A Molecular Phylogeny of the Bivalve Mollusks

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A phylogenetic reconstruction based on 506 nucleotides near the 5′ end of the 18S subunit of ribosomal DNA (rDNA) in 2 gastropod, 3 chiton and 28 bivalve mollusks supported the monophyly and sister group relationship of the subclasses Heterodonta and Palaeoheterodonta but could not confidently establish either the monophyly or the phylogenetic relationships of the morphologically well defined subclasses Pteriomorphia, Protobranchia, and Anomalodesmata. When both gastropods and chitons were included in the analysis, one or the other invariably emerged within Bivalvia. Some evidence indicates that this apparent polyphyly may be the consequence of unequal rates of evolution and of rapid changes in the protobranch and anomalodesmatan lineages. The taxa usually included in Pteriomorpha emerge as a grade rather than a clade, although in a sequence that differs from morphologically based phylogenies.

Introduction

One of the most powerful uses of molecular characters in systematics is the ability to examine ancient divergences for which morphology is a weak guide. Thus, many workers have used sequence data to investigate the relationships among animal phyla and the classes within them (e.g. Philippe, Chenuil, and Addoute 1994; Boore et al., 1995; Halanych et al. 1995; Winnepenninckx, Backeljau, and DeWachter 1996). Of the protostome phyla, Mollusca are among the most ancient and diverse. Living representatives of this phylum are usually divided into seven classes: (1) Aplacophora (solenogasters and caudofoveates), (2) Polyplacophora (chitons), (3) Monoplacophora (pseudometameric, limpet-like animals), (4) Scaphopoda (tusk shells), (5) Bivalvia (oysters, scallops, clams), (6) Gastropoda (snails), and (7) Cephalopoda (octopus, squid). Classes 3–7 are grouped in the clade Conchifera. The commonest and most speciose classes in the modern fauna are the bivalves and the gastropods. To date, molecular investigations of the mollusks have focused primarily on relationships of other phyla to the Mollusca, represented by the classes Gastropoda, Bivalvia, and the Polyplacophora, the latter being regarded as basal.

In an analysis of molluscan phylogeny that included five other animal phyla, Winnepenninckx, Backeljau, and DeWachter (1994) compared complete sequences of 18S rDNA from representatives of three molluscan classes: two gastropods, two bivalves, and a chiton. Based on both distance and parsimony analyses, they concluded that both gastropod and bivalve classes, as well as the phylum Mollusca, were monophyletic. At the same time, they commented on the unorthodox position of the chiton in their phylogeny, where it formed a clade with the gastropods, this clade being the sister group to the bivalves, contrary to morphological evidence. Given their very few molluscan taxa, Winnepenninckx, Backeljau, and DeWachter called for the examination of more taxa to resolve molluscan relationships. Kenchington et al. (1994), also using 18S rDNA data, did examine many more bivalve taxa. Using all complete sequences available at the time, this group compared 13 bivalves belonging to four superfamilies in two subclasses, in addition to an arthropod, a chiton, and a gastropod (these last two taxa and five of the bivalves are included in the present study). Kenchington's study produced a phylogeny in which the gastropod was sister taxon to the bivalves and the chiton was included among the pteriomorph bivalves, leading Kenchington et al. to conclude that the Bivalvia were polyphyletic with respect to the Polyplacophora. They also recommended the acquisition of sequence data for many more taxa.

These contradictions concerning relationships among the classes of mollusks will not be resolved until much denser taxonomic coverage is available, and such coverage must begin with a thorough examination of relationships within each single class, with sufficient taxa to guard against the distorting effects of any particular species. Lecointre et al. (1993) have demonstrated the large and unpredictable effect that choice of taxa can have on a phylogeny and have strongly supported the view that increasing the number of taxa is as or more important than extending the length of sequence per taxon. To that end, it was the purpose of this present study to increase greatly the number of bivalve taxa and the breadth of coverage of the class represented by partial sequences of the 18S rDNA gene. This would allow us to examine more powerfully the relationships among members of Bivalvia, which are interesting in their own right. We have also included additional polyplacophorans and gastropods in an effort to determine the relationships among the three classes.

Systematists are in broad agreement about divisions at the highest taxonomic levels of Bivalvia. All recognize five major subdivisions of the class, each of which is represented in this study: (1) Protobranchia (a small group of deposit-feeding deep-sea clams), (2) Pteriomorphia (mussels, scallops, and oysters), (3) Anomalodesmata (a small group of marine clams including all septibranch clams), (4) Palaeoheterodonta (including all

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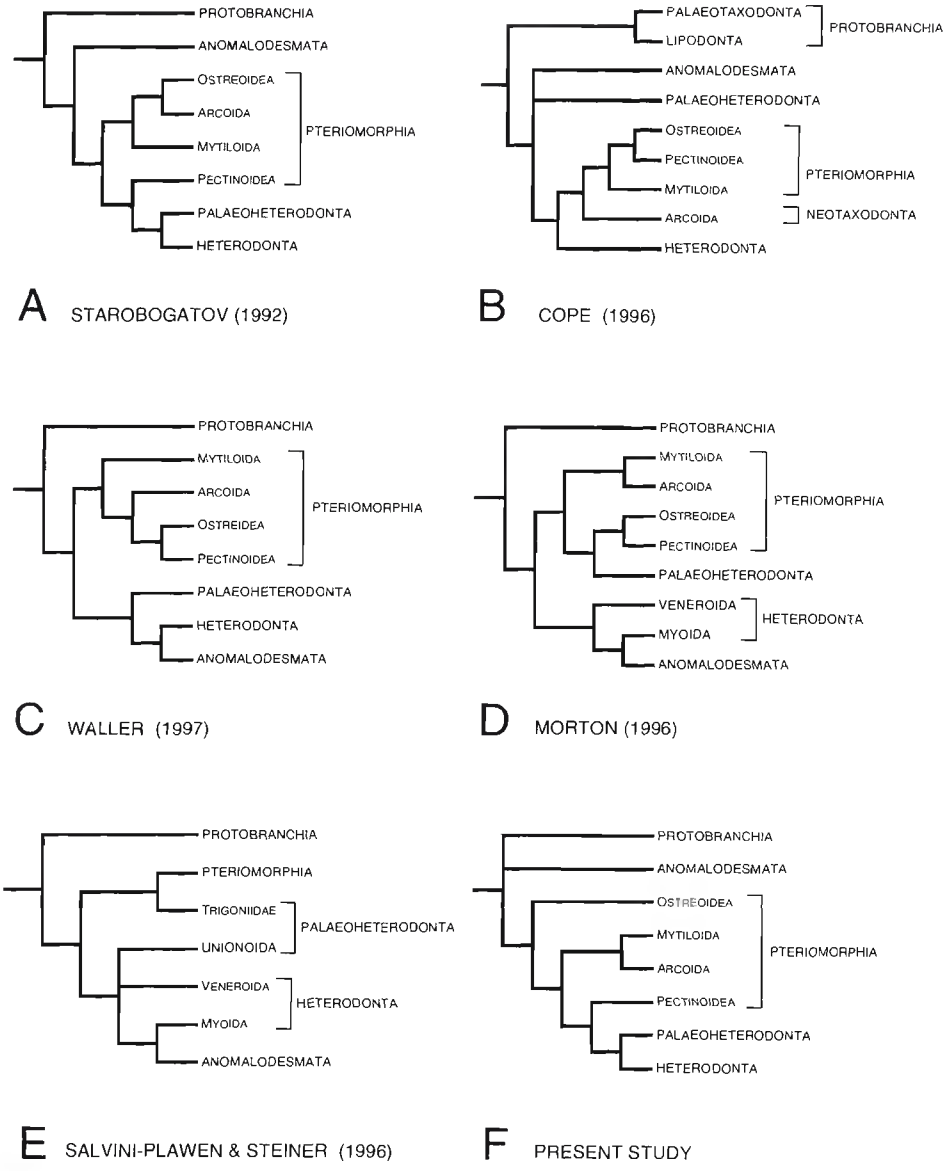

FIG. 1.—Summaries of five recent morphology-based hypotheses of phylogenetic relationships among major groups within the molluscan class Bivalvia compared with the results of our molecular study. The nomenclature, taxonomic rank, and exact composition of some higher groups vary among these authors but have been standardized here for purposes of comparison. Earlier classifications of Bivalvia are summarized in Newell (1969; fig. 102).

freshwater mussels), and (5) Heterodonta (most marine clams). The Protobranchia are believed to be the most ancient clade and to comprise the sister taxon to the remaining bivalves. The majority of living species belong to the Pteriomorphia, the Palaeoheterodonta, or the Heterodonta, the latter representing the most recent major radiation. So different are the Protobranchia that Waller (1997) preferred to recognize only one other subclass, the Autobranchia, containing the other four major groups. Others, such as Abbott and Dance (1986) recognized each of the five groups as a subclass. Disagreement begins with the relationships among the five groups, particularly with regard to which group diverged first from the protobranchs and how many distinct clades exist within the Bivalvia (fig. 1). Some systematists, including Waller (1997), Morton (1996), and Salvini-Plawen and Steiner (1996), regard the Heterodonta and Anomalodesmata as closely related and recently diverged. Others, such as Cope (1996) and Starobogatov (1992), hypothesize that Anomalodesmata is an ancient group that diverged from the base of the bivalve tree. There is little consensus as to the placement of the Palaeoheterodonta or as to whether the Pteriomorphia form a grade or a clade. All of these views were based on careful morphological and paleontological studies, making the addition of molecular information of great interest.

Bowman (1989), in a prodigious effort relying on direct sequencing of both 28S and 18S rRNA, was the first to investigate these relationships within a broad sampling of bivalves. She combined data from two published sequences with 19 new sequences from species

in 15 superfamilies representing 4 subclasses, but she was unable to achieve a uniform data matrix, instead using sequences from different genes/regions for different groups of taxa (Bowman 1989, pp. 80–81). We have attempted to emulate Bowman's efforts at very broad taxonomic coverage using PCR cycle sequencing of DNA to produce a uniform data matrix for all species. In order to maximize the number of species represented, sequencing effort was confined to the 5′ region of the 18S rDNA gene corresponding to positions 26–582 of Winnepenninckx, Backeljau, and DeWachter's (1994) complete sequence for the gastropod *Onchidella celtica* (Cuvier, 1817). For this restricted portion of the gene, we obtained data from 21 previously unsequenced taxa (20 bivalves and 1 chiton), obtained new data from 7 taxa for which published sequences are available (6 bivalves and 1 chiton), and included published sequence for 6 taxa (3 bivalves, 2 gastropods, and 1 chiton). Altogether our data matrix contained 33 taxa: 28 bivalves, 2 gastropods and 3 chitons (table 1). These 28 bivalves represent 9 of the 10 orders recognized by Abbott and Dance (1986) and 22 of the 41 extant superfamilies recognized by Morton (1996).

While this coverage is far from complete, each major lineage of the Bivalvia is represented by multiple taxa, permitting a more thorough testing of phylogenetic hypotheses. The questions that we have attempted to investigate are: (1) What are the relationships among the bivalve superfamilies represented in our data? (2) To which morphologically based bivalve phylogeny, if any, do the molecular data conform most closely? (3) What are the relationships among the three molluscan classes represented in our data?

Materials and Methods

Sample Collection and DNA Extraction

DNA was extracted from foot, adductor muscle, or whole animal by the method of Doyle and Doyle (1987) as communicated to us by Andrew McArthur (University of Victoria) and modified in our laboratory (Adamkewicz and Harasewych 1996). This method has proven to be both simple and superior to most others in the quality of DNA produced from mollusks. Once extracted, all DNA was stored frozen at −20°C. With the exceptions of *Cuspidaria* and *Periploma,* which were preserved specimens maintained in alcohol, all of the species sequenced for this project were collected living, frozen immediately, and maintained frozen until their DNA was extracted. All identifications were confirmed by one of us (M.G.H.) and the shells have been placed in the collections of the National Museum of Natural History. Collection localities and catalog numbers for the voucher specimens are given in table 1. Data on voucher specimens are also available via the World Wide Web (http://www.tigr.org) in the Sequences, Sources, Taxa (SST) database (Bult et al. 1997).

Primer Design

Initial PCR amplification of the target region of the gene for the small subunit of ribosomal DNA (18S rDNA) was always performed with the primers developed by Holland, Hacker, and Williams (1991), which were: (forward) 5′-GCC AGT AGC ATA TGC TTG TCT C and (reverse) 5′-AGA CTT GCC TCC AAT GGA TCC. The sequencing reactions were then generated with these primers and also with another set of internal primers designed by one of us (C.J.B.) as follows: (forward) 5′-GCC GGC GAC G(T/C)A TCT TTC and (reverse) 5′-GAA AGA T(A/G)C GTC GCC GGC. Used in combination, these four primers produced double coverage of the entire region.

Production of Sequencing Template

Initial PCR amplifications were performed using a Perkin-Elmer 480 thermal cycler and a reaction mix of 50 μl containing 500 ng of genomic DNA, 1.23 U of Amplitaq *Taq* polymerase (Perkin-Elmer), 0.25 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 5 μl of 10 × Perkin-Elmer PCR reaction buffer. All PCR reactions were performed as "hot start" reactions with the following parameters: 5 min at 95°C, addition of *Taq* polymerase, 25 cycles of the pattern 45 s at 94°C for denaturing, 2 min at 50°C for annealing, and 1 min at 72°C for extension. Following the 25 cycles, an 8-min final extension was performed at 72°C, after which the reaction mix was held at 4°C. Five microliters of each PCR product was evaluated on a 1% (w/v) agarose gel and the remaining reaction mix was transferred to a Microcon 100 ultrafiltration device (Amicon Inc., Beverly, Mass.) for removal of primers, unreacted dNTPs, and salts. An initial wash with 200 μl of 40% (v/v) isopropanol was performed prior to a wash with 450 μl of sterile distilled water.

DNA Sequencing and Data Management

Five microliters of cleaned PCR product were used as a template for sequencing on an Applied Biosystems 373A automated DNA sequencer using fluorescence dye terminator cycle sequencing kits (Applied Biosystems/Perkin Elmer). Sequence data were edited and then assembled into a consensus sequence using modified TIGR Assembler software (Fleischmann et al. 1995). Each consensus sequence was then edited using modified ABI Sequence Editor software.

Multiple Sequence Alignment

The confirmed consensus sequences of our 27 taxa were translated into multiple FASTA file format (Pearson and Lipman 1988), combined with GenBank published sequences for six taxa, and viewed with Genetic Data Environment (GDE) version 2.0 (Smith et al. 1994). The sequences were aligned using the multiple sequence alignment algorithm (MSA) of Sutton (Bult et al. 1997).

Phylogenetic Analyses

Phylogenetic analyses using parsimony were conducted using the heuristic search function of PAUP 4.0.0d49 (Swofford 1996) with and without character reweighting using the rescaled consistency index. When characters were reweighted, a base weight of 100 was used. All bootstrap analyses (2,000 replicates) used the

Table 1
Locality Data, Tissues Extracted, Voucher Specimen Information, and Sequence Accession Numbers for Taxa Used in this Study

Taxonomic Position of Each Species[a]	Collection Locality	Tissue Extracted	USNM[b] Catalog Number	GSDB[c] Accession Number
Class Polyplacophora				
Order Neoloricata				
Suborder Ishnochitonina				
Acanthopleura granulata (Gmelin, 1791)	Pt. Antonio, Jamaica	Buccal mass	888684	L78872
Acanthopleura japonica (Lischke, 1873)	NA	NA	NA	X70210[d]
Suborder Acanthochitonina				
Cryptochiton stelleri (Middendorff, 1847)	Bamfield, Canada	Buccal mass	888657	L78873
Class Gastropoda				
Subclass Gymnomorpha				
Order Systellommatophora				
Superfamily Onchidoidea				
Onchidella celtica (Cuvier, 1817)	NA	NA	NA	X70211[d]
Subclass Pulmonata				
Order Stylommatophora				
Superfamily Achatinoidea				
Limicolaria kambeul (Bruguière, 1792)	NA	NA	NA	X66374[d]
Class Bivalvia				
Subclass Protobranchia				
Order Solemyoida				
Superfamily Solemyoidea				
Solemya velum Say, 1823	Woods Hole, Mass.	Siphon	888665	L78846
Order Nuculoida				
Superfamily Nuculoidea				
Nucula proxima Say, 1822	Woods Hole, Mass.	Foot	888666	L78847
Superfamily Nuculanoidea				
Yoldia limatula (Say, 1831)	Woods Hole, Mass.	Adductor and foot	888668	L78848
Subclass Anomalodesmata				
Order Pholadomyoida				
Superfamily Pandoroidea				
Periploma fragile (Totten, 1835)	New Jersey	Whole animal	832796	L78845
Superfamily Poromyoidea				
Cuspidaria glacialis (G. O. Sars, 1878)	New Jersey	Whole animal	857559	L78844
Subclass Pteriomorphia				
Order Arcoida				
Superfamily Arcoidea				
Anadara ovalis (Bruguière, 1789)	Sanibel, Fla.	Adductor	888681	L78852
Order Mytiloida				
Superfamily Mytiloidea				
Mytilus edulis Linnè, 1758	Lewis, Del.	Adductor	888682	L78854
Geukensia demissa (Dillwyn, 1817)	Lewis, Del.	Adductor	888683	L78853
Order Pterioida				
Superfamily Pterioidea				
Pteria brevialata (Dunker, 1872)	Minabe, Japan	Adductor	888685	L78849
Superfamily Pinnoidea				
Atrina rigida (Lightfoot, 1786)	Ft. Pierce, Fla.	Adductor	888609	L78850
Order Ostreoida				
Superfamily Ostreoidea				
Crassostrea virginica (Gmelin, 1791)	Lewis, Del.	Adductor	888693	L78851
Superfamily Pectinoidea				
Argopecten irradians (Lamark, 1819)	NA	NA	NA	L11265[d]
Placopecten magellanicus (Gmelin, 1791)	NA	NA	NA	X53899[d]
Subclass Palaeoheterodonta				
Order Unionoida				
Superfamily Unionoidea				
Anodonta imbecilis Say, 1829	Lake Worth, Fla.	Adductor	888686	L78858
Elliptio complanata (Lightfoot, 1786)	Fairfax, Va.	Adductor	888687	L78857
Subclass Heterodonta				
Order Veneroida				
Superfamily Galeommatoidea				
Divariscintilla yoyo Mikkelsen and Bieler, 1989	Ft. Pierce, Fla.	Whole animal	888688	L78869
Superfamily Chamoidea				
Arcinella cornuta Conrad, 1866	Sanibel, Fla.	Adductor	888651	L78866
Superfamily Mactroidea				
Mulinia lateralis (Say, 1822)	NA	NA	NA	L11268[d]
Superfamily Solenoidea				
Ensis directus Conrad, 1843	Lewis, Del.	Adductor	888689	L78871

Table 1
Continued

Taxonomic Position of Each Species[a]	Collection Locality	Tissue Extracted	USNM[b] Catalog Number	GSDB[c] Accession Number
Superfamily Tellinoidea				
Donax variabilis Say, 1922	St. George's Island, Fla.	Foot	888679	L78867
Asaphis deflorata (Linnè, 1758)	Andros Island, Bahamas	Foot	888680	L78868
Superfamily Corbiculoidea				
Corbicula leana Prime, 1864	Japan	Adductor	888690	L78861
Superfamily Veneroidea				
Mercenaria mercenaria (Linnè, 1758)	Lewis, Del.	Adductor	888692	L78864
Dosinia discus (Reeve, 1850)	Sanibel, Fla.	Adductor	888653	L78863
Order Myoida				
Superfamily Myoidea				
Mya arenaria Linnè, 1758	Maryland	Adductor	888691	L78859
Corbula contracta Say 1822	Ft. Pierce, Fla.	Adductor	888667	L78860
Superfamily Hiatelloidea				
Panopea japonica A. Adams 1850	Japan	Adductor	888626	L78870
Superfamily Pholadoidea				
Cyrtopleura costata (Linné, 1758)	Sanibel, Fla.	Adductor	888608	L78865

[a] The systematics and nomenclature follow those of Vaught (1989).
[b] National Museum of Natural History, Smithsonian Institution.
[c] Genome Sequence Data Bank.
[d] GenBank accession number.

"fast" stepwise addition feature of the same program. Initial analyses were performed with random addition of taxa (10 addition sequences, then another 10 using a different seed value). Because the order of addition did not affect the results, thereafter addition was simple and always in the same order. Both the data matrix and some trees were viewed/edited using MacClade version 3.01 (Maddison and Maddison 1992). When aligned with the sequence of the gastropod *Onchidella celtica* as published by Winnepenninckx, Backeljau, and DeWachter (1994), our sequence is near the 5′ end of the 18S gene, from position 26 to position 582 of *Onchidella,* and includes four of the nine areas cited by Kenchington et al. (1994) as informative among families of bivalves. Any nucleotide whose identity was uncertain was changed to "missing" in the final data matrix and only those positions determined in at least 23 of the 33 taxa were included in our analysis. All other nucleotides were excluded with the "exclude charset" function of PAUP but are present in the aligned data matrix, available in Nexus format by sending a formatted Macintosh diskette to the corresponding author. In addition to individual bases present in only one taxon, one region, corresponding to positions 308–421 of our sequence, was excluded from analysis because it was unalignable. Our final data matrix consisted of 756 aligned positions, of which 506 were used for phylogenetic inference.

Results

Of the 506 nucleotides analyzed, 115 (22.7%) were informative. However, these characters were not uniformly distributed throughout the sequence. Of the 115, 27 occurred within the first 100 nucleotides, 31 within the second hundred, 31 within the third hundred, 11 within the fourth hundred, and 15 within the last hundred and six. Thus, the last 206 nucleotides, containing 40% of all aligned nucleotides and 26/115 (23%) of the informative characters, appeared to be much more conserved than the first 300 nucleotides, which contained 60% of the sequence and 89/115 (77%) of all informative characters.

An initial heuristic search of all 33 taxa, with the chiton *A. japonica* designated as the outgroup and using informative characters only, produced 37 equally parsimonious trees of length 315 with a consistency index (CI) of 0.546, a retention index (RI) of 0.743, and a rescaled consistency index (RC) of 0.406. A consensus of these trees is shown in figure 2*A*. To improve resolution, the search was repeated with characters reweighted using the RC. This search produced 2 trees, identical to 2 of the 37 original trees, with CI = 0.767, RI = 0.885, and RC = 0.678, that differed only in the relative positions of *Ensis* and *Panopea* within the heterodonts. The phylogram with bootstrap proportions (BP) shown in figure 2*B* has *Ensis* diverging before *Panopea,* the same order as in the unique tree in figure 3. Many of the branches in figure 2*B* are short and have BP values below 70% (the value corresponding to a probability of 0.95 that the corresponding clade is real—see Hillis and Bull 1993). The tree's most surprising feature is the appearance of the two gastropods not as a separate, outlying clade but among the bivalves as a sister group (BP = 100) to two taxa, *Solemya,* a protobranch, and *Periploma,* an anomalodesmatan, that are usually assigned to separate subclasses of bivalves. *Nucula* and *Yoldia,* the other two protobranchs in the data set, appeared in separate lineages, although morphologists consider the protobranchs deeply separated from all other bivalves.

Because the position of the gastropods was unexpected and contradicts all morphologically based phylogenies, this set of analyses was repeated designating the gastropod *Onchidella* as the outgroup. These search-

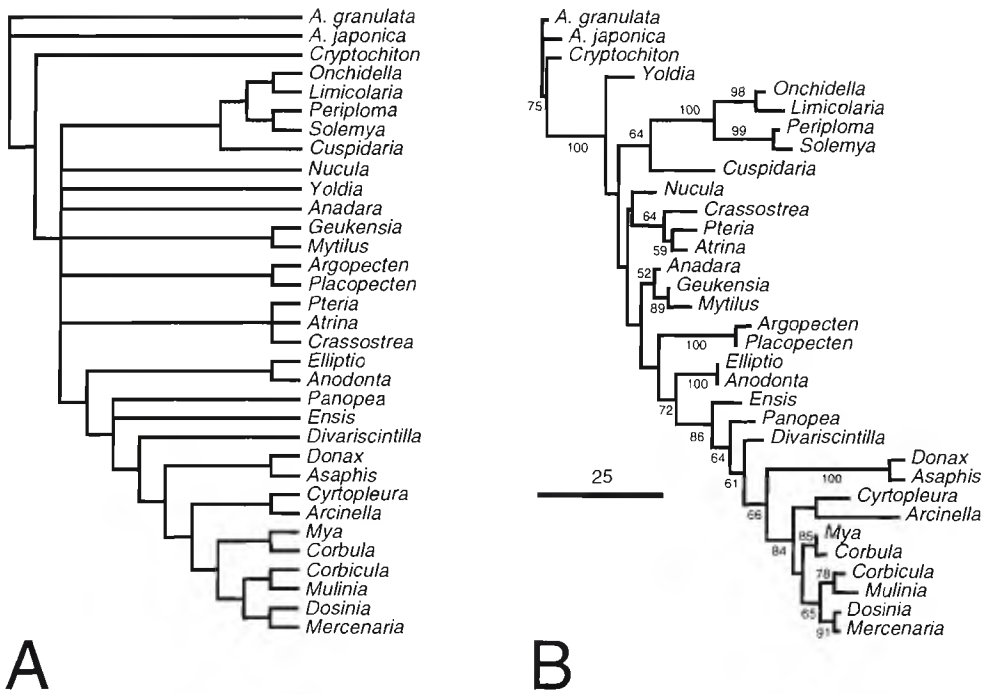

FIG. 2.—Phylogenetic relationships among 28 Bivalvia, 3 Polyplacophora, and 2 Gastropoda based on 506 bp of the 18S rDNA gene. *A*, Consensus of 37 most-parsimonious trees (length = 315; CI = 0.546; RI = 0.743; RC = 0.406) generated in a heuristic search with *Acanthopleura japonica* designated as the outgroup. *B*, One of two most-parsimonious trees (CI = 0.767; RI = 0.885; RC = 0.678) resulting from a heuristic search following reweighting of the data using the rescaled consistency index. This tree is identical to one of the 37 trees of length 315 produced by the unweighted search. Bootstrap values are shown only for nodes with BP of >50%.

es again produced 37 trees topologically identical to the first set, differing only in the position of the root. The three chitons still behaved as a monophyletic group (BP = 100) but appeared among the bivalves in the position previously occupied by the gastropods.

The tree shown in figure 2*B* contains only nine branches with lengths greater than eight, and four are in the clade containing the gastropods and *Cuspidaria,* as are three of the nodes with BP of >95. To test the hypothesis that the gastropod placement was an artifact of long-branch attraction, the analysis leading to figure 2*B* was repeated with *Periploma* and *Solemya* excluded from the data matrix. In this search, the two gastropods emerged as sister group to the scallops on the next nearest long branch.

The incongruity of the position of the gastropods made us reluctant to proceed further with this data set. Because morphological opinion unanimously considers chitons to be more distantly related to the bivalves than are gastropods, additional analyses aimed only at investigating the relationships within the class Bivalvia were performed excluding both gastropods and using only the chitons as the outgroup. A heuristic search like the ones above but using the reduced data set yielded 14 trees of minimum length 296 with CI = 0.557, RI = 0.740, and RC = 0.412. Reweighting using the RC produced a unique tree (length = 11,940, CI = 0.757, RI = 0.879, RC = 0.665), shown in figure 3, which was identical to one of the 14 unweighted trees.

In this reweighted tree, the relationships among the more derived taxa were unchanged and the sister group status of the subclasses Palaeoheterodonta and Heterodonta, each well supported as a separate lineage, received good support (BP = 72, $P > 0.95$). The relationships within the Heterodonta did not reflect their morphological subdivision into two orders, the Veneroida and Myoida. Taxa referred to these two orders are grouped in a single clade with BP = 84. The subclasses Pteriomorphia, Anomalodesmata and Protobranchia were not well resolved with respect to one another. The Pteriomorphia appeared to form a grade rather than a single clade but the relationships among its subgroups were not well resolved and bootstrap support for clades other than the scallops (*Argopecten* + *Placopecten*) and the mussels (*Mytilus* + *Geukensia*) was low. The Protobranchia and Anomalodesmata were not segregated at all.

The sensitivity of this phylogeny to selection of taxa was investigated by repeating both unweighted and reweighted heuristic searches while systematically excluding one bivalve taxon at a time, always using the chitons as the outgroup. Effects on both the number of minimum-length trees and the degree of resolution were tabulated. As shown in table 2, six taxa had major effects when removed, three more than doubling the number of minimum-length trees and three decreasing the number to less than half. Despite having major effects on the numbers of minimum-length trees, none of these removals affected branch lengths or BP values by more than a few percent (±3%). The removal of most taxa produced either no change in the number of trees (13 of 28 taxa) or minor change. The average for all trials was

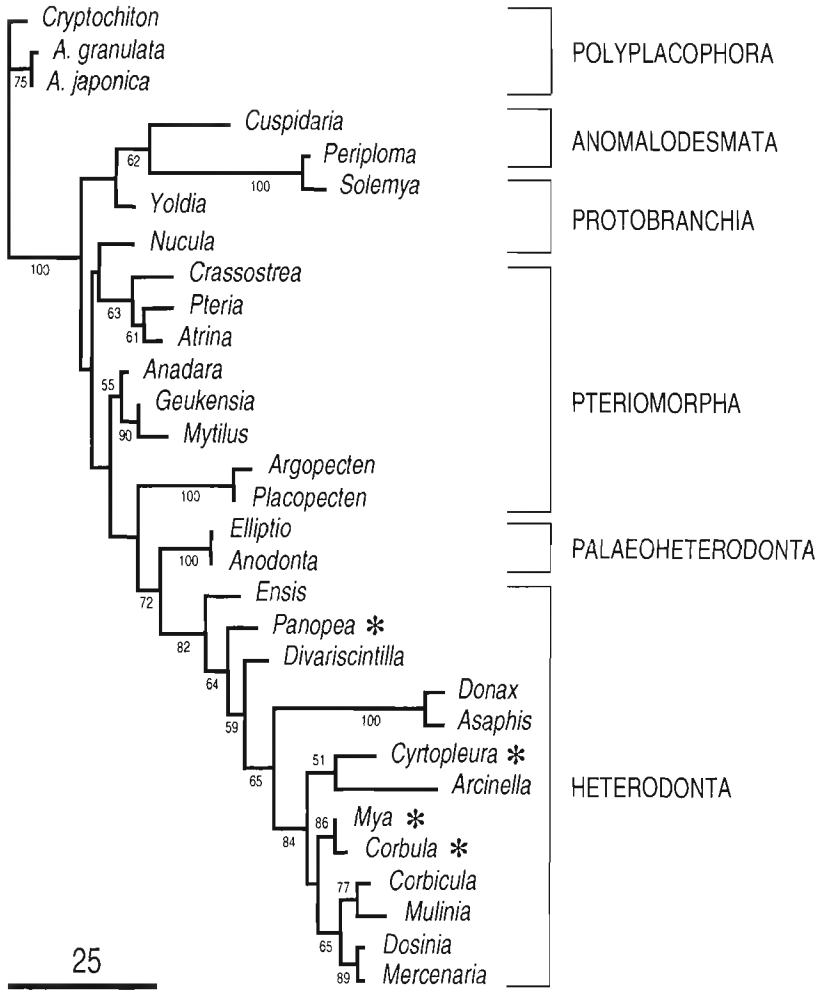

FIG. 3.—The single most-parsimonious tree (length = 11,940; CI = 0.757; RI = 0.879; RC = 0.665) (length = 296; CI = 0.557; RI = 0.740; RC = 0.412) resulting from a heuristic search, characters reweighted using the rescaled consistency index, when gastropods are excluded from the data set and the three polyplacophorans are designated as the outgroup. Branch lengths shown are for an unweighted tree of length 296 identical to the reweighted tree. Bootstrap values are shown only for nodes with BP of >50%. An asterisk following the taxon identifies those heterodonts placed in the Myoida in most classifications.

14.75 trees, or an increase of approximately one tree from the original search. The effect on the lengths of the shortest trees was modest in all cases, the range being −25 to −1, with an average change of −9 steps (−3%).

As could be expected from the results of removing single taxa, removing single clades had little effect except for a few cases. Deleting any of the basal clades, whose five taxa included three of the most sensitive, left the more derived portions of the tree intact. Also to be expected, removing most clades simply caused fewer, shorter trees to be found. The results of removing some of the inner clades were, however, unexpected. Removing either the scallops (*Argopecten* + *Placopecten*), the tellins (*Donax* + *Asaphis*), or the venerid clams (*Mercenaria* + *Dosinia*) caused a substantial increase in the number of equally parsimonious trees, from 14 to 104, 49, or 56 respectively. Each of these tests removed only two taxa. While none of the species had a high impact individually, in pairs the effect of their removal was substantial.

The data showed clear signs of mutational saturation. Of the original 115 informative characters, 107 involved bivalve taxa only. When these were examined using three categories (transitions [TS] only, transversions [TV] only, or a mixture of both), 39 characters contained only transitions, 33 contained only transversions, and 35 contained both, yielding a TS : TV ratio of 39:33 or 1.2:1. If a somewhat less stringent definition was used (not more than one taxon nonconforming), the ratio was 47:43 or 1.1:1. When the character changes reported by PAUP as supporting each node were also classified by type, the TS : TV ratio was 137:151 or 1:1.1, again nearly unity. Such TS : TV ratios were lower than would be expected if most sites had experienced only a single mutational change. The consistency of each character was also related to the type of change. The 33 transversions had an average CI of 0.79 and 22 of them (67%) had CI = 1. The 39 transitions had an average CI of 0.66 and only 38% had a CI = 1. The 35 mixed characters had an average CI of 0.58 and only 23% had a CI = 1. This pattern suggested that weighting

Table 2
Taxa Whose Removal Had a Major Effect on the Phylogeny

Deleted Taxon	Change in Number of Trees (and length)[a]	Effect on Resolution[b]	Effect of Reweighting
Mya	14 → 56 (294)	Heterodonts poorly resolved	Unique tree, only *Arcinella* moved from original tree
Cyrtopleura	14 → 56 (283)	Same as *Mya*	Same as *Mya*
Atrina	14 → 31 (287)	Oyster clade disappears	Unique tree, protobranchs and anomalodesmaceans united
Nucula	14 → 4 (288)	Resolution improved	Same as *Atrina*
Cuspidaria	14 → 2 (272)	Well resolved, oysters with protobranchs and anomalodesmaceans	Same as *Atrina*
Yoldia	14 → 2 (287)	Same as *Cuspidaria*	Same as *Atrina*

[a] Comparisons are to an unweighted tree of length 296 identical to the tree in figure 3 which was produced by reweighting.
[b] Prior to reweighting.

characters by type of change should produce a result similar to reweighting characters with the RC. However, when this experiment was tried with two weighting schemes, a ratio of mixed : TS : TV of 1:2:3 or of 1:3:10, multiple trees were produced and these trees were both more different from morphologically based trees and less well resolved than those generated by reweighting with the RC.

As would be expected with saturated data, the deeper nodes, uniting the more distantly related taxa, were not well resolved unless a weighting or reweighting scheme was applied. At the same time, the data also showed that the 18S sequence had not changed rapidly enough to resolve differences within single families or genera. Only one step separated the two species of chitons in the genus *Acanthopleura,* although one occurs in the northern Pacific and the other occurs in the Caribbean. Similarly, branch lengths for closely related bivalves were short, reflecting few changes within these more recent divergences. When published sequences for other bivalves (Mactridae, eight species; *Mytilus,* four species) were examined for the same 506 bases as our taxa, few or no differences were found within either group. Because PAUP could not resolve them with only 506 bases, these species were not included in this study.

An attempt was also made to repeat as nearly as possible Bowman's (1989, p. 191) analysis of 21 bivalves using the amphibian *Xenopus* as the outgroup. Although neither our set of sequences nor our set of taxa was identical to Bowman's, 18 of our taxa were from the same superfamilies (9 from the same genera), with only 3 of her superfamilies not represented in our data set. Neither Bowman's original analysis nor our reconstruction produced tree topologies concordant with morphologically based hypotheses. Unlike our analysis of 33 taxa, our reconstruction of Bowman's tree using a subset of 18 of our bivalves plus *Xenopus* failed to resolve the heterodonts from the pteriomorphs. Bowman's tree did show the heterodonts as a distinct clade, but the pteriomorphs appeared to be polyphyletic and the single palaeoheterodont was most closely allied with the Protobranchia. The contrast between the two analyses lessened, however, when bootstrap results were compared. Bowman's bootstrap test of her tree showed significance support only for the heterodont clade and for the pairing of the scallops *Argopecten* and *Placopecten,* both supported by our analysis. Neither Bowman's tree nor our reconstruction corresponded with either our most resolved reweighted tree or with any of the phylogenetic hypotheses outlined in figure 1.

The phylogenetic hypotheses represented in figure 1 were compared by bringing our most resolved tree (fig. 3) into the MacClade version 3.01 program (Maddison and Maddison 1992) and then altering it to represent each hypothesis and noting the effect on tree length, CI, RI, and RC (Harasewych et al. 1997, table 2). This comparison required that all of the published hypotheses be greatly simplified, primarily by restricting them to relationships among the major subgroups of the Bivalvia. Otherwise, the taxonomic units considered by each author were too diverse for reasonable comparisons. As table 3 shows, the largest increase in tree length and decrease in index values was caused by resolving the Anomalodesmata and the Protobranchia in our tree, a move also required by every other hypothesis. Once that change was in effect, our tree had statistics virtually identical to those of Starobogatov (1992) and only slightly different from those of Cope (1996) and Waller (1997). Most of the additional increases in length and decreases in indices for the hypotheses of Morton (1996) and Salvini-Plawen and Steiner (1996) were caused by the separation of the orders Myoida and Veneroida rather than by the positions of the subclasses.

Discussion

Our data strongly support the assertion of Lecointre et al. (1993) that dense representation of taxa is as important as length of sequence for stable phylogenetic reconstruction. The effect of removing one species from our matrix was unpredictable and could be large, as could the effect of removing one clade. Furthermore, these effects did not depend on which order or superfamily was represented, because sensitive clades often did not contain a sensitive species. The only protection against such effects is broad taxonomic coverage. Our failure to reproduce the results of Bowman (1989), even with similar taxonomic coverage at the level of the superfamily, also supports the conclusions of Lecointre et al. (1993). Because neither our selection of species nor our exact gene region was identical to Bowman's, we can only conclude that the incongruence was caused by the inclusion of different taxa and sequences. Because our sequences already included both slower and faster changing regions, expanding the data set to include complete sequences of the 18S rDNA gene may not in-

Table 3
A Comparison of the Phylogenetic Hypotheses in Figure 1

Hypothesis	Length[a] (Δ%)[b]	CI[c] (Δ%)	RI[d] (Δ%)	RC[e] (Δ%)
Present study (fig. 3),[f] Anomalodesmacea not separated	296	0.56	0.74	0.41
Present study (fig. 1*F*), Anomalodesmacea separated	326 (+10%)	0.51 (−9%)	0.68 (−8%)	0.34 (−17%)
Starobogatov (1992) (fig. 1*A*)	327 (+10%)	0.50 (−11%)	0.68 (−8%)	0.34 (−17%)
Cope (1996) (fig. 1*B*)	329 (+11%)	0.50 (−11%)	0.67 (−9%)	0.34 (−17%)
Waller (1977) (fig. 1*C*)	330 (+11%)	0.50 (−11%)	0.67 (−9%)	0.34 (−17%)
Morton (1996) (fig. 1*D*)	358 (+21%)	0.46 (−18%)	0.62 (−16%)	0.28 (−32%)
Salvini-Plawen and Steiner (1996)	351 (+18%)	0.47 (−16%)	0.63 (−15%)	0.30 (−27%)

[a] Minimum length of the tree in MacClade.
[b] Change from the minimum tree in percent.
[c] Consistency index.
[d] Retention index.
[e] Rescaled consistency index.
[f] Length and indices are for an unweighted tree identical to the reweighted tree shown in figure 3.

sulate against the effects of particular species. Ideally, one wants complete sequences from many genes and many taxa, but, given the limits on time, effort, and expense, we believe that the inclusion of partial sequences from several different genes will be more informative than longer sequences from fewer genes and that the inclusion of more species may be more informative still.

One use for which complete 18S sequences appear to be important is to distinguish among closely related species. Rice and Singh (1993), using complete 18S sequences, were able to fully resolve groups of closely related species within individual families and genera, taxa which we were unable to resolve using partial sequences. Based on its resolution of relationships within orders as well as within the most recently radiated subclass, the Heterodonta, the 5′ region of the 18S gene used in our study appears best suited to resolving intermediate relationships among the Bivalvia and should be used to broaden taxonomic coverage by including representatives of more families rather than more genera or species. That this familial and ordinal level coverage is desirable is made clear by Salvini-Plawen and Steiner (1996), who have called for systematic coverage at all levels of hierarchy to advance phylogenetic knowledge.

Our findings resemble those of Kenchington et al. (1994) in that chitons can be made to appear within the Bivalvia. In our results, their position is interchangeable with that of the gastropods, whichever is designated as the outgroup causing the other to appear within Bivalvia. Clearly, the chitons themselves are monophyletic regardless of their position in the phylogeny, but we consider the relationships among the three molluscan classes much more difficult to assess. Distantly related taxa may behave as random outgroups, joining the ingroup at the longest branch and making the root position for the tree unreliable (Wheeler 1990). This the gastropods appear to do. Until the effects of long-branch attraction can be excluded, the issue of bivalve polyphyly, and the relationships among the molluscan classes, is unlikely to be resolved using the 18S rDNA gene. Using the largest set of complete 18S sequences to date, Winnepenninckx, Backeljau, and DeWachter (1996) have been unable to do so and have concluded that the task may not be possible. A more extensive set of species, containing many more basal taxa, is clearly needed. Additionally, the problem of suitable outgroups must be solved, perhaps, on the evidence of substantial homoplasy in our data, by examining different, more highly conserved sequences.

Within Class Bivalvia, our data clearly support the Palaeoheterodonta as the sister group of the Heterodonta. Although the BP = 72 value for this node is modest, it should correspond to a probability of 0.95 that the clade is real (Hillis and Bull 1993) and the node was consistently supported throughout our analysis. The Pteriomorphia appear to form a grade rather than a separate clade. Because branch lengths among the Pteriomorphia are short and BP values are low, except those supporting the monophyly of the scallops, our results are suggestive rather than persuasive. Another surprise in our results is the apparently polyphyletic nature of the heterodont orders Myoida and Veneroida. *Mya, Corbula,* and *Cytropleura,* all myoids, and the veneroid clams such as *Mercenaria* and *Corbicula* form an inner clade with good bootstrap support (BP = 84). Other taxa referred to Veneroida, such as *Divariscintilla* and *Ensis,* as well as the myoid *Panopea,* all emerge outside this clade. The veneroid superfamily Tellinoidea, represented by *Donax* and *Asaphis,* is also outside the clade, although this superfamily is regarded as being closely related to the superfamily containing *Mercenaria.* Only additional work by both morphologists and molecular systematists can resolve these issues. Molecular data for other genes as well as for more taxa are needed to confirm these findings, but our data do challenge the conventional view of relationships within the subclass Heterodonta.

The inability of 18S sequence data to resolve the monophyly of the subclass Protobranchia is especially surprising, because morphologists are united in regarding the Protobranchia to be the sister group of all other Bivalvia. This lack of resolution may be caused by unequal rates of change in different bivalve lineages, as

suggested by widely varying branch lengths in our trees. The uncertainty surrounding the relationship between the protobranchs and the anomalodesmatans is part of our larger inability to distinguish decisively among the various phylogenetic hypotheses put forward by morphologists (fig. 1). Our results conform most closely to the hypothesis of Starobogatov (1992), who views the Heterodonta and Palaeoheterodonta as sister groups arising from the Pteriomorpha. However, differences in tree lengths and character indices between our sequence-based results and the other morphology-based hypotheses were minor. This absence of large differences is probably a reflection of the relatively short time during which all the molluscan classes and subclasses radiated. Proably not more than 40–80 Myr separated the earliest record of a mollusk in the Cambrian from the latest divergence of a class in the Ordovician (Waller 1997), and the short branch lengths at the base of our tree (fig. 3) may reflect this. Philippe, Chenuil, and Adoutte (1994) have already discussed the difficulty of using molecular data to distinguish among phyla that diverged over a relatively short period of time, a difficulty that Winnepenninckx, Backeljau, and DeWachter (1996) has confirmed using complete 18S sequences. Reweighting characters with the RC reduced this problem, but definitive evidence may require new sequences from more slowly evolving genes before a consensus of the evidence will tell us what the order of divergence of subclasses within the Bivalvia actually was.

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