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A multigene framework for polychaete phylogenetic studies

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Abstract

Producing a robust phylogenetic reconstruction for Polychaeta using either morphological or molecular data sets has proven very difficult. There remain many conflicts between morphological analyses and hypotheses based on DNA data, the latter principally derived from 18S rRNA sequences. For the present study a data set covering a broad range of polychaete diversity was assembled, including 38 new sequences from 21 species. Besides available 18S rRNA data, five additional gene segments were examined: the D1 and D9-10 expansion regions of 28S rRNA, histone H3, snU2 RNA and cytochrome *c* oxidase subunit I. Maximum parsimony, maximum likelihood and Bayesian analyses were conducted.

Annelida and Mollusca were reciprocally monophyletic in maximum likelihood analyses, but Polychaeta included a cephalopod in maximum parsimony analyses, and a patellogastropod in Bayesian analyses. When rooted on the Mollusca, optimal topologies from maximum likelihood analyses showed a recognisable basal group of taxa, including Oweniidae, Chaetopteridae and Amphinomidae. The six studied phyllodocidan families plus Orbiniidae (as the sister group of the scale-worms) formed the next most basal group. All analyses support the inclusion of Echiura, Clitellata and Siboglinidae within polychaetes. Bayesian analyses show Echiura as the sister group of Capitellidae, in agreement with previous 18S rRNA results, In contrast, Echiura formed the sister group to Trichobranchidae in maximum likelihood and maximum parsimony analyses.

Supra-familial groupings consistently recovered within Polychaeta in the analyses are: (i) Terebellida without Ampharetidae; (ii) Scolecida (excepting Orbiniidae); (iii) Eunicidae, Lumbrineridae and Clitellata; and (iv) ''Cirratuliformia'' (including Sternaspidae) plus Sabellidae, Serpulidae and Spionidae.

Keywords: Basal polychaetes; Rooting; 28S rRNA; U2 snRNA; Histone H3; Cytochrome c oxidase

Introduction

The interaction of molecular and morphological approaches has produced substantial progress in understanding the deeper phylogeny of most major invertebrate groups. This is only partly true for the

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Lophotrochozoa which, although first identified as a clade by molecular analyses (Halanych et al. 1995), have not been resolved into monophyletic phyla by subsequent investigations of 18S ribosomal DNA (Winnepenninckx et al. 1995; Giribet et al. 2000; Halanych et al. 2002). Polychaeta is a particularly problematic taxon. Consequently, there has been extensive recent interest in the group's membership and higher classification from the perspectives of both morphology (e.g. Bartolomaeus 1995; Nielsen 1995; Eibye-Jacobsen and Nielsen 1996; Rouse and Fauchald 1997; Rouse and Pleijel 2001, 2003; Purschke 2002; Bartolomaeus et al. 2005) and DNA sequencing (e.g. McHugh 1997, 2000; Brown et al. 1999; Martin 2001; Struck et al. 2002a, b; Bleidorn et al. 2003a, b; Hall et al. 2004).

Discussion about the membership of Polychaeta has recently focussed on the inclusion of Clitellata (Oligochaeta and Hirudinea), Pogonophora, Vestimentifera and Echiura. There are good morphological (Purschke et al. 1993; Westheide 1997; McHugh 2000) and molecular data (Moon et al. 1996; Erséus et al. 2000; Martin 2001; Siddall et al. 2001) that Clitellata forms a monophyletic group, as do several of its constituent taxa (Erséus and Källersjö 2004).

The division of Annelida into two reciprocally monophyletic sister groups, Polychaeta and Clitellata, was maintained by Rouse and Fauchald (1997), Rouse (1999) and Rouse and Pleijel (2001). In contrast, Purschke et al. (2000) suggested an unspecified subclade of Polychaeta as the sister taxon of Clitellata. Molecular studies support this suggestion but have not clarified potential relationships with polychaete subgroups (McHugh 2000; Martin et al. 2000; Purschke et al. 2000; Rota et al. 2001; Martin 2001; Struck et al. 2002a, b; Bleidorn et al. 2003a, b; Hall et al. 2004; Erséus 2005).

Taxa previously recognised as the phyla Pogonophora and Vestimentifera are now generally considered, with some dissent (Salvini-Plawen 2000), to form a single clade within the polychaete family Siboglinidae (Bartolomaeus 1998; Rouse 2001; Halanych et al. 2001). Molecular studies generally concur but have not been able to establish its sister taxon (Halanych et al. 2001; Halanych 2005; Bleidorn et al. 2003b).

Recent studies by Hessling and Westheide (2002) and Hessling (2003) on the development of the nervous system of *Bonellia* (Echiura) have shown elegantly that these worms are derived from segmented ancestors and belong within Annelida. This is supported by molecular studies (Aguinaldo et al. 1997; McHugh 1997, 2000; Bleidorn et al. 2003a, b; Hall et al. 2004). Studies using in situ hybridisation (Hessling and Westheide 1999; Hessling 2003) strongly suggest that the absence of segmentation in echiurans is secondary, as the organisation of characters such as neuronal ganglia and the organisation of the suboesophageal ganglion is consistent with that found in most annelids. This implies that the trunk is a modified segmental structure and not a single large segment. Hessling and Westheide (1999) suggest that the pattern of the nervous system in larval and juvenile Echiura is homologous with that of Annelida. This was supported by Hessling and Westheide (2002) who used antibodies against the neurotransmitters serotonin (5-hydroxytryptamine) and FMRFamide to demonstrate the presence of paired, discrete repetitive units in the ventral nerve cord during echiuran larval development. Hessling (2003) used computer-aided 3-D reconstruction to show that the organisation of the nervous system is truly metameric, supported by a corresponding arrangement of peripheral nerves. Echiuran cleavage patterns, chaetal formation and sperm ultrastructure closely resemble those found in polychaetes (Newby 1940; Franzén and Ferraguti 1992; Pilger 1993). In 18S rRNA analyses, Echiura is shown as the sister group of the Polychaete family Capitellidae, with considerable bootstrap support (Bleidorn et al. 2003a, b; Hall et al. 2004).

While a lot of progress has been made during the past decade using morphological and/or molecular data to investigate relationships within the annelidans, many questions remain. These concern the relationships among the polychaete annelids, what group is sister to the Clitellata, and what extant group is the most basal on the annelid tree. The low resolution of annelid phylogenies may be due to a rapid radiation of the group. This has been discussed at length by McHugh (2000) and Rota et al. (2001). McHugh (2000) commented on the problems with using morphological characters for interpreting deep annelid relationships. These include determining homology between character states and the prevalence of convergence or parallel secondary losses. Halanych (1998) has argued that the hypothesised rapid radiation resulted in short internodes with few informative sites at the molecular level. Over time, subsequent evolution has led to an erosion of even this relatively small amount of information (Abouhelf et al. 1998). This may explain why analyses of single genes, such as the 18S rRNA data, have not robustly resolved the branching patterns among annelid lineages. The solution may be to use combined data from multiple genes, hoping that phylogenetic signals from the different data sets will be concordant and noise not correlated. This strategy has been adopted in the present study.

Rouse and Fauchald (1997) studied the majority of the approximately 80 polychaete families, including all those for which data were available to code most characters. They separated polychaetes into two main divisions, Scolecida and Palpata. The latter was divided into two groups: (i) Aciculata containing Phyllodocida and Eunicida, and (ii) Canalipalpata containing the major subclades Sabellida, Terebellida and Spionida.

Phyllodocida, Eunicida and Aciculata were well supported. Support for the other clades was low, however, and all were contradicted by at least one of the analyses of Rouse and Fauchald (1997).

Rouse (1999) added 12 egg and larval characters to the morphological data matrix. The placement of a number of families in this analysis differed from the one in Rouse and Fauchald (1997), particularly where characters were unweighted. In Rouse and Fauchald (1997), Chaetopteridae was placed in Spionida, whereas Rouse (1999) found the family within Sabellida. In Rouse (1999), Oweniidae was removed from Sabellida and placed with four minor families not coded by Rouse and Fauchald (1997), forming a distinct, deep-branching clade.

An alternative approach in morphological systematics has been the detailed investigation of particular organs or tissues, thereby increasing confidence in the homology hypotheses underlying character state coding. In particular, investigation of chaetae has been intensive (Hausen 2005). Bartolomaeus et al. (2005) summarise arguments for a monophyletic group within the polychaetes that includes all taxa with chaetae in the form of hooded hooks and uncini. Development and structural details have been used to sustain the hypothesis of the homology of the hooked chaetae and uncini in Oweniidae (Meyer and Bartolomaeus 1996), Arenicolidae (Bartolomaeus and Meyer 1997), Terebellidae and Sabellidae (Bartolomaeus 1995), and Pogonophora (Bartolomaeus 1995). Schweigkofler et al. (1998) investigated the formation of hooded hooks in capitellids and suggested that they are homologous to these hooked chaetae.

No substantial DNA sequence data set unequivocally supports monophyly of any of the major clades defined by Rouse and Fauchald (1997) or the "hooked chaetae" clade discussed in detail by Bartolomaeus et al. (2005). However, some groups within these clades have found support with increases in the number of the available 18S rRNA sequences, especially following the addition of large data sets for Eunicida (Struck et al. 2002a, b), Scolecida (Bleidorn et al. 2003a, b) and Terebellida (Hall et al. 2004).

Whether the levels of conflict between morphological and molecular inferences regarding polychaete phylogeny are as great for other genes as they are for 18S rRNA is unknown. Data for other genes are relatively few, Brown et al. (1999) being the only study attempting a substantial coverage of polychaete diversity prior to the publication of Jördens et al. (2004). Brown et al. (1999) studied two segments of 28S ribosomal RNA, histone H3 and snU2 RNA. Jördens et al. (2004) examined 18S rRNA, part of 28S rRNA and part of cytochrome *c* oxidase subunit I, but did not use the same region of 28S rRNA as Brown et al. (1999). Here, we augment the non-18S rRNA molecular data for polychaetes, compiling sequences from the segments used in Brown et al. (1999), plus mitochondrial cytochrome *c* oxidase subunit I (hereafter abbreviated as COI). This latter gene is too variable to resolve, by itself, the major groups in higher-level phylogenetic analyses of polychaetes (Nylander et al. 1999) but may be useful in combination with other data, or as an adjunct, notably recovering clitellate monophyly in Jördens et al. (2004). New evidence collected for the present compilation includes data missing in Brown et al. (1999), COI sequences from taxa not included in Colgan et al.'s (2001) study of Terebellida, three species not previously sequenced (one a phoronid), and a new gene segment scored in Mollusca to broaden the diversity of the species used as outgroups.

Material and methods

Specimen collection and DNA sequencing

Specimens were collected by the second author or as specified in Brown et al. (1999), Colgan et al. (2001) or Hall et al. (2004). Morphological vouchers, fixed in formalin, were used for identification.

DNA extractions for the investigations of Brown et al. (1999), Colgan et al. (2000, 2001) and Hall et al. (2004) were used as specified in Table 1.

PCR was performed on various dilutions of the DNA, using the primers specified in Brown et al. (1999), except that COI was amplified using the universal primer pair specified in Folmer et al. (1994). Amplification reactions were usually prepared to contain 1.0–2.0 units of QIAGEN Taq Polymerase^{TM}, 1 × QIAGEN PCR buffer, 200 μM of each dNTP, between 2.5 and 4.5 mM $MgCl_2$ (final, allowing for the amount in the QIAGEN buffer), and 0.125 μL of each primer (at a concentration of 100 pM/μL) in a 50 μL volume. Negative controls were performed in each reaction array. Reactions for templates that were difficult to amplify were modified by the inclusion of QIAGEN Q-solution^{TM} (final concentration of 1 ×). Repeated attempts with varied reaction conditions were performed where successful PCRs could not be obtained. PCR products were visualised on 2% agarose gels containing ethidium bromide (0.1 μg/40 mL). PCR products were purified using the QIAquick^{TM} PCR Purification Kit, or using AMPURE magnetic beads (Agencourt) processed by a liquid handling system (Corbett Engineering CAS-3800). Direct sequencing was performed in both directions using the BigDye^{TM} version 3 sequencing kit according to manufacturer's instructions, except that only 2 μL BigDye reaction mix was used in the reactions. Sequencing primers (1 μL) were used at a concentration of 3.2 pM/μL. Reactions

Table 1. Taxa included in the sequence compilation, GenBank accessions and Australian Museum voucher numbers

Classification	Identification at lowest possible rank	U2 snRNA	H3	D1 28S	D9-10 28S rRNA	COI	18S rRNA	Voucher no.
POLYCHAETA								
Sabellida								
Sabellariidae	*Idanthyrsus pennatus*	AF185197 (a)	AF185231 (a)	AF185149 (a)	AF185174 (a)			W 24395
Sabellidae	*Amphiglena terebro*	AF185198 (a)		AF185150 (a)		AF342670 (b)		W 21360
Serpulidae	*Galeolaria caespitosa*	AF185199 (a)	AF185233 (a)	AF185151 (a)			AB106257 (c)	W 24394
Siboglinidae	*Lamellibrachia satsuma*	AF185201 (a)	AF185235 (b)	AF185153 (b)	AF185175 (b)	AF342671 (b)	X79876 (h;F)	W 23600
Oweniidae	*Owenia australis*	AF185200 (a)	AF185234 (a)	AF185152 (a)	AY583709	AY583696	AB106256 (c)	W 23407
"Cirratuliformia"								
Fauveliopsidae	*Fauveliopsis banyuls*	AF185209 (a)	AF185243 (a)	AF185160 (a)	AF185182			
Sternaspidae	*Sternaspis* sp.	AF185210 (a)	AF185244 (a)					
		AF185161 (a)	AF185183 (a)	AB106264 (c)				
Cirratulidae	*Dodecaceria* sp.	AF185203 (a)	AF185237 (a)	AF185155 (a)	AF185177 (a)	AF342673 (b)	AF448154 (i;G)	W 23009
Cirratulidae	*Cirratulus* sp.	AF185204 (a)	AF185238 (a)	AF185156 (a)	AF185178 (a)	AF342672 (b)	AB106262 (c)	W 24391
Terebellida								
Ampharetidae	*Amphicteis dalmatica*	AF342706 (b)	AF342700 (b)	AF342687 (b)	AF342693 (b)	AF342678 (b)		W 199656
Trichobranchidae	*Trichobranchus* sp.	AF342704 (b)	AF342698 (b)	AY583704	AY583711	AF342674 (b)		W 24401
Terebellidae	Amphitritinae (new genus)	AF185206 (a)	AF185240 (a)	AF185157 (a)	AF185179 (a)	AF342683 (b)	AB106260 (c)	W 24400
Terebellidae	*Amphitritides harpa*	AF185207 (a)	AF185241 (a)	AF185158 (a)	AF185180 (a)	AF342676 (b)		W 24398
	Lysilla pacifica	AF342709 (b)	AF342703 (b)	AF342690 (b)	AF342696 (b)	AF342681 (b)	AB106259 (c)	W 23933
	Amaeana trilobata	AF342708 (b)	AF342702 (b)	AF342689 (b)	AF342695 (b)	AF342680 (b)		EBU W50
	Loimia sp.	AF342710 (b)		AF342691 (b)	AF342697 (b)	AF342685 (b)		EBU W70
	Pista australis	AF185208 (a)	AF185242 (a)	AF185159 (a)	AF185181 (a)	AF342682 (b)	AB106261 (c)	W 24390
	Rhinothelepus lobatus	AF342707 (b)	AF342701 (b)	AF342688 (b)	AF342694 (b)	AF342679 (b)		W 201640
Spionida								
Spionidae	*Malacoceros* sp.	AF185211 (a)	AF185245 (a)	AF185162 (a)	AF185184 (a)		AF448149 (i)F	W 8761
	Scololepis squamata					AF138956	AF448164 (i)F	
Chaetopteridae	*Mesochaetopterus* sp.	AF185212 (a)	AF185246 (a)	AF185163 (a)	AF185185 (a)		M20103-5 (k)F	W 22402
Phyllodocida								
Phyllodocida	*Phyllodoce* sp.	AY58372 (a)	AY583719	AY583705	AY583712	AY583697	AB106249 (c)	W 28494
Polynoidae	*Paralepidonotus ampulliferus*	AF185213 (a)	AF185247 (a)	AF185164 (a)	AF185186 (a)	AY583698	AB106253 (c)	W 196405
Sigalionidae	*Sigalion bandaensis*	AF185214 (b)	AF185248 (b)	AF185165 (b)	AF185187 (b)	AY583699	AB106254 (c)	W 23401
Glyceridae	*Glycera tridactyla*	AF185215 (a)	AF185249 (a)	AY583706	AF185176 (a)	AY583700	U19519 (l;G)	W196835
Nephtyidae	*Nephtys australiensis*	AF185216 (a)	AF185250 (a)	AF185166 (a)	AF185188 (a)			W 23425
Myzostomida	*Myzostoma cirriferum*	:	AY218149 (m)			AY218082 (m)	AY210446 (n;G)	
Nereididae	*Ceratonereis longiceratophora*	AF185217 (a)	AF185251 (a)	AY859731	AF185189 (a)	AY583701	AB106251 (c)	W 24392
Amphinomida								
Amphinomidae	*Eurythoe* sp.	AF185218 (a)	AF185252	AF185167 (a)	AF185190	AY364857 (o;G)	AY364851 (o;G)	W 4444
Eunicida								
Lumbrineridae	*Lumbrineris latreilli*	AF185219 (a)	AF185253 (a)	AF185168 (a)	AF185191 (a)		AB106247 (c)	W 194794
Eunicidae	*Marphysa macintoshi*	AF185220 (a)	AF185254 (a)		AY583713		AF412789 (p;G)	W 196557
	Eunice australis	AF185221 (a)	AF185255 (a)	AF185169 (a)	AF185192 (a)		AF412790 (p;G)	W 10076

Table 1. (*continued*)

Classification	Identification at lowest possible rank	U2 snRNA	H3	D1 28S	D9-10 28S rRNA	COI	18S rRNA	Voucher no.
Scolecida								
Maldanidae	*Euclymene trinalis*	AF185222 (a)	AF185256 (a)	AF185170 (a)	AF185193 (a)	AY583702	AF448152 (i;F)	W 24100
Capitellidae	*Notomastus torquatus*	AF185224 (a)	AF185258 (a)		AY859739		AB106265 (c;F)	W 23426
Scalibregmatidae	*Hyboscolex* sp.	AY583723 (a)	AY583720	AY583707	AY583714		AB106268 (c)	
Opheliidae	*Polyophthalmus pictus*	AF185225 (a)	AF185259 (a)	AF185171 (a)	AF185194 (a)		AB106267 (c)	
Orbiniidae	*Phylo felix*	AF185226 (a)	AF185260 (a)		AY583715	AY583703	AY612619 (q;G)	W 8266
CLITELLATA								
Lumbricidae	*Lumbricus terrestris*	AF185228 (a)	AF185262 (a)	AF185172 (a)	AF185195 (a)	U24570 (g;G)	Z83753 (r;G)	W 24393
Glossiphoniidae	*Glossiphonia complanata*					AY047321 (s)	AF099943 (t)	
Branchiobdellida	*Cambarincola pamelae*					AF310712 (u)	AF310695 (u)	
Haemadipsidae	*Haemadipsa sumatrana*				AY425373 (v;G)	AY425446 (v)	AY425464 (v)	
ECHIURA	*Bonellia* sp.	AF185196 (a)	AF185263 (a)	AF185173 (a)	AF185229 (a)		X79875 (h;F)	W 5506
MOLLUSCA								
Polyplacophora	*Ischnochiton australis*	AY296851 (e)	AF033694 (d)	AY296873 (e)	AY859732	AY296815 (e)		C203201
	Liolophura japonica		AY377756 (w)			AY377717 (w)	AY377652 (w)	
Cephalopoda	*Nautilus scrobiculatus*	AY296854 (e)	AF033704 (d)			AF000054 (f)		USNM885678
Bivalvia	*Anadara trapezia*	AY296853 (e)	AF033677 (d)	AY296875 (e)	AY859734	AY296817 (e)		C203202
	Trichomya hirsuta	AY296852 (e)	AF033714 (d)	AY296874 (e)	AY859733	AY296816 (e)		C203203
	Anodonta SWH2004		AY579132 (x)			AY579122 (x)	AY579106 (x)	
	Mytilus edulis		AY377769 (w)			AY377727 (w)	L33448 (y)	
Gastropoda	*Cellana tramoserica*	AY296855 (e)	AF033687 (d)	AY296877 (e)	AY859736	AY296818 (e)		C203204
	Depressigyra globulus	AY296861 (e)	AF033689 (d)	AY296886 (e)	AY859738	AY296825 (e)		Tunnicliffe: HYS202
	Notocrater houbricki	AY859729	AF033700 (d)	AY296883 (e)	AY859737	AY296822 (e)		USNM888656
	Austrocochlea porcata	AY296859 (e)	AF033676 (d)	AY296881 (e)		AY296821 (e)		C203207
	Epitonium cf. *jukesianum*	AY296865 (e)	AF033691 (d)	AY296896 (e)	AY583716	AY296836 (e)	AY583724	C203218
PHORONIDA	*Phoronis* sp.		AF033691	AY859730	AY583717		PVU12648 (l;F)	W 17650
SIPUNCULA	Unidentified sipunculan	AF185230 (a)	AF185264 (a)	AY583708	AY583718		AJ389664 (z;F)	W 24397

The taxonomy is adapted from Rouse and Fauchald (1997) as modified by Rouse and Pleijel (2003). New sequences were determined from DNA extracted for Brown et al. (1999), Hall et al. (2004; *Polyophthalmus*, *Paralepidonotus*, *Sigalion* and *Phyllodoce*) and Colgan et al. (2000, 2003; molluscs), respectively. Accession numbers not followed by any bracketed reference term denote sequences newly generated for the present study. Lower-case letters in the reference terms indicate sequence provenance as follows: a = Brown et al. (1999); b = Colgan et al. (2001), c = Hall et al. (2004); d = Colgan et al. (2000); e = Colgan et al. (2003); f = Carlini and Graves (1999) (from *Nautilus pompilius*); g = Boore and Brown (1995), h = Winnepenninckx et al. (1995); i = Bleidorn et al. (2003b); j = Schulze et al. (2000); k = Field et al. (1988); l = Halanych et al. (1995); m = Giribet et al. (2004); n = Y.J. Passamaneck and K.M. Halanych, unpublished; o = Jördens et al. (2004); p = Struck et al. (2002a); q = Persson and Pleijel (2005); r = Kim et al. (1996); s = Light and Siddall (1999); t = Trontelj et al. (2001); u = Gelder and Siddall (2001); v = Borda and Siddall (2004); w = Okusu et al. (2003); x = Huff et al. (2004); y = Kenchington et al. (1995); z = Steiner and Hammer (2000). A capital G in the bracketed reference term indicates that the sequence is not from the listed species but from the same genus, a capital F indicates that it is from the same family only.

were purified by ethanol precipitation, or using CleanSeq magnetic beads (Agencourt) on the robot, and run on an Applied Biosystems (ABI Prism)^{TM} 310 automated DNA sequencer.

Sequence adjustment and alignment

Sequences were edited using Sequence Navigator^{TM}. Sequences for each gene fragment were aligned with ClustalX (Thompson et al. 1997), assuming the following values for parameters. The "slow-accurate" algorithm was used for pairwise alignment, with costs of 10.0 for gap opening and 0.10 for gap extension. For multiple alignment, the cost for gap opening was set at 10.0, and gap extension at 0.20, with a DNA transitions weight of 0.50 and a "delay divergent sequences" percentage of 30. Manual changes to restore the alignment of clearly homologous base positions were made in MacClade (Maddison and Maddison 1992). This was necessary where an additional base in a sequence caused the displacement of a long run of bases when compared to the alignment of the other taxa. The base was removed from the alignment where rechecking indicated that its inclusion was erroneous.

Phylogenetic analysis

With some exceptions, taxa were included in the analysis if data were available for at least four of the six gene segments under investigation. The polychaete exceptions were *Scololepis* which was included as a second spionid, and the sabellid *Amphiglena terebro* and the myzostome as members of families representing important lineages. Mollusca were included if sequenced for two or more gene segments, in order to maximise the number of outgroups. To summarise the results, we report on three pairs of analyses: maximum parsimony (MP), maximum likelihood (ML), and Bayesian (BY) Where third-position data are excluded this is indicated by "−p3" in the analysis designation. Areas of uncertain alignment in the 28S rRNA sequences were excluded from all of these analyses, but were included in some series among the many performed in addition to those reported here. Optimal trees for all analyses were rooted using Mollusca as the outgroup.

MP analyses of sequence data were performed using PAUP* 4.0b10 (Swofford 2001). Parsimony analysis used a heuristic search with 200 replicates and the following assumptions: gaps were treated as missing data; branch swapping used tree-bisection reconnection; the addition sequence was random; characters were unordered and unweighted. Bootstrapping was performed using heuristic searches (addition sequence random; 10 replicates) for 500 pseudosample repetitions.

ML analyses were conducted with PAUP*. MODELTEST (Posada and Crandall 1998) was used to select a model (based on the Akaike information criterion) for searches. Heuristic searches were conducted with 20 random addition replicates for ML, and five for ML−p3. For the ML analysis, the model selected was (GTR + I + G), with the following settings: estimated base frequencies (A = 0.2560, C = 0.2227, G = 0.2561, T = 0.2652); number of substitution types = 6; substitution rate matrix (A–C: 2.0489, A–G: 3.3309, A–T: 2.7564, C–G: 1.5921, C–T: 7.7006, G–T: 1.0000); discrete gamma distribution with an α parameter of 0.3738; and proportion of invariant sites 0.2082.

BY analyses were conducted with the programme MrBayes 3.1 (Huelsenbeck and Ronquist 2001), estimating the likelihood parameters for initial base frequencies, the number of substitution types and rates, the shape of the gamma distribution and the proportion of invariable sites. Likelihood parameters were estimated separately for each gene (and each codon position within coding sequences) using a character partition and the "unlink" command in MrBayes. Trees were sampled every 100 steps along a 400,000 step Markov Chain. Four simultaneous chains were run. To allow for convergence to an area of stable likelihood (burn-in), the first 200,000 steps were discarded in the BY analysis, and the first 150,000 in the BY-p3 analysis.

Results

The alignment for the complete data set included 56 taxa with 4023 aligned positions. Regions of uncertain alignment in the 18S rRNA and 28S rRNA sequences totalled 558 bases. After excluding areas of uncertain alignment, the data set had 1218 parsimony-informative sites and 630 sites that were variable but parsimony-uninformative. The percentage GC composition including all taxa was homogeneous for all gene segments except COI: for D1 28S rRNA, $P = 1.000$; for D9-10 rRNA, $P = 1.000$; for 18S rRNA, $P = 1.000$; for COI, $P = 0.000$; for COI excluding third-base positions, $P = 0.998$; for H3, $P = 0.656$; for H3 excluding third-base positions, $P = 1.0000$; and for U2 snRNA, $P = 1.000$.

Details of the respective number of bases in the alignments for individual genes are given in Table 2. This also lists the bootstrap-supported clades in MP analyses of the individual genes (excluding areas of uncertain alignment).

Topologies illustrating some of the analyses for this paper are shown in Figs. 1–3. Fig. 1 shows the ML tree. Fig. 2 shows the majority rule consensus tree of the topologies sampled during the BY analysis.

Table 2. Analyses of individual genes

Analysis	Aligned bases	Uncertain alignment	Parsimony informative	Variable	Bootstrap-supported clades	Trees	CI	Length
D1 28S rRNA	377	96	96	56	(*Dodecacaria*, *Cirratulus*)-94; (*Lysilla. Amaeana*)-90; (*Sigalion*, *Paralepidonotus*)-80	2033	0.403	665
D9-10 28S rRNA	688	130	186	128	(*Anadara*, *Epitonium*)-97; ((*Trichobranchus*, Patellogastropoda)-95, *Bonellia*)-75; (*Dodecacaria*, *Cirratulus*)-65; (((*Lysilla. Amaeana*)-93, *A. harpa*)-87, *Amphitritides*)-57; (*Sigalion*, *Paralepidonotus*)-68, *Phylo*)-92; (*Marphysa*, *Eunice*)-89	2200	0.489	1140
Histone H3	299	–	22	125	(*Trichomya*, *Mytilus*)-94; (*Lysilla*, *Amaeana*)-98; (*Marphysa*, *Eunice*)-74; (*Malacoceros*, *Hyboscolex*)-54; (*Liolophura*, *Ischnochiton*)-95	400	0.205	1226
SnU2 RNA	136	–	52	26	((*Lysilla*, *Amaeana*)-99, *Amphitritides*)-61; ((*Malacoceros*, *Galeolaria*)-99, *Amphiglena*)-59; ((*Lysilla. Amaeana*)-99, *Amphitritides*)-71; (*Sigalion*, *Paralepidonotus*)-97; (*Austrochlea*, *Ischnochiton*, *Depressigyra*)-59	12,000	0.387	377
18S rRNA	1981	332	417	338	(*Dodecacaria*, *Cirratulus*)-100; ((*Lysilla*, *Amphitritides*)-59, *Pista*)-90; (*Sigalion*, *Paralepidonotus*)-100; (*Phyllodoce*, *Glycera*)-90; (*Marphysa*, *Eunice*)-100; (((*Haemadipsa*, *Glossiphonia*)-90, *Cambarincola*)-65, *Lumbricus*)-94; (Myzostomida, *Galeolaria*)-75 [a]	13	0.481	2441
COI	542	–	346	61	((*Trichomya*, *Mytilus*)-100, *Anadara*)-97; (*Dodecacaria*, *Cirratulus*)-87; (*Lysilla*, *Amaeana*)-100; (Maldanidae, *Hyboscolex*)-63; ((*Cambarincola*, *Glossiphonia*)-83, *Haemadipsa*)-65	2	0.243	3464

Columns two to five give the number of bases in the respective gene alignment, the number excluded from the analysis owing to uncertain alignment, the number of parsimony informative sites and the number of variable sites that are not parsimony informative. Column six shows the bootstrap percentages for clades supported by each analysis. The last three columns specify the number of trees found in 100 replicates (keeping at most 200 in each replicate, with an overall maximum of 12,000), the consistency index (excluding uninformative characters), and the tree length; [a]: these two taxa have very long branches.

Only one MP tree was found in heuristic searches (Fig. 3). This had a length of 9858 and a consistency index of 0.324. A single most parsimonious tree was also found when third-base positions were excluded (MP−p3). This had a length of 6237 and a consistency index of 0.408.

Annelida and Mollusca were monophyletic in ML and distinct in all analyses, but Annelida included *Nautilus* (Cephalopoda) in MP and MP−p3, and *Cellana* (Patellogastropoda) in BY and ML−p3. In MP−p3, ML and BY, there was a recognisably basal group of polychaete taxa, consisting of Oweniidae, Chaetopteridae and Amphinomidae. The sipunculan and the phoronid were interspersed with these polychaetes in the MP−p3 and ML trees. *Nautilus* and the myzostome were also included in this basal assemblage in MP−p3. In ML, MP−p3 and BY, the next higher branching clade of polychaetes included the six phyllodocidan families and Orbiniidae. In ML−p3, Amphinomidae and Oweniidae were basal, with the positions of Chaetopteridae and Phyllodocida reversed. Polychaetes other than Oweniidae, Chaetopteridae and Amphinomidae (and Myzostomida in MP−p3) were monophyletic in BY, ML and MP−p3.

The basal topology of the MP tree was different from those obtained by the other analyses. *Nautilus*, the clitellates, the sipunculan, the myzostome, Oweniidae plus Serpulidae formed a clade that was the sister to a monophyletic group containing all other polychaetes. The next higher branching clade in this topology contained most Terebellida and the siboglinid. The topology of this is likely to be affected by long branch attraction problems for at least the serpulid and myzostome.

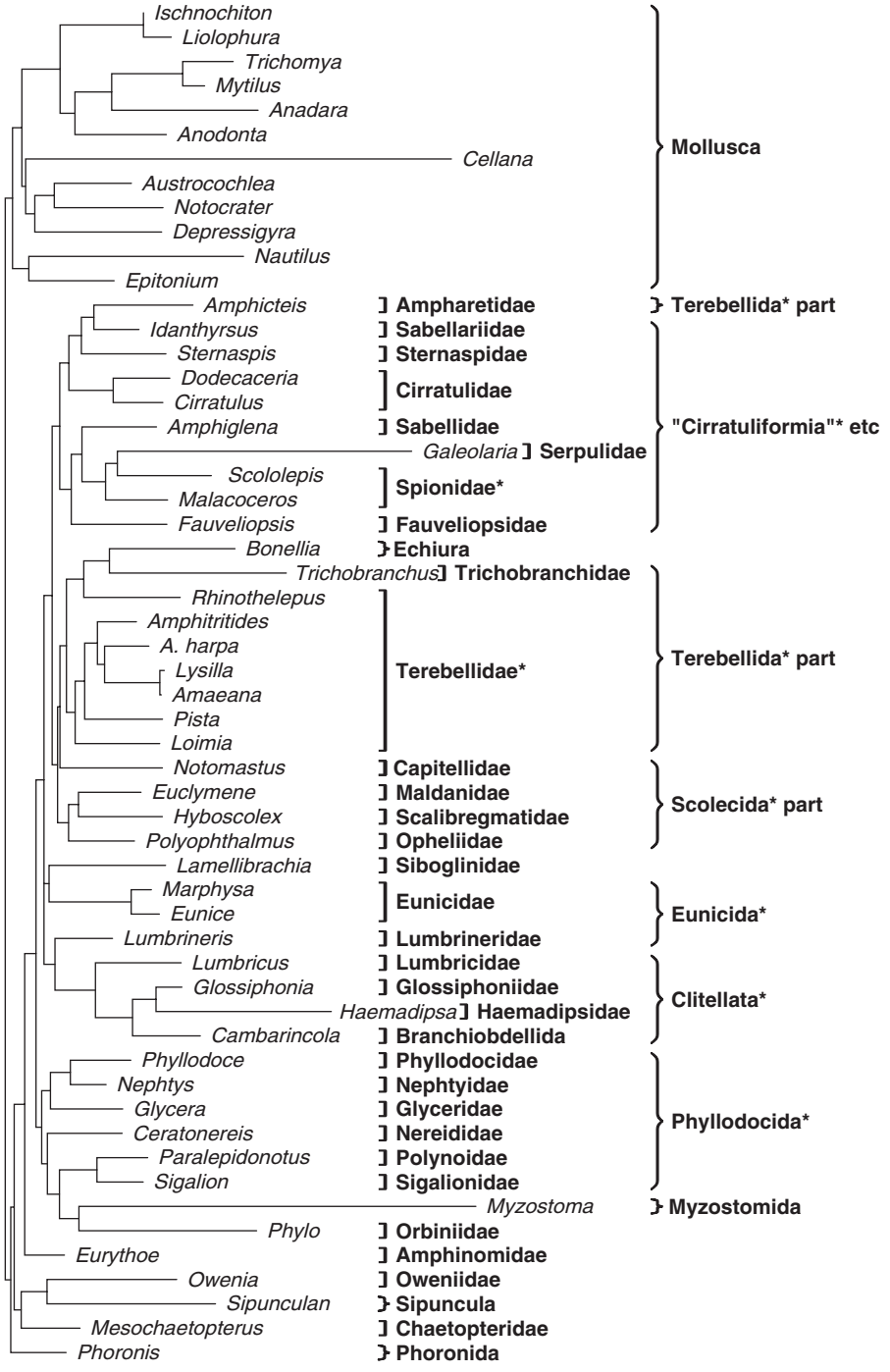

Fig. 1. Phylogram of maximum likelihood tree rooted on Mollusca. Branch length indicated by scale bar graduated in units of 0.01 changes per site; families identified by braces to the right of generic names; higher-level groups identified by brackets; asterisk indicates that group is not monophyletic; where a named group occurs in multiple regions of the topology this is indicated by ''part''; generic names specify the taxon from which most segments were sequenced.

Notable relationships within Polychaeta were observed in a number of analyses. These included (i) the inclusion of Echiura in Terebellida; (ii) support for the Phyllodocida, but including Orbiniidae; (iii) ''Cirratuliformia'' (Cirratulidae, Sternaspidae, Fauveliopsidae; sensu Rouse and Pleijel 2003) plus Sabellidae, Sabellariidae and Spionidae; and (iv) Scolecida except Orbiniidae.

The echiuran was shown as the sister group of *Trichobranchus* (Trichobranchidae) in all analyses ex-

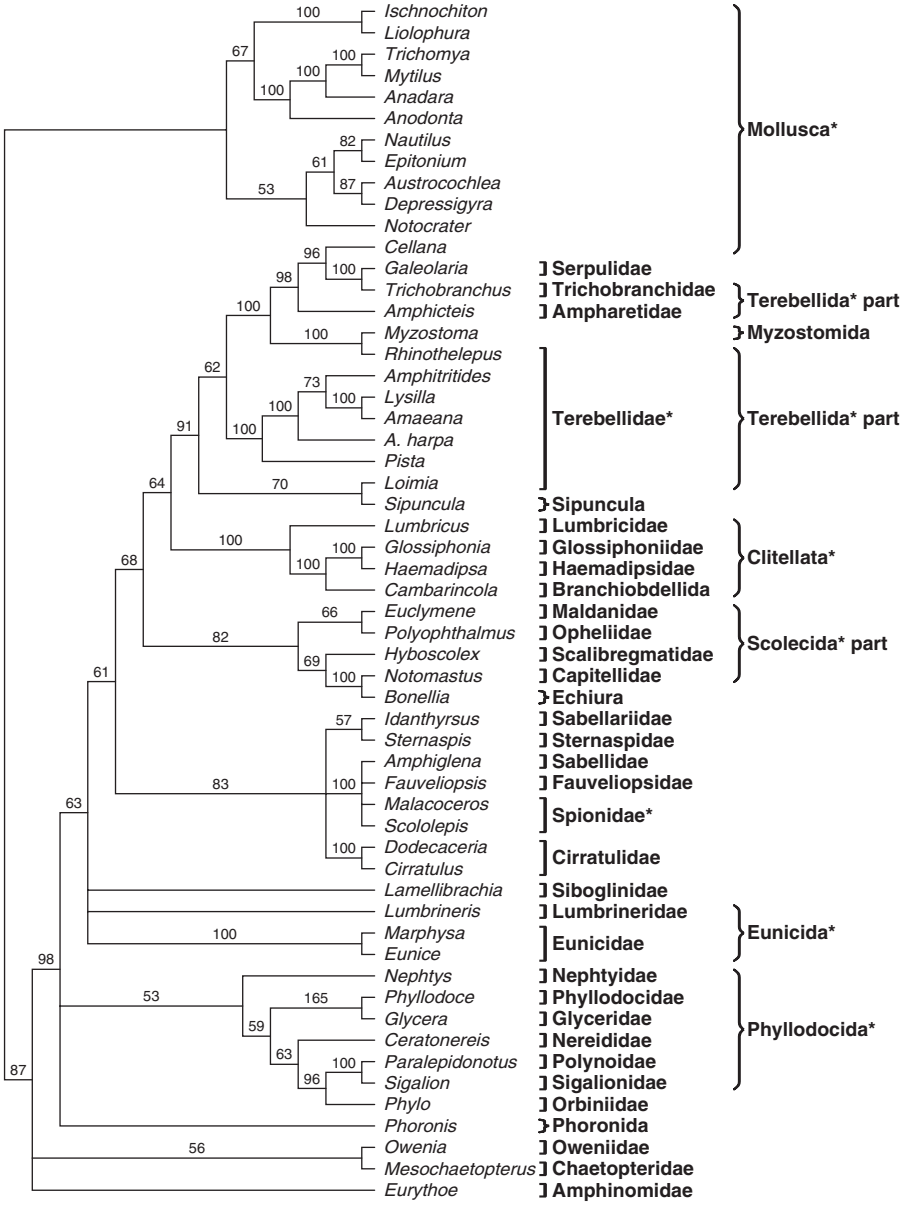

Fig. 2. Majority-rule consensus of trees sampled during Metropolis-coupled Monte Carlo Markov Chain simulation for Bayesian analysis. Tree rooted on Mollusca, except for *Cellana*; figures above branches indicate posterior probabilities; families identified by braces to the right of generic names; higher-level groups identified by brackets; asterisk indicates that group is not monophyletic; where a named group occurs in multiple regions of the topology this is indicated by ''part''; generic names specify the taxon from which most segments were sequenced.

cept BY where its placement as the sister group to *Notomastus* (Capitellidae) had a posterior probability of 100%. Conversely, in BY−p3, the probability was 100% for the pairing of *Trichobranchus* and the echiuran. When Trichobranchidae were excluded, MP and MP−p3 analyses showed the echiuran as sister group to *Rhinothelepus* (Terebellidae).

Three terebellidan families (Ampharetidae, Terebellidae and Trichobranchidae) were included in this study. With the exception of BY, Ampharetidae were generally excluded from Terebellida. Terebellidae and Trichobranchidae were generally included with the echiuran in a monophyletic clade that had moderate bootstrap and BY support. The siboglinid was also included in this clade in MP and MP−p3 analyses.

The studied ''Cirratuliformia'' were grouped with Sabellidae, Sabellariidae and Spionidae in BY. In ML and ML−p3, the group also included Spionidae, Capitellidae, Serpulidae and Ampharetidae. In BY−p3, a group comprising Cirratuliformia plus Siboglinidae,

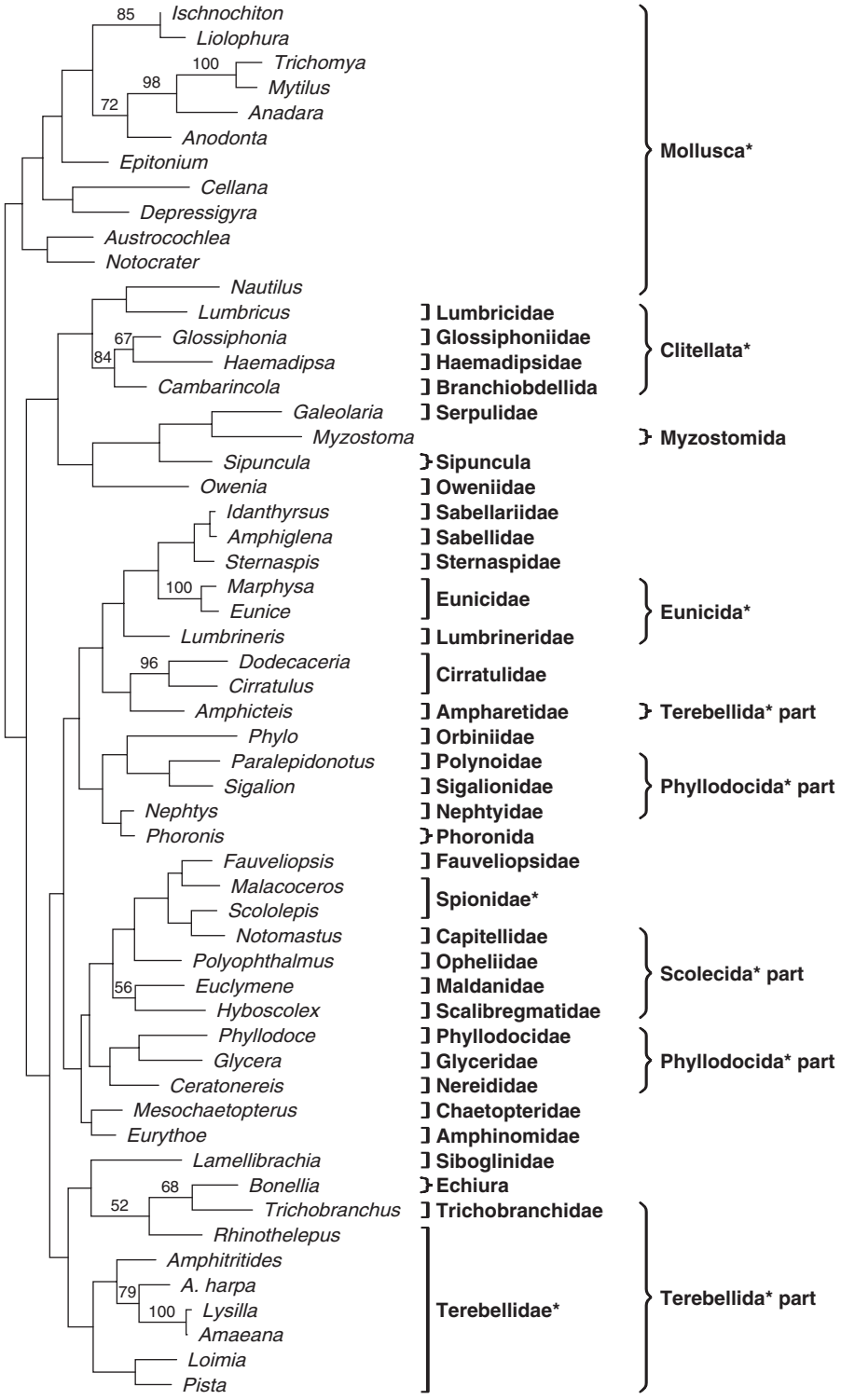

Fig. 3. Phylogram of maximum parsimony tree rooted on Mollusca (except for *Nautilus*). Bootstrap support percentages greater than 50 shown above branches; number of changes indicated by scale bar, graduated in units of 10 steps; families identified by braces to the right of generic names; higher-level groups identified by brackets; asterisk indicates that the group is not monophyletic; where a named group occurs in multiple regions of the topology this is indicated by ''part''; generic names specify the taxon from which most segments were sequenced.

Serpulidae, Lumbricidae and both families of Eunicida was supported by a posterior probability of 69%. Sternaspidae was shown as the sister group to Fauveliopsidae in MP−p3, but was more closely related to members of Sabellida in most other analyses. The placement of Serpulidae was notably variable in the analyses, apparently owing to its great difference from other taxa in the 18S sequence. The family is the sister group of Trichobranchidae in parsimony analyses of D1, but is associated with one or more families of the (Cirratuliformia, Sabellidae, Sabellariidae, Spionidae) group in other single-gene analyses.

Within Phyllodocida, Orbiniidae was shown as the sister group to the scale-worms (Sigalionidae and Polynoidae) in all analyses, with high posterior probabilities in BY and BY−p3, but limited bootstrap support. The association was not due to the dominating effect of a single gene, as shown in MP analyses of individual data sets. Orbiniidae were not scored for D1 28S rRNA. In D9-10 28S rRNA, Orbiniidae was a sister group to Sigalionidae and Polynoidae with high bootstrap support, although inspection of sequences revealed that the scale-worm pair were much more similar to each other (0.02 Kimura 2-parameter genetic distance) than either was to Orbiniidae (genetic distances 0.06 and 0.08, respectively). In histone H3 analyses, Orbiniidae was basal within polychaetes. In U2 snRNA, Orbiniidae was sister group to Maldanidae in 56% of MP trees but otherwise not closely associated with any taxon. In COI analyses, Orbiniidae and Glyceridae were shown as sisters, and in 18S rRNA, the orbiniid and sipunculan were sister taxa, but each with a long branch.

Eunicida was recovered only in ML−p3. In some other analyses (MP, MP−p3), Lumbrineridae was sister to a clade comprising Eunicidae and other families. Lumbrineridae and Clitellata were sister groups in ML. Lumbrineridae was the sister group to *Lumbricus* (but not the other clitellates) in BY−p3. Eunicidae and Clitellata were sister groups in MP−p3.

Discussion

The use of multiple genes in the present analysis has increased the concordance of molecular and morphological perspectives on polychaete phylogenetics and the degree of statistical support for at least some clades, particularly in the BY analyses. Whilst a great deal of more investigation is necessary to achieve a satisfactory understanding of this enigmatic taxon, consideration of combined molecular analyses in the light of recent morphological investigations outlines a framework to direct future investigations. Aspects of this framework are discussed below.

Identifying basal polychaetes

Monophyly of the supposed outgroups to Annelida was not observed in any of the present analyses. Annelida and Mollusca were recovered as reciprocally monophyletic groups in some analyses (e.g. ML; Fig. 1), but not in others where the patellogastropod *Cellana* (BY; Fig. 2) or the cephalopod *Nautilus* (MP; Fig. 3) was included in Annelida. Of the two other studied lophotrochozoan outgroups, the phoronid was sometimes excluded from Annelida (Fig. 1) and sometimes included (Figs. 2 and 3), and the sipunculan was generally included. Sipuncula have also been closely associated with annelid groups in previous analyses of DNA sequences (Martin 2001; Brown et al. 1999; Bleidorn et al. 2003a; Hall et al. 2004). Gene order in the studied section (approximately half) of the mitochondrial DNA of the sipunculan *Phascolopsis gouldii* (Boore and Staton 2002) is notably similar to that in the oligochaete *Lumbricus terrestris* (Boore and Brown 1995), differing only by one inversion and one transposition. Bartolomaeus and Quast (2005) identify a ''remarkable similarity in the podocyte lining'' among Sipuncula, some Sabellida and Terebellida, that they consider is unlikely to be homoplasious as a result of functional constraints. Sipuncula may eventually prove to be a suitable outgroup to Polychaeta in molecular analyses. To root the current topologies on this group would, however, imply paraphyly of the Polychaeta with respect to all other supposed outgroups, a doubtful proposition if based on only one representative.

In the absence of outgroup monophyly, we have chosen to root topologies presented herein on the largest possibly monophyletic clade of Mollusca in each analysis.

Using Mollusca to root the polychaetes in likelihood analyses and MP−p3 consistently identifies a basal group of polychaete taxa comprising Chaetopteridae, Amphinomidae and Oweniidae. The phoronid and sipunculan were usually included within this group in an assemblage that was basal to the main polychaete lineage. The derived position of most polychaetes is strongly supported, with a BY posterior probability of 98 for the clade including the main lineage and the phoronid. Other taxa that may need to be studied in subsequent molecular attempts to resolve relationships within the basal polychaetes include Aphroditidae, Chrysopetalidae, and a variety of interstitial polychaetes, especially Diurodrilidae (Worsaae and Kristensen 2005).

The distribution of characters in the three basal taxa identified here is, as discussed below, too confusing to specify which of these taxa is the most basal. One example, however, may be considered here. Recently, Chrysopetalidae has been shown to lack the organisation of body-wall musculature presumed to characterise

Annelida (i.e. the possession of both outer circular fibres and inner longitudinal fibres) (Tzetlin et al. 2002). Other taxa that lack circular muscle elements include Oweniidae, Opheliidae, Spionidae, Aphroditidae, scale-worms and several other phyllodocidan families (Tzetlin et al. 2002). The distribution of this character is difficult to reconcile with any previously proposed taxon.

The two main morphological approaches to establishing the root of Polychaeta have been (i) cladistic analyses (Rouse and Fauchald 1997; Rouse and Pleijel 2001), and (ii) the search for the taxon's ground plan using selected characters and considering their particular evolutionary patterns (Westheide 1997; Purschke 2002). Purschke (2002) and Bartolomaeus et al. (2005) emphasise that the condition in putative ancestors must be functional.

By assuming Clitellata as the sister group to Polychaeta, Rouse and Fauchald (1997) found the root in their preferred topology to lie between Scolecida and Palpata. Neither of these clades were, however, monophyletic in all of their analyses. Rouse and Pleijel (2001) also treated Clitellata as the sister group to Polychaeta, with the basal division in Polychaeta being a polytomy of Scolecida, Aeolosomatidae, Parergordrilidae, Psammodrilidae and the remaining taxa (as a monophyletic clade). As molecular studies have shown that Clitellata is probably a derived taxon within Polychaeta, the root cannot be identified correctly by this approach.

Investigations of the ground plan have generally not specified a root of the overall annelid/polychaete topology but have indicated a number of families as possibly basal. Amphinomidae (''traditionally''; Westheide 1997) and Oweniidae (Bubko 1973 fide Westheide 1997) have been regarded as morphologically primitive by previous authors. Westheide (1997) also notes the exclusively monociliated nature of the epithelia of some (but not all) oweniid genera, quoting Rieger's opinion (1976) that this character is primitive among the Spiralia. Salvini-Plawen (2000) suggested that *Owenia*'s paedomorphic retention of the juvenile intra-epithelial nervous system and its possession of a ''deuterostome-like'' nephridium in its mitrarian larva (Smith et al. 1987) may possibly be primitive characters.

In the MP analysis of Struck et al. (2002a), one of the few 18S rRNA studies in which Annelida and Mollusca were not topologically intermingled, the basal division in Polychaeta separates Chaetopteridae and the remaining taxa (Oweniidae was not included). The development of Chaetopteridae is quite distinct from other polychaetes during both embryonic (Mead 1897; Rouse 1999) and larval stages (Irvine et al. 1999). However, this character is not likely to be informative for polychaete phylogeny as is it most probably an autapomorphy (Irvine et al. 1999; Rouse 1999).

Scolecida and the "hooked chaetae" clade

Scolecida was not strongly supported in the morphological analyses of Rouse and Fauchald (1997), with only two synapomorphies supporting the group: presence of parapodia with similar rami, and presence of two or more pairs of pygidial cirri. Both characters were homoplasious. Another homoplasious character (the presence of sensory ''lateral'' organs) supports the monophyly of the taxa scored here. Orbiniidae differ from all other Scolecida scored here in the possession of dorsal flat branchiae and an eversible ventral buccal organ. This organ is axial in the other families scored here.

Families referred to the Scolecida by Rouse and Fauchald (1997) have generally exhibited extremely variable placement in molecular analyses although, with the exception of Orbiniidae and Questidae (Bleidorn et al. 2003a; Hall et al. 2004), most are closely related to each other (Bleidorn et al. 2003a; Persson and Pleijel 2005). The close association of Capitellidae and Maldanidae with Scalibregmatidae and Opheliidae was observed in all present analyses except MP−p3 and ML−p3. In the ML analyses of Bleidorn et al. (2003a), a group of four scolecid families was paired with Cirratulidae. Arenicolidae and Maldanidae were associated with Terebellida in Hall et al. (2004), in agreement with Brown et al. (1999). Scolecida has never been recovered in a basal position in molecular analyses.

The concept that there is a monophyletic group within the polychaetes that includes all taxa with chaetae in the form of hooded hooks and uncini (Bartolomaeus et al. 2005) finds some support in the present analyses. There is a clear tendency in all of the analyses for the remnant Scolecida (i.e. excepting Orbiniidae) to associate with Terebellida, Sabellida, etc. A clade including all of the scored taxa (except Oweniidae and Siboglinidae) included in the ''hooked chaetae'' clade by Bartolomaeus et al. (2005) was observed in ML. However, this clade also included scolecidan families without hooked chaetae (Scalibregmatidae and Opheliidae), and Echiura.

Phyllodocida

The results reported here support recognition of the Phyllodocida (including Orbiniidae). In previous molecular analyses, the taxon Phyllodocida was closest to recognition in Struck et al. (2002a) where it was paraphyletic with respect to the clitellates, and in Hall et al. (2004) where the monophyly of Phyllodocida excluding Myzostomida was not statistically significantly rejected. In most of our analyses (BY, MP−p3, ML), a monophyletic clade including at least six of the seven scored Phyllodocida was recovered. In ML, this

clade also included the seventh, the morphologically highly modified Myzostomida, although other analyses agree with Zrzavý et al. (2001) in the exclusion of this group.

Our analyses place Orbiniidae with the scale-worms Polynoidae and Sigalionidae. Although based on a more restricted taxonomic sample, analyses of combined 28S and 18S rRNA data by Jördens et al. (2004) suggest that Orbiniidae is more closely related to Syllidae (the sole representative of Phyllodocida) than to Opheliidae or Capitellidae, the other studied Scolecida. The possession of aciculae was used to define one of the largest clades in Polychaeta by Rouse and Fauchald (1997). Aciculae were encoded as absent from Orbiniidae in those authors' data matrix but recorded as present in their family descriptions. This was corrected in the matrix for Rouse (1999), who placed Orbiniidae in Scolecida. The inclusion of Orbiniidae in Phyllodocida in the present molecular topologies suggests that the aciculae of the two taxa may be homologous. Hausen's (2005) comprehensive survey of chaetae in Polychaeta does not, however, specify whether fine-scale investigation of orbiniid aciculae supports this inference.

"Cirratuliformia" and Terebellida

Terebellida has generally been regarded as comprising five families (Alvinellidae, Ampharetidae, Pectinariidae, Terebellidae and Trichobranchidae) (Hatschek 1893; Hessle 1917; Holthe 1986). Although Ampharetidae is not associated with the group in the present analyses, Terebellida is often monophyletic in molecular studies (Brown et al. 1999; Hall et al. 2004). The concept of Terebellida was expanded by Rouse and Fauchald (1997) to include taxa later included in Cirratuliformia, but there is no evidence in the present data that these families are more closely related to the traditional Terebellida than to Sabellidae, Sabellariidae, etc. The traditional concept of Terebellida is generally supported by more recent morphological analyses (Glasby et al. 2004).

Echiura is generally included in Terebellida in the present analyses, usually with strong support, in contrast to 18S rRNA analyses that strongly support the hypothesis that echiurans and Capitellidae are sister groups (Bleidorn et al. 2003a, b; Hall et al. 2004). Echiura was paired with Capitellidae in the ML analysis and in the combined 18S and 28S rRNA ML analysis of Jördens et al. (2004), although the two taxa are separated in their individual 28S rRNA analysis.

Sternaspidae was included in "Cirratuliformia" by Rouse and Pleijel (2003), a suggestion supported by previous non-18S rRNA molecular analyses (Brown et al. 1999) which placed the family as the sister group to Fauveliopsidae. 18S rRNA data (Hall et al. 2004) suggest an alternative association with Lumbrineridae, although this is not strongly supported by bootstrap values or Bremer decay indices. Here, the combined data associate Sternaspidae most closely to Sabellariidae or Sabellidae, although the family is clearly related to the "Cirratuliformia".

Clitellata and Eunicida

Clitellata is sometimes associated with the Lumbrineridae or Eunicidae in our analyses (except MP), but the results are not consistent enough to be confident of this placement. Likewise, Clitellata was placed as the sister group to Lumbineridae in the 18S rRNA MP analysis of Hall et al. (2004) and in the 18S rRNA ML analysis of Jördens et al. (2004). Another family, Dinophilidae, was included with the serpulid *Galeolaria caespitosa* in the sister group to Clitellata in the likelihood analysis of Hall et al. (2004). The placement of the serpulid was doubtful owing to its high divergence from other taxa. Lumbrineridae was the sister group to (Clitellata + Dinophilidae + Serpulidae). In Struck et al. (2002a), Dinophilidae was included in the sister group of Clitellata in the strict consensus of MP trees, although not in ML analyses. The suggestion of a close association of Clitellata and Dinophilidae is complicated by the taxonomic uncertainty regarding the latter. A detailed morphological study by Eibye-Jacobsen and Kristensen (1994) suggested that Dinophilidae would be best classified within Dorvilleidae, differences between them being ascribed to the loss of characters in some species of dinophilids due to their interstitial life style. Struck et al (2002b) included seven dorvilleids and four dinophilids in their analyses. The groups were distinct and, moreover, Dorvilleidae was neither monophyletic nor closely related to Clitellata.

Envoi

Obtaining robust support for the relationships within Polychaeta and its potential members or outgroups using DNA sequence data is likely to require the sequencing of many additional taxa and multiple additional genes. This process may take some time. In the interim, however, sufficient data are now available to inform the decisions of morphologists as to which taxa are critical for intensive investigation. This study suggests, for instance, that Oweniidae, Chaetopteridae and Amphinomidae are of prime interest for determining the root of the polychaete tree.

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