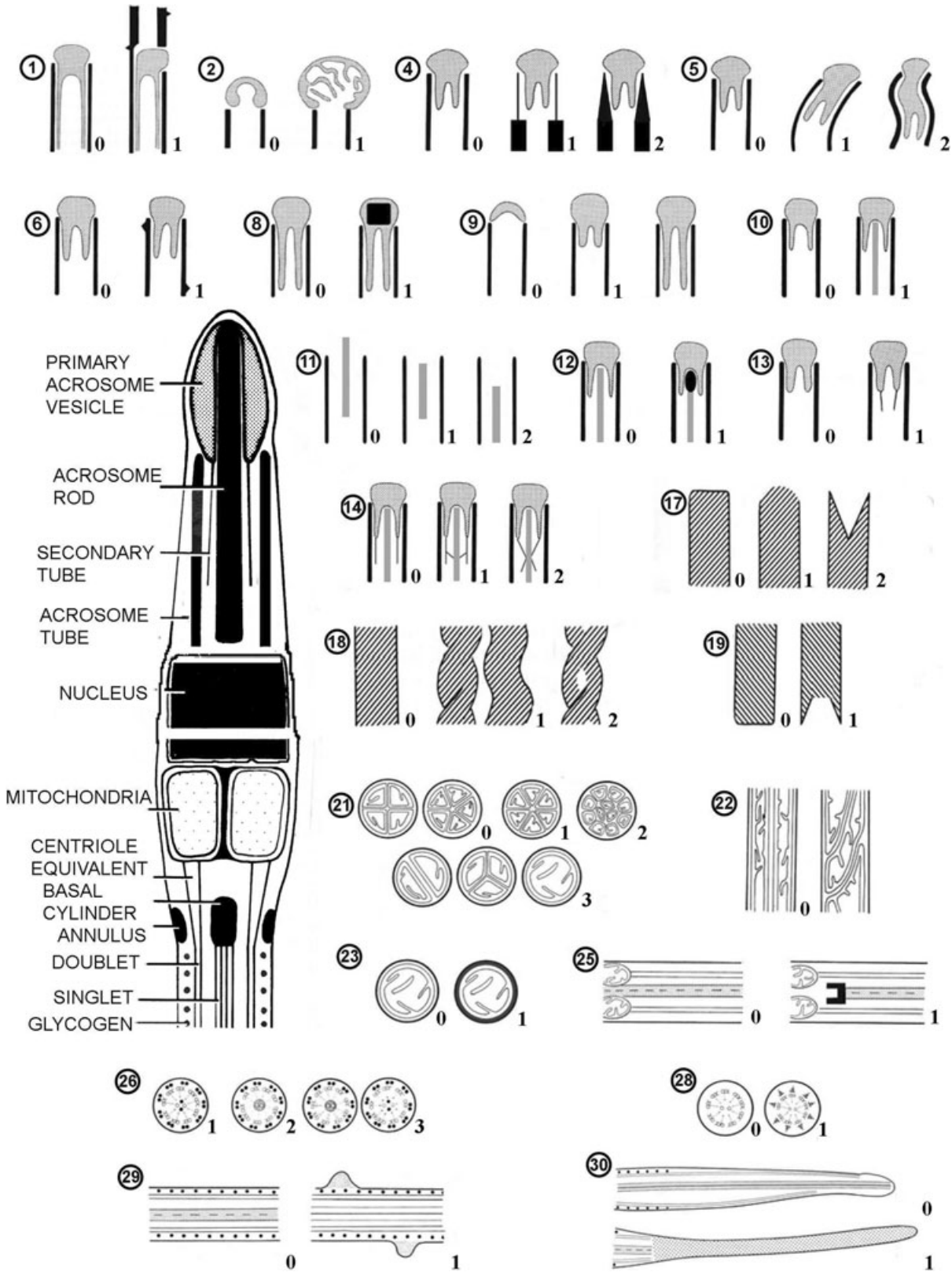


Figure 1. Schematic representation of some of the 34 considered spermatozoal characters. Inset, hypothetical plesiomorphic spermatozoon for the Clitellata, as inferred from ancestral-state reconstruction analysis (modified from Jamieson *et al.*, 1987).

reversal (GTR) model, with an estimate of invariable site (I) and a discrete (four rate categories) Γ-distribution model among site rate heterogeneity, was selected for the combined data set, both when analyzed alone and when in combination with the other data sets. For the somatic and spermatological data sets the Mk (Markov K) model (Lewis, 2001) was selected, with equal state frequencies and Γ-distributed rates across characters. For each of the four data sets three replicate analyses were performed. Each analysis was initiated from a random starting tree, and the program was set to run four (three heated and one cold) Markov chain Monte Carlo iterations simultaneously for at least three million generations, with trees sampled every 100th generation. The trees generated before the stabilization (burn-in) were discarded, and posterior probabilities for clades were estimated by a majority-rule consensus tree based on the saved trees used to indicate branch support.

Nodal support for the combined data set, as well as for the 18S rDNA and morphological data sets, was evaluated under the parsimony criterion with a bootstrap analysis (Felsenstein, 1985), using PAUP. The support values were estimated with 1000 bootstrap replicates, each with five random additions of taxa.

To assess – using the combined data set – whether the Bayesian topology is significantly different from that generated by the parsimony analysis, constrained analyses were performed using maximum parsimony (MP): the lengths, and characters changes, of the most parsimonious solutions were then compared against the unconstrained MP solution, and Templeton's test (Templeton, 1983) was run, as implemented in PAUP. To determine whether the molecular data could discriminate between the total evidence hypotheses obtained using different optimization criteria, the Kishino-Hasegawa (KH) test was performed as implemented in PAUP (Kishino & Hasegawa, 1989).

Additional phylogenetic analyses were performed to check the effect of combining the 18S rDNA of one species with the morphological data of another species belonging to the same genus. We ran new combined-data parsimony and Bayesian analyses with the same search settings (trees not shown), but including only the 18S rDNA for the taxa where we combined molecular data with morphological data. The morphological characters for these taxa were coded as missing (?). The resulting phylogenetic trees were largely congruent with those obtained from our previous analyses, thus corroborating our results.

Parsimony unequivocal reconstruction of somatic and spermatozoal characters, all treated as unordered, was performed on the combined-data set Bayesian tree using Mesquite (Maddison & Maddison, 2006). Likelihood somatic and spermatozoal apomorphic trends and ancestral state were reconstructed using the MK1 model (Lewis, 2001), the rate of change being estimated on the character distribution on a corrected combined-data Bayesian topology. Indeed, to avoid wrong interpretations, we decided to exclude *I. bifidus* and *Haplotaxis* sp. from the uncorrected topology, because both taxa were grouped in an unsupported position at the base of the tree that was not corroborated by other phylogenetic analyses.

RESULTS

Analysis of individual data sets

18S rDNA data

The three Bayesian inference runs resulted in identical majority-rule consensus trees (Fig. 2), with small variations in posterior probabilities for each clade, and were congruent with the results obtained by the parsimony analysis (tree not shown). This tree identified 35 groups, with 27 supported by a posterior probability of ≥ 0.85 in all runs, and it yielded strong support for the monophyly of the clitellate ingroup as a whole, as well as for the following higher taxa within Clitellata: Enchytraeidae, Lumbricidae, Branchiobdellida, Hirudinida, and Tubificidae (all with posterior probabilities of 1.00). The relationship between Lumbriculidae and *Acanthobdella* (posterior probability for lumbriculids + *Acanthobdell*a, 0.99–1.00) was, however, unresolved (see Table 2). With regard to the basal branching of Clitellata, the capilloventrid *Capilloventer australis* is the sister group of all other considered clitellates (posterior probability 1.00). The other clitellates, with the exclusion of the phreodrilid *I. bifidus* and the haplotaxid *Haplotaxis* sp., group into two main clades: (1) an aquatic 'microdrile' clade consisting of Tubificidae, including Naidinae (posterior probability 1.00), and (2) a clade (posterior probability 1.00), consisting of *Propappus*, Enchytraeidae, Lumbricidae, Lumbriculidae (unresolved from *Acanthobdella*), Branchiobdellidae, and Hirudinida, arranged in this nesting order on the tree. Inside Tubificidae, Tubificinae (posterior probability 0.91–0.94) and Limnodriloidinae (posterior probability 1.00) are monophyletic groups. The rhyacodrilines *Ainudrilus* sp. and *Heterodrilus minisetosus* group, respectively, with the naidines (posterior probability 1.00) and the phallodriline species considered, except *Bathydrilus formosus* (also 1.00); these two assemblages may be sister groups, but this has a posterior probability < 0.85.

Morphological data: somatic and spermatological characters

The results of the Bayesian analysis of the somatic and spermatological data sets (Fig. 3) are largely con-

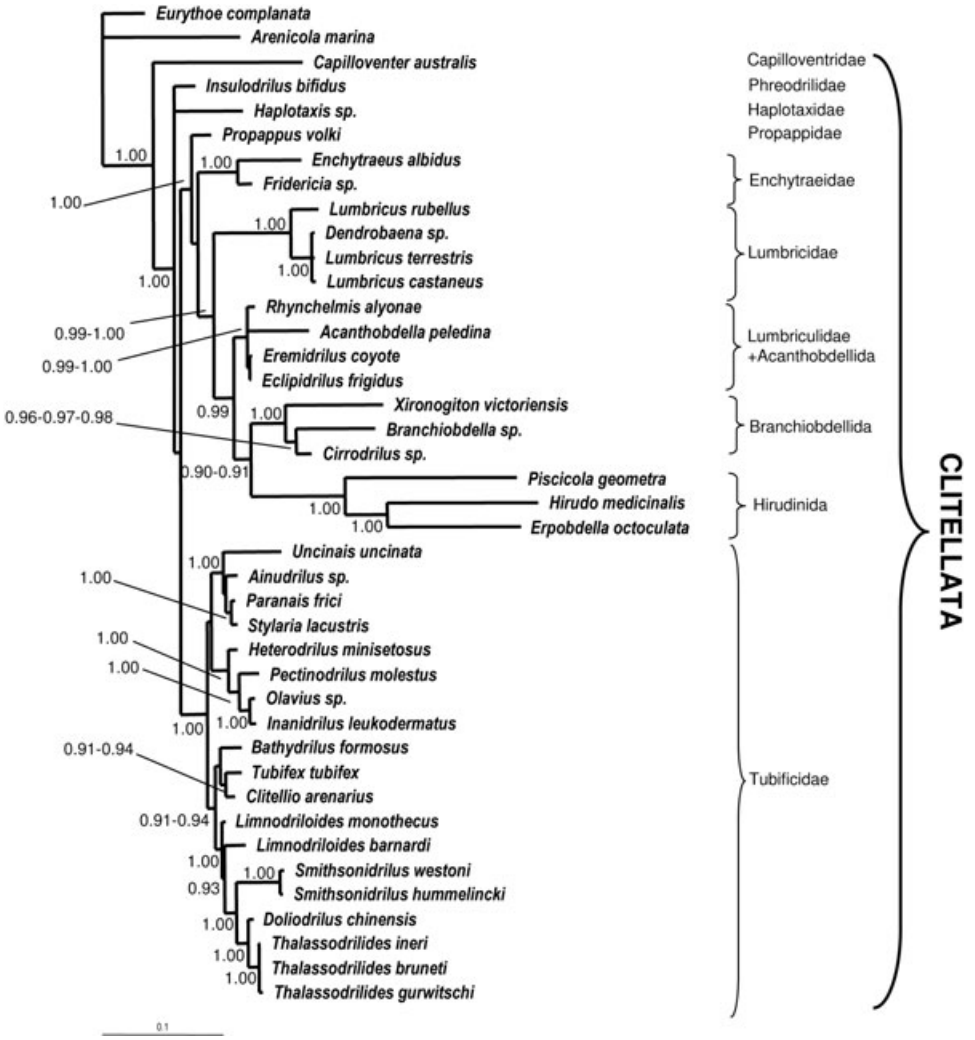


Figure 2. Phylogenetic tree obtained from one of the three replicate Bayesian inference runs of the 18S rDNA sequences. Posterior probabilities ≥ 0.85 are indicated in front of the nodes.

sistent with those obtained by parsimony analysis (not shown). The three runs of each of the somatic and spermatological data sets resulted, respectively, in one identical majority-rule consensus tree, with only small variations in the posterior probabilities for each clade. The two Bayesian trees (Fig. 3A,B) are poorly resolved, with only ten (somatic) and thirteen (spermatozoal) nodes with posterior probabilities of ≥ 0.85 (see Table 2). Both data sets strongly support the monophyly of clitellates (posterior probability 1.00), and inside clitellates support Hirudinida (posterior probability 0.98 and 1.00, respectively), Lumbricidae (posterior probability 1.00 and 0.91, respectively) and Enchytraeidae (posterior probability of 0.97 and 0.88, respectively). The somatic and spermatozoal characters also support the sister-group relationship between Hirudinida and *Acanthobdella* (posterior probability of 1.00 and 0.92–0.93, respectively); note that this was not supported by the 18S data set (see above). Somatic characters alone support Branchiobdellida (posterior probability 0.96) and Limnodriloidinae (posterior probability 0.99–1.00). Spermatological characters alone support Lumbriculidae (posterior probability 0.90–0.91) and the monophyly of Naidinae (posterior probability 0.86–0.87).

Analysis of the combined data set

Parsimony analysis

The combined data set contains 2062 characters, and 448 of them are parsimony informative. The parsimony analysis yielded four equally most parsimonious trees (MPTs); the consensus of them is shown in Figure 4. Each MPT is 1962 steps long, with a consistency index (CI), excluding parsimony uninformative characters, of 0.45, and a retention index (RI) of

Table 2. Status and support values obtained for some of the clades resulting from phylogenetic analyses of Clitellata using 18S rDNA, somatic, and spermatological data sets, with different optimality criteria. Values shown are bootstrap support (> 50%) and posterior probabilities (> 0.85)

	Parsimony – Bayesian analysis			
Taxa	18S rDNA	Somatic characters	Spermatological characters	Total evidence
Enchytraeidae	*100 – 1.00*	*85 – 0.97*	*66 – 0.88*	*100 – 1.00*
Lumbricidae	*100 – 1.00*	*100 – 1.00*	*87 – 0.91*	*100 – 1.00*
Lumbriculidae	*80 – unresolved*	*67 – unsupported*	*65 – 0.90–0.91*	*99 – 1.00*
Branchiobdellida	*98 – 1.00*	*90 – 0.96*	*unresolved – unresolved*	*100 – 1.00*
Hirudinida	*100 – 1.00*	*92 – 0.98*	*97 – 1.00*	*100 – 1.00*
Tubificidae	*98 – 1.00*	*unresolved – unresolved*	*unresolved – unresolved*	*100 – 1.00*
Rhyacodrilinae	*unresolved – unresolved*	*unresolved – unresolved*	*unresolved – unresolved*	*unresolved – unresolved*
Phallodrilinae	*unresolved – unresolved*	*56 – unsupported*	*unresolved – unresolved*	*unresolved – unresolved*
Limnodriloidinae	*82 – 1.00*	*78 – 0.99–1.00*	*unresolved – unresolved*	*99 – 1.00*
Tubificinae	*paraphyletic – 0.91–0.94*	*unresolved – unresolved*	*unresolved – unresolved*	*unsupported – 1.00*
Naidinae	*unresolved – unresolved*	*unresolved – unresolved*	*unresolved – 0.86–0.87*	*65 – 0.95*
Naidinae + *Ainudrilus*	*85 – 1.00*	*unresolved – unresolved*	*unresolved – unresolved*	*93 – 1.00*
Gutless Phallodrilinae + *Heterodrilus*	*94 – 1.00*	*unresolved – unresolved*	*unresolved – unresolved*	*85 – 1.00*
Tubificinae + Limnodriloidinae	*unresolved – unresolved*	*unresolved – unresolved*	*unresolved – unresolved*	*unsupported – 0.97*
Propappus + Enchytraeidae	*unresolved – unresolved*	*unresolved – 0.97*	*unresolved – unresolved*	*unresolved – 0.86*
Enchytraeidae + *Propappus* + Lumbricidae + Lumbriculidae + leech-like taxa	*unsupported – 1.00*	*unresolved – unresolved*	*unresolved – unresolved*	*unresolved – 1.00*
Lumbricidae + Lumbriculidae + leech-like taxa	*unresolved – 0.99–100*	*unresolved – unresolved*	*unresolved – unresolved*	*unresolved – 1.00*
Lumbriculidae + *leech-like* taxa	*unresolved – 0.99*	*unresolved – unrsolved*	*unresolved – 0.97–0.98*	*83 – 1.00*
Acanthobdellida + Hirudinida	*unresolved – unresolved*	*79 – 1.00*	*unresolved – 0.92–0.93*	*82 – 1.00*
Propappus + Enchytraeidae + Lumbricidae + Lumbriculidae + leech-like taxa + Tubificidae	*unsupported – unsupported*	*unresolved – unresolved*	*unresolved – unresolved*	*unsupported – unsupported*
Capilloventer australis + other clitellates	*82 – 1.00*	*unresolved – unresolved*	*unresolved – unresolved*	*85 – 0.99–1.00*

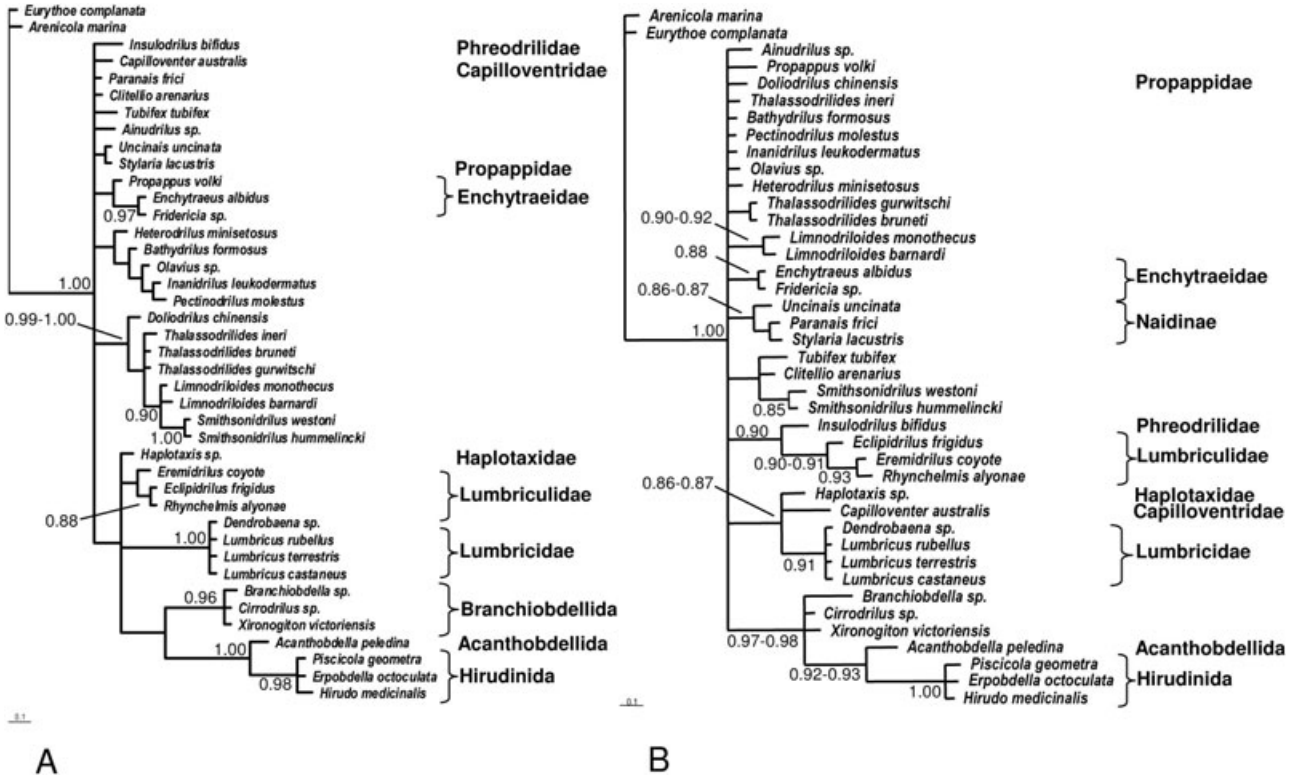


Figure 3. Phylogenetic trees obtained from morphological data. A, phylogenetic tree obtained from one of the three replicate Bayesian inference runs of the somatic data set. Posterior probabilities of ≥ 0.85 are indicated in front of the nodes. B, phylogenetic tree obtained from one of the three replicate Bayesian inference runs of the spermatozoal data set. Posterior probabilities ≥ 0.85 are indicated in front of the nodes.

0.64. The consensus tree (Fig. 4) identified 26 groups supported by bootstrap frequencies over 50% (the most important of these groups are listed in Table 2). Most of the well-supported nodes from the parsimony analysis are recovered in the Bayesian inference, with a few main contradictions (see Table 2). The parsimony analysis resulted in the placement of Lumbricidae and *Propappus* differing from that of the Bayesian analysis (see below), but these positions have no support. The basal branching of Tubificidae is in many ways also different from that obtained by the Bayesian analysis, but none of this has a bootstrap support ≥ 50%.

Bayesian inference

The three runs of the combined data set resulted in an identical majority-rule consensus tree, with small variation in posterior probabilities for each clade. The results of this analysis are largely consistent with those obtained from the analysis of 18S rDNA: most of the well-supported nodes from the Bayesian analysis of the 18S data set are recovered with high support by the total-evidence analysis (see Table 2). However, the total-evidence tree (Fig. 5) differs from that obtained with the 18S rDNA data alone in three main points. First, *Propappus* and Enchytraeidae are sister taxa, with some support (posterior probability 0.86), and they are placed as the sister (with posterior probability 1.00) to the Lumbricidae + Lumbriculidae + Hirudinida assemblage. Second, the lumbriculid species considered form a well-supported clade (posterior probability 1.00), and *Acanthobdella* is the sister group to Hirudinida, with strong support (posterior probability 1.00). Third, inside Tubificidae, the Naidinae (i.e. *Paranais*, *Uncinais*, and *Stylaria*) forms a clade (posterior probability 0.95–0.96), and Tubificinae (*Tubifex* and *Clitellio*) and Limnodriloidinae (from *Limnodriloides* through to *Thalassodrilides*) are sister groups, supported by a posterior probability of 1.00.

Constraint analyses

The Templeton's test on the combined data set indicated that the Bayesian topology, which was six steps longer, was not significantly different ($P < 0.05$) from the unconstrained MP solution. Both KH and Templeton's tests on the 18S data set indicated that the two

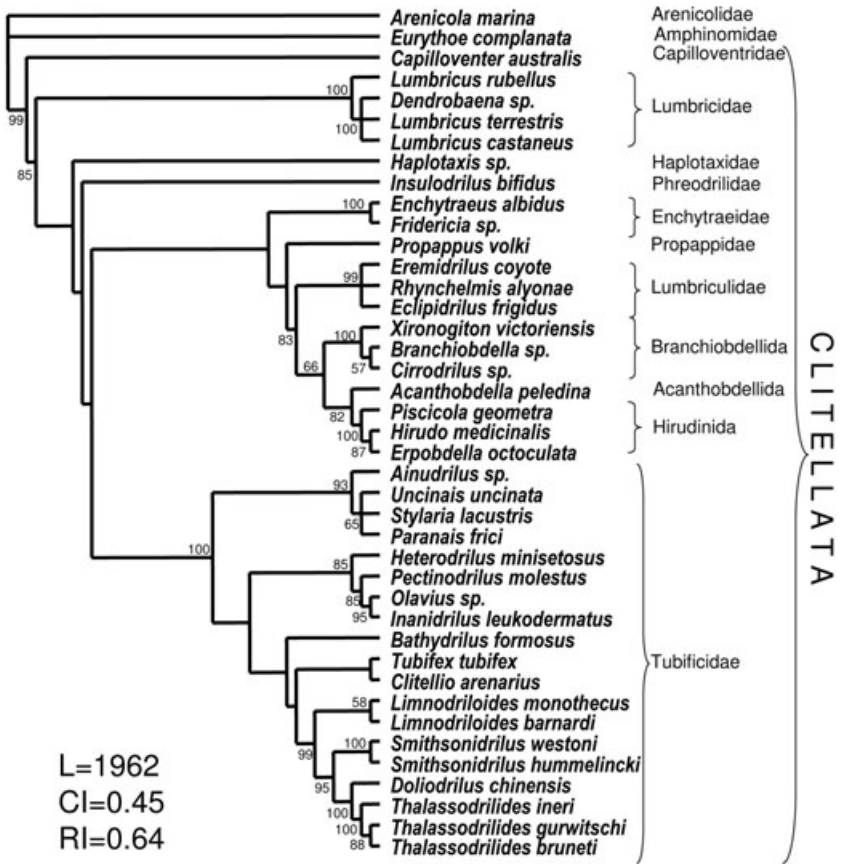


Figure 4. Parsimony consensus tree of the combined (18S rDNA, somatic, and spermatozoal) data set. Bootstrap frequencies ≥ 50% are indicated above the branches.

Table 3. Results of constraint analyses. $P < 0.05$ indicates that the maximum parsimony (MP) solutions are significantly different from the unconstrained solution

Constraint	Length	CI*	*P* (KH)†	*P* (Templeton's)
Unconstrained, combined data (MP, Fig. 4)	1962		–	–
Constrained, combined data (Bayesian Inference, Fig. 5)	1968		–	0.446
Unconstrained, 18S rDNA (MP, tree not illustrated)	1730		–	–
Constrained, 18S rDNA (Bayesian Inference, Fig. 2)	1733		0.654	0.649

*CI: consistency index.
†*P*(KH): *P* value derived from the Kishino-Hasegawa (KH) test.

topologies obtained using MP and Bayesian inference optimization criteria were not significantly different (Table 3).

DISCUSSION

COMBINED-DATA PHYLOGENETIC ANALYSIS

To get the best hypothesis for phylogenetic relationships inside Clitellata, we prefer to consider all of the available information, and therefore mainly results from the total-evidence analyses, under both parsimony and Bayesian criteria, will be further discussed here.

The results of the Bayesian analysis are largely congruent with those obtained from the MP analysis. Both analyses indicate the same terminal monophyletic groups, support the monophyly of Clitellata, and, inside Clitellata, a deep position of Capilloventridae, corroborating the 18S analysis of Erséus & Källersjö (2004), and the previous conclusions based on mor-

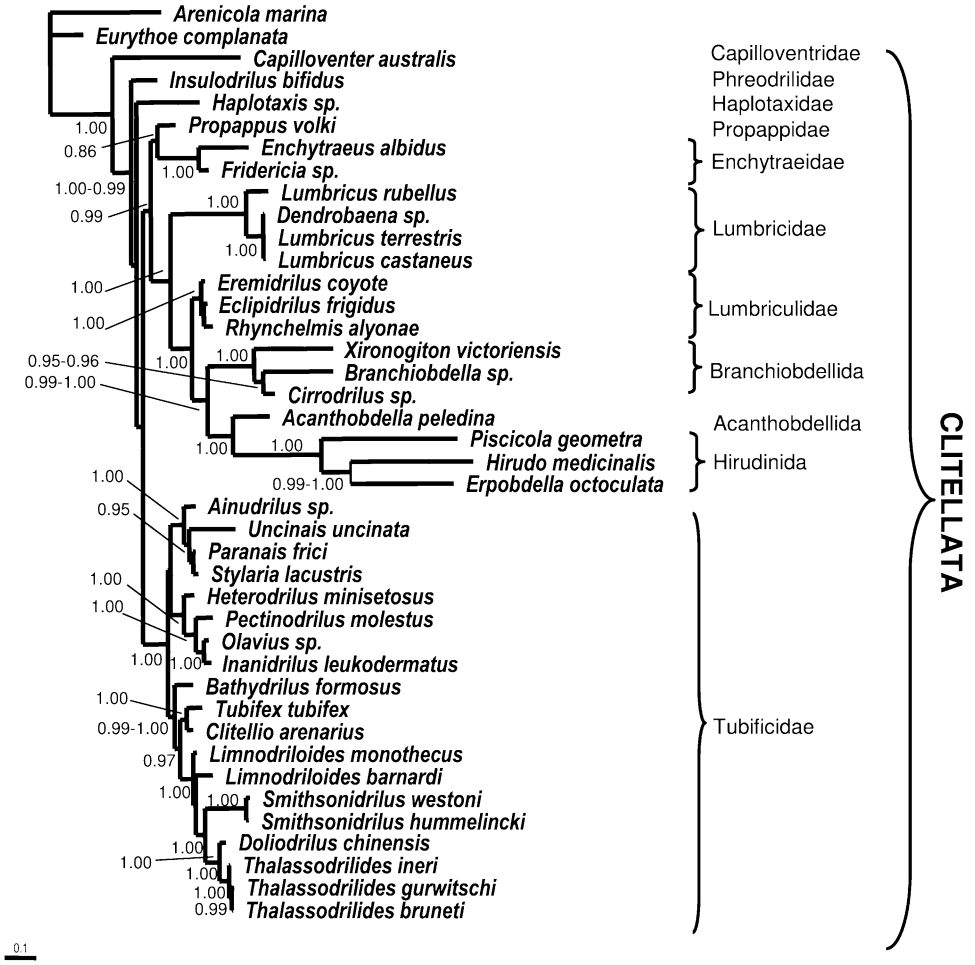


Figure 5. Phylogenetic tree obtained from one of the three replicate Bayesian inference runs of the combined (18S rDNA, somatic, and spermatozoal) data set. Posterior probabilities ≥ 0.85 are indicated in front of the nodes.

phology (Harman & Loden, 1984; Erséus, 1993) and sperm ultrastructure (Ferraguti, Erséus & Pinder, 1996).

There are two main contradictions between the Bayesian and parsimony analyses: (1) in the parsimony analysis Lumbricidae groups in an unsupported basal position, whereas in the Bayesian analysis it forms, with strong support, the sister group of the Lumbriculidae + leech-like taxa; (2) in the parsimony analysis, *Propappus* groups, without support, at the base of the Lumbriculidae–Hirudinida assemblage, whereas in the Bayesian analysis it is, with support, the sister group of Enchytraeidae.

Our Bayesian analysis is congruent with the phylogenetic pattern proposed by recent molecular studies (reviewed in Jamieson, 2006), in which two main clitellate clades are found: an aquatic 'microdrile' clade, mainly consisting of Tubificidae, including Naidinae (*sensu* Erséus & Gustavsson, 2002); and a larger clade consisting of both terrestrial and non-tubificid freshwater families, ranging from enchytraeids to hyrudineans.

Acanthobdellida

The grouping of leeches and their allies with Lumbriculidae on molecular grounds was already shown by Siddall *et al.* (2001) and Erséus & Källersjö (2004). Our phylogenetic analyses based on both the individual 18S gene data set and on the combined data set, under both optimality criteria, strongly support this result. Siddall *et al.* (2001) and Erséus & Källersjö (2004) also found strong support for the sister group relationship between Hirudinida and Branchiobdellida and, consequently, for *Acanthobdella* as their plesiomorphic sister group. On the contrary, our phylogenetic analyses of the combined data set, under both optimality criteria, strongly suggest a sister-group relationship between *Acanthobdella* and Hirudinida, with Branchiobdellida as their plesiomorphic sister group. A close affinity

between leeches and *Acanthobdella* was already proposed by Livanow (1906), who considered *A. peledina* to be an ancient hirudinean. This view, despite several hypotheses that the similarities between leeches and *Acanthobdella* have been convergently acquired in relation to commensalism (summarized in Siddall & Burreson, 1996), has been supported by a phylogenetic analysis on morphological grounds by Purschke *et al.* (1993). These authors found a large set of somatic characters restricted to both taxa. Independent support for a close relationship between *Acanthobdella* and leeches comes from sperm ultrastructure (Westheide & Purschke, 1996; Ferraguti & Erséus, 1999).

Enchytraeidae

A sister-group relationship between enchytraeids and earthworms emerged at first in the combined 18S and COI analysis of Siddall *et al.* (2001). Although from general morphology (Jamieson, 1988) and sperm ultrastructure (Jamieson, 1983; Ferraguti & Erséus, 1999) Enchytraeidae has been considered a basal group without close affinity to crassiclitellates (= oligochaetous clitellates with a multilayered clitellum; see Jamieson, 1988), this relationship has been corroborated, with good support, by the 18S phylogeny of Erséus & Källersjö (2004). The results of this Bayesian analysis, corroborates – with strong support – the close relationship between enchytraeids and earthworms, but differ from those of Siddall *et al.* (2001) and Erséus & Källersjö (2004). In our study, enchytraeids do not form the sister group of Lumbricidae, but together with *Propappus* group at the base of a large assemblage ranging from Lumbricidae to Hirudinida, in accord with the results of a combined nuclear 18S, 28S, and mitochondrial COI genes Bayesian analysis performed by Hugall *et al.* (unpublished, in Jamieson, 2006). Such a position is also supported in the Bayesian analysis of 18S only (Fig. 2).

Propappus

In their parsimony analysis based on 18S, Erséus & Källersjö (2004) found that *Propappus* groups far from Enchytraeidae, near the tubificid clade. On the contrary, our Bayesian analysis supports (posterior probability 0.86) a sister-group relationship between *Propappus* and enchytraeids. Also in our 18S analyses under both parsimony and Bayesian criteria, *Propappus* groups close to Enchytraeidae. A close relationship between enchytraeids and *Propappus* has been suggested on morphological grounds straight from its description (Michaelsen, 1916). *Propappus* has been arranged inside Enchytraeidae, as the stem species for the family, because its peculiar characters were shared with members of wholly aquatic oligochaete families (Stephenson, 1930). Then it was separated from Enchytraeidae by Coates (1986), and was recognized as a member of a new monotypic family Propappidae, as the possible sister group to enchytraeids (Coates, 1989). A sister-group relationship between *Propappus* and enchytraeids has been suggested in several phylogenetic analyses based on somatic characters (Coates, 1987; Brinkhurst, 1994). A detailed ultrastructural study of the cuticle and the spermatozoon of *Propappus volki* (Gustavsson, Ferraguti & Marotta, 2008) supports, on one hand, a close relationship between Propappidae and Enchytraeidae, and, on the other hand, suggests a possible phylogenetic relationship of propappids with megadriles and leech-like taxa.

Tubificidae

Both parsimony and Bayesian analyses indicate with strong support (100%, posterior probability 1.00) the monophyletic status of Tubificidae, corroborating previous phylogenetic analyses on morphological (Erséus, 1990a; Brinkhurst, 1994) and molecular grounds (Erséus *et al.*, 2002; Sjölin *et al.*, 2005). Our analyses, under both optimization criteria, recognized the monophyletic status of three of the five tubificid subfamilies (Limnodriloidinae, Tubificinae, and Naidinae), although without support for Tubificinae in the parsimony analysis (see Table 2). The Bayesian topology suggests an initial split of Tubificidae in two main lineages. One including Tubificinae and Limnodriloidinae, the other one rhyacodrilines, phallodrilines, and naidines, as suggested previously on morphological grounds (Erséus, 1990a). In both parsimony and Bayesian analyses, Tubificinae and Limnodriloidinae are monophyletic sister groups, as already suggested in molecular and morphological phylogenetic analyses (Erséus, 2005). In both analyses Rhyacodrilinae are unresolved. The rhyacodriline *Ainudrilus* sp. groups in a well-supported clade with the Naidinae (93%, probability 1.00), supporting previous results based on molecules (Erséus *et al.*, 2002) and morphology (Erséus, 1990a), and corroborating the hypothesis that naidines are specialized Rhyacodrilinae (Sjölin *et al.*, 2005; Envall *et al.*, 2006). Moreover, *Heterodrilus*, which was previously classified as a member of Rhyacodrilinae, is positioned within Phallodrilinae (85%, probability 1.00), in agreement with recent phylogenetic analyses (Erséus & Gustavsson, 2002; Siddall *et al.*, 2001). In both of our analyses the Phallodrilinae are paraphyletic, with *Bathydrilus* grouping far from the rest of the subfamily, close to Tubificinae. Inside Phallodrilinae, the gutless genera *Olavius* and *Inanidrilus*, both characterized by the lack of a normal alimentary system associated with possession of symbiotic bacteria (Dubilier *et al.*, 2001), form a well-supported monophyletic group.

Table 4. Parsimony-unambiguous somatic and spermatozoal autapomorphies for clitellate groups. Shaded lines indicate autapomorphies; white lines indicate autapomorphies with a low level of generality

Clitellata	#6. Absence of nuchal organs;	#3. Acrosome tube;
	#17. Longitudinal muscle as a continous sheet;	#20. Mitochondria interpolated between nucleus and flagellum;
	#20. Absence of parapodia;	#24. Single centriole;
Hirudinida	#1. Anterior sucker;	#1. Anterior acrosome and lateral button;
	#14. Coelomic organization reduced to lacunae;	#6. Acrosome tube ornamentation;
	#32. Absence of sperm funnel;	#11. Acrosome rod deeply withdrawal inside the tube;
Branchiobdellida	#2. Posterior sucker made of 1–2 segments;	#17. Nuclear apex with fossa;
	#3. Segment number fixed to about 15;	#29. Helical marginal fiber around the flagellum;
	#9. Jaws dorsal and ventral;	
	#46. Spermathecae present and unpaired;	
Lumbriculidae	#41. Diffuse or broadly attached prostate glands;	#5. Acrosome tube bent or spiral;
		#8. Electron dense area in the acrosome vesicle;
		#27. Axonemal doublet helically coiled;
Lumbricidae	#4. Clitellum multilayered;	#11. Acrosome rod deeply withdraval inside the tube;
	#5. Number of cltellar segments more than 3;	#12. Capitulum;
	#7. Tubercula pubertatis;	#14. Connective-like structure;
	#8. Lateral lines;	#18. Nuclear shape straight;
	#13. Intestinal typhlosole;	
	#15. Subneural vessels;	
	#27. Grooved genital chaetae;	
	#31. Male duct opisthopore;	
Enchytraeidae	#10. Pharyngeal peptonephridia;	#11. Acrosome rod protuberant from tube;
	#48. Spermathecal connection with gut;	
Tubificidae	#36. Presence of atria;	
Tubificinae	#45. Copulatory organ: penis;	#34. Basal cylinder of paraspermatozoa;
Limnodriloidinae	#39. Atrial duct;	#34. Absence of the basal cylinder of paraspermatozoa;
	#41. Diffuse or broadly attached prostate glands;	#33. Vestigial acrosome in paraspermatozoa;
	#43. Prostatic pad;	
Naidinae	#45. Abence of copulatory organs;	#13. Secondary acrosome tube absent;
		#28. Complex network connecting doublets and plasma membrane;

The contribution of morphological characters on the combined-data topology

Both somatic and spermatozoal characters contribute to the 18S rDNA phylogeny under both optimization criteria: first, in resolving the 18S topology and adding new extra nodes; second, in increasing the support for many groups (see Table 2). Somatic and spermatological characters, under both optimization criteria, support many of the groupings obtained by the 18S rDNA analyses, and suggest new relationships: the sister-group relationship between *Acanthobdella* and Hirudinida, and between *Propappus* and Enchytraeidae; within Tubificidae, the monophylum of Naidinae and the sister-group relationship between Tubificinae and Limnodriloidinae.

Evolution of morphological characters

Morphological character patterns will be discussed here on the basis of the Bayesian topology. Under both optimality criteria our analyses support similar patterns for somatic and spermatozoal characters, but the Bayesian tree is more resolved than the parsimony-based tree.

Spermatozoal autapomorphies

Unambiguous spermatozoal autapomorphies for clitellate orders and families are summarized in Table 4. Autapomorphic spermatozoal features supporting the sister-group relationship between *Acanthobdella* and Hirudinida are the presence of coiled fibers around the nucleus (#18, marginal probability 0.96), and the midpiece formed by a single mitochondrion (#21, marginal probability 0.97), surrounded by an electron-dense sheath (#23, marginal probability 0.98). A secondary loss of the axonemal basal cylinder (*sensu* Ferraguti, 1984a) (#25, marginal probability 0.97), and a flagellum end piece filled with dense material (#30, marginal probability 0.99) are autapomorphies for the Branchiobdellida + *Acanthobdella* + Hirudinida

Figure 6. Maximum likelihood spermatozoal character state reconstructions on a corrected combined data Bayesian topology, using the ancestral-state reconstruction packages as implemented in Mesquite v1.12 (Maddison & Maddison, 2006). The shaded spots at the nodes indicate the different character states, and their relative likelihoods (marginal probabilities) are indicated by pie diagrams. A, acrosome rod withdrawal (#11) character state reconstruction. Note the two reversions from an acrosome rod partially withdrawn inside the tube (in green) to an acrosome rod protuberant from tube, occurring in Enchytraeidae and among Tubificidae. B, number of mitochondria (#21) character state reconstruction. Note the reduction in mitochondria number from four or five mitochondria (in white) to a single mitochondrion (in black) in *Acanthobdella* and hirudineans. C, central apparatus of the axoneme (#26) character state reconstruction. Inset: visual key for tree.

assemblage. The single autapomorphism for the Lumbriculidae + Branchiobdellida + *Acanthobdella* + Hirudinida assemblage is a prominent central sheath as a modification of the central apparatus of the axoneme (#26, marginal probability 0.96).

The sister-group relationship between *Propappus* and enchytraeids is corroborated by two autapomorphies: an acrosome vesicle protuberant from the acrosome tube (#9, marginal probability 0.93) and a basal nuclear shape that is flat or convex (#19, marginal probability 0.95). The grouping of *Propappus* + Enchytraeidae close to the Lumbricidae + Lumbriculidae + leech-like taxa is supported, although with low marginal probability (0.52), by a single autapomorphy: a spiral arrangement of mitochondria (#22). In accord with the great diversity in tubificid sperm morphologies (Erséus & Ferraguti, 1995), there are no autapomorphies for the family. However, several autapomorphies characterize tubificid subfamilies (see Table 4). The presence of tetragonal fibers and a prominent central sheath as a modification of the central axonemal apparatus (#26, marginal probability 0.95) is the single autapomorphy supporting the sister-group relationship between Naidinae and the rhyacodriline *A. nharna*. A double sperm line (#31, marginal probability 0.97), i.e. the production of two types of spermatozoa (Ferraguti, 2000) and the presence of complex spermatozeugmata (#32, marginal probability 0.97), are synapomorphies for Tubificinae and Limnodriloidinae, corroborating previous phylogenetic analyses based on somatic and spermatological characters (Erséus, 1990a; Marotta *et al.*, 2003).

Plesiomorphic spermatozoon and spermatological apomorphic trends

Jamieson *et al.* (1987) proposed an intuitive hypothetical plesiomorphic spermatozoon for oligochaetous clitellates. Our ancestral state reconstruction corroborates, with various marginal probability scores, Jamieson *et al.*'s (1987) hypothetical model, pointing out some differences (see Fig. 1 inset and Table 5). In our plesiomorphic spermatozoon model the thickness of the acrosome tube wall is uniform throughout its length (#4, marginal probability 0.99); the diameter of the nucleus is constant from the base to the apex (#16, marginal probability 0.86), the nuclear apex is flat or concave (#17, marginal probability 0.96), and the single modification of the central axonemal apparatus is the prominent central sheath (#26, marginal probability 0.74).

Starting from the hypothetical plesiomorphic spermatozoon, Jamieson *et al.* (1987) first proposed spermatological apomorphic trends in the evolution of oligochaetous clitellates. The progressive withdrawal of the axial rod into the acrosomal tube (#11) (See Fig. 6A), the appearance of ornamentations on the axial rod (#12) taking contact with the secondary tube with connectives (#14), and the progressive spiralization of the nucleus (#18) are all confirmed by this analysis, but show reversions and convergences. Some other proposed trends, i.e. the withdrawal of the acrosome vesicle inside the acrosome tube (#9) and the increasing in the number of mitochondria (#21) did not evolve gradually (See Fig. 6B). Our analysis confirms the evolutionary pattern of the clitellate sperm tail (#26): the prominent central sheath is the plesiomorphic state, and its loss is a secondary event, leading to a model of mature sperm axoneme with tetragonal fibers only (see Fig. 6C), as was previously suggested (Ferraguti, 1984b).

Pattern of spermatological characters among clitellates

The pattern of spermatological characters show that, at least in oligochaetous clitellates, spermatozoal character patterns are 'complex and contain elements of convergence and probably also reversals' (Erséus & Ferraguti, 1995). The wide gap between RI and CI obtained constraining the sperm characters to fit the total-evidence topologies obtained under both optimization criteria reveals that most spermatozoal characters are homoplasic characters that give support to tree topology. Such a pattern could be explained assuming that the morphology of spermatozoa among clitellates is strongly constrained, compared for example with those of polychaetes (Jamieson &

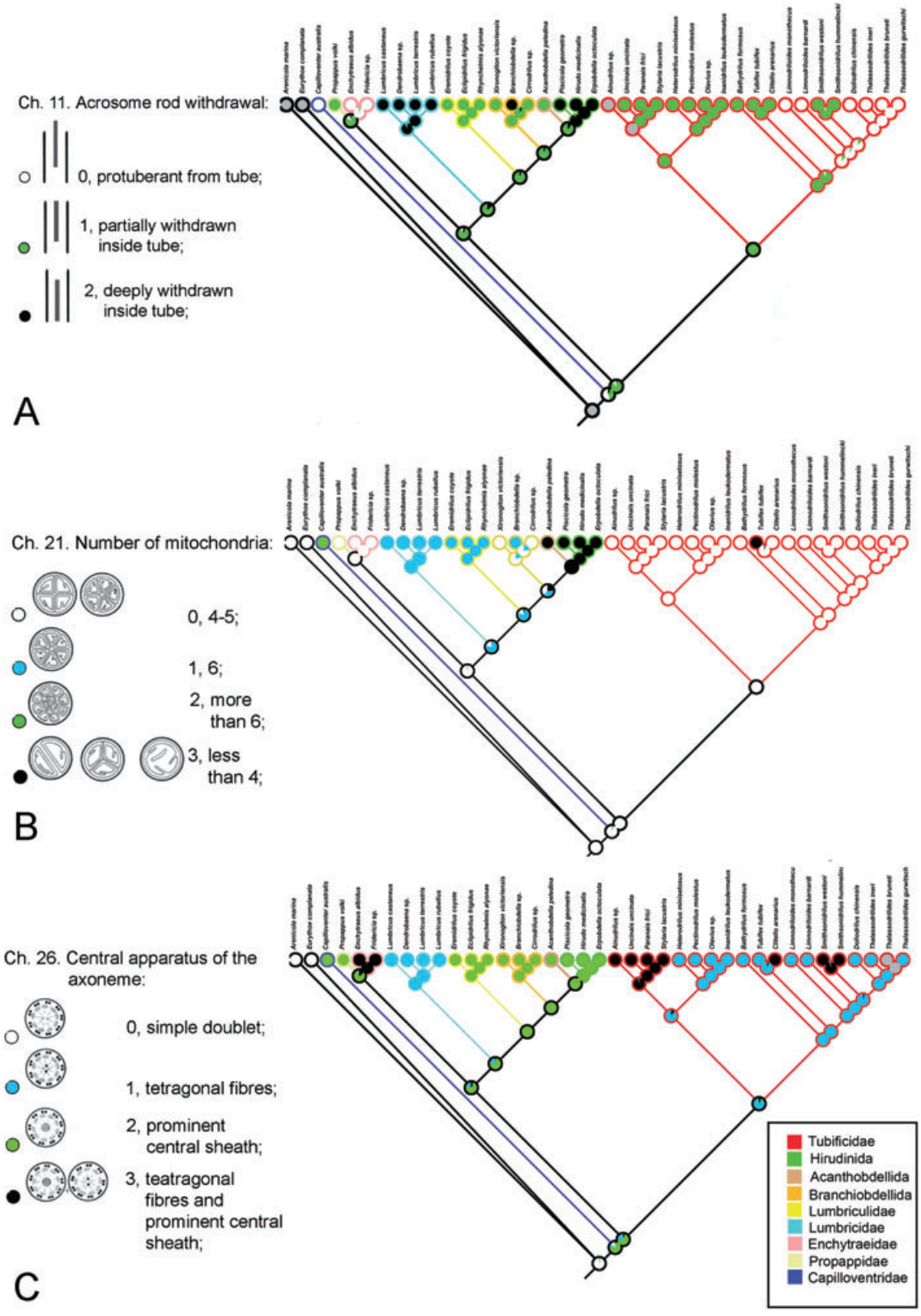


Figure 7. Maximum likelihood somatic character state reconstructions on the corrected combined data Bayesian topology using the ancestral state reconstruction packages, as implemented in Mesquite v1.12 (Maddison & Maddison, 2006). The differently coloured spots at the nodes indicate the different character states, and their relative likelihoods (marginal probabilities) are indicated by pie diagrams. Each tree summarizes the evolution of correlated characters, represented as spots placed side by side. A, evolution of male genital apparatus (characters considered: #29, #31, and #36). B, evolution of chaetal bundles (characters considered: #22, #23, and #24). Inset: visual key for tree.

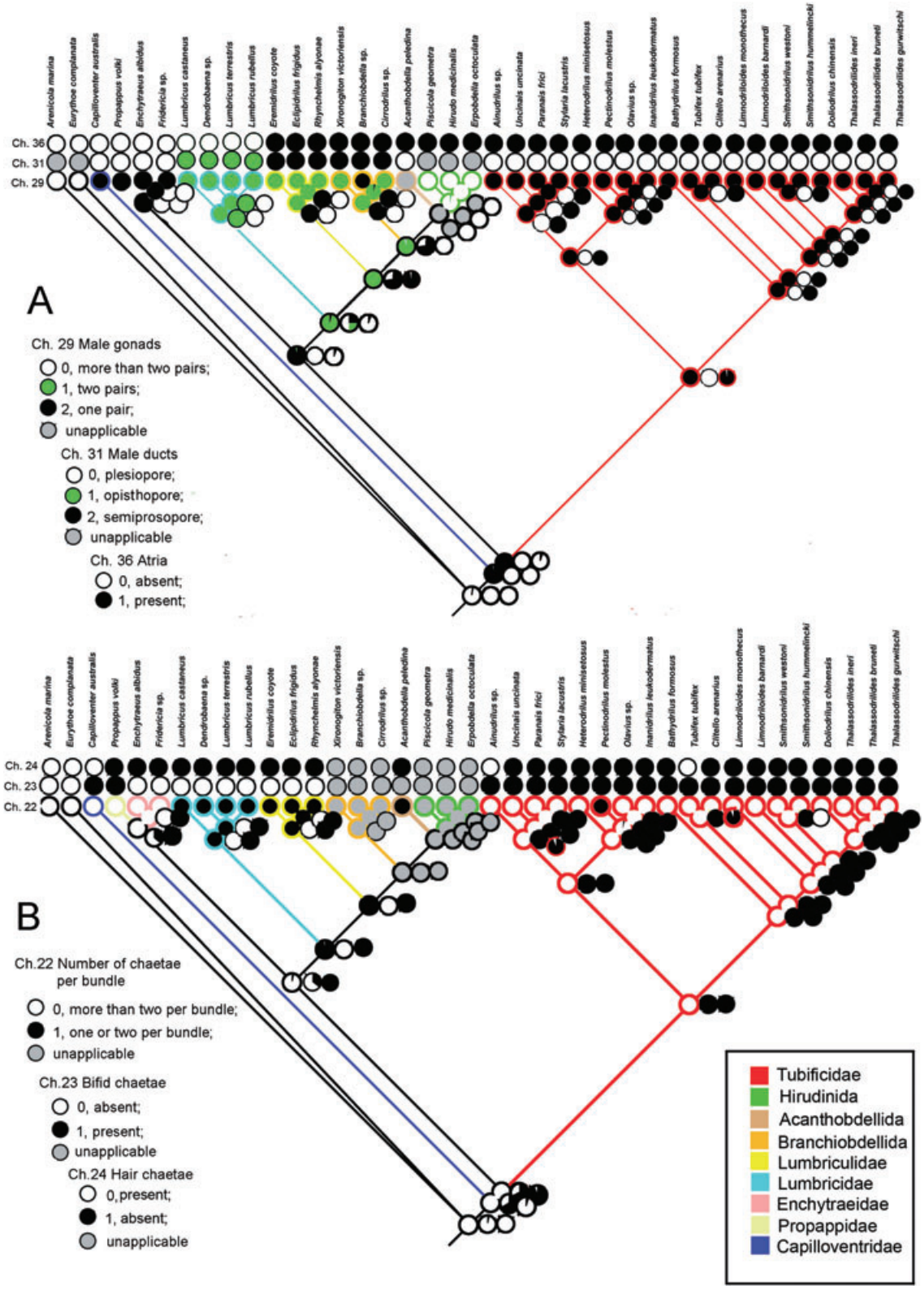
Table 5. Plesiomorphic spermatozoon characters and marginal probability values. Characters in *italics* differ from the plesiomorphic model proposed by Jamieson *et al.* (1987). Characters in **bold** have low marginal probability values

1. Anterior extension of acrosome and lateral button	Absent	0.99
2. Formation of acrosome	From Golgi cisterna	0.99
3. Acrosome tube	Present	0.98
4. Thickness of acrosome tube wall	Uniform	0.99
5. Acrosome tube shape	Straight or slightly bent	0.98
6. Acrosome tube ornamentation	Absent	0.99
7. Acrosome diameter	Uniform throughout length	0.99
8. Electron-dense area in the acrosome vesicle	Absent	0.99
9. Acrosome vesicle withdrawal into the acrosome tube	**Absent**	**0.66**
10. Acrosome rod (or perforatorium)	**Visible**	**0.71**
11. Acrosome rod withdrawal	**Protuberant from tube**	**0.54**
12. Capitulum	Absent	0.96
13. Secondary acrosome tube	**Present**	**0.76**
14. Connective-like structures between secondary tube and rod	Absent	0.99
15. Basal chamber	Absent	0.92
16. Diameter of nucleus	**More or less the same along its length**	**0.86**
17. Nuclear apex	Flat or concave	0.95
18. Nuclear shape	Straight	0.96
19. Basal shape of nucleus	**Flat or convex**	**0.67**
20. Position of mitochondria	Interpolated between nucleus and Flagellum	0.98
21. Number of mitochondria	4–5	0.96
22. Arrangement of mitochondria	Parallel	0.99
23. Dense sheath around mitochondria	Absent	0.99
24. Centrioles inside mature spermatozoon	1	0.98
25. Basal cylinder in the inner end of the axoneme	Present	0.97
26. Central apparatus of the axoneme	**Prominent central sheath**	**0.73**
27. Arrangement of axonemal doublets	Parallel	0.99
28. Complex network connecting doublets and plasma membrane	Absent	0.99
29. Helical marginal fiber around the flagellum	Absent	0.99
30. Flagellum endpiece filled with dense material	Absent	0.99
31. Double sperm line	Absent	0.99

Rouse, 1989); thus, in their long evolutionary history, similar spermatozoal traits originated several times independently at the base of large clitellate groups.

Somatic characters

Unambiguous somatic autapomorphies for clitellate families and subfamilies are summarized in Table 4. Somatic characters support the sister-group relationships between *Acanthobdella* and hirudineans. Autapomorphies for the *Acanthobdella* + Hirudinida assemblage are: a posterior sucker made of more than three segments (#2, marginal probability 0.99); a fixed number of 30–34 segments in adults (#3, marginal probability 0.99); an extensive development of an oblique muscle layer (#18, marginal probability 0.99); specific nephridia (#19, marginal probability 0.99); and sperm sac as sperm proliferation sac (#30, marginal probability 0.99). Two autapomorphies support the sister-group relationship between branchiobdellids and the *Acanthobdella* + Hirudinida assemblage: fused unpaired male pores (#44, marginal probability 0.95) and a single-median position of



the atria (#38, marginal probability 0.98). A well formed penis (#45) and the absence of chaetae (#21) are characters present in both branchiobdellids and hirudineans. There is uncertainty if both characters evolved independently in branchiobdellids and hirudineans (average marginal probability for both characters 0.50), or if they evolved once at the basis of the Branchiobdellida + *Acanthobdella* + Hirudinida assemblage. Two or more vasa deferentia entering in well-defined atria (#35 and #36, marginal probabilities of 0.98 and 0.96, respectively) are the two autapomorphies supporting the sister-group relationship between Lumbriculidae and leech-like taxa. The presence of bundles formed by one or two chaetae (#22, marginal probability 0.96) is the single autapomorphy for the Lumbricidae and Lumbriculidae assemblage. Vas deferens thick entally (#34, marginal probability 0.98) is the single autapomorphy supporting the *Propappus* + enchytraeids assemblage. Although externally homogeneous, tubificids exhibit great morphological variation in their internal organization, especially with regard to the male genitals (Erséus, 1990a). The presence of a well-defined atrium (#36, marginal probability 0.97) is the single autapomorphy for the family, in agreement with previous morphological analyses (Erséus, 1987). The grouping of the rhyacodriline *Ainudrilus* together with Naidinae is supported by a single autapomorphism: the presence of vas deferens entering the ectal parts of atria (#33, marginal probability 0.96). As previously noted (Erséus, 1990a), a single autapomorphism supports the Phallodrilinae (gutless) + *Heterodrilus* assemblage: heavily ciliated atria (#37, marginal probability 0.94).

Somatic apomorphic trends

An octogonadial male gonad arrangement has been considered, although tentatively, as the plesiomorphic condition for oligochaetous clitellates, from which all other clitellate families may be derived by a reduction in the number of gonads (Brinkhurst, 1984). Our analysis, on the contrary, strongly suggests that a monotesticulate arrangement (#29, marginal probability 0.95) is the plesiomorphic condition, and is retained in all tubificids and in the *Propappus* + Enchytraeidae assemblage. The presence of more than one pair of testes, occurring in Lumbricidae and leech-like taxa, thus occurred for secondary multiplication of the gonad number (Fig. 7A). This analysis supports the hypothesis that a plesioporous arrangement of male gonoducts (#31) (male pores on the segment immediately behind the corresponding testes) is the plesiomorphic condition among oligochaetous clitellate (marginal probability 0.99), as indicated by Brinkhurst (1984). Both the semiprosoporous condition (male pores in the same segment as the segment containing the testes), originated at the base of the Lumbriculidae–leech-like assemblage (marginal probability 0.72), and the opistoporous condition (male pores more than one segment behind the segment containing the posteriormost testes) of megadriles thus originated secondarily from a plesioporous condition (Fig. 7A). Atria with prostate glands are apomorphic characters (#36, #42) that evolved convergently in tubificids and leech-like taxa. According to Brinkhurst (1984), male genital tracts without atria and prostate glands is the plesiomorphic condition for clitellates, and is retained in lumbricids and in the *Propappus* + Enchytraeidae assemblage (marginal probability 0.99) (Fig. 7A). A forward location of spermathecae (#47) with respect to the male openings has been considered to be an apomorphic state among oligochaetous clitellates (Erséus, 1987: fig. 5). On the contrary, in this analysis, spermathecal pores opening several segments anterior to the male pores is the plesiomorphic condition, and is maintained in *Propappus*, enchytraeids, and lumbricids (marginal probability 0.95 or 0.62).

Trends regarding the evolution of external clitellate features have also been proposed (Timm, 1981; Brinkhurst, 1984). Our analysis supports a trend of clitellar modifications (#4, #5) from a plesiomorphic single-layered clitellum, a few segments long (marginal probability of respectively 0.98 and 0.99), characterizing all clitellates excluding 'megadriles', towards an apomorphic multilayered clitellum, more than three segments long, characterizing 'earthworms' (Lumbricidae, in our analysis). Concerning the evolution of chaetae, the plesiomorphic clitellates were characterized by bifid crotchets (#23, marginal probability 0.66 or 0.94), hair chaetae (#24, marginal probability 0.99), and more than two chaetae per bundle (#22, marginal probability 0.99), in agreement with the hypothesis of Timm (1981) (Fig. 7B). Thus, single pointed chaetae (#23, marginal probability 0.51) numbering two per bundle (#22, marginal probability 0.90) evolved secondarily at the base of the *Propappus*–Hirudinida assemblage.

This pattern is thus compatible with the hypothesis of a trend from primarily aquatic forms, with bifid chaetae of indefinite number, towards a more terrestrial mode of life, leading to a simplification of the chaetae, and thus supporting the hypothesis that the first clitellate was an aquatic annelid (Timm, 1981; Erséus, 1987).

ACKNOWLEDGEMENTS

Financial support for RM was provided by the European Union HIGHLAT project and by the Swedish Institute for work at the Swedish Museum of Natural

History, Invertebrate Department; financial support for CE was provided by The Swedish Research Council, grant no. 621-2004-2397.

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APPENDIX 1

SOMATIC CHARACTERS AND CHARACTER STATES: ALL CHARACTER STATES ARE UNORDERED

1. *Anterior sucker*:
   absent (0); present (1).
2. *Posterior sucker*:
   absent (0); present, made of one or two segments (1); present, made of three or more segments (2).
3. *Segment number in adults*:
   variable (0); fixed at about 15 (1); fixed at about 30–34 (2).
4. *Clitellum*:
   absent (0); unilayered (1); multilayered (2).
5. *Number of clitellar segments*:
   up to three (0); more than three (1).
6. *Nuchal organs*:
   present (0); absent (1).
7. *Tubercula pubertatis*:
   absent (0); present (1).
8. *Lateral lines*:
   absent (0); present (1).
9. *Jaws*:
   absent (0); dorsal and ventral (1); more than two (2).
10. *Pharyngeal peptonephridia of enchytraeid type*:
    absent (0); present (1).
11. *Barrel-shaped, dilated portion of oesophagus*:
    absent (0); present (1).
12. *Paired oesophageal diverticula:*
    absent (0); present (1).
13. *Intestinal typhlosole*:
    absent (0); present (1).
14. *Coelomic organization*:
    open with complete septa (0); reduced to lacunae without complete septa (1).
15. *Subneural vessel*:
    absent (0); present (1).
16. *Blind posterior lateral blood vessels*:
    absent (0); present (1).
17. *Longitudinal muscles*:
    as four distinct bands (0); as continuous sheath around body (1).
18. *Extensive development of oblique muscle layer*:
    absent (0); present (1).
19. *Specific nephridia*:
    absent (0); present (1).
    (Separation of nephrostome and nephridial duct, nephridial duct without cilia; see Purschke *et al.*, 1993.)
20. *Parapodia*:
    present (0); absent (1).
21. *Chaetae*:
    present (0); absent (1).
22. *Number of chaetae per bundle*:
    more than two per bundle (0); one or two per bundle (1).
23. *Bifid chaetae*:
    absent (0); present (1).
24. *Hair chaetae*:
    present (0); absent (1).
25. *Pectinate chaetae*:
    absent (0); present (1).
26. *Modified, but not grooved, genital chaetae*:
    absent (0); present (1).
27. *Grooved genital chaetae*:
    absent (0); present (1).
28. *Subdental ligaments on bifid chetae:*
    absent (0); present (1).
    (Subdental ligaments: thin structures connecting tip of lower tooth with chaetal shaft.)
29. *Male gonads*:
    more than two pairs (0); two pairs (1); one pair (2).
30. *Sperm proliferation sac*:
    seminal vesicle (0); sperm sac (1).
    (Seminal vesicles: sac-like structures formed by an enlargement of the septa, maintaining an open connection with the coelom. Sperm sacs: sac-like structures, delimited by a proper epithelium, and without connection with the coelom.)
31. *Male ducts:*
    plesiopore (0); opisthopore (1); semiprosopore (2).
    (Terminology following Brinkhurst & Jamieson, 1971.)
32. *Sperm funnels*:
    absent (0); present (1).
33. *Vas deferens*:
    entering ectal parts of atria (0); entering ental parts of atria (1).
34. *Entalmost part of vas deferens*:
    not enlarged (0); enlarged and glandular, as a direct cylindrical continuation of sperm funnel (1).
35. *Number of vasa deferentia per atrium*:
    one (0); two or more (1).
36. *Atria*:
    absent (0); present (1).
37. *Ciliation in atria*:
    absent or sparse (0); dense (1).
38. *Atrial position*:
    paired lateral (0); single median (1).

39. *Atrial duct*:
absent (0); present (1).
(Atrial duct: a long, slender, outer part of the atrium, well set-off from inner part.)
40. *Granulation of atrial duct epithelium*:
absent or sparse (0); dense (1).
41. *Diffuse or broadly attached prostate glands, probably of mesodermal origin*:
absent (0); present (1).
(See Gustavsson & Erséus, 1997.)
42. *Stalked prostate, probably of ectodermal origin*:
absent (0); present (1).
(see Gustavsson & Erséus, 1997.)
43. *Prostatic pad*:
absent (0); present (1).
(Prostatic pad: terminology following Erséus, 1982a & b.)
44. *Male pores*:
paired (0); unpaired by fusion (1).
45. *Copulatory organ*:
absent (0); copulatory sac or pseudopenis (1); penis (2).
46. *Spermathecae*:
absent (0); present and paired (1); present and unpaired (2);
47. *Position of spermathecae*:
in segments immediately anterior to that of male openings (0); in anterior segment, separated by several segments from male openings (1); posterior to male opening (2).
48. *Spermathecal connection with gut*:
absent (0); present (1).

APPENDIX 2

SPERMATOZOAL CHARACTERS AND CHARACTER STATES: ALL CHARACTER STATES ARE UNORDERED – FOR ADDITIONAL INFORMATION SEE FIGURE 1

1. *Anterior extension of acrosome and lateral button:*
absent (0); present (1).
(Lateral button: a lateral protruding part of the acrosome vesicle. See Ferraguti, 2000.)
2. *Formation of acrosome:*
from Golgi cisterna (0); from a skein of Golgi tubules (1).
3. *Acrosome tube:*
absent (0); present (1).
(Acrosome tube: a supporting structure with the shape of a tube or a truncated cone, containing, to a variable extent, or supporting, an acrosome vesicle. See Ferraguti, 2000.)
4. *Thickness of acrosome tube wall:*
uniform (0); basally thick then abruptly thinner (1); basally thick, gradually becoming thinner towards apex (2).
5. *Acrosome tube shape:*
straight or slightly bent (0); bent or spiral (1); corkscrew-shaped or flanged (2).
6. *Acrosome tube ornamentation:*
absent (0); present (1).
7. *Acrosome diameter:*
uniform throughout length (0); apically reduced (1).
8. *Electron-dense area in the acrosome vesicle:*
absent (0); present (1).
9. *Acrosome vesicle withdrawal into the acrosome tube:*
absent (0); partial (1); complete (2).
10. *Acrosome rod (or perforatorium):*
not visible (0); visible (1).
11. *Acrosome rod withdrawal:*
protuberant from tube (0); partially withdrawn inside tube (1); deeply withdrawn inside tube (2).
12. *Capitulum:*
absent (0); present (1).
13. *Secondary acrosome tube:*
absent (0); present (1).
(Secondary acrosome tube: a tubular structure of varying thickness and length inside the acrosome tube, and surrounding the acrosome rod. See Ferraguti, 2000.)
14. *Connective-like structures between secondary tube and rod:*
absent (0); present but weak (1); present and well formed (2).
15. *Basal chamber:*
absent (0); present and small (1); present and large (2).
(Basal chamber: an empty space inside the acrosome tube, between the rod and the base of the acrosome. See Ferraguti, 2000.)
16. *Diameter of nucleus:*
more or less the same along its length (0); gradually reduced from basis to apex of nucleus (1).
17. *Nuclear apex:*
flat or concave (0); convex (1); with fossa (2).
18. *Nuclear shape:*
straight (0); corkscrew-shaped or twisted (1); coiled fibers (2).
19. *Basal shape of nucleus:*
flat or convex (0); concave (1).
20. *Position of mitochondria:*
flanking flagellum (0); interpolated between nucleus and flagellum (1).
21. Number of mitochondria:
four or five (0); six (1); more than six (2); less than four (3).
22. *Arrangement of mitochondria:*
parallel (0); twisted or spiral (1).
23. *Dense sheath around mitochondria:*
absent (0); present (1).

24. *Centrioles inside mature spermatozoon:*
one (0); two (1).
25. *Basal cylinder in the inner end of the axoneme:*
absent (0); present (1).
(Basal cylinder: structure growing during spermiogenesis inside the basal body from which the central apparatus of the flagellum emerges. See Ferraguti, 2000.)
26. *Central apparatus of the axoneme:*
absent (0); tetragonal fibres (1); prominent central sheath (2); tetragonal fibres and prominent central sheath (3).
(The central apparatus of the flagellum has, in Clitellata, two autapomorhic modifications. In the tetragonal fibres modification, two dense fibres run along the two central tubules, forming a tetragonal structure. In the prominent central sheath, the central sheath involving the two central tubules assumes a dense appearance, and the microtubules appear embedded in a cylinder. See Ferraguti, 2000.)
27. *Arrangement of axonemal doublets:*
parallel (0); helically coiled (1).
(In some clitellates, e.g. some lumbriculids, phreodrilids, and hirudineans, the external axonemal microtubules surrounding the two central microtubules are helically coiled.)
28. *Complex network connecting doublets and plasma membrane:*
absent (0); present (1).
(In some naidines the axoneme is surrounded by a complex network generating rays connecting the doublets to the plasma membrane. See Ferraguti *et al.*, 1999: fig. 1E.)
29. *Helical marginal fiber around the flagellum:*
absent (0); present (1).
[The flagellum, in several branchiobdellids, is characterized by the presence of a helical ridge of electron-dense material, called the marginal fibre (Ferraguti, 2000). See Ferraguti & Erséus, 1999: fig. 1, character 20.]
30. *Flagellum end piece filled with dense material:*
absent (0); present (1).
(In some hirudineans, the axoneme is followed by an end piece with a characteristic pattern. In it, distally, the microtubules disappear gradually, and a dense material fills the whole section of the tail. See Ferraguti, 2000.)
31. *Double sperm line:*
absent (0); present (1).
[The production of two types of spermatozoa (euspermatozoa and paraspermatozoa), each with a proper role in fertilization. See Ferraguti, 2000.]
32. *Spermatozeugmata:*
absent (0); two sperm types grouped together to form common spermatozeugmata (1); two sperm types grouped separately to form two different kinds of spermatozeugmata (2).
(Spermatozeugmata are 'sperm aggregates implanted in the spermatheca by the concopulant, characterized by a repetitive order of the spermatozoa and the presence of some sort of cementing agent, but lacking a proper capsule'. See Ferraguti, 2000.)
33. *Vestigial acrosome in paraspermatozoa:*
absent (0); present (1).
[For additional information see Marotta *et al.*, 2003: fig. 7, character 14.)
34. *Basal cylinder of paraspermatozoa:*
absent (0); present (1).
[See Marotta *et al.*, 2003: fig. 7, character 26.]

APPENDIX 3

MORPHOLOGICAL DATA MATRIX: SOMATIC CHARACTERS, FROM 1 TO 48 (SEE APPENDIX 1); SPERMATOZOAL CHARACTERS, FROM 49 TO 82 (SEE APPENDIX 2)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Dendrobaena sp.	0	0	0	2	1	1	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	–	0	–	0	–	–	–	–	0	0	0	0	0	1	1	0
Lumbricus castaneus	0	0	0	2	1	1	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	–	0	–	0	–	–	–	–	0	0	0	0	0	1	1	0
Lumbricus terrestris	0	0	0	2	1	1	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	–	0	–	0	–	–	–	–	0	0	0	0	0	1	1	0
Lumbricus rubellus	0	0	0	2	1	1	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	1	1	–	0	–	0	–	–	–	–	0	0	0	0	0	1	1	0
Eremidrilus coyote	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	0	2	1	1	0	1	1	0	0	0	0	1	0	0	0	0	1	2	0
Eclipidrilus frigidus	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0	0	0	0	1	0	2	1	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0
Rhynchelmis alyonae	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0	0	0	0	1	0	2	1	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0
Branchiobdella sp.	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	–	–	–	–	–	–	–	2	0	2	1	1	0	1	1	0	1	0	0	0	0	0	1	2	2	0	0
Xironogiton victoriensis	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	–	–	–	–	–	–	–	1	0	2	1	1	0	1	1	0	1	0	0	0	0	0	1	2	2	0	0
Cirrodrilus sp.	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	–	–	–	–	–	–	–	1	0	2	1	1	0	1	1	0	1	0	0	0	0	0	1	2	2	0	0
Acanthobdella peledina	0	2	2	?	?	1	0	0	0	0	0	0	?	0	0	1	1	1	1	0	1	0	1	0	0	0	0	?	1	0	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	–	–
Piscicola geometra	1	2	2	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	–	–	–	–	–	–	–	0	1	?	0	?	?	1	1	0	1	0	0	0	0	0	1	2	0	–	–
Hirudo medicinalis	1	2	2	1	0	1	0	0	2	0	0	0	0	0	1	0	0	1	1	1	1	1	–	–	–	–	–	–	–	0	1	?	0	?	?	1	1	0	1	0	0	0	0	0	1	2	0	–	–
Erpobdella octoculata	1	2	2	1	?	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	–	–	–	–	–	–	–	0	1	?	0	?	?	1	1	0	1	0	0	0	0	0	1	2	0	–	–
Enchytraeus albidus	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	2	0	0	1	–	1	–	0	–	–	–	–	0	0	0	0	0	1	1	1
Fridericia sp.	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	2	0	0	1	–	1	–	0	–	–	–	–	0	0	0	0	0	1	1	1
Insulodrilus bifidus	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	2	1	2	0
Haplotaxis sp.	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1	0	0	0	0	1	0	0	1	–	0	–	0	–	–	–	–	0	0	0	0	0	1	0	0
Ainudrilus sp.	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	1	0	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0
Heterodrilus minisetosus	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	0	2	0	0	1	1	0	0	1	1	0	0	0	1	0	0	0	1	1	0	0
Olavius sp.	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	1	2	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	1	1	0	0
Inanidrilus leukodermatus	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	1	2	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0
Pectinodrilus molestus	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1	0	1	0	1	2	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0
Bathydrilus formosus	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	0	2	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	1	1	0	0
Limnodriloides monothecus	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	0	0	1	1	1	0	1	0	1	1	0	0
Limnodriloides barnardi	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1	1	0	0	1	0	2	0	0	1	1	0	0	1	0	0	1	1	1	0	1	0	1	1	0	0
Smithsonidrilus westoni	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	1	0	1	1	1	0	1	1	1	2	0	0
Smithsonidrilus hummelincki	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	1	0	1	1	1	0	1	1	1	2	0	0
Thalassodrilides ineri	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	0	0	1	1	1	0	1	1	1	1	0	0
Thalassodrilides gurwitschi	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	0	0	1	1	1	0	1	0	1	1	0	0
Thalassodrilides bruneti	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	0	0	1	1	1	0	1	0	1	1	0	0
Doliodrilus chinensis	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	0	0	1	0	1	0	1	0	0	1	0	0
Tubifex tubifex	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	2	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	2	1	0	0
Clitellio arenarius	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	2	1	0	0
Uncinais uncinata	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	0	2	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0
Paranais frici	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0
Stylaria lacustris	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	1	0	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0
Propappus volki	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	2	0	0	1	–	1	–	0	–	–	–	–	0	0	0	0	0	1	1	0
Capilloventer australis	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	2	0	0	1	–	0	–	0	–	–	–	–	0	0	0	0	0	1	1	0
Eurythoe complanata	0	0	?	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	0	–	–	–	0	–	–	–	–	0	0	0	?	0	?	–	–
Arenicola marina	0	0	?	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	0	–	–	–	0	–	–	–	–	0	0	0	?	0	0	–	–

APPENDIX 3 *Continued*

	4	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	8	8	8
	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2
Dendrobaena sp.	0	0	1	0	0	0	1	0	2	1	2	1	2	0	?	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	–	–
Lumbricus castaneus	0	0	1	0	0	0	1	0	2	1	2	1	1	2	0	?	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	–	–
Lumbricus terrestris	0	0	1	0	0	0	1	0	2	1	2	1	1	2	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	–	–
Lumbricus rubellus	0	0	1	0	0	0	1	0	2	1	2	1	1	2	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	–	–
Eremidrilus coyote	0	0	1	2	1	0	1	1	2	1	1	0	1	0	?	1	0	1	1	1	0	0	0	1	2	1	0	0	0	0	0	0	–	–
Eclipidrilus frigidus	0	0	1	0	1	0	1	1	2	1	1	0	1	1	2	?	0	1	1	0	0	0	1	1	2	?	0	0	0	0	0	0	–	–
Rhynchelmis alyonae	0	0	1	2	1	0	1	1	2	1	1	0	1	0	0	1	2	1	0	1	1	0	0	0	1	2	1	0	0	0	0	0	–	–
Branchiobdella sp.	0	1	1	0	2	0	1	0	2	1	2	0	1	0	0	1	2	0	1	1	0	0	0	0	2	0	0	1	1	0	0	0	–	–
Xironogiton victoriensis	0	0	1	1	1	0	1	0	2	1	1	0	0	0	0	1	2	1	0	0	0	0	0	0	2	0	0	1	1	0	0	0	–	–
Cirrodrilus sp.	0	1	1	1	?	1	0	?	?	2	1	1	0	0	0	1	2	1	0	0	1	0	0	0	2	0	0	1	1	0	0	0	–	–
Acanthobdella peledina	0	1	1	1	?	2	0	1	1	0	0	0	0	2	?	?	2	1	1	3	1	1	0	0	2	0	0	1	1	0	0	0	–	–
Piscicola geometra	1	0	1	0	0	1	1	0	2	1	2	0	0	0	0	0	1	2	?	1	3	1	1	0	0	2	1	0	0	1	0	0	–	–
Hirudo medicinalis	1	0	1	0	0	1	1	0	2	1	2	0	0	0	0	0	0	2	?	1	3	1	1	0	0	2	?	0	0	1	0	0	–	–
Erpobdella octoculata	1	0	1	0	0	0	1	1	0	2	1	2	0	0	0	1	0	2	0	1	3	1	1	0	0	2	?	0	0	1	0	0	–	–
Enchytraeus albidus	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	1	3	0	0	0	0	0	0	–	–
Fridericia sp.	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	1	3	0	0	0	0	0	0	–	–
Insulodrilus bifidus	0	0	1	0	0	0	1	0	2	1	1	0	1	1	1	0	1	1	0	1	1	0	0	0	1	3	1	0	0	0	0	0	–	–
Haplotaxis sp.	0	0	1	0	0	0	0	0	1	1	0	1	1	2	2	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	–	–
Ainudrilus sp.	0	0	1	0	2	0	0	0	2	?	?	0	?	0	1	1	1	1	1	0	0	0	0	0	1	3	0	0	0	0	0	0	–	–
Heterodrilus minisetosus	0	0	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	–	–
Olavius sp.	0	0	1	0	2	0	0	0	2	1	1	0	1	0	0	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	–	–
Inanidrilus leukodermatus	0	0	1	0	0	0	0	0	2	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	–	–
Pectinodrilus molestus	0	0	1	0	0	0	0	0	2	1	1	0	?	0	0	0	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	–	–
Bathydrilus formosus	0	0	1	0	0	0	0	0	2	0	1	0	0	0	0	?	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	–	–
Limnodriloides monothecus	0	0	1	1	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	1	1	1	1	0
Limnodriloides barnardi	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	1	1	0	0	0	1	1	1	1	0
Smithsonidrilus westoni	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0	0	1	1	0	1	0	0	0	0	1	3	0	0	0	1	2	1	0	0
Smithsonidrilus hummelincki	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	0	0	1	3	0	0	0	1	2	1	0	0
Thalassodrilides ineri	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	2	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	–	–
Thalassodrilides gurwitschi	0	0	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	–	–
Thalassodrilides bruneti	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	1	?	0	0	0	0	0	–	–
Doliodrilus chinensis	0	0	1	1	2	0	0	0	1	1	0	0	1	0	0	2	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	–	–
Tubifex tubifex	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1	1	1	3	0	0	0	0	1	1	0	0	0	1	1	0	1
Clitellio arenarius	0	0	1	0	0	0	0	0	2	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	3	0	0	0	1	1	0	1
Uncinais uncinata	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	?	1	1	1	1	0	0	0	0	0	1	3	0	1	0	0	0	–	–
Paranais frici	0	0	1	0	1	0	0	0	2	0	1	0	0	0	0	1	1	1	1	1	0	0	0	0	0	1	3	0	1	0	0	0	–	–
Stylaria lacustris	0	0	1	0	0	1	0	0	0	2	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	1	3	0	1	0	0	0	–	–
Propappus volki	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	2	0	0	0	0	0	0	–	–
Capilloventer australis	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	–	–
Eurythoe complanata	0	0	0	–	–	–	0	0	0	–	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	–	–
Arenicola marina	0	0	0	0	–	–	–	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	–	–