

# On the origin of the Hirudinea and the demise of the Oligochaeta

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The phylogenetic relationships of the Clitellata were investigated with a data set of published and new complete 18S rRNA gene sequences of 51 species representing 41 families. Sequences were aligned on the basis of a secondary structure model and analysed with maximum parsimony and maximum likelihood. In contrast to the latter method, parsimony did not recover the monophyly of Clitellata. However, a close scrutiny of the data suggested a spurious attraction between some polychaetes and clitellates. As a rule, molecular trees are closely aligned with morphology-based phylogenies. Acanthobdellida and Euhirudinea were reconciled in their traditional Hirudinea clade and were included in the Oligochaeta with the Branchiobdellida via the Lumbriculidae as a possible link between the two assemblages. While the 18S gene yielded a meaningful historical signal for determining relationships within clitellates, the exact position of Hirudinea and Branchiobdellida within oligochaetes remained unresolved. The lack of phylogenetic signal is interpreted as evidence for a rapid radiation of these taxa. The placement of Clitellata within the Polychaeta remained unresolved. The biological reality of polytomies within annelids is suggested and supports the hypothesis of an extremely ancient radiation of polychaetes and emergence of clitellates.

**Keywords:** Annelida; Clitellata; Oligochaeta; Hirudinea; molecular phylogeny; 18S rRNA gene

## 1. INTRODUCTION

While there is general agreement about the monophyletic nature of the Clitellata, if only due to their possession of a clitellum (Michaelsen 1928; de Bauchamp 1959; Sawyer 1986; Purschke *et al.* 1993; Rouse & Fauchald 1995; Brinkhurst 1999a; Ferraguti & Erséus 1999; Purschke 1999; Westheide *et al.* 1999), the phylogenetic relationships within the group still remain a matter of considerable debate (Brinkhurst 1999a,b; Martin *et al.* 2000).

Clitellates are most often divided into the Oligochaeta and Hirudinea on the basis of morphological features, the latter group including true leeches, i.e. the Euhirudinea and leech-like worms, i.e. the Branchiobdellida and the Acanthobdellida (Sawyer 1986; Brusca & Brusca 1990; Rouse & Fauchald 1995). However, the monophyly of the Oligochaeta has been regularly questioned and many authors have proposed schemes in which the Hirudinea are contained within the Oligochaeta (Erséus 1987; Jamieson *et al.* 1987; Jamieson 1988; Gelder & Brinkhurst 1990; Brinkhurst & Gelder 1991; Ferraguti & Gelder 1991; Purschke *et al.* 1993; Brinkhurst 1994, 1999a; Siddall & Burrenson 1996; Ferraguti & Erséus 1999).

The position of acanthobdellids and branchiobdellids within the Clitellata has always been controversial because these organisms share a puzzling combination of synapomorphies with both oligochaetes and leeches (Gelder & Brinkhurst 1990; Purschke *et al.* 1993; Brinkhurst 1999a). Recent morphological observations have strongly supported a close relationship between the Acanthobdellida and the Euhirudinea (Purschke *et al.* 1993; Ferraguti & Erséus 1999). In addition, sperm characters have suggested a sister relationship of this assemblage with the Branchiobdellida and the possibility of a link between this 'leech' group and the lumbriculid oligochaetes (Ferraguti & Erséus 1999). Interestingly, the latter relationship was also indicated by a cladistic analysis

limited to lumbriculids, branchiobdellidans, acanthobdellidans and leeches following a reappraisal of the characters used by Purschke *et al.* (1993; Brinkhurst 1999a).

The position of the Clitellata in relation to the Annelida also remains an unresolved issue. It has long been admitted that the Polychaeta constitute the group closest to the Clitellata. However, it is still not clear whether they are to be considered as two separate clades or whether the clitellates have a sister group among the polychaetes (Rouse & Fauchald 1997; Westheide 1997). Molecular studies have suggested that clitellates are derived polychaetes, but most of these investigations included too few species for assessing the position of this taxon properly (among others McHugh 1997; Kojima 1998; Winnepeninckx *et al.* 1998).

The phylogenetic relationships within the clitellates have benefited from several molecular works to date based on a nuclear gene (18S rRNA), as well as on two mitochondrial genes (cytochrome oxidase I (COI) and 12S rRNA) (Kim *et al.* 1996; Moon *et al.* 1996; Siddall & Burrenson 1998; Apakupakul *et al.* 1999; Nylander *et al.* 1999; Trontelj *et al.* 1999; Erséus *et al.* 2000; Martin *et al.* 2000). However, most of these studies focused on resolving the phylogeny of one particular group within the clitellates, resulting in an underrepresentation of the other clitellates. These analyses were also limited to a small number of taxa and relied almost exclusively on parsimony methods. It is known that, with small numbers of taxa, the choice of species can profoundly affect the phylogenetic reconstruction (Lecointre *et al.* 1993). Martin *et al.* (2000) recently showed that, among the Clitellata, the leeches and leech-like worms have significantly faster rates of molecular evolution for the 18S and COI genes. Such taxa hamper phylogenetic analysis and can also mislead the parsimony method (Felsenstein 1978; Hendy & Penny 1989; Huelsenbeck 1995; Hillis *et al.* 1996; Maley & Marshall 1998).

Table 1. *List of species used in the 18s rRNA analysis, binomial names and EMBL accession numbers*

(Taxa marked with an asterisk were omitted from the phylogenetic analyses (see §2(d)).)

species	binomial name	EMBL
Arthropoda		
Chelicerata	<i>Eurypelma californica</i> Hausserer, 1871	X13457
Insecta	<i>Tenebrio molitor</i> Linnaeus, 1758	X07801
Mollusca		
Pectinidae	<i>Placopecten magellanicus</i> (Gmelin, 1791)	X53899
Achatinidae	<i>Limicolaria kambeul</i> (Bruguère, 1789)	X66374
Chitonidae	<i>Acanthopleura japonica</i> (Lischke, 1873)	X70210
Echiura		
Echiuridae	<i>Ochetostoma erythrogrammon</i> Rueppell and Leuckart, 1830	X79875
Pogonophora		
Siboglinidae	<i>Siboglinum fiordicum</i> Webb, 1963	X79876
Sipuncula		
Phascolosomatidae	<i>Phascolosoma granulatum</i> Leuckart, 1828	X79874
Vestimentifera		
Ridgeiidae	<i>Ridgeia piscesaei</i> Jones, 1985	X79877
Polychaeta		
Terebellidae	<i>Lanice conchilega</i> (Pallas, 1766)	X79873
Aphroditidae	<i>Aphrodita aculeata</i> Linnaeus, 1761	Z83749
Glyceridae	<i>Glycera americana</i> Leidy, 1855	U19519
Nereididae	<i>Neanthes succinea</i> (Frey and Leuckart, 1847)	U36270
	<i>Neanthes virens</i> (Sars, 1835)	Z83754
Capitellidae	<i>Capitella capitata</i> (Fabricius, 1780)	U67323
Chaetopteridae	<i>Chaetopterus variopedatus</i> (Renier, 1804)	U67324
Cirratulidae	<i>Dodecaceria concharum</i> Ørsted, 1843	U50967
Polynoidae	<i>Harmothoe impar</i> (Johnston, 1839)	U50968
Magelonidae	<i>Magelona mirabilis</i> (Johnston, 1865)	U50969
Nephtyidae	<i>Nephtys hombergii</i> Savigny, 1818	U50970
Spionidae	<i>Polydora ciliata</i> (Johnston, 1838)	U50971
Spionidae	<i>Pygospio elegans</i> Claparède, 1863	U67143
Serpulidae	<i>Protula</i> sp.	U67142
Sabellidae	<i>Sabella pavonina</i> Savigny, 1822	U67144
Orbiniidae	<i>Scoloplos armiger</i> (Müller, 1776)	U50972
Aphanoneura		
Aeolosomatidae	<i>Aeolosoma</i> sp.	Z83748
	<i>Aeolosoma hemprichi</i> Ehrenberg, 1828	AJ310500
Oligochaeta		
Lumbricidae	<i>Lumbricus rubellus</i> Hoffmeister, 1843	Z83753
Lumbricidae	<i>Eisenia fetida</i> (Savigny, 1826)	X79872
Enchytraeidae	<i>Enchytraeus</i> sp. 1	Z83750
	<i>Enchytraeus</i> sp. 2	U95948
Lumbriculidae	<i>Rhynchelmis shamanensis</i> Martin <i>et al.</i> , 1998	AJ308512
	<i>Styloscolex baicalensis</i> Michaelsen, 1902	AJ308513
Naididae	<i>Stylaria</i> sp.	U95946
Tubificidae	<i>Tubifex</i> sp.	U67145
Branchiobdellida		
Branchiobdellidae	<i>Sathodrilus attenuatus</i> Holt, 1981	Z83755*
	<i>Xironogiton victoriensis</i> Gelder and Hall, 1990	Z83756*
	<i>Xironogiton victoriensis</i> Gelder and Hall, 1990	AF115977
	<i>Cambarincola holti</i>	AF115975
	<i>Cronodrilus ogygius</i>	AF115976
Acanthobdellida		
Acanthobdellidae	<i>Acanthobdella peledina</i> Grube, 1851	AF099948
	<i>Acanthobdella peledina</i> Grube, 1851	AF115978*
Euhirudinea		
Erpobdellidae	<i>Erpobdella octoculata</i> (Linnaeus, 1758)	AF099949
Glossiphoniidae	<i>Glossiphonia complanata</i> (Linnaeus, 1758)	AF099943
	<i>Glossiphonia</i> sp.	Z83751
Haemadipsidae	<i>Haemadipsa</i> sp.	AF099944
Hirudinidae	<i>Hirudo medicinalis</i> Linnaeus, 1758	Z83752
Ozobranhidae	<i>Ozobranhus margoi</i> (Apathy, 1890)	AF115991
Piscicolidae	<i>Piscicola geometra</i> (Linnaeus, 1761)	AF099946
Salifidae	<i>Barbronia weberi</i> (Blanchard, 1897)	AF099951
Xerobdellidae	<i>Xerobdella lecomtei</i> Frauenfeld, 1868	AF099947

Recently, Martin *et al.* (2000) applied the maximum-likelihood method, which is renowned for its robustness against violations of rate constancy among lineages (Hasegawa & Kishino 1994), to 18S and COI data sets. The COI gene was too homoplastic for resolving high-level relationships of the clitellates, in spite of the diversity and the high number of species considered in the data set. Instead, the 18S gene suggested a close relationship between the branchiobdellidans, leeches and lumbriculids, as well as the incorporation of the former two taxa in oligochaetes *sensu stricto*. These results were not conclusive due to the lack of key taxa (acanthobdellidans) and partial sequence data for the lumbriculids.

Since then, the first 18S sequences of acanthobdellidans and many new sequences of leeches, branchiobdellidans and non-clitellate annelids have been made available in the EMBL databank. Preliminary analyses have shown that the new sequences from leech-like worms were not fast evolving. For the first time an important possible source of bias in phylogenetic inference has been excluded. New complete 18S sequences of the key taxon lumbriculid were also obtained in the present study.

With these new data, we are now in possession of an 18S database of 51 taxa that enables us to reconsider the problem of high-level relationships within the clitellates (oligochaetes, leeches and leech-like worms) and, in parallel, their position with respect to other annelids.

## 2. MATERIAL AND METHODS

### (a) Taxa

We analysed a data set from the EMBL databank consisting at that stage, of most-available 18S sequences for the clitellates representatives of the Polychaeta, Pogonophora, Echiura, Sipuncula, Vestimentifera, Aphanoneura and Mollusca, as well as new sequences of the oligochaete family Lumbriculidae (*Rhynchelmis shamanensis* Martin *et al.*, 1998 and *Styloscolex baicalensis* Michaelsen, 1902) (table 1).

The choice of an outgroup for the Annelida was problematic because so far molecular studies have failed to recover the monophyly of this taxon (McHugh 1997; Westheide *et al.* 1999) and because putatively valid outgroups, such as the Mollusca, are scattered among annelids or have even appeared as an ingroup within polychaetes (Siddall *et al.* 1998; Winnepenninckx *et al.* 1998). For this reason, two Arthropoda were designated as an unambiguous outgroup in accordance with their molecular placement among the Ecdysozoa, the sister group of the Lophotrochozoa to which all other taxa herewith considered belong (Aguinaldo *et al.* 1997; Adoutte *et al.* 2000).

The new oligochaete material was sampled from Lake Baikal (Siberia, Russia) and was preserved in 75% alcohol. The sexual segments of the worms were dissected for identification after hardening the tissues in 7% formalin. Voucher material was deposited in the collection of the Institut Royal des Sciences Naturelles de Belgique (IRScNB), Brussels (IG no. 28235, slides 97.148.3 and 99.049.1). The remaining segments were used for DNA extraction according to a Chelex<sup>TM</sup> procedure (Singer-Sam *et al.* 1989; Hillis *et al.* 1996).

### (b) DNA amplification and sequencing

18S rRNA gene fragments were amplified using the primers 4F 5'-CTGGTTGATYCTGCCAGT-3' (forward) and 16R 5'-CYGCAGGTTCCACCTACRG-3' (reverse) (Winnepenninckx

*et al.* 1994). The thermal cycling profile was set up according to a touch-down sequence (Palumbi 1996): 30 cycles at 94 °C for 60 s, 60 °C for 60 s, 72 °C for 120 s, -0.5 °C per cycle at the second step, ten cycles at 94 °C for 60 s, 48 °C for 60 s and 72 °C for 90 s. The amplification product was used in cycle sequencing reactions with the 16 primers of Winnepenninckx *et al.* (1994), which were kindly granted to me by the authors, according to the manufacturers' protocols (Amersham Pharmacia Biotech, BJ Roosendaal, The Netherlands). Twenty-five cycles at 94 °C for 36 s, 52 °C for 36 s and 72 °C for 80 s were used. The sequencing reactions were electrophoresed on an ALFexpress-DNA sequencer (Amersham Pharmacia Biotech).

### (c) Alignment

Sequences were aligned using Dedicated Comparative Sequence Editor (De Rijk & De Wachter 1993; De Rijk 1995), which considers a secondary structure model (most EMBL sequences are available already aligned in a database that is accessible via the Internet at <http://rrna.uia.ac.be/>) (Van de Peer *et al.* 1997). Some regions of the gene were objectively impossible to align due to their hypervariability (in domain 23 in particular) (Van de Peer *et al.* 1997) and were discarded from the final alignment.

### (d) Phylogenetic analyses

The resulting alignment (EMBL accession no. ALIGN\_000077) was analysed with maximum parsimony and maximum likelihood using PAUP\* v. 4.0b2 (Swofford 1998). Gaps at the beginning and the end of the alignment, which resulted from unequal sequence lengths, were removed prior to analyses. Maximum-parsimony analyses were performed with unweighted and unordered characters, heuristic search, 100 random additions of sequence, tree bisection-reconnection (TBR) branch swapping and 'Multrees', and gaps were treated as missing. Five hundred replicate bootstrap analyses were performed using an heuristic search and the same settings (except for a random addition of sequence with 50 replicates in order to make computations in reasonable limits of time). The Bremer support index, which examines how many extra steps are needed before a branch is not recovered (Bremer 1994), was calculated with PAUP\* v. 4.0b2 (Swofford 1998) in connection with the program Auto-Decay 4.0 (Eriksson & Wikström 1996). It is well known that different types of substitution can occur at different evolutionary rates which may, in some cases, justify differential weighting (Milinkovitch *et al.* 1996). We therefore checked for the possibility of saturation of the nucleotide substitution by plotting transitions (Tr) against transversions (Tv) and the Tr:Tv ratio against the whole range of pairwise distances.

Maximum-likelihood analyses were conducted with a quartet-puzzling tree search (PAUP\* v. 4.0b2) assuming an HKY (Hasegawa, Kishino and Yano) model of evolution, which enables rate differences between transitions and transversions and possible biases in nucleotide composition to be taken into account (Hasegawa *et al.* 1985; Milinkovitch *et al.* 1996). The quartet-puzzling method has proved to be a valid alternative to the prohibitive computational costs of an heuristic tree search with maximum-likelihood analyses when the number of taxa is particularly large (51 in the present study) (Strimmer & Von Haeseler 1996; Strimmer *et al.* 1997). As it is well known that there is a pronounced between-site rate variation in the 18S rRNA molecule (Abouheif *et al.* 1998), which can lead to incorrect reconstruction of phylogenies (Yang 1996), we used a discrete gamma distribution model of rate heterogeneity

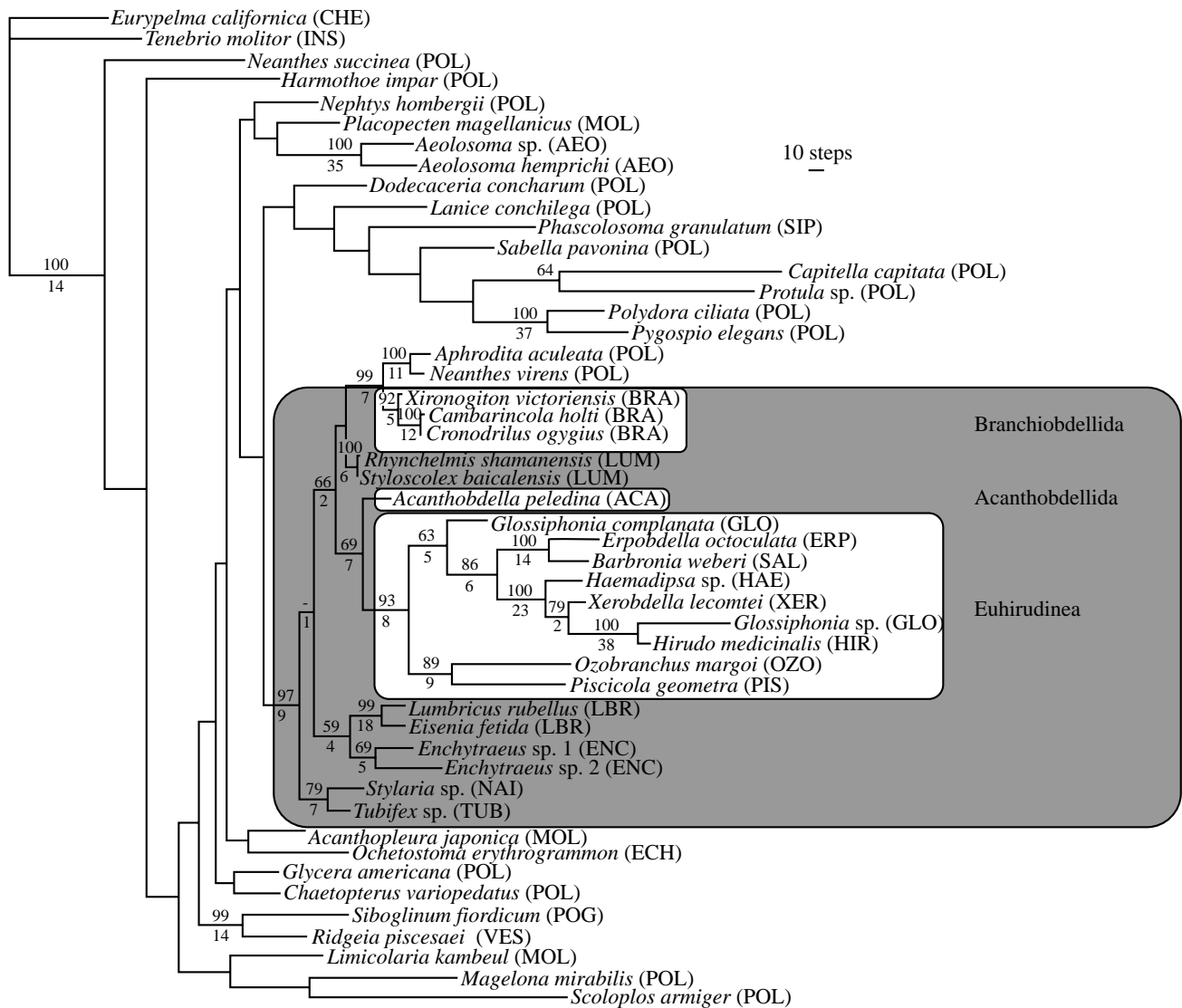


Figure 1. Clitellate relationships based on 18S rRNA. One of the 17 most-parsimonious trees. The length of the tree is 3619 steps. CI (consistency index) (excluding uninformative characters) = 0.3265 and RI (retention index) = 0.4371. The numbers above and below internodes indicate bootstrap and Bremer support values, respectively. Bremer supports for outgroup branches are not shown (never higher than unity), with an occasional exception, in addition to bootstrap values inferior to 50%. The shaded area corresponds to Clitellata. Branchiobdellida, Acanthobdellida and Euhirudinea are isolated from the latter in white boxes. ACA, Acanthobdellida; AEO, Aphanoneura; BRA, Branchiobdellida; CHE, Chelicerata; ECH, Echiura; ENC, Enchytraeidae; ERP, Erpobdellidae; GLO, Glossiphoniidae; HAE, Haemadipsidae; HIR, Hirudinidae; INS, Insecta; LBR, Lumbricidae; LUM, Lumbriculidae; MOL, Mollusca; NAI, Naididae; OZO, Ozobranchidae; PIS, Piscicolidae; POG, Pogonophora; POL, Polychaeta; SAL, Salifidae; SIP, Sipunculata; TUB, Tubificidae; VES, Vestimentifera; XER, Xerobdellidae.

(Yang 1996). Eight gamma rate categories were considered as they were shown to give a good fit of the continuous gamma model without the computational drawbacks (Yang 1996; Yang & Kumar 1996). The shape parameter ( $\alpha$ ) of the gamma distribution and the Tr:Tv ratio were first estimated via maximum likelihood (HKY model) on a preliminary tree topology obtained by neighbour-joining analysis using Tamura & Nei (1993) distances. Ten thousand puzzling steps were performed using approximate likelihoods. The number of puzzling steps was optimized in order to ensure that increasing this parameter did not modify the tree topology and quartet-puzzling values.

The likelihoods of resulting and constrained trees (see § 4) were calculated with the Lscores command of PAUP\* v. 4.0b2 (Swofford 1998) (HKY model with  $\alpha$  and the Tr:Tv ratio estimated by

maximum likelihood and eight gamma rate categories) and were tested for significant differences by the Kishino–Hasegawa test (Kishino & Hasegawa 1989) implemented in Lscores.

As long-branched lineages can mislead phylogenetic methods, particularly parsimony, a preliminary neighbour-joining analysis was conducted on the full data set (51 taxa) (Tamura & Nei (1993) distances). Interestingly, the analysis indicated that the newly published sequences of branchiobdellidans did not produce long branches, in contrast to Moon *et al.*'s (1996) sequences. The older ones unexpectedly differed from the new ones by an insertion of 46 bp (between bases 411 and 458 of the final alignment), consisting of a mostly similar duplication of the preceding bases in the sequence. The uniqueness of this event in the 18S database of most sequences available to date (Van de

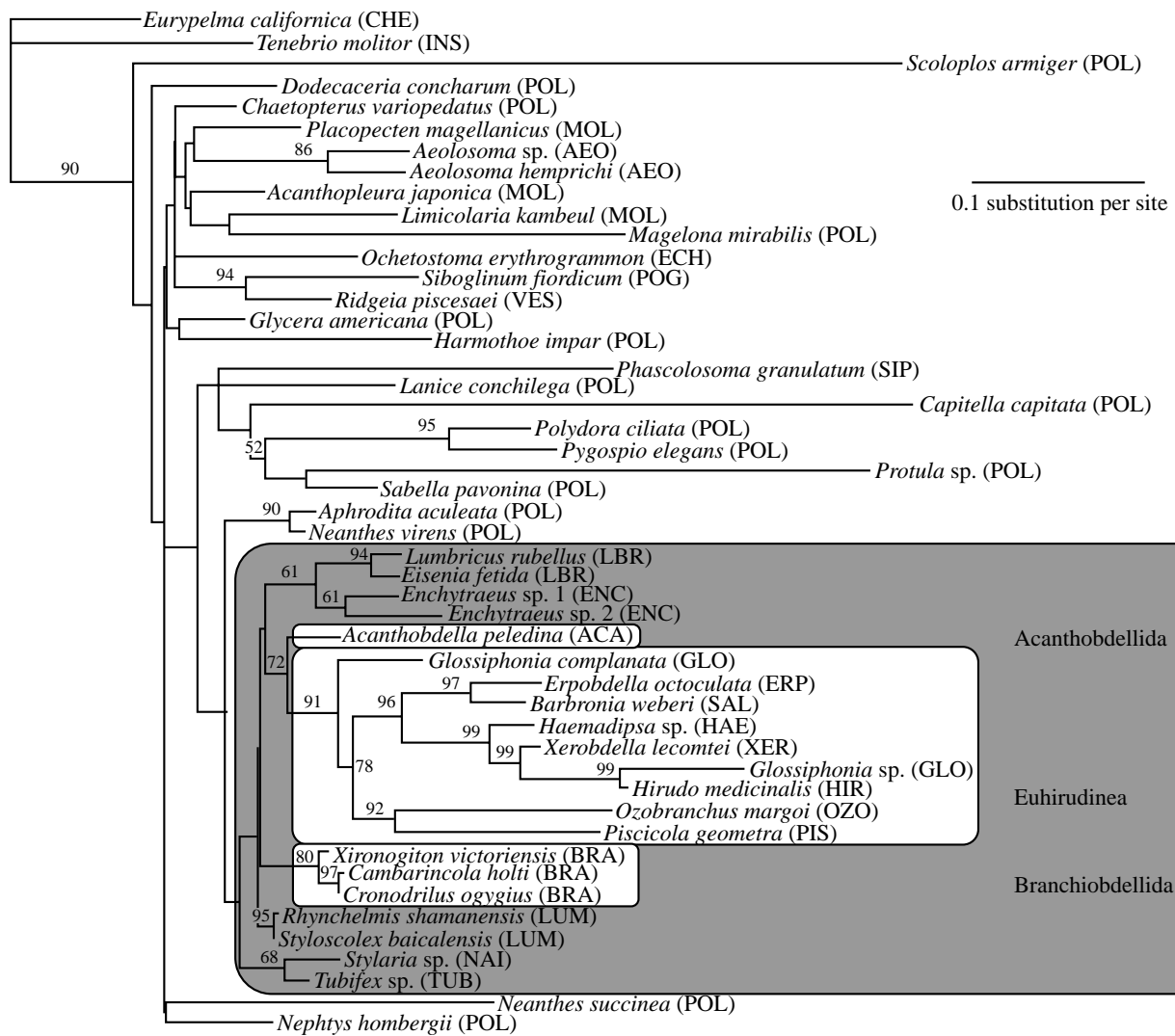


Figure 2. Quartet-puzzling tree ( $-\ln L = 18654.6$ ) reconstructed using a maximum-likelihood criterion assuming a Hasegawa *et al.* (1985) model of substitution (parameters estimated from the data set) and a gamma model of rate heterogeneity. The numbers indicate the quartet-puzzling values. Values inferior to 50% are not shown. The shaded area corresponds to Clitellata. Branchiobdellida, Acanthobdellida and Euhirudinea are isolated from the latter in white boxes. Abbreviations are as in the legend to figure 1.

Peer *et al.* 1997) strongly suggested a methodological bias in the sequences of Moon *et al.* (1996) and provided additional support for their exclusion from the final alignment. These branchiobdellidans were discarded from all subsequent phylogenetic analyses. No leeches were discarded from the final alignment since they constitute a homogeneous cluster of long-branched taxa, leaving no possibility of eliminating possible biases due to long-branch attraction. The new sequence of *Acanthobdella peledina* (Apakupakul *et al.* 1999) (AF115978) was also removed from subsequent analyses, as its aberrant clustering with a mollusc (*Acanthopleura japonica*), which was far removed from the other representative of the species (Trontelj *et al.* 1999) (AF099948), as well as from all other clitellates, strongly suggested that the sequence available from the EMBL databank is inaccurate.

### 3. RESULTS

Saturation plots showed no obvious transition or transversion saturation for the whole range of pairwise distances (data not shown); thus, maximum-parsimony analyses with a weighting scheme could not be justified.

A final alignment of 48 taxa and 2355 characters was obtained, of which 611 characters were excluded. Out of the 1744 included characters, 900 were constant and 540 parsimony informative. Parsimony analysis resulted in 17 equally most-parsimonious trees (figure 1). The analysis under maximum likelihood yielded a very low maximum-likelihood estimate of the shape parameter  $\alpha$  of the gamma distribution (0.27), confirming previous studies (Abouheif *et al.* 1998; Martin *et al.* 2000) and the importance of taking between-site rate variation into account in phylogenetic analyses of the 18S rRNA gene with this method.

The relationships between non-clitellate taxa were mostly unresolved, whatever the method considered (figures 1 and 2). Trivial associations between taxa belonging to the same family (e.g. Aeolosomatidae and Spionidae) were recovered, as expected, with the notable exception of the two nereidid species *Neanthes succinea* and *Neanthes virens*.

In contrast, the phylogenetic reconstructions of the clitellates provided resolution for most relationships. Both

methods agreed on the monophyletic nature of the Branchiobdellida and Euhirudinea, as well as on considering *Acanthobdella* as the well-supported sister group to the Euhirudinea. However, the results were conflicting as far as the monophyly of the clitellates was concerned. In contrast to the quartet-puzzling tree, the most-parsimonious trees made this taxon paraphyletic due to an unexpected placing of a group of two polychaetes, *N. virens* and *Aphrodita aculeata*, close to the Branchiobdellida, within the clitellates. These polychaetes appear, although unsupported, as the closest outgroup to the clitellates in the quartet-puzzling tree. Importantly, leeches and leech-like worms were placed well within the oligochaetes, making the latter taxon paraphyletic. However, the exact position of the Branchiobdellida and the (Acanthobdellida, Euhirudinea) clade remained uncertain and conflicting according to the trees considered. These discrepancies disappeared when the lack of support for these different patterns was taken into account. A sister relationship between a clade including Tubificidae and Naididae together and all other 'oligochaetes' was suggested in the two analyses but support was almost non-existent.

#### 4. DISCUSSION

##### (a) *Monophyly of the Clitellata*

The monophyly of the Clitellata is strongly supported by morphological data and is not seriously questioned by any specialist of this group (see, among others, Purschke *et al.* 1993; Rouse & Fauchald 1995; Brinkhurst 1999a; Ferraguti & Erséus 1999; Purschke 1999; Westheide *et al.* 1999). Accordingly, the placement of some polychaetes (*N. virens* and *A. aculeata*) close to branchiobdellidans in the maximum-parsimony tree, thus making the clitellates paraphyletic, was surprising and probably erroneous (figure 1). We believe that this was because parsimony, by neglecting back substitutions, is more sensitive to homoplasies. The monophyly of the clitellates in the quartet-puzzling tree (figure 2) suggested that *N. virens* and *A. aculeata* were spuriously attracted by branchiobdellidans due to random rather than historical similarity. The quartet-puzzling tree was inferred according to a maximum-likelihood criterion, a method that explicitly considers undetected changes and is less likely to be affected by such an artefact when the assumed model of evolution is adequate (Bruno & Halpern 1999) (here an HKY85 model of evolution coupled with a gamma model of rate heterogeneity).

The fact that the position of the polychaetes *A. aculeata* and *N. virens* was so unstable according to the method considered suggests that their placement in the maximum-parsimony tree should be suspicious (figure 1). Moreover, the surprising segregation of the two *Neanthes* species into two distant clusters, in contrast to all other taxa belonging to the same family, provided additional support for excluding this taxon at least in subsequent phylogenetic considerations. If the polychaetes *A. aculeata* and *N. virens* are misleading, it is most surprising that no particular characteristic of the group, such as the presence of long branches, leads us to expect that the group is a problem. In fact, recent theoretical studies have shown that the problem of inconsistency in parsimony

is much more complex than expected, making the detection of spurious attraction very difficult (Kim 1996; Poe & Swofford 1999): long branches do not necessarily result in inconsistent estimates, while short or even identical branches can lead to inconsistency. Moreover, fast-evolving lineages do not always display very long branches when complete data sets are used (Philippe & Laurent 1998; Philippe *et al.* 2000). The presence of between-site rate variation in the gene exacerbates the problem (Yang 1996).

##### (b) *Phylogenetic relationships within the Clitellata*

It is noteworthy that both the maximum-parsimony and maximum-likelihood methods agreed on the phylogenetic relationships of the clitellates when supported branches were considered (figures 1 and 2). Taxa cluster according to their respective families, with the notable exception of the leech family Glossiphoniidae. The fact that the location of *Glossiphonia complanata* (AF099943) (Trontelj *et al.* 1999) is corroborated by either morphological (Siddall & Burreson 1996) or other independent molecular data (Siddall & Burreson 1998; Apakupakul *et al.* 1999) points to an erroneous sequence of *Glossiphonia* sp. (Z83751) (Moon *et al.* 1996) (e.g. wrong identification of the taxon or errors in the sequence).

The relationships among the Euhirudinea agreed with other leech molecular phylogenies, based on a more extensive data set (Siddall & Burreson 1998; Apakupakul *et al.* 1999; Trontelj *et al.* 1999). The equivocal position of the Glossiphoniidae relative to the Piscicolidae and Ozobranchidae was again noticeable, as exemplified by the contradiction between the maximum-parsimony and quartet-puzzling trees, as far as these families are concerned. This problem was discussed by Trontelj *et al.* (1999) for 18S- and 12S-based leech phylogenies.

With regard to 'oligochaetes', the relationships between families did not contradict the most recent morphology-based phylogeny (Brinkhurst 1994), except that a different rooting of the morphological tree had to be assumed (Martin *et al.* 2000). The 18S gene remained consistent with the COI-based phylogeny (Martin *et al.* 2000) in suggesting a close relationship between the Enchytraeidae and the Lumbricidae, as well as between the Tubificidae and the Naididae. The first grouping was noteworthy because the Enchytraeidae are a primarily terrestrial family, with some freshwater and marine species (Brinkhurst & Jamieson 1971), while the Lumbricidae are purely terrestrial, suggesting that the adaptation to a terrestrial life is a derived ecological feature among oligochaetes. The second association was not unexpected since many lines of evidence suggest that tubificids are paraphyletic unless they include naidids (Erséus 1990; Brinkhurst 1994; Christensen & Theisen 1998; Martin *et al.* 2000).

The monophyly of the Branchiobdellida and Euhirudinea was confirmed, but the respective positions of the Acanthobdellida (A), Branchiobdellida (B), Euhirudinea (E) and Lumbriculidae (L) remained unresolved, whatever the method of tree inference. However, there was strong support for a sister relationship between *Acanthobdellida* and euhirudinids, which has been corroborated by morphological evidence (Purschke *et al.* 1993; Brinkhurst 1999a; Ferraguti & Erséus 1999) but challenged by other classifications (Brusca & Brusca

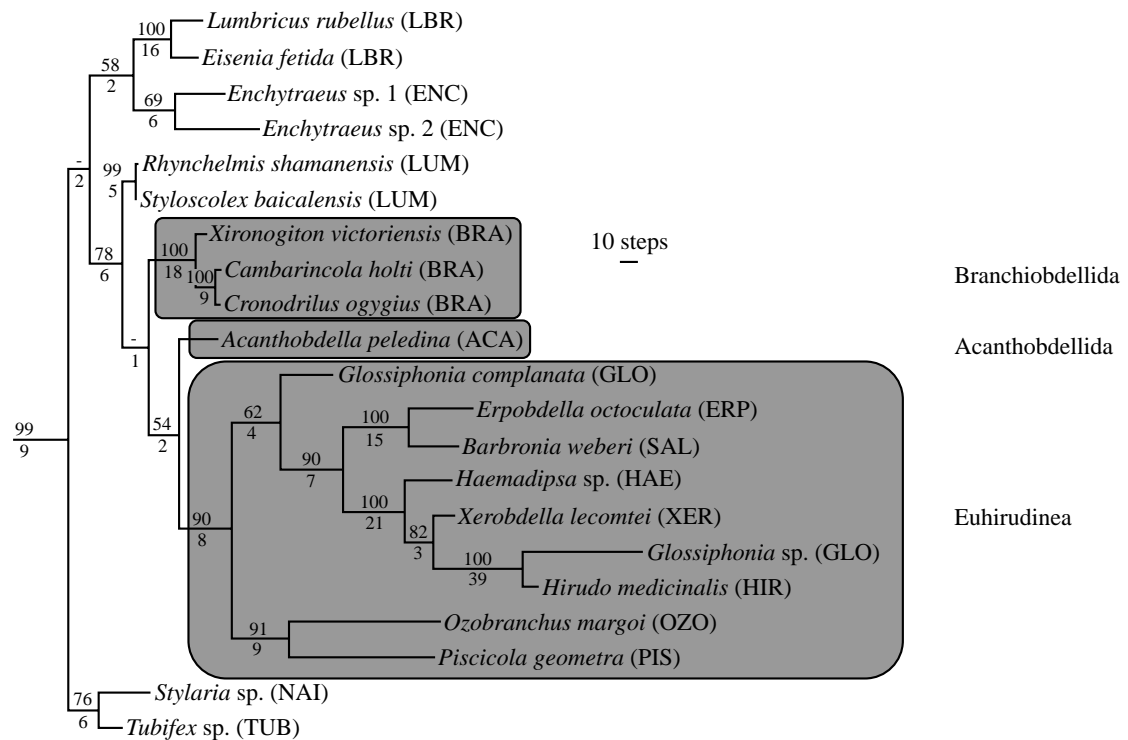


Figure 3. Clitellate relationships based on 18S rRNA after removal of the polychaetes *N. virens* and *A. aculeata* from the data set (clitellate topology only shown). One of the ten most-parsimonious trees. The length of the tree is 3566 steps. CI (consistency index) (excluding uninformative characters) = 0.3291 and RI (retention index) = 0.4301. The numbers above and below internodes indicate bootstrap and Bremer support values, respectively. Bremer supports for outgroup branches are not shown (never higher than unity), with an occasional exception, in addition to bootstrap values inferior to 50%. The shaded area corresponds to Branchiobdellida, Acanthobdellida and Euhirudinea.

1990; Siddall & Burreson 1998; Apakupakul *et al.* 1999). In the COI-based phylogeny of Siddall & Burreson (1998), *Acanthobdellida* was the sister taxon to a branchiobdellidans plus leech clade. This pattern collapsed when more 'oligochaetes' were incorporated in the data set (including lumbriculids) (Martin *et al.* 2000). Apakupakul *et al.* (1999) reaffirmed the placing of *Acanthobdellida* by Siddall & Burreson (1998) on the basis of separate COI and 18S phylogenies, as well as a total evidence analysis combining both genes and morphological characters. As oligochaetes were also underrepresented (only two taxa) and, with even the key family Lumbriculidae absent from the data set, these results are questionable for the same reasons. Interestingly, support for the placement of *Acanthobdellida* was obtained only in the total evidence analysis. One morphological character, the presence or absence of setae, the homoplastic nature of which was recently reaffirmed (Brinkhurst 1999a), appears responsible for that.

In this context, it was interesting to note the incidence of the removal of the presumed misleading *A. aculeata* and *N. virens* polychaetes from the database on phylogenetic inferences. In that case, relationships already supported (figures 1 and 2) remained unchanged, the support values being, as a rule, slightly higher (figure 3 and data not shown). In contrast, while the respective positions of the lumbriculid, branchiobdellidan and the (A,E) clade remained unresolved in the quartet-puzzling tree (data not shown), a well-supported (L(B(A,E))) relationship was

obtained in the maximum-parsimony trees (bootstrap and Bremer support values of 78 and 6, respectively) (figure 3). However, there was no support for the sister relationship between Branchiobdellida and the (A,E) clade.

If a heuristic tree search with the maximum-likelihood method is computationally prohibitive for a final alignment of almost 50 taxa, the quartet-puzzling tree, without the assumed misleading *A. aculeata* and *N. virens* polychaetes (data not shown), can be used as a basic topology in which only (L,B,A,E) relationships are variously combined, according to the relationships proposed so far, on a morphological and molecular basis. The resulting topologies can be tested for significant differences in their log likelihood estimated from the 18S data set using a Kishino & Hasegawa (1989) test (implemented in PAUP\*) (table 2). Surprisingly, the topology that received the lowest log-likelihood value corresponded to the relationships suggested in the quartet-puzzling tree. The fact that the quartet-puzzling tree search found a significantly different tree from the maximum-likelihood tree is striking. However, this was not unexpected because, if the quartet-puzzling method is a valid alternative to a fully maximum-likelihood approach when the number of taxa is large (Strimmer & Von Haeseler 1996; Strimmer *et al.* 1997), it is well known that this method does not always yield the highest likelihood tree (Cao *et al.* 1998). In this case, because resolution is lacking when lumbriculids, leeches and

Table 2. *Kishino & Hasegawa (1989) test of significance of differences ( $\Delta$ ) in log likelihoods ( $\ln L$ ) of currently proposed clitellate topologies*

(The  $p$ -values are the probability of obtaining a more extreme  $t$ -value under the null hypothesis of no difference between the two trees (two-tailed test). The test is significant when  $p < 0.05$  (denoted by an asterisk). Maximum-likelihood values were computed assuming a Hasegawa *et al.* (1985) model of substitution and a gamma model of rate heterogeneity (parameters estimated from the data set). A, Acanthobdellida; B, Branchiobdellida; E, Euhirudinea; L, Lumbriculidae; Lb, Lumbricidae; En, Enchytraeidae.)

tree	$-\ln L$	$\Delta - \ln L$	$p$ -value	references
(L,(B,(A,E)))	18 284.8	best	—	Purschke <i>et al.</i> (1993), Siddall & Bureson (1996), Ferraguti & Erséus (1999), Brinkhurst (1999a), Martin <i>et al.</i> (2000) and this study (maximum-parsimony tree) (figure 3)
((L,B),(A,E))	18 285.2	0.35	0.93	Purschke <i>et al.</i> (1993)
(L,(A,(B,E)))	18 288.8	4.03	0.57	Brusca & Brusca (1990), Siddall & Bureson (1998) and Apakupakul <i>et al.</i> (1999)
(L,((B,A),E))	18 292.8	7.95	0.14	Trontelj <i>et al.</i> (1999) and Martin <i>et al.</i> (2000)
(L,((Lb,En), (B,(A,E))))	18 306.7	21.87	0.02*	this study (puzzle tree)

leech-like worms are considered, the method is probably unable to find an optimal tree in all possible quartet combinations and, thus, is forced to choose randomly among the available quartet topologies.

No constrained topology was significantly different from the maximum-likelihood tree. In spite of this, there was a notable gap in the log-likelihood values between the first two topologies and the others. It was remarkable that the (L,B,A,E) topology which received the best likelihood value was recovered in the maximum-parsimony tree when the two polychaetes *A. aculeata* and *N. virens* were eliminated from the analysis and is favoured by the most recent morphological studies. Of note is that the alternative '(L,B),(A,E)' once suggested by Purschke *et al.* (1993) had an almost indistinct log-likelihood value as the best maximum-likelihood tree. However, it was clear that, in favouring an independent evolution of the Branchiobdellida within the 'oligochaetes', this hypothesis shifts the problem of the 'oligochaetous' sister group of the Hirudinea from the Lumbriculidae to an as yet unknown taxon. Both topologies were almost equally probable in the present study. However, whatever the alternative, *Acanthobdella* and the Euhirudinea were reconciled in their traditional Hirudinea clade (Harant & Grassé 1959; Sawyer 1986; Purschke *et al.* 1993).

### (c) *The Clitellata in relation to the Annelida*

Due to its assumed proximity to polychaetes, the position of the Clitellata within the Annelida is intimately connected to the resolution of the phylogenetic relationships within this group. No less than five genes have been studied so far with this aim in view, but none of them has convincingly recovered groupings (McHugh 1997; Giribet & Ribera 1998; Kojima 1998; Siddall *et al.* 1998; Brown *et al.* 1999; Erséus *et al.* 2000). If the lack of resolution among polychaetes was initially interpreted to result from poor taxonomic sampling (Brown *et al.* 1999), the careful study of Brown *et al.* based on three different genes did not significantly improve the resolution, despite a broad sampling of polychaete diversity, including clitellates. The biological reality of these polytomies thus appears

corroborated by independent evidence supporting the hypothesis of an ancient radiation of polychaetes and emergence of clitellates.

The placement of the Clitellata within the Polychaeta has received no support from most studies and there exist as many candidate sisters to Clitellata as there are molecular studies addressing the issue. To date, only two of them has identified a supported although contradictory polychaete sister group to the Clitellata. In the analyses of Brown *et al.* (1999), the pairing of the Siboglinidae with the Clitellata had notable Bremer and some bootstrap support. However, it is significant that this support was only obtained when the three genes studied (histone H3, U2 snRNA and 28S rDNA) were combined together; each gene taken separately yielded a different and unsupported sister group to the clitellates. The H3 data alone placed the clitellates out of the polychaetes. The 18S-based phylogeny of Tubificidae of Erséus *et al.* (2000) gave support to a sister relationship between the clitellates and the polychaetes *Neanthes* (Nereidae) and *Aphrodita* (Aphroditidae). This grouping was not refuted by the quartet-puzzling tree in the present study, while not supported but, given that *Neanthes* and *Aphrodita* are probably misleading as far as the clitellate monophyly is concerned, this relationship is questionable.

The fact that the position of the Clitellata in relation to the Annelida remains inconclusive, despite the amount of molecular data available, is in accordance with the hypothesis of an ancient or very fast emergence of this clade. Although the 18S gene proved to yield a meaningful historical signal for determining relationships within the clitellates, the exact position of leeches and leech-like worms within oligochaetes is still mostly unresolved. Similarly, this lack of phylogenetic signal could be interpreted as a rapid radiation of these groups. In that case, the time interval separating the emergence of these clades was too short, with respect to the time elapsed since the event, for enough mutations to accumulate in the internal branches connecting these taxa (Philippe *et al.* 1994). The study of other genes is imperative if this hypothesis is to receive some support. However, in this



event the resolution of the respective position of leeches and leech-like worms among oligochaetes will be fraught with extreme difficulties in future studies, whatever the gene under scrutiny.

## 5. CONCLUSION

Regardless of the method of tree inference considered, 18S rDNA sequences support an oligochaete ancestor for the Euhirudinea, Branchiobdellida and Acanthobdellida, suggesting that the Oligochaeta are paraphyletic. Molecular data further suggest that the Lumbriculidae is probably the sister group to this clade, in accordance with morphological evidence. This has a profound significance on the taxonomic rank of the Oligochaeta. The taxon name for a clade including oligochaetes, leeches and leech-like worms could be the Oligochaeta or Clitellata. However, in accordance with Rouse & Fauchald (1995), although Oligochaeta is an older name (Grube 1850) than Clitellata (Michaelsen 1928), the latter was explicitly formulated in order to include the Hirudinea and, hence, follows the priority principle for the phylogenetic taxon names of de Queiroz & Gauthier (1990, 1992).

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