

# Investigation of Molluscan Phylogeny on the Basis of 18S rRNA Sequences

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The 18S rRNA sequences of 12 molluscs, representing the extant classes Gastropoda, Bivalvia, Polyplacophora, Scaphopoda, and Caudofoveata, were determined and compared with selected known 18S rRNA sequences of Metazoa, including other Mollusca. These data do not provide support for a close relationship between Platyhelminthes (Turbellaria) and Mollusca, but rather suggest that the latter group belongs to a clade of eutrochozoan coelomates. The 18S rRNA data fail to recover molluscan, bivalve, or gastropod monophyly. However, the branching pattern of the eutrochozoan phyla and classes is unstable, probably due to the explosive Cambrian radiation during which these groups arose. Similarly, the 18S rRNA data do not provide a reliable signal for the molluscan interclass relationships. Nevertheless, we obtained strong preliminary support for phylogenetic inferences at more restricted taxonomic levels, such as the monophyly of Polyplacophora, Caenogastropoda, Euthyneura, Heterodonta, and Arcoidea.

## Introduction

Mollusca is one of the most diverse and speciose animal phyla. Ontogenetic features clearly indicate that molluscs are Spiralia, which include the phyla Platyhelminthes, Nemertea, Mollusca, Sipuncula, Echiura, Annelida, and probably Gnathostomulida and Entoprocta (e.g., Götting 1980a, 1980b; Wingstrand 1985; Brusca and Brusca 1990; Willmer 1990; Erwin 1991; Haszprunar 1992, 1996), as well as the lophophorate phyla Brachiopoda, Ectoprocta, and Phoronida (Ghiselin 1988; Halanych et al. 1995; Mackey et al. 1996). However, the molluscan origin is still debated and the sister group of the Mollusca remains unknown (e.g., Wingstrand 1985; Haszprunar 1996). Several competing hypotheses dealt with this issue, only two of which are still widespread. The first suggests that Mollusca have an acoelomate Turbellaria-like ancestor (e.g., Clark 1964, 1979; Stasek 1972; von Salvini-Plawen 1990a; von Salvini-Plawen and Steiner 1996) and relies on the common presence of a flat, often ciliated, ventral creeping sole. The alternative hypothesis interprets Mollusca as protostome coelomate animals with as sister group either Annelida (e.g., Götting 1980a, 1980b), Sipuncula (e.g., Inglis 1985; Conway Morris 1993; Scheltema 1993, 1996), Arthropoda (Bergström 1986), a cluster of Echiura + Sipuncula + Pogonophora + Annelida (Eernisse, Albert, and Anderson 1992), Echiura + Sipuncula (Haszprunar 1992), Arthropoda + Annelida (Nielsen 1995), or Entoprocta (Haszprunar 1996).

Hitherto, molluscan phylogeny has not been adequately addressed by molecular studies. As summarized in table 1, most sequence data either suffered from a too-limited information content of the investigated genes (e.g., Ohama et al. 1984; Hendriks et al. 1986) or from a too-limited taxon sampling (e.g., Lyddiatt, Peacock, and Boulter 1978; Holland, Hacker, and Williams 1991; Lenaers and Bhaud 1992; Winnepenninckx et al. 1992;

Winnepenninckx, Bacheljau, and De Wachter 1994). Moreover, analyses using the very same data but different analytical methods yielded contradictory results. For example, partial 18S rRNA sequences (covering approximately 900 nucleotides and 4 molluscs) suggested either molluscan monophyly (Ghiselin 1988), polyphyly (Field et al. 1988), or paraphyly (Patterson 1989; Lake 1990) depending on the tree construction methods used. This controversy is even enhanced when results on the basis of different molecules are compared. Complete 18S rRNA sequences preliminarily suggested molluscan monophyly (Winnepenninckx et al. 1992; Winnepenninckx, Bacheljau, and De Wachter 1994; but see Winnepenninckx, Bacheljau, and De Wachter 1995), whereas very preliminary mitochondrial DNA data put it in doubt (e.g., Ballard et al. 1992; Lecanidou, Douris, and Rodakis 1994; Wägele and Stanjek 1995). On the basis of different mitochondrial gene sequences, Lecanidou, Douris, and Rodakis (1994) suggested a possible non-monophyletic origin of Mollusca. Comparison of a single data set of mitochondrial 12S rRNA genes indicated that Mollusca may either be mono- (Ballard et al. 1992), para- (Ballard et al. 1992), or polyphyletic (Wägele and Stanjek 1995), depending on the alignment and the tree construction method used.

Also, the phylogenetic relationships between the molluscan classes are still controversial. The views of different morphologists are summarized in figure 1. There is general agreement about the monophyly of the subphylum Conchifera (e.g., Milburn 1960; Götting 1980a, 1980b; von Salvini-Plawen 1985; Wingstrand 1985; Eernisse, Albert, and Anderson 1992; Nielsen 1995; Ivanov 1996) but less agreement about the branching pattern of the constituent classes. The Conchifera, or shell-bearing molluscs, include the extant classes Bivalvia (clams), Gastropoda (snails and slugs), Cephalopoda (squids and octopuses), Scaphopoda (tusk shells), and Monoplacophora (*Neopilina*) (e.g., Götting 1980a; von Salvini-Plawen 1990a; Nielsen 1995; von Salvini-Plawen and Steiner 1996). Several authors divide the Conchifera into Diasoma (=Loboconcha = Bivalvia + Scaphopoda + the fossil rostroconchs) and Cyrtosoma (=Monoplacophora + Gastropoda + Ce-

Key words: molecular phylogeny, Gastropoda, Bivalvia, Polyplacophora, Caudofoveata, Scaphopoda.

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**Table 1**  
**Previous Results on Molluscan Phylogeny on the Basis of Molecular Data**

AUTHOR(S)	GENE OR PROTEIN	NUMBER OF SEQUENCES		TREE CONSTRUCTION METHOD <sup>a</sup>	RESULT
		Mol-lusca	Other Meta-zoa		
Lyddiatt, Peacock, and Boulter (1978) . . . . .	Cytochrome <i>c</i>	1	13	MP	
Ohama et al. (1984) . . . . .	5S rRNA	5	49	WPGMA	Polyphyly
Hendriks et al. (1986) . . . . .	5S rRNA	5	72	WPGMA/UPGMA	Polyphyly
Field et al. (1988) . . . . .	18S rRNA, partial	4	9	Fitch and Margoliash	Polyphyly
Ghiselin (1988) . . . . .	18S rRNA, partial	4	17	Signature approach	Monophyly
Goodman et al. (1988) . . . . .	Globins	6	25	MP	Monophyly
Patterson (1989) . . . . .	18S rRNA, partial	4	21	MP	Paraphyly
Lake (1990) . . . . .	18S rRNA, partial	4	17	Evolutionary parsimony	Paraphyly
Holland, Hacker, and Williams (1991) . . . . .	18S rRNA, partial	1	6	ML, MP, Fitch and Margoliash	
Ballard et al. (1992) . . . . .	Mitochondrial 12S rRNA	2	38	NJ, MP	Mono-/paraphyly
Lenaers and Bhaud (1992) . . . . .	28S rRNA, partial	1	9	NJ	
Winnepenninckx et al. (1992) . . . . .	18S rRNA	3	43	NJ	Monophyly
Lecanidou, Douris, and Rodakis (1994) . . . . .	Several mitochondrial genes	2	9	NJ, MP	Paraphyly
Winnepenninckx, Bacheljau, and De Wachter (1994) . . . . .	18S rRNA	5	21	NJ, MP	Monophyly
Wägele and Stanjek (1995) . . . . .	Mitochondrial 12S rRNA	2	21	NJ	Polyphyly
Winnepenninckx, Bacheljau, and De Wachter (1995) . . . . .	18S rRNA	5	17	NJ, MP	Polyphyly

<sup>a</sup> ML: maximum likelihood; MP: maximum parsimony; NJ: neighbor joining.

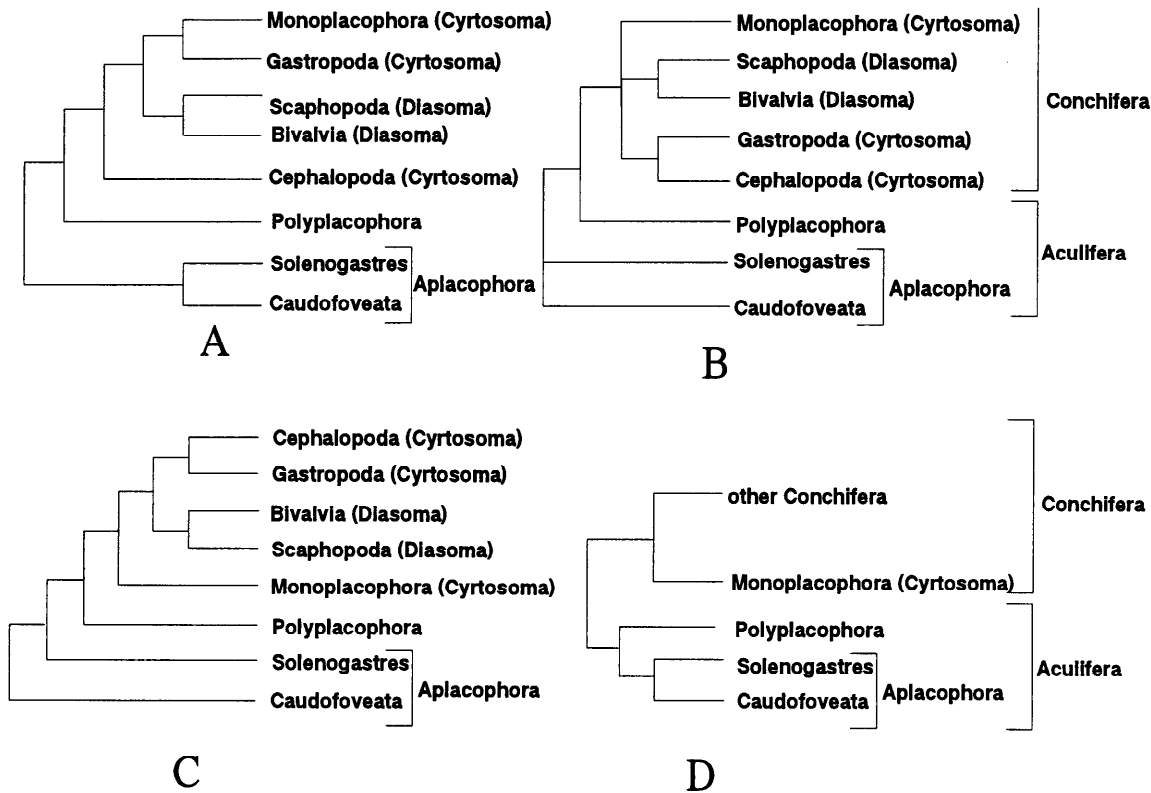


FIG. 1.—Four hypotheses on molluscan phylogeny based on morphological and larval features. A, Götting (1980a, 1980b). B, von Salvini-Plawen (1990a, 1990b). C, Brusca and Brusca (1990). D, Scheltema (1993, 1996). For the conchiferan classes, it is indicated in brackets whether they belong to the Diasoma or Cyrtosoma, a division which is based on paleontological data (e.g., Runnegar and Pojeta 1974, 1985; Pojeta 1980; Steiner 1992).

phalopoda) (Runnegar and Pojeta 1974, 1985; Pojeta and Runnegar 1976; Pojeta 1980), while a variant of this scheme distinguishes the Diasoma from the Visceroconcha (=Gastropoda + Cephalopoda), thus keeping the Monoplacophora (i.e., Tryblidia) as a separate group (Steiner 1992; von Salvini-Plawen and Steiner 1996). Yet the monophyly of both Diasoma and Cyrtosoma has been questioned by Peel (1991), and the position of the Monoplacophora is uncertain.

Figure 1 also illustrates the uncertainties concerning the remaining three molluscan classes, viz. Polyplacophora (chitons), Solenogastres (narrowfoot gliders), and Caudofoveata (mud moles). It is generally accepted that Conchifera and Polyplacophora are sister groups (e.g., Stasek 1972; Götting 1980a, 1980b; Wingstrand 1985; Scheltema 1988; von Salvini-Plawen 1990a; Nielsen 1995; von Salvini-Plawen and Steiner 1996). Furthermore, Caudofoveata and Solenogastres are either considered as a monophyletic group (Aplacophora) (e.g., Milburn 1960; Stasek 1972; Götting 1980a, 1980b; Pojeta 1980; Scheltema 1988, 1996; Nielsen 1995; Ivanov 1996) (fig. 1A and B) or as a paraphyletic group (von Salvini-Plawen 1990a, 1990b) (fig. 1C), while Scheltema (1993, 1996) (fig. 1D) and Ivanov (1996) suggested that the monophyletic Aplacophora and the Polyplacophora are sister taxa. The latter hypothesis supports the existence of a second molluscan subphylum, the Aculifera, which unifies the classes Polyplacophora (chitons), Solenogastres (narrowfoot gliders) and Caudofoveata (mud moles) (e.g., Götting 1980a; Brusca and Brusca 1990; von Salvini-Plawen 1990a; Nielsen 1995).

In an attempt to address the phylogenetic position of the Mollusca and their interclass relationships, we determined 12 new nearly complete molluscan 18S rRNA gene sequences, including the first Caudofoveata and Scaphopoda and additional Gastropoda, Polyplacophora, and Bivalvia. These data were processed for tree construction with selected published nearly complete 18S rRNA sequences of molluscs and several representatives of other metazoan phyla.

Throughout this paper we apply a "traditional" nomenclature. We refer to several papers in Taylor (1996) for an overview of the different points of view on this matter.

## Materials and Methods

### Animals and DNA Extraction

Table 2 lists the sampling locations, DNA sources, and EMBL accession numbers of the 12 mollusc species whose 18S rRNA genes were sequenced. All animals were frozen alive and kept at  $-70^{\circ}\text{C}$ . Voucher material was deposited in the collections of the Koninklijk Belgisch Instituut voor Natuurwetenschappen (Brussels). DNA was extracted as described by Winnepeninckx, Bäckeljau, and De Wachter (1993), except for *Scutopus ventrolineatus* and *Littorina littorea*, of which, respectively, one complete individual and the foot and digestive gland were cut into pieces and incubated in 100 mM Tris (pH = 7.4), 1 mM EDTA, 0.5% SDS, and 3

mg/ml Proteinase K. The DNA was then purified in phenol, phenol/chloroform/isoamylalcohol, and chloroform/isoamylalcohol extractions followed by isopropanol precipitation (Sambrook, Fritsch, and Maniatis 1989).

### 18S rDNA Amplification and Sequencing

The 18S rRNA genes were PCR-amplified in two parts and cloned afterward, as described by Winnepeninckx, Bäckeljau, and De Wachter (1995). The 5' part of the gene (24 nucleotides), where the forward PCR primer anneals, could not be amplified by this method. The alkaline lysis method (Birnboim and Doly 1979) and spin columns (Quiagen, Inc., Chatsworth, Calif.) were used to isolate plasmids from recombinant DH5 $\alpha$ -*E. coli* cells. Dideoxysequencing of both strands was performed with the USB (Cleveland, Ohio) and Pharmacia (Uppsala, Sweden) sequencing kits using primers specific for 18S rRNA genes (Winnepeninckx, Bäckeljau, and De Wachter 1994).

### Data Analysis

18S rRNA sequences were aligned by means of the DCSE program (De Rijk and De Wachter 1993) taking into account the 18S rRNA secondary structure (Van de Peer et al. 1996; this alignment is available on ftp site [rrna.uia.ac.be](http://rrna.uia.ac.be) or on WWW site <http://rrna.uia.ac.be/rrnssuform.html>).

Phylogenetic analyses were performed with the distance matrix program TREECON (Van de Peer and De Wachter 1993) and the maximum-parsimony (MP) program PAUP version 3.1.1 (Swofford 1993). Since the alignment was based on a recent, well-established secondary structure model, derived on the basis of 1,023 18S rRNA sequences (Van de Peer et al. 1996), we judged that all alignment positions could be used for the phylogenetic analyses. Neighbor-joining (NJ) trees were derived from distance matrices calculated by three different methods.

The Jukes and Cantor (1969) method assumes that all substitutions occur at the same rate. The distance ( $d$ ) in function of the dissimilarity ( $f$ ) is given by

$$d = -\frac{3}{4} \ln \left( 1 - \frac{4}{3} f \right).$$

In actual sequence data the transitional substitution rate is often higher than the transversional substitution rate. In this case the Jukes and Cantor formula may give an underestimation of the evolutionary distance. The Kimura two-parameter distance model (1980) assumes that the transitional substitution rate is different from the transversional substitution rate. The distance is given by

$$d = -\frac{1}{2} \ln \left[ (1 - 2P - Q) \sqrt{1 - 2Q} \right],$$

where  $P$  is the fraction of transitional differences and  $Q$  is the fraction of transversional differences observed.

Previous methods tend to underestimate large evolutionary distances, making distant species seem close to one another than they actually are. Van de Peer, Va

**Table 2**  
**Data on the 12 Mollusca for Which 18S rRNA Sequences Were Determined in this Study (Marked by \*), and Taxonomic Position of All Molluscan Species Considered**

Class Subclass or Order Species	Sampling Location	DNA Source	EMBL Accession No.	Chain Length <sup>a</sup>
Caudofoveata				
<i>Scutopus ventrolineatus*</i> .....	Trondheim (Norway)	Total body	X91977	1,878
Polyplacophora				
<i>Lepidochitona corrugata*</i> .....	Bahar Ic-Caghaq (Malta)	Digestive gland	X91975	1,824
<i>Acanthopleura japonica</i> .....				
Gastropoda				
Neritimorpha				
<i>Nerita albicilla*</i> .....	Hong Kong	Foot	X91971	1,823
Caenogastropoda				
<i>Littorina littorea*</i> .....	Oostende (Belgium)	Foot + digestive gland	X91970	1,834
<i>Thais clavigera*</i> .....	Hong Kong	Reproductive organs	X91979	1,825
Euthyneura				
<i>Onchidella celtica</i> .....				
<i>Siphonaria pectinata*</i> .....	Benalmadena (Spain)	Foot	X91973	1,845
<i>Helix aspersa*</i> .....	Oostende (Belgium)	Reproductive organs	X91976	1,840
<i>Limicolaria kameul</i> .....				
Scaphopoda				
<i>Antalis vulgaris*</i> .....	Roscoff (France)	Total body minus gut	X91980	1,865
Bivalvia				
Pteriomorpha				
<i>Barbatia virescens*</i> .....	Hong Kong	Foot, adductor muscle	X91974	1,815
<i>Glycymeris glycymeris*</i> .....	Malaga (Spain)	Foot	X91978	1,814
<i>Placopecten magellanicus</i> .....				
<i>Chlamys islandica</i> .....				
<i>Argopecten irradians</i> .....				
<i>Mytilus edulis</i> .....				
<i>Crassostrea virginica</i> .....				
Heterodonta				
<i>Galeomma takii*</i> .....	Hong Kong	Total body	X91969	1,815
<i>Tridacna</i> sp.* .....	Indonesia	Foot	X91972	1,873
<i>Spisula solida</i> .....				
<i>Mactromeris polynyma</i> .....				
<i>Tresus capax</i> .....				
<i>Mulinia lateralis</i> .....				

<sup>a</sup> Chain lengths are calculated assuming that the missing 5' end is 24 nucleotides long.

der Auwera, and De Wachter (1996) recently developed a method for avoiding this long branch attraction. The estimation of distances takes into account the spread of relative substitution rates at different sites of a molecule. It is given by

$$d = p \left[ \left( 1 - \frac{4}{3}f \right)^{-3/4p} - 1 \right],$$

where  $f$  is the overall dissimilarity between two sequences and  $p$  is an empirically derived value, which for 18S rRNA has been estimated at 0.26 (Van de Peer, Van der Auwera, and De Wachter 1996).

Kimura distances were obtained with the MEGA version 1.01 software (Kumar, Tamura, and Nei 1993). Jukes and Cantor (1969) and Van de Peer, Van der Auwera, and De Wachter (1996) distances were computed with the TREECON software (Van de Peer and De Wachter 1993). MP trees were constructed via the heuristic search option of PAUP combined with tree-bissection-reconnection branch swapping. Searches were conducted 100 times with multiple random addition of sequences. Only parsimony-informative sites were included. Both the NJ and MP trees were tested by boot-

strapping over 1,000 or 100 replicates. The number of bootstrap replicates of MP trees was limited to 100 because of computer time limitations. The robustness of the MP tree was further assessed by calculating a "decay index" (Bremer 1988; Donoghue et al. 1992), which is the number of steps that must be added before a particular cluster in the MP tree is no longer unequivocally supported. To calculate the index, cladograms that are a few steps longer than the MP tree were generated and combined into strict consensus trees. Finally, alternative MP trees, reflecting generally accepted relationships, were interactively constructed and evaluated using the computer program MACCLADE version 3.0 (Maddison and Maddison 1992).

## Results

The length of the newly determined sequences ranged from 1,814 (*Glycymeris glycymeris*) to 1,878 (*Scutopus ventrolineatus*) nucleotides (table 2). No deviations from the general eukaryotic secondary structure model (Van de Peer et al. 1996) were observed.

An NJ tree of 47 metazoan 18S rRNA sequences, of which 24 are molluscs, is shown in figure 2. The tree

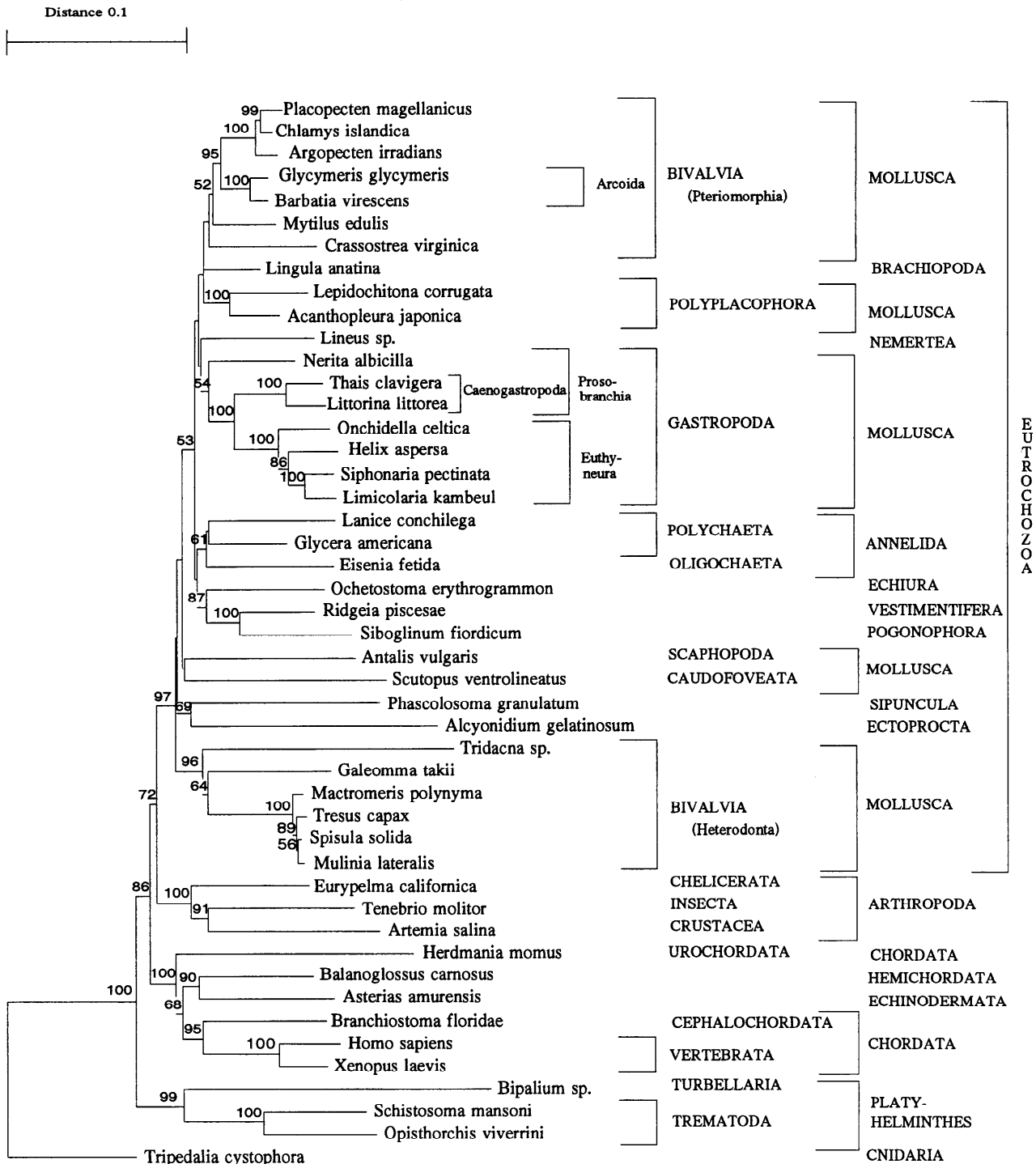


FIG. 2.—NJ tree of 47 nearly complete metazoan 18S rRNA sequences, using *Tripedalia cystophora* as outgroup. Bootstrap percentage based on 1,000 replicates.

suggests that Mollusca belong to a strongly bootstrap-supported eutrochozoan (i.e., protostomes excluding arthropods) clade, which includes Vestimentifera, Pogonophora, Echiura, Annelida, Nemertea, Sipuncula, Ectoprocta, and Brachiopoda. However, it does not support the widely accepted monophyly of the molluscs or that of the bivalves, and it provides virtually no bootstrap support for gastropod monophyly. Furthermore, the tree

suggests that the bivalve subclass Heterodonta and the order Arcoida, within the subclass Pteriomorpha, are both monophyletic. Among the gastropods, the Euthyneura are monophyletic but the Prosobranchia are not. Parts of the tree which are not bootstrap-supported were subject to change when individual in-group taxa were removed. The NJ tree was also reconstructed with two other methods for converting dissimilarities into dis-

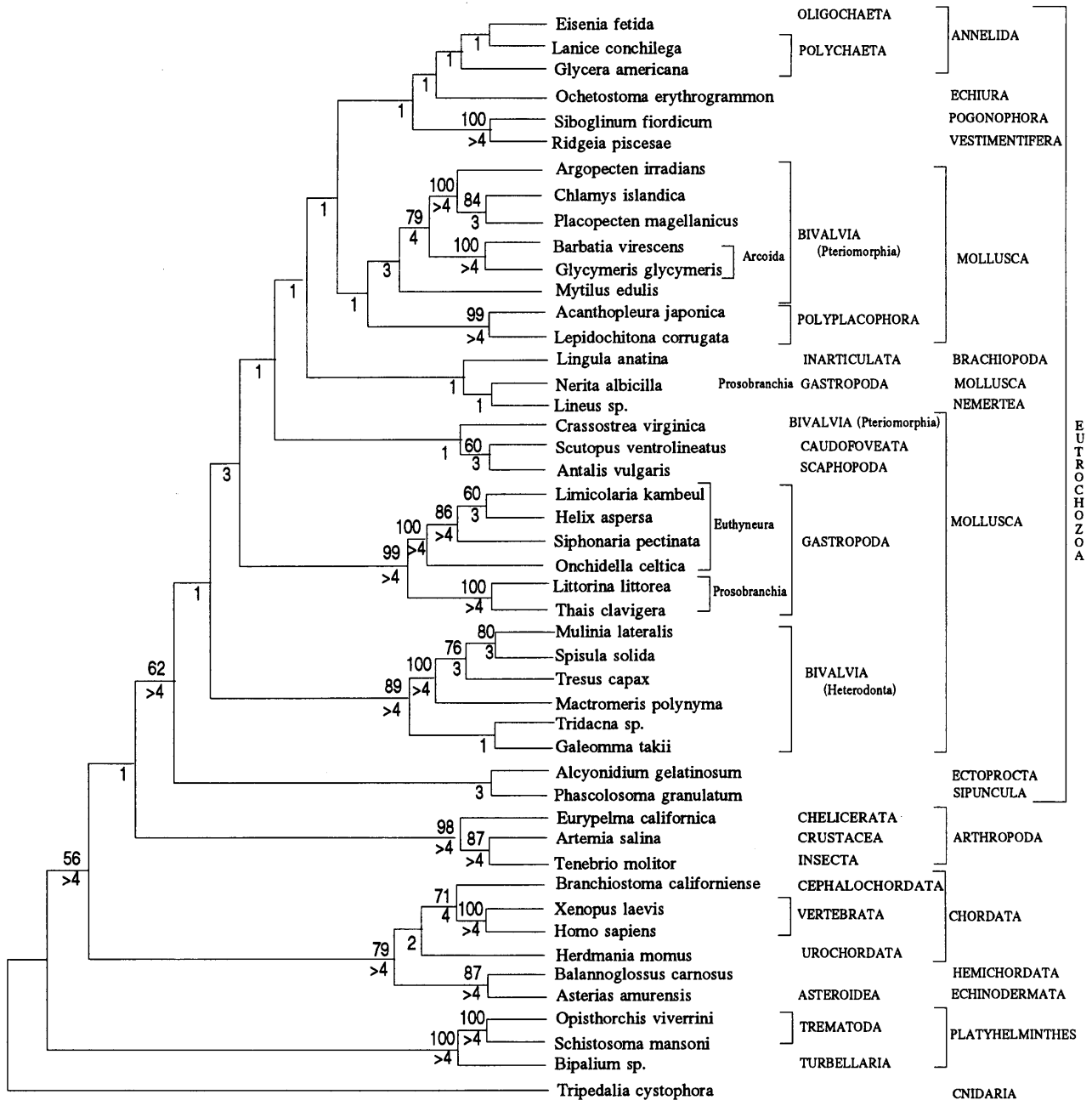


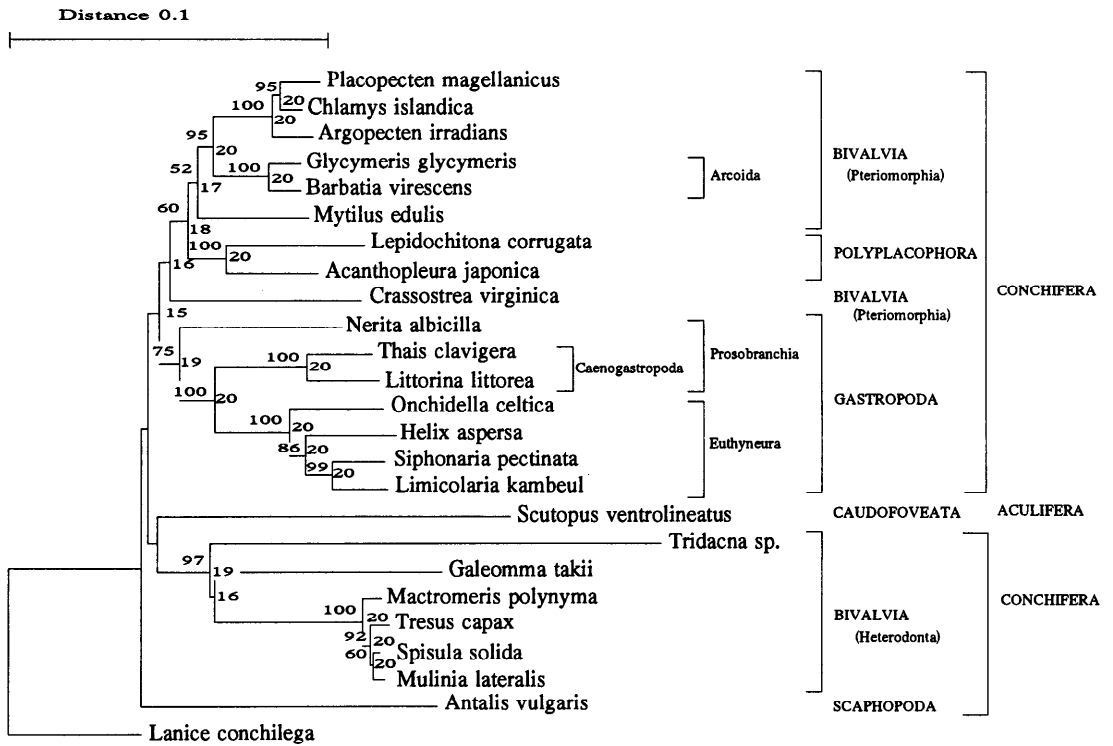
FIG. 3.—MP tree ( $L = 4,712$ ) found on the basis of 806 informative sites of an alignment of 47 nearly complete metazoan 18S rRNA sequences, using *Tripedalia cystophora* as outgroup. Bootstrap analysis was run with 100 replicates and values above 50% are indicated above the nodes. Figures below the nodes indicate how many additional steps were necessary for this clade not to be unequivocally supported (decay index).

tances (results not shown). Computing the distance according to Kimura (1980) instead of Jukes and Cantor (1969) yielded the same topology as in figure 2 except for the branching order of Gastropoda, Nemertea, and Annelida-Vestimentifera-Pogonophora-Echiura. Computation of distances according to Van de Peer, Van der Auwera, and De Wachter (1996) only affected nodes that were not bootstrap-supported but did not influence the conclusions based on the tree in figure 2.

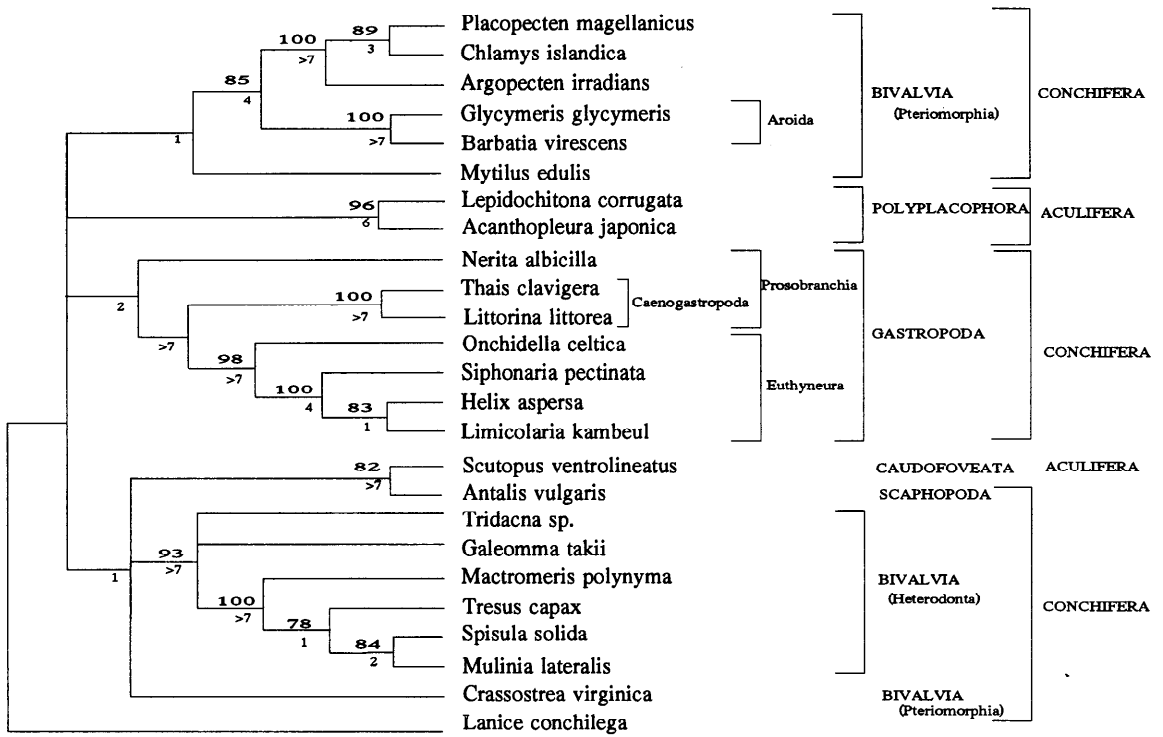
The MP analysis of the same data set involved 806 informative sites and yielded a single tree of 4712 steps.

The MP tree, which is shown in figure 3, confirms all the points suggested by the NJ tree of figure 2, with the exception that eutrochozoan monophyly is no longer bootstrap-supported.

In an attempt to focus on relationships within the molluscs, we assumed that the poorly resolved protostome topology and the lack of molluscan monophyly are "artifacts" of the 18S rRNA gene caused by the rapid divergence of the major metazoan phyla in the early Cambrian (see *Discussion*; Erwin 1991; Adoutte and Philippe 1993). Therefore, we performed a restricted



A



B

FIG. 4.—Phylogenetic relationships within the Mollusca. A, NJ tree found on the basis of an alignment of 24 molluscan nearly complete 18S rRNA sequences. B, Strict consensus tree of the four MP trees ( $L = 1,534$ ) found on the basis of the 466 informative sites of the same alignment. *Lanice conchilega* was used as outgroup. A bootstrap analysis running 1,000 replicates was performed and values higher than 50% are indicated above the nodes. In addition to the tree in A, 20 NJ trees were constructed in which *L. conchilega* was subsequently replaced by

analysis by NJ of the 24 molluscan sequences only, with the polychaete *Lanice conchilega* as outgroup. However, in the tree shown in figure 4A none of the molluscan interclass relationships show high bootstrap values. Moreover, the bivalves appeared to be polyphyletic and so did the Pteriomorphia, although the latter result was not bootstrap-supported. Yet there was a signal for gastropod monophyly and sound bootstrap support for the monophyly of Polyplacophora, Caenogastropoda, Euthyneura, Heterodonta, and Arcoida. The use of Kimura (1980) distances yielded exactly the same topology as in figure 4A. With Kimura (1980) or Van de Peer, Van der Auwera, and De Wachter (1996) distances, topological shifts of the non-bootstrap-supported nodes were observed (not shown) but the monophyletic clusters observed in figure 4A persisted. Removing ingroup taxa of figure 4A one by one affected the positions of *Crassostrea virginica*, *Tridacna* sp., *Galeomma takii*, *Antalis vulgaris*, and *Scutopus ventrolineatus* but did not affect the aforementioned monophyletic clusters. Using the program TREECON, *Lanice conchilega* was replaced by 20 other metazoan species as outgroup (table 3). The number of outgroups supporting each branching point is indicated in figure 4A. The method supported each of the monophyletic clusters observed with sufficient bootstrap support in figure 4A and pointed to the monophyly of the Gastropoda in 19 of the 20 outgroups.

An MP analysis was also performed on the alignment of the 24 molluscan taxa with *Lanice conchilega* as outgroup. This analysis included 466 informative sites and yielded four MP trees of 1,534 steps. Their strict consensus tree (fig. 4B) did not provide bootstrap or decay index support for any interclass relationship except for a sister group status of Caudofoveata and Scaphopoda. For the remainder, the MP tree largely confirmed the results obtained by the NJ analysis, i.e., there is bootstrap and decay index support for the monophyly of Polyplacophora, Caenogastropoda, Euthyneura, Heterodonta, and Arcoida but there is no indication of bivalve monophyly and pteriomorphs are perceived as polyphyletic. Furthermore, in contrast to the NJ tree, there is no bootstrap or decay index support for gastropod monophyly, even though all gastropods are clustered together.

We assessed whether current opinions on molluscan relationships (i.e., Bivalvia, Diasoma, and Conchifera monophyly; the sister group relationship between Polyplacophora and Conchifera; and the basal position of the Caudofoveata) are compatible with our results by successively forcing the assumed relationships on the MP tree and counting the number of extra steps this implies. Forcing Bivalvia into a monophyletic cluster required only five additional steps (length [ $L$ ] = 1,539). In order to achieve conchiferan monophyly as well, 23 additional steps ( $L$  = 1,557) were necessary when Poly-

**Table 3**  
List of 20 Metazoan Species Which Were Successively Used to Root the NJ Tree of Figure 4A

Species	Phylum
<i>Eisenia fetida</i> .....	Annelida
<i>Glycera americana</i> .....	Annelida
<i>Eurypelma californica</i> .....	Arthropoda
<i>Artemia salina</i> .....	Arthropoda
<i>Tenebrio molitor</i> .....	Arthropoda
<i>Lingula anatina</i> .....	Brachiopoda
<i>Branchiostoma floridae</i> .....	Chordata
<i>Herdmania momus</i> .....	Chordata
<i>Xenopus laevis</i> .....	Chordata
<i>Asterias amurensis</i> .....	Echinodermata
<i>Ochetostoma erythrogrammon</i> .....	Echiura
<i>Alcyonidium gelatinosum</i> .....	Ectoprocta
<i>Balanoglossus carnosus</i> .....	Hemichordata
<i>Lineus</i> sp. ....	Nemertea
<i>Opisthorchis viverrini</i> .....	Platyhelminthes
<i>Schistosoma mansoni</i> .....	Platyhelminthes
<i>Bipalium</i> sp. ....	Platyhelminthes
<i>Siboglinum fiordicum</i> .....	Pogonophora
<i>Phascolosoma granulatum</i> .....	Sipuncula
<i>Ridgeia piscesae</i> .....	Vestimentifera

placophora were a sister group to the Conchifera. Yet the number of extra steps increased to 28 ( $L$  = 1,562) when the caudofoveate *Scutopus ventrolineatus* was set as a sister group to the Conchifera. Respecting the Diasoma concept in addition to conchiferan monophyly required 28 additional steps ( $L$  = 1,562) with Polyplacophora, and 32 additional steps ( $L$  = 1,566) with the caudofoveate *Scutopus ventrolineatus* set as a sister group to the Conchifera. Thus, a tree reflecting the widely accepted molluscan relationships is only 28 steps (i.e., less than 2%) longer than the MP tree of figure 4B.

## Discussion

Our NJ analysis (fig. 2) strongly favors the Eutrochozoa concept, in which molluscs share a common ancestor with the Annelida, Echiura, Sipuncula, Pogonophora, Vestimentifera, Nemertea, and the lophophorate phyla Ectoprocta and Brachiopoda, but not with the Arthropoda (Ghiselin 1988; Eernisse, Albert, and Anderson 1992; see also Halanych et al. 1995; Mackey et al. 1996). The coelomate molluscan origin is supported by several characters, such as the presence of an anus, a gonopericardial complex, metanephridia, metamery, and trochophore larvae (cf. von Salvini-Plawen 1980). These features suggest that molluscs are derived from a coelomate spiralian ancestor (e.g., Hammarsten and Runnström 1925; Siewing 1976; Götting 1980a, 1980b; Wingstrand 1985; Morse and Reynolds 1996; Scheltema 1996). The protostome coelomate position of the Mollusca further agrees with paleontological (e.g., Runnegar and Pojeta 1974), physiological (e.g., Florey 1951;

←

20 other metazoan outgroups, given in table 3. The figures to the right of the nodes in A indicate how many of these 20 NJ trees contained a cluster of this composition. Nodes having no figure at the right were only found in less than 10/20 trees. Figures below the nodes in B indicate the number of steps required for this clade not to be unequivocally supported (decay index).

Stang-Voss 1970, 1971; Peters 1972; Wurzinger and Hartenstein 1974; Kilby, Crichton, and Lafferty 1973), and molecular data (Field et al. 1988; Ghiselin 1988; Lake 1989, 1990; Patterson 1989; Ballard et al. 1992; Lenaers and Bhaud 1992). If this result is correct, then one must conclude that the flattened shape and the ventral, often ciliated, creeping sole (e.g., Stasek 1972; von Salvini-Plawen 1990a) are either convergent features of Mollusca and Turbellaria or spiralian plesiomorphies which are highly conserved in both groups.

Because of the extreme morphological and anatomical diversity of the Mollusca, the monophyly of this phylum has been based on a combination of characters that are not necessarily present in all molluscan classes, e.g., the presence of a chambered heart lying in a pericardium, a radula, dorsal mantle glands producing spicules or a shell, and a muscular foot (e.g., Brusca and Brusca 1990; von Salvini-Plawen 1990a; von Salvini-Plawen and Steiner 1996). The present 18S rRNA data seem to indicate molluscan polyphyly, but molluscan monophyly is not disproven since the deeper branches of the Eutrochozoa divergence have low bootstrap values and the branching pattern is strongly dependent on the choice of ingroup taxa (figs. 2 and 3). The monophyly of the Annelida is not proven by either the NJ tree or the MP tree (figs. 2 and 3). These results are in line with other molecular sequence analyses, which failed to provide convincing evidence for molluscan monophyly (see table 1). Recently, molluscan nonmonophyly was also tentatively inferred on the basis of paleontological data (Runnegar 1996).

It has been suggested that the inability of 18S rRNA genes (and other molecular data) to resolve protostome relationships may reflect the rapid metazoan radiation in the late Precambrian or lower Cambrian (Erwin 1991; Adoutte and Philippe 1993; Philippe, Cheneuil, and Adoutte 1994). Indeed, most extant animal phyla, including molluscs, suddenly appeared in the fossil record around the Precambrian/Cambrian boundary (e.g., Valentine 1980, 1994; Runnegar and Pojeta 1985) and subsequently diversified explosively in a time span of some tens of millions of years or even less than 10 million years (e.g., Bergström 1991; Valentine 1991; Bowring et al. 1993; Graham et al. 1995; but see Durham 1971 for arguments for a more gradual evolution). In this scenario, only nucleotide positions which change fast enough to accumulate substitutions within the short time span of the Cambrian radiation will contain information about the branching pattern of the eutrochozoan phyla. However, after the radiation, these fast-evolving sites most probably changed further, so that the original informative substitutions were obliterated. The Cambrian radiation is visible in the NJ tree (fig. 2) as a set of short deep internodes leading to clusters with low bootstrap support. On the other hand, two observations seem to conflict with this saturation scenario: (1) the monophyly of the arthropods, which are more speciose and diverse than molluscs or annelids, is unequivocally supported by 18S rRNA and other molecular data (e.g., Turbeville et al. 1991; Ballard et al. 1992; Boore et al. 1995; Friedrich and Tautz 1995), and (2) globin amino acid

sequences do provide evidence for annelid and molluscan monophyly (Goodman et al. 1988). However, the globin data must be regarded with caution for (1) they involved too few molluscan and other eutrochozoan representatives, (2) the globin trees were presented without proper testing of the robustness of the branching pattern, and (3) the globin trees were constructed without considering the fact that globins are a very diverse multi-gene family which, particularly in the Mollusca and Annelida, is represented by an impressive array of different types (Terwilliger 1980; Terwilliger and Terwilliger 1985; Vinogradov, Walz, and Pohajdak 1992). Of course, it is still possible that the addition of more spiralian 18S rRNA sequences will improve resolution.

The fact that the results of our 18S rRNA data analyses were too unstable to decide on the mono- or polyphyly of the bivalves (see also Kenchington et al. 1994) and gastropods or on the relationships between the molluscan classes (fig. 4) suggests that the molluscan classes also radiated rapidly. On the other hand, our 18S rRNA sampling of molluscs is still too limited, so that a more comprehensive taxonomic coverage of the phylum, including Cephalopoda, Solenogastres, and Monoplacophora, may perhaps solve some of the remaining controversies. Unfortunately, hitherto we did not succeed in producing reliable complete 18S rRNA sequences of Cephalopoda and Solenogastres and we were unable to obtain monoplacophoran samples. Of course, in addition, the representation of the other molluscan class is probably still insufficient. Yet we suspect that the addition of more taxa of these classes will not solve the aforementioned problems, because our conclusions were not affected by ingroup changes. Also suggestive in this respect is the fact that an increase in the number of examined molluscs from 5 (Winnepeninckx, Bäckeljau, and De Wachter 1994) to 24 (this work) disrupted molluscan, bivalve, and gastropod monophyly instead of confirming it.

Although the 18S rRNA gene seems hitherto unsuitable to (1) reliably recover the monophyly of Mollusca, Bivalvia, and Gastropoda; (2) assess molluscan relationships within the Eutrochozoa; and (3) resolve molluscan interclass relationships, it seems as if the gene does contain sufficient signal to infer molluscan relationships at more restricted taxonomic levels. This follows from the preliminary support we found (fig. 2) for the monophyly of Polyplacophora, Euthyneura, Caenogastropoda, Heterodonta, and Arcoidea.

In conclusion, the available 18S rRNA sequences seem to indicate that the molecule contains insufficient information to solve molluscan and spiralian higher-level relationships but these sequences provide stable results at more restricted taxonomic levels. However, the value of the molecule in assessing molluscan relationships at these different levels still needs further examination with more taxa.

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