Molecular Phylogenetics of the Metazoan Clade Lophotrochozoa

By

Yale J. Passamaneck

B.A., University of California, Santa Cruz, 1996

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at the

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and the

WOODS HOLE OCEANOGRAPHIC INSTITUTION

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Signature of Author

Joint Program in Biological Oceanography
Massachusetts Institute of Technology
and Woods Hole Oceanographic Institution
September 2003

Certified by

Kenneth M. Halanych, PhD.
Thesis Supervisor

Accepted by

John Waterbury, Ph.D.
Chair, Joint Program for Biological Oceanography
Massachusetts Institute of Technology
and Woods Hole Oceanographic Institution

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ABSTRACT

DNA sequencing and phylogenetic analyses were conducted to investigate evolutionary relationships between taxa within the metazoan clade Lophotrochozoa. Chapter 1 presents an introduction to phylogenetics of the Metazoa and the clade Lophotrochozoa. Chapter 2 analyzes higher level relationships between the major groups within the phylum Mollusca using sequences of the nuclear ribosomal large-subunit RNA gene (LSU rDNA). Results presented provide the first molecular evidence for a close relationship between the Scaphopoda and Cephalopoda. Phylogenetic trees with this topology were found to have likelihood scores significantly better than those for phylogenies constrained to fit the Diasoma hypothesis grouping Scaphopoda and Bivalvia as sister taxa. Chapter 3 utilizes LSU rDNA sequences to analyze relationships between diverse phyla within the clade Lophotrochozoa. LSU rDNA sequences were found to provide greater resolution than has been provided by previous analyses of the nuclear small-subunit ribosomal RNA gene (SSU rDNA). Analysis of LSU rDNA sequences recovered the monophyly of several phyla, such as Mollusca and Annelida, whose members are found to be paraphyletic using SSU rDNA sequences alone. Results also suggest that the clade Platyzoa, including rotifers and platyhelminthes, may have arisen within the Lophotrochozoa, rather than as a sister group to lophotrochozoans. Chapter 4 investigates the Hox gene complement of the bryozoan *Bugula turrita*. Six Hox genes were recovered, including an ortholog of the posterior class gene *Post2*, which is a synapomorphy for the Lophotrochozoa. The identification of a *Post2* ortholog provides evidence of a close relationship between the Bryozoa and other lophotrochozoan phyla.

Thesis Supervisor: Kenneth M. Halanych
Title: Assistant Scientist, Biological Oceanography, Woods Hole Oceanographic Institution

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Table of Contents

Chapter 1

Introduction to metazoan phylogenetics and the clade Lophotrochozoa

The work presented in this thesis explores the phylogenetic relationships between major groups within the metazoan clade Lophotrochozoa. This clade, which encompasses a many animal phyla, including bryozoans, brachiopods, annelids and mollusks, was first identified from analyses of nuclear ribosomal small-subunit gene (SSU rDNA) sequences (Halanych et al., 1995). Although the clade has been supported by additional markers, such as Hox genes (de Rosa et al., 1999), resolution of relationships among lophotrochozoan phyla remains uncertain. The Lophotrochozoa encompasses a broad diversity of body plans, developmental modes and life histories. A greater understanding of the evolutionary relationships amongst taxa within the clade is crucial to understanding the origins of morphological and developmental novelties. The work presented here builds upon the current body of knowledge by employing sequence data from the nuclear ribosomal large-subunit gene (LSU rDNA) and Hox genes to explore the evolution of lophotrochozoans.

To appreciate the context in which this thesis has developed, it is valuable to understand historical and current views of metazoan evolution. Traditionally, hypotheses of metazoan evolution have been based upon researchers' knowledge of the animals under study and their personal interpretation of similarities between them. The dominant view has long been one of increasing complexity over the course of metazoan evolution (e.g. Haeckel, 1874; Hyman, 1940) (Figure 1), where animals moved from a simple organization with two cell layers (diplobastic) to a more advanced state with three cell layers (triploblastic).

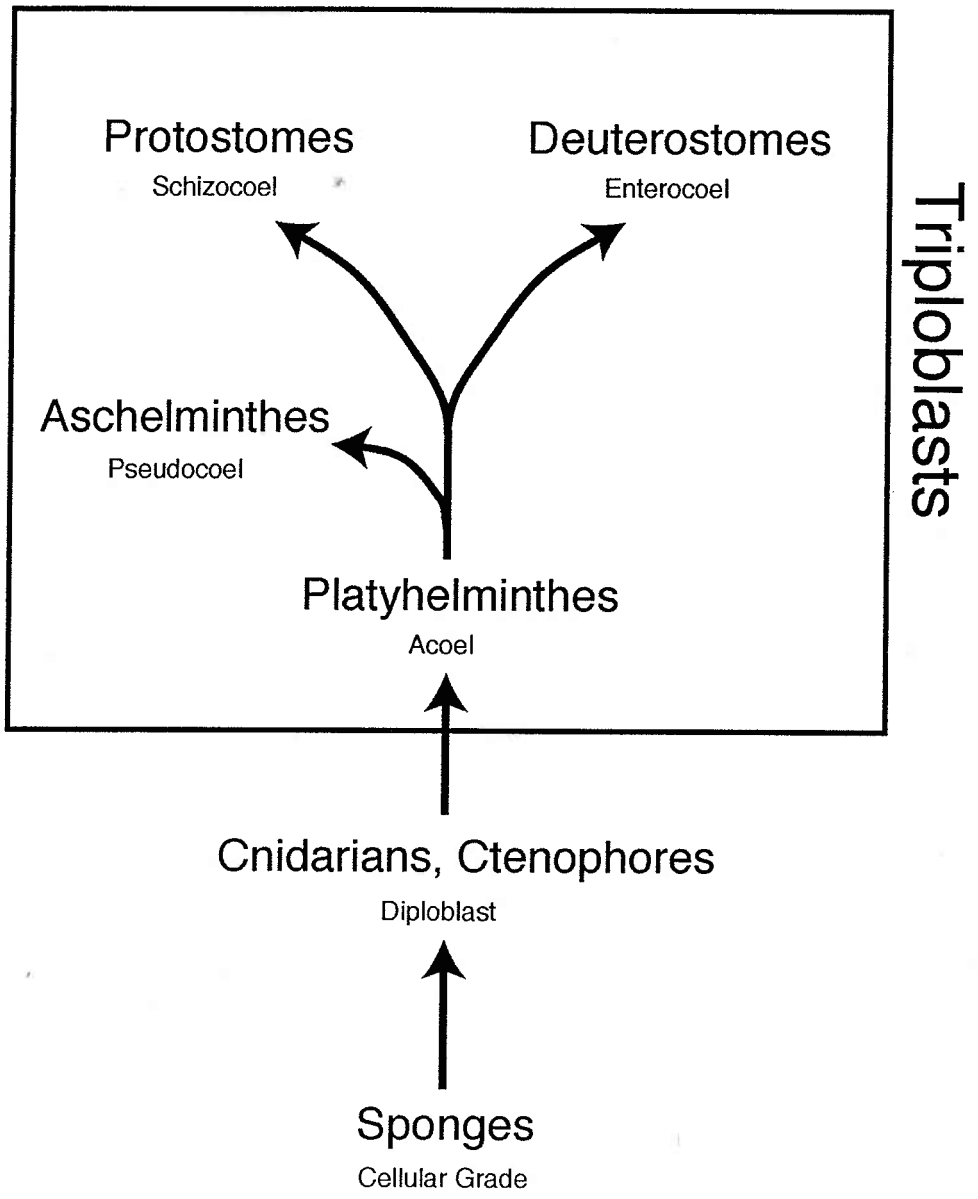

Figure 1: Traditional view of metazoan phylogeny, based upon interpretation of increasing morphological complexity. Tissue organization or pattern of coelom fomation is listed for each group. Modified from Halanych and Passamaneck (2001).

Under this view, relationships among bilaterian triploblasts were defined based upon the nature and origin their coelomic cavities, with organisms having more complex coeloms being viewed as more derived. Platyhelminthes were descibed as acoelomate, and therefore the most primitive of bilaterian triploblasts. Several taxa, such as rotifers and nematodes, have simple body cavities derived from the embryonic blactocoel. Such animals have been described as pseudocoelomate, and grouped together by some authors under the name Aschelminthes (e.g. Hyman, 1951).

Taxa with fully developed coeloms surrounded by mesodermal tissue were viewed as the most advanced metazoans. Among such taxa a further distinction was drawn based upon the mode of coelom formation. Deuterostomes were characterized by coelom formation through invagination of the endoderm, termed enterocoely, while protostomes formed coeloms by means of schizocoely, a splitting of mesodermal bands.

Other researchers have also posited the phylogenetic significance of features such as cleavage pattern during early development (e.g. Siewing, 1976; 1980), fate of the blastopore in relation to the mouth and anus of the adult (Grobben, 1908), and larval type (e.g. Jägersten, 1972; Nielsen, 1985). However, each of these hypotheses is limited by the potential bias in the investigator's perspective on what small set characters are phylogenetically important. The major problem with these approaches is that reliance on a small number of features to infer evolutionary relationships limits the potential for rigorous comparison of alternative hypotheses.

With the advent of cladistics, Willi Hennig (1966) provided the groundwork for a systematic approach to analyzing the evolutionary relationships between metazoan phyla that answers the limitation of traditional analyses. Cladistics bases determination of

phylogenetic relationships upon the identification of synapomorphies, shared derived characters present in related organisms and absent in unrelated organisms. Identification of synapomorphies allows determination of monophyletic clades of organisms.

In recent, years cladistic methods have been employed to analyze several large datasets of metazoan morphological and embryological datasets. Cladistic analyses by Eernissee et al., (1992) provided evidence contradicting the widely held Articulata hypothesis, which viewed annelids and arthropods as sharing a common segmented ancestor. More recent analyses (e.g. Zrzavy et al., 1998; Giribet et al., 2000; Peterson and Eernissee, 2001) have incorporated large datasets that include nearly all known extant metazoan phyla. While there are many similarities in the results from each of these studies, the position of some taxa, such as the lophophorate taxa (brachiopods, phoronids, and bryozoans), varies depending upon what characters are chosen and how they are coded. Recently, Jenner (2001) has urged caution in analysis of morphological characters, as many studies have included characters from previous studies without critical appraisal as to whether these characters are coded correctly.

Resurgent interest in the evolution of development during the last decade may provide a valuable tool for identifying phylogenetically informative characters. More detailed understanding of ontogenetic processes and the molecular mechanisms underlying them has the potential to aid determination of homology between structures. For example, recent evidence suggests that the molecular mechanisms underlying formation of the blastopore are conserved across bilaterians (Arendt et al., 2001). These findings are important because comparisons of blastopore fate are predicated on a presumption that all blastopores are homologous. Detailed studies of cell fate have also

helped to establish the homology of cell lineages among taxa with spiral cleavage (Henry, 2002). Spiral cleavage may therefore have had a single origin during the course of metazoan evolution. While utilizing such an approach may be produce phylogenetically informative results, great care must be exercised, as homologous processes often do not produce homologous structures (Abouheif et al., 1997; Wray and Abouheif., 1998).

Recent advances in DNA sequencing techniques have provided the ability of use gene sequences as an independent dataset for inferring evolutionary relationships among metazoans. To date, many molecular phylogenetic analyses of the relationships between metazoan phyla have relied upon sequence of the nuclear small-subunit ribosomal RNA gene (SSU rDNA or 18S rDNA; e.g. Field et al., 1988; Halanych et al, 1995; Aguinaldo et al., 1997; Giribet et al., 2000; Peterson and Eernissee, 2001). SSU rDNA has been valuable because portions of the gene sequence appear to evolve quite slowly, creating the potential for conservation of changes accrued during the diversification of metazoan phyla. Such conserved changes would then allow insight into the relationships between phyla. Analysis of SSU sequence has provided independent verification of many hypotheses of metazoan evolution, including the monophyly of the Bilateria (Field et al., 1988), and the division of Bilateria into protostome and deuterostome lineages (Lake, 1990). However, SSU sequence has also revealed unexpected relationships.

One dramatic finding has been that the three lophophorate phyla (bryozoans, brachiopods, and phoronids) are more closely related to protostome annelids and mollusks, than they are to deuterostomes, as has traditionally been believed (Halanych et al., 1995; Table 2). Halanych et al. (1995) named this new clade "Lophotrochozoa", for the lophophore of bryozoans, brachiopods, and phoronids, and the trochophore type larva

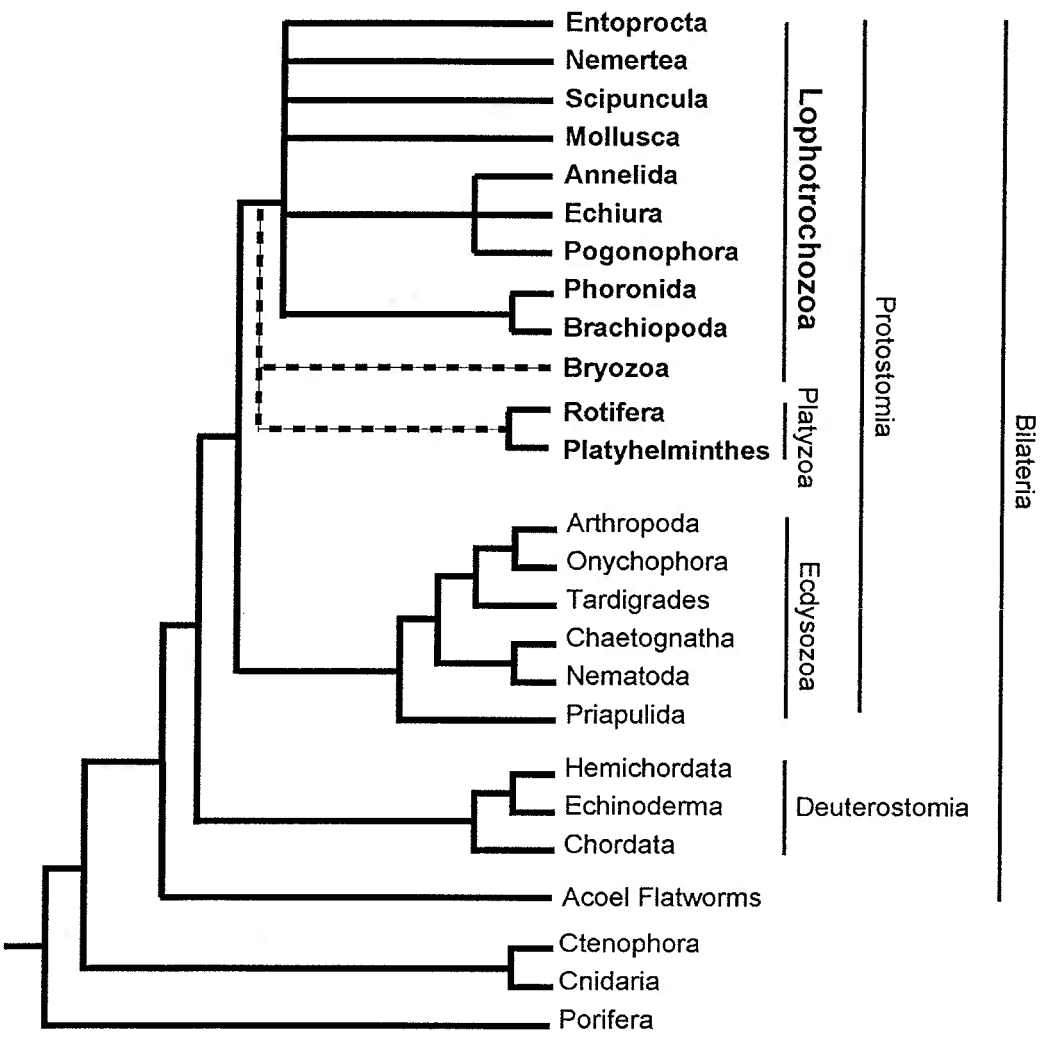

Figure 2: Current understanding of evolutionary relationships among metazoan phlya. Members of the clade Lophotrochozoa are highlighted. Phyla with uncertain phylogentic affinities in the tree are denoted with dashed branches. Relationships presented in the tree are primarily derived from analyses of small-subunit ribosomal rRNA gene (SSU rDNA) sequences. Modified from Halanych and Passamaneck (2001).

shared by annelids and mollusks. The results presented in this study also suggested that lophophorates might not comprise a monophyletic clade, as the bryozoan sequence branched basally to that of the other lophotrochozoans. The relationships between brachiopods, phoronids, annelids and mollusks were not resolved in this study.

Subsequent analysis of SSU sequence from additional taxa suggests that the clade Lophotrochozoa encompasses a broad diversity of metazoan phyla, including sipunculans, nemerteans, and entoprocts (e.g. Winnepenninckx et al., 1995; Mackey et al., 1996). Rotifers and platyhelminthes also appear to be closely related to lophotrochozoans, either as members of the clade (as discussed in Chapter 3), or as members of a sister group termed the Platyzoa (Giribet et al., 2000). Despite the diversity of the Lophotrochozoa, the relationships among the phyla within the group have not been extensively studied, and are not well understood. While SSU rDNA provided the initial evidence for the clade Lophotrochozoa, it does not appear to be able to resolve relationships among phyla within the clade.

Although the utility of SSU rDNA for elucidating metazoan evolution has been criticized (Abouheif et al., 1998), simulation studies have suggested that additional sequence with evolutionary properties like that of SSU rDNA for each taxon would be sufficient to increase resolution (Halanych, 1998). Multiple copies of the SSU gene are present in the genome of metazoans, however, their sequences remain homogeneous through a process of concerted evolution (Hillis and Dixon, 1991). Other molecular markers must therefore be explored to obtain additional sequence data for phylogenetic reconstructions.

The nuclear large-subunit ribosomal RNA gene (LSU rDNA) provides a potential source of information for metazoan phylogenetics because it has properties similar to that of SSU rDNA (Hillis and Dixon, 1991). Both are part of the ribosomal DNA tandem repeat, and like SSU rDNA, LSU rDNA displays rate heterogeneity among sites. Highly conserved sites therefore allow for design of universal primers for amplification and sequencing, while changes accumulated at less conserved sites may hold information regarding the evolutionary relationships among taxa. Several recent studies have evidenced the utility of LSU sequence for analyzing phylum level relationships within the Metazoa, particularly when combined with SSU sequence (Medina et al., 2001; Winchell et al., 2002; Mallatt and Winchell, 2002).

In the following chapters I present work done to assess the ability of LSU rDNA and Hox sequences to inform our understanding of lophotrochozoan phylogenetics. Chapter 2 focuses on higher-level relationships within the phylum Mollusca. The Mollusca represents the most diverse of lophotrochozoan phyla, in terms of both morphology and numbers of species. Despite this diversity, the relationships between the major groups of mollusks has received relatively little attention from the standpoint of molecular phylogenetics. Work presented here provides the first molecular evidence of a close evolutionary relationships between scaphopods and cephalopods. This finding challenges the widely held Diasoma hypothesis, which suggests scaphopods to be closely related to bivalves. Chapter 2 also explores heterogeneity in the rate of LSU evolution between molluscan taxa, and its potential impact of phylogenetic reconstruction.

Chapter 3 utilizes LSU sequence to investigate the relationships among lophotrochozoan phyla. LSU sequence is found to improve resolution of phylum level

relationships from the standpoint that most phyla are recovered as monophyletic. Although bootstrap branch support values are low, this finding is a dramatic advance over analyses of SSU sequence alone, which generally fail to recover the monophyly of phyla such as the Mollusca and Annelida. Results in Chapter 3 also suggest that rotifers and platyhelminthes may have emerged as part of the lophotrochozoan radiation, rather than diverging prior to it.

Chapter 4 of the thesis utilizes Hox gene sequences to explore the phylogenetic affinities of the enigmatic phlyum Bryozoa. The Bryozoa are part of the Lophotrochozoa, as it was originally defined. However, recent analyses of SSU sequences have failed to recover a close relationship between bryozoans and other lophotrochozoans, and have called the phylogenetic position of the Bryozoa into question. Recent identification of Hox genes which appear to be present only in lophotrochozoans presents the possibility that these genes may have utility as synapomorphies for members of the clade (de Rosa et al., 2001). In this chapter evidence is presented for a bryozoan ortholog of one such gene, *Post2*, which is also present in annelids, mollusks, brachiopods, nemerteans, and platyhelminthes. This finding provides strong evidence of a close relationship between the Bryozoa and other lophotrochozoans. The potential utility of Hox genes in elucidating metazoan phylogenetics in discussed further in Halanych and Passamaneck (2001), which is included as an Appendix to this thesis.

Chapter 2

Investigation of Molluscan Phylogeny Using Large-Subunit and Small-Subunit Nuclear rRNA Sequences, and Analysis of Rate Variation Across Lineages.

Abstract

The Mollusca represent one of the most morphologically diverse animal phyla, prompting a variety of hypotheses on relationships between the major lineages within the phylum based upon morphological, developmental, and paleontological data. Analyses of small-ribosomal RNA (SSU rRNA) gene sequence have provided limited resolution of higher-level relationships within the Mollusca. Recent analyses suggest large-subunit (LSU) rRNA gene sequences are useful in resolving deep-level metazoan relationships, particularly when combined with SSU sequence. To this end, LSU (~ 3.5kb in length) and SSU (~ 2kb) sequences were collected for 33 taxa representing the major lineages within the Mollusca to improve resolution of intraphyletic relationships. In contrast to phylogenetic analyses base on SSU, the Polyplacophora, Gastropoda, and Cephalopoda were each recovered as monophyletic clades with the LSU + SSU dataset. Analyses of LSU sequences strongly contradict the widely accepted Diasoma hypotheses that bivalves and scaphopods are closely related to one another. The data are consistent with recent morphological analyses suggesting scaphopods are more closely related to gastropods and cephalopods than to bivalves. While the Bivalvia were not recovered as monophyletic clade in analyses of the SSU, LSU, or LSU + SSU, the Shimodaira-Hasegawa test showed that likelihood scores for these results did not differ significantly from topologies where the Bivalvia were monophyletic. Although the LSU and combined LSU + SSU datasets appear to hold potential for resolving branching order within the recognized molluscan classes, low bootstrap support was found for relationships between the major lineages within the Mollusca. LSU + SSU sequences also showed significant levels of rate heterogeneity between molluscan lineages. The dataset also presents the first published DNA sequences from a neomeniomorph

aplacophoran, a group considered critical to our understanding of the origin and early radiation of the Mollusca.

Introduction

Recent phylogenetic research on major metazoan lineages has relied heavily on the nuclear small subunit ribosomal rRNA gene (SSU rRNA or 18S), and prompted reevaluation of traditional theories of animal evolution (e.g. Halanych et al., 1995; Agiunaldo et al., 1997; Balavoine and Adoutte, 1998). Although rate variation between sites within SSU rRNA has made the gene useful for resolving relationships between organisms with varying degrees of relatedness, SSU rRNA alone has not been sufficient to resolve some higher-level relationships among metazoans. For example, major relationships within the Mollusca have proven difficult to resolve with SSU rRNA gene data (Winnepenninckx et al., 1996; Steiner and Hammer, 2000). Winnepenninckx et al., (1996) suggested two hypotheses to account for this lack of resolution. Rates of evolution within the gene may be inappropriate for the relationships being investigated, because changes accumulated during divergence of the molluscan classes have been subsequently masked by multiple substitutions. Alternatively, the Mollusca may have diversified rapidly, not allowing sufficient changes in SSU to permit accurate reconstruction of major relationships.

Simulations by Halanych (1998) have suggested that in such cases where SSU rRNA alone is inadequate to resolve relationships, additional sequence data with similar properties may provide greater signal and thus greater resolving power. The large-subunit (LSU) rRNA gene is linked to the SSU gene in a tandem repeat, having a shared evolutionary history. Several recent studies (Medina et al., 2001; Winchell et al., 2002; Mallatt and Winchell, 2002) have investigated the utility of LSU rRNA gene sequence for resolving higher-level relationships within the Metazoa. Each of these studies has

shown that combined datasets of LSU and SSU may provide greater resolution of higher-level relationships among metazoans than is achieved by analysis of SSU sequences alone. The present study investigates the ability of a combined LSU + SSU dataset to provide information regarding class level relationships within the Mollusca not available from SSU sequence alone.

The Mollusca represent one of the most diverse metazoan phyla both in terms of species number as well as in range of body plans. The diversity of the phylum is represented by seven or eight extant clades, commonly recognized as "classes". The Neomeniomorpha and Chaetodermomorpha (often referred to collectively as the Aplacophora), along with the Polyplacophora, are believed to be basally divergent lineages of the Mollusca (Wingstrand, 1985; Salvini-Plawen and Steiner, 1996). Together the three groups are referred to as the Aculifera (Scheltema, 1993). The Conchifera, comprised of the Monoplacophora, Bivalvia, Scaphopoda, Gastropoda, and Cephalopoda, appear to have arisen from a univalved common ancestor (Wingstrand, 1985).

Although the Aculifera are widely agreed to have diverged prior to the diversification of the Conchifera, relationships between the basal molluscs have been variously interpreted. Based upon morphological data, the Chaetodermomorpha (=Caudofoveata) have been described as the earliest diverging lineage within the Mollusca (Salvini-Plawen, 1972; 1980; 1985) (Figure 3A). Cladistic analyses of morphological datasets have evidenced the Neomeniomorpha (=Solenogastres) as the most basal of extant lineages (Salvini-Plawen and Steiner, 1996; Haszprunar, 2000) (Figure 3B). Under either scenario the Aplacophora and Aculifera are viewed as paraphyletic grades, with the Polyplacophora branching as the sister group to the Conchifera to form the Testaria

(Salvini-Plawen, 1972; 1980). Alternative interpretations of morphological and developmental characters have maintained the monophyly of the Aculifera, with the Neomeniomorpha and Chaetodermomorpha as members of a monophyletic Aplacophora forming the sister group to the Polyplacophora (Scheltema, 1993; 1996; Ivanov, 1996) (Figure 3C).

The Conchifera has been divided into two major clades, the Diasoma containing the Bivalvia and Scaphopoda, and the Cyrtosoma (sensu lato) including the Monoplacophora, Gastropoda, and Cephalopoda (Figure 3D). This widely accepted view (e.g. Brusca and Brusca, 1992; Meglitsch and Schram, 1991) is based primarily on paleontological evidence (Runnegar and Pojeta, 1974). The term Cyrtosoma is used herein to refer only to the Gastropoda and Cephalopoda, due to the likely paraphyly of the Monoplacophora (sensu Wingstrand, 1985). The Diasoma hypothesis, based upon inferred common origins of bivalves and scaphopods has come into question. Waller (1998) has proposed close relationship between the Scaphopoda and Cephalopoda based upon inferred developmental commonalities (Figure 3E). A cladistic analysis by Haszprunar (2000) also contradicts the Diasoma hypothesis, finding the Scaphopoda to be the sister group to the Cyrtosoma (Figure 3F).

To gain further understanding of molluscan diversification, we have sequenced LSU and SSU genes for all extant major lineages of the Mollusca, except monoplacophorans. Herein we evaluate the phylogenetic signal present in these rRNA genes, and their utility in resolving higher level molluscan relationships. Analyses found short internal branch lengths and variability in branching order among the major molluscan lineages. High levels of rate heterogeneity were also found between taxa sampled. However,

reconstructions grouping scaphopods with cephalopods were found to have likelihood scores significantly better than those for reconstructions constrained to fit the Diasoma + Cyrtosoma hypothesis of conchiferan evolution.

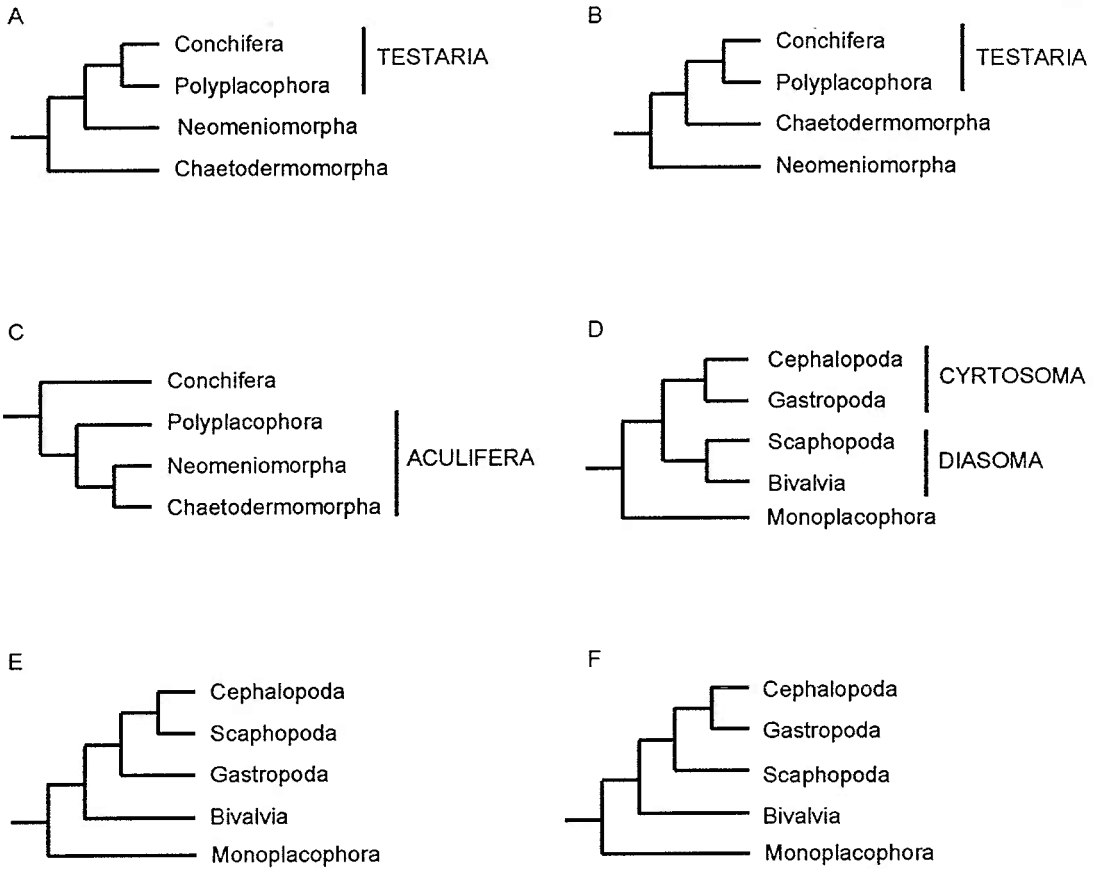

Figure 3: Hypothesis of molluscan class relationships. (A)-(C) Hypotheses of basal molluscan relationships. (A) Basal position of Chaetodermomorpha (Salvini-Plawen, 1972, 1980, 1985); (B) Basal position of Neomeniomorpha (Salvini-Plawen and Steiner, 1996; Haszprunar, 2000); (C) Aculifera hypothesis (Scheltema, 1993, 1996; Ivanov, 1996). (D)-(F) Hypotheses of Conchiferan relationships. (D) Diasoma and Cyrtosoma hypothsis (Runnegar and Pojeta, 1974); (E) Scaphopoda as sistergroup of Cephalopoda (Waller, 1998); (F) Scaphopoda as sistergroup of Cephalopoda + Gastropoda (Haszprunar, 2000).

Materials and Methods

Taxon sampling

Molluscan taxa were chosen from available material to provide the broadest representation of extant lineages. Genomic DNA was isolated from 32 mollusk and 1 outgroup taxa (Table 1) using the DNeasy Tissue Kit (Qiagen), with an additional two sequences obtained from GenBank. Monica Medina kindly provided tissue and LSU rDNA sequence for *Dialula* sp. Akiko Okusu kindly provided samples of *Cryptoplax japonica*, *Dentalium octangulatum*, *Ischnochiton comptus*, and *Nordotis discus*. Janet Voight kindly provided samples of *Arboliopsis* sp., *Benthoctopus yaquinae*, *Graneledone pacifica*, *Histioteuthis* sp., and *Vampyroteuthis infernalis* from the collection of the Field Museum of Natural History. DNA extractions of molluscan samples were taken from mantle or muscle tissue, with the exception of *Chaetoderma* sp. and *Helicoradomenia* sp. where, due to size, whole animals were used. DNA extraction for the outgroup taxon *Cerebratulus lacteus* was taken from sperm. Outgroups were chosen based on knowledge of lophotrochozoan phylogeny (e.g. de Rosa et al., 1999; Giribet et al., 2001; Peterson and Eernisse, 2001) and the presence of low nucleotide substituion rates.

SSU sequence for *Crassostrea gigas* available from GenBank was combined with LSU sequence from *C. virginica* collected for this study. A 381 nucleotide fragment of *C. virginica* SSU (accession number L78851) was 98% similar to that of *C. gigas*, suggesting minimal difference in the complete sequence of the gene from the two species.

Table 1: Taxa sampled for SSU and LSU rDNA sequences

Species	Collection location	Accession numbers LSU	SSU
Mollusca			
Aplacophora			
Helicoradomenia acredema	18°N - East Pacific Rise	AY145409[a]	AY145377[a]
Chaetoderma sp.	Tjärnö, Sweden	AY145397[a]	AY145369[a]
Bivalvia			
Arctica islandica	Maine	AY145390[a]	AIU93555
Argopecten irradians	Cape Cod, MA	AY145391[a]	L11265
Crassostrea virginica	North Falmouth, MA	AY145400[a]	AB064942
Geukensia demissa	North Falmouth, MA	AY145405[a]	L33450
Nuculuna pernula	Tjärnö MBL, Sweden	AY145419[a]	AY145385[a]
Phaxas pellucidus	Tjärnö MBL, Sweden	AY145420[a]	AY145386[a]
Placopecten magellanicus	?	AF342798	X53899
Solemya velum	Cape Cod, MA	AY145421[a]	AF120524
Yoldia limulata	Cape Cod, MA	AY145424[a]	AF120528
Cephalopoda			
Arbaliopsis sp.	FMNH 962-69[b]	AY145389[a]	AY145364[a]
Benthoctopus yaquinae	FMNH 278119[b]	AY145393[a], AY145394[a]	AY145366[a]
Graneledone pacifica	FMNH 278306[b]	AY145407[a]	AY145376[a]
Histioteuthis sp.	FMNH 962-69[b]	AY145410[a]	AY145378[a]
Loligo paeli	Woods Hole, MA	AY145415[a], AY145416[a]	AY145383[a]
Nautilus pompilius	MBL, Woods Hole, USA	AY145417[a]	AY145384[a]
Vampyroteuthis infernalis	FMNH 286569[b]	AY145422[a], AY145423[a]	AY145387[a]
Gastropoda			
Arion silvaticus	Sandwich, MA	AY145392[a]	AY145365[a]
Boonea seminuda	Woods Hole, MA	AY145395[a]	AY145367[a]
Deroceras reticulatum	Connecticut	AY145404[a]	AY145373[a]
Diaulula sandiegensis	California	AY144352[a]	AY145374[a]
Gibbula magnus	Vigo Harbor, Spain	AY145406[a]	AY145375[a]
Haminoea solitaria	West Falmouth, MA	AY145408[a]	AF249221
Ilyanassa obsoleta	North Falmouth, MA	AY145411[a]	AY145379[a]
Lepetodrilus elevatus	9°N - East Pacific Rise	AY145413[a]	AY145381[a]
Nordotis discus	Japan	AY145418[a]	AF082177
Polyplacophora			
Chaetopleura apiculata	North Falmouth, MA	AY145398[a]	AY145370[a]
Cryptoplax japonica	Japan	AY145402[a]	AY145371[a]
Ischnochiton comptus	Japan	AY145412[a]	AY145380[a]
Leptochiton acellus	Kristineberg MRS, Sweden	AY145414[a]	AY145382[a]
Scaphopoda			
Antalis entalis	Tjärnö MBL, Sweden	AY145388[a]	AY145363[a]
Dentalium octangulatum	Japan	AY145403[a]	AY145372[a]

Nemertea			
Cerebratulus lacteus	Woods Hole, MA	AY145396[a]	AY145368[a]
Brachiopoda			
Terebratalia transversa	?	U12650	AF342802

[a] Sequences collected for this study
[b] Voucher numbers of Field Museum of Natural History samples provided by Janet Voight.

Data Collection

All oligonucleotide primers used in this study are listed in Table 2. LSU fragments were amplified using the primers F63.2 and R3264.2 and SSU fragments were amplified using the primers 18e and 18p. Molluscan specific primers were designed to avoid contamination of extraneous genomic DNA in *Helicoradomenia* sp. and *Chaetoderma* sp. extractions. For these species, the SSU region was amplified as two overlapping fragments, using the primer pairs 18e and Mollusc18R1, and Mollusc18F1 and 18p. LSU was amplified in these species using F63.2 and Mollusc28R2, which amplified all but ~400 bases at the 3' end of the gene.

Both genes were isolated using a long PCR protocol to facilitate amplification of nearly complete gene fragments. PCR reactions contained 15µl 3.3x rTth buffer, 2.5µl 10 µM primer, 5µl 2mM dNTPS, 0.4 µl rTth (PE Applied Biosystems), 1µl Vent polymerase (New England BioLabs) (diluted 1:100 in a buffer composed of 50% glycerol, 20mM HEPES, 10mM KCL, 1mM DTT, 0.1mM Na_2EDTA, 0.0025% Tween-20, and 0.0025% NP-40), with genomic DNA and water to a final volume of 45µl. Following a 5 minute denaturation, 5µl of 25mM $Mg(OAc)_2$ was added to each reaction. PCR involved 30 cycles of denaturation at 94°C for 30 sec, annealing at 45-55°C for 1 min, and extension at 65°C for 12 min LSU or 8 min for SSU. A final extension was carried out at 72°C for 10 min. PCR products were cleaned with QIAquick PCR Purification Kit (Qiagen) and incubated at 70°C for 10 minutes in the presence of *Taq* polymerase (Promega) and 0.4mM dATP to create adenine overhangs. PCR fragments were cleaned a second time with the QIAquick PCR Purification Kit and cloned using the pGEM-T Vector System (Promega).

Table 2: Primers used for PCR amplification and sequencing

Primer	Reference	Sequence 5' > 3'
PCR amplification		
LSU		
F63.2	Medina (personal communication)	ACCCGCTGAAYTTAAGCATAT
R3264.2	Medina (personal communication)	TWCYRMCTTAGAGGCGTTCAG
Mollusc28R2	Present study	GCGAGGTTTCCGTCCTCGC
SSU		
18e	Hillis & Dixon, 1991	CTGGTTGATCCTGCCAGT
18p	Halanych et al., 1998	TAATGATCCTTCCGCAGGTTCACCT
Mollusc18F1	Present study	TTTAGCCACRCGAGAWTGA
Mollusc18R1	Present study	GTTATTGCTCAWTCTCGYG
Sequencing		
LSU		
28ee	Hillis & Dixon, 1991	ATCCGCTAAGGAGTGTGTAACAACTCACC
28ff	Hillis & Dixon, 1991	GGTGAGTTGTTACACACTCCTTAGCGG
28gg	Hillis & Dixon, 1991	GACGAGGCATTTGGCTACCTTAAG
28nn	Present study	GGAACCAGCTACTAGATGGTTCG
28F1-2	Present study	GYWGGGACCCGAAAGATGGTGAAC
28F2-2	Present study	GCAGAACTGGCGCTGAGGGATGAAC
28F4	Present study	CGCAGCAGGTCTCCAAGGTGMACAGCCTC
28F5	Present study	CAAGTACCGTGAGGGAAAGTTG
28R2	Present study	GAGGCTGTKCACCTTGGAGACCTGCTGCG
28V	Hillis & Dixon, 1991	AAGGTAGCCAAATGYCTCGTCATC
28X	Hillis & Dixon, 1991	GTGAATTCTGCTTCACAATGATAGGAAGAGCC
28 MT4.1	Present study	TCCTTGGTCCGTGTTTCAAGACG
28R3	Present study	GATGACGAGGCATTTGGCTACC
28R4	Present study	GAGCCAATCCTTATCCCAAAGTTACGGATC
SSU		
18h	Hillis & Dixon, 1991	AGGGTTCGATTCCGGAGAGGGAGC
18L	Halanych et al., 1998	GAATTACCGCGGCTGCTGGCACC
18M	Halanych et al., 1998	GAACCCAAAGACTTTGGTTTC
18M0	Halanych et al., 1998	GAAACCAAAGTCTTTGGGTTC
18O	Halanych et al., 1998	GGAATRATGGAATAGGACC
18Q	Halanych et al., 1998	TGTCTGGTTAATTCCGATAAC
18Q0	Halanych et al., 1998	GTTATCGGAATTAACCAGACA
18R	Present study	GTCCCCTTCCGTCAATTYCTTTAAG
18F3	Present study	CGAAGACGATCAGATACCG
Vector		
M13f		GTAAAACGACGGCCAGT
M13r		CAGGAAACAGCTATGAC

Sequencing was conducted with BigDye Terminator v2.0 Sequencing Reaction chemistry (Applied Biosystems), using the primers listed in Table 2. Sequencing reactions were purified using Centri-Sep (Princeton Separations) purification columns. Sequencing reactions were analyzed using an ABI 377 automated sequencer (Applied Biosystems) using 48cm plates and 4.75% Long Ranger (FML BioProducts) polyacrylamide gels. For each taxon, each gene was sequenced in both directions.

Phylogenetic analyses

Sequences were aligned by the profile alignment function of ClustalW (Thompson et al., 1994), using previously aligned sequences from the Ribosomal Database Project II (Maidak et al., 2001) as guides. Alignments were checked manually with MacClade 4 (Maddison and Maddison, 2000), and regions that could not be unambiguously aligned were excluded.

In order to better understand the relative contribution of each rDNA gene, analyses were carried out on SSU data alone, the LSU data alone, and the combined LSU + SSU data. To evaluate consistency in results between phylogenetic reconstruction methods, minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) analyses were conducted using PAUP* version 4.0 b10 (Swofford, 2002). Appropriate models for maximum likelihood analyses were determined using the hierarchical likelihood ratio test (LRT) implemented in Modeltest (Posada and Crandall, 1998).

Support in the datasets for previously published hypotheses of relationships between molluscan clades was evaluated by explicit hypothesis testing. Unresolved trees conforming to *a priori* hypotheses were used to constrain maximum likelihood heuristic searches with TBR. Resultant trees were compared with unconstrained maximum

likelihood trees using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 2000) implemented in PAUP*4.0b10.

Results

Alignment and Base Composition

Total lengths of the alignments, number of unambiguously aligned characters included in analyses, number of variable characters, and number of parsimony informative characters for the SSU, LSU and LSU + SSU data are shown in Table 3.

Stationarity of base frequencies is an assumption of parsimony and likelihood based methods of phylogenetic reconstruction (Swofford et al., 1996). Therefore, the relative nucleotide composition of the datasets was evaluated using the "basefreqs" command in PAUP. The LSU + SSU dataset shows high proportions of A and G among most of the sampled taxa (Table 4). This pattern is reflected in the dataset for each gene when analyzed separately (not shown). Five of the cephalopods sampled (*Arboliopsis*, *Benthoctopus*, *Graneledone*, *Loligo*, and *Vampyroteuthis*) differed from this pattern, having high levels of G and low levels of T. Inclusion of these taxa results in significant ($P << 0.0001$) rejection of c^2 test of homogeneity of base frequencies across taxa, as implemented in PAUP*4.0b10. This result is exhibited in both the SSU and LSU datasets, suggesting the variation in nucleotide usage is lineage specific, rather than gene specific. Such a pattern might be expected in genes which are linked and share evolutionary history. However, Winchell et al. (2002) found LSU sequences displayed differences in base proportions across deuterostome lineages, while SSU sequences did not. Exclusion of the nucleotide biased cephalopods from the datasets results in acceptance of stationarity of base frequencies under the c^2 test ($P = 0.7704$) in the LSU + SSU dataset (Table 4), as well as in the SSU and LSU datasets individually (not shown).

Table 3: Total, Included, Variable, and Parsimony Informative characters for alignmentsof SSU, LSU, and combined LSU + SSU datasets

	Total	Included	Variable	Informative
SSU	2605	1603	651	399
LSU	4076	2615	1054	517
LSU + SSU	6681	4218	1705	916

Table 4: Average base frequencies in the combined LSU + SSU dataset with c^2 tests of stationarity for complete and trimmed dataset

Data set with all taxa

	A	C	G	T	# Sites
Mean	0.2606	0.2276	0.2899	0.2219	3513

c^2 = 350.866 (d.f.=102), P = 0.00000000

Arboliopsis, *Benthoctopus*, *Graneledone*, *Loligo*, and *Vampyroteuthis* alone

	A	C	G	T	# Sites
Mean	0.2329	0.2629	0.3147	0.1896	3627

c^2 = 0.377 (d.f.=12), P = 0.99999995

Data set without *Arboliopsis*, *Benthoctopus*, *Graneledone*, *Loligo*, and *Vampyroteuthis*

	A	C	G	T	# Sites
Mean	0.2729	0.2223	0.2808	0.2240	3629

c^2 = 76.977 (d.f.=87), P = 0.77037135

Datasets including sequences for all cephalopods sampled were initially analyzed with minimum evolution (ME) (Figure 4) using LogDet-Paralinear distances (Lake, 1994; Lockhart et al., 1994), which is less biased by variability in base frequencies across taxa than are parsimony (Lockhart et al., 1994) and likelihood (Swofford et al., 1996) based methods. Monophyly of the Cephalopoda was strongly supported (bootstrap support = 100%) by ME analysis of the LSU + SSU dataset (Figure 4), as well by the individual SSU and LSU datasets (not shown). *Nautilus* and *Histioteuthis*, having base frequencies consistent with other mollusks sampled, were retained as representatives of the Cephalopoda for parsimony and likelihood analyses. Therefore, subsequent discussion will assume that nucleotide biased cephalopod lineages were not included in the analyses unless otherwise stated.

Relative Rates

Variation in relative rates of nucleotide substitution across taxa and its potential impact on phylogenetic reconstructions are well-documented issues with rDNA genes (e.g. Stiller and Hall, 1999; Philippe et al., 2000; Peterson and Eernisse, 2001). To help identify taxa with relatively elevated rates of nucleotide substitution, we conducted relative rates tests of all pairwise comparisons of the ingroup taxa to the reference outgroup taxa using the HYPHY program (Muse and Kosakovsky Pond, 2002) with a Tamura-Nei (1993) model. A Tamura-Nei model was the best fit to the data as determined in Modeltest. The analysis found for 432 of the 528 (82%) ingroup comparisons showed significant rate variation ($P < 0.05$; including all cephalopod taxa) for at least one of the two outgroups. Additionally, 70% (371/528) of the comparisons

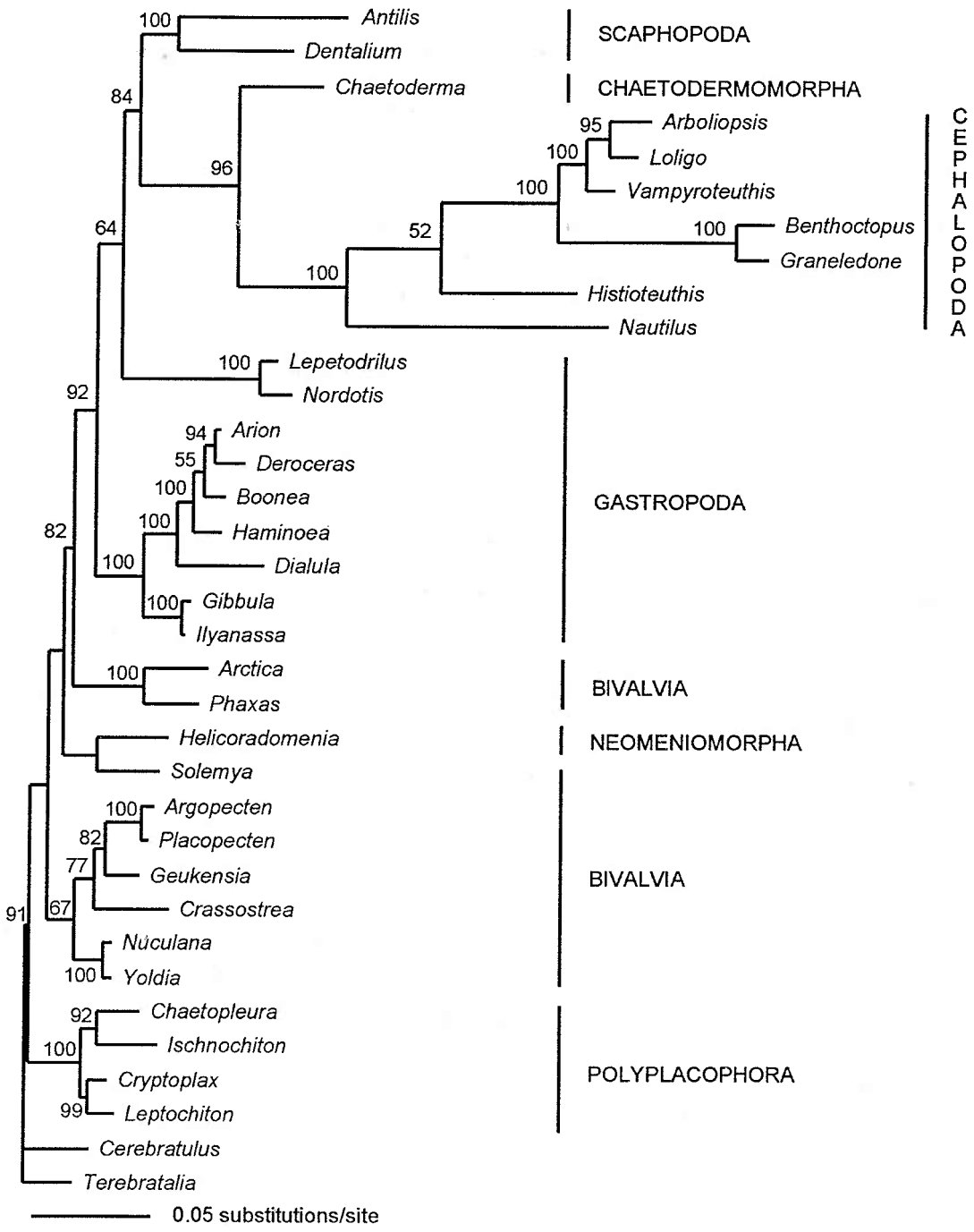

Figure 4: ME tree of the combined LSU+SSU dataset, including all seven cephalopods, calculated using LogDet-Paralinear distances. Bootstrap values from 1000 replicates are shown for nodes with support values of <50%.

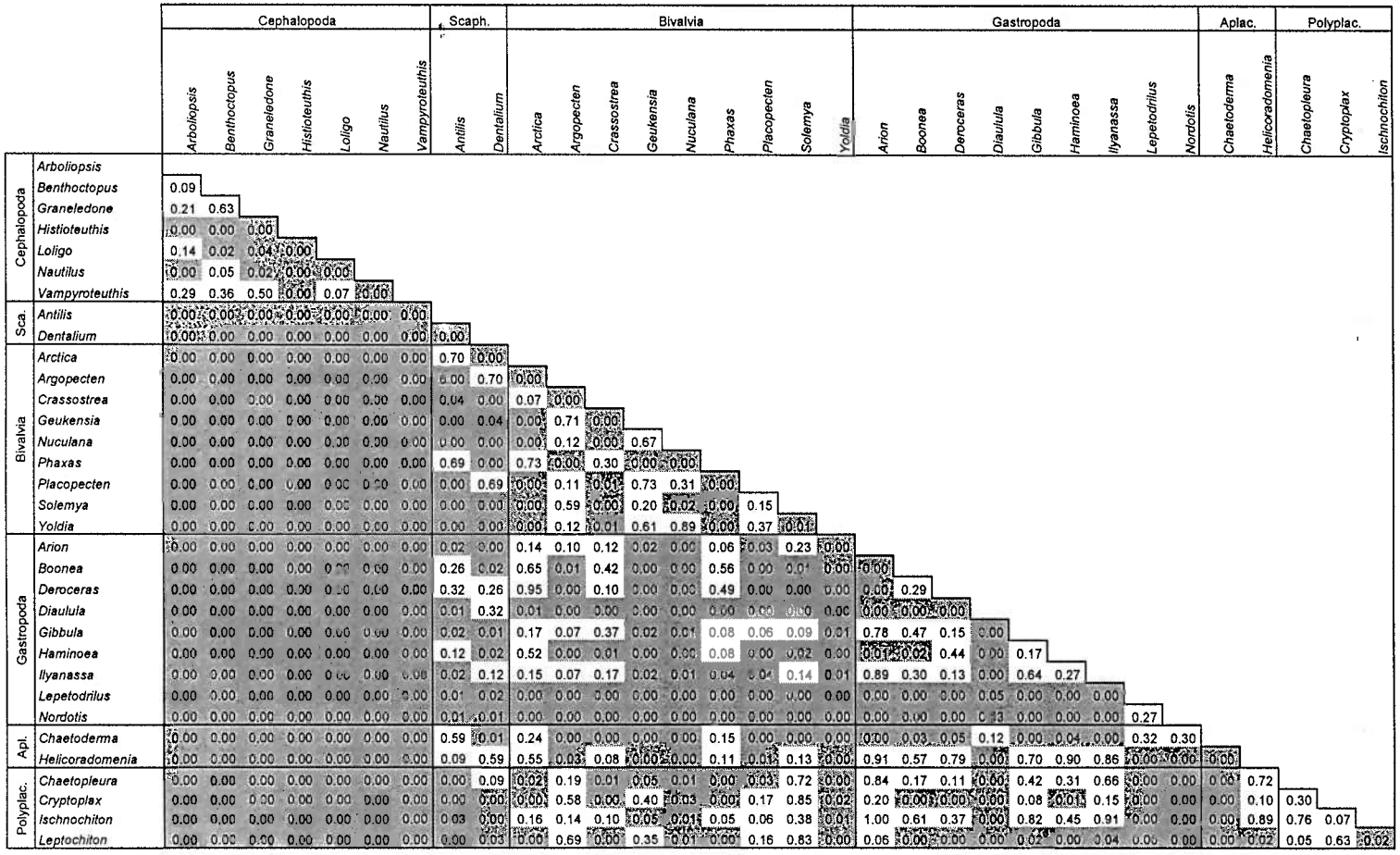

Figure 5: Relative rates for pairwise comparisons of combined LSU + SSU sequences, using Terebratalia as the outgroup. Number in each cell is the p-value for a given comparison. Shaded cells denote taxon pairs with a significant difference in substitution rates ($P < 0.05$).

showed significant variation for both outgroups. As an example of the results, Figure 5 shows the relative rates test result when the brachiopod, *Terebratalia*, was used as outgroup. Clearly, rate variation across taxa is a serious concern for these data. However, exclusion of all the taxa that showed significantly elevated rates of nucleotide substitution would eliminate representation from several mollusk clades (e.g. cephalopods, chaetoderms, and scaphopods), rendering the dataset useless for trying to gain a deeper understanding about mollusk phylogeny.

Phylogenetic Reconstruction

The reconstructed topologies for the SSU dataset alone are shown in Figues 6 and 7, the LSU dataset alone in Figures 8 and 9, and the LSU + SSU dataset in Figures 10 and 11. For each dataset, MP (A) and ML (B) are presented with the parameter and search details in the figure legends. Because available evidence suggests the phylogenetic signal in the SSU alone is limited for mollusks (e.g., Winnepenninckx et al., 1996; Steiner and Hammer, 2000), and in an effort to maximize the amount of available data, the discussion herein will emphasize the LSU + SSU data.

Several features are immediately obvious on inspection of the resultant trees: internal branch-lengths are short, bootstrap support tends to be higher near the tips of the tree, the exact topology is dependent upon the reconstruction method, and variation in nucleotide substitution rates is notable. Despite these pitfalls, the data still represent the most comprehensive molecular perspective of mollusk phylogeny to date and provide insight on several long-standing hypotheses about molluscan evolution.

Consistent with expectations, many of the traditionally recognized molluscan "classes" were found to be monophyletic in the best trees recovered under all or most

reconstruction conditions (e.g. Gastropoda, Cephalopods, Polyplacophora, and Scaphopods; admittedly the taxon sampling for some of these groups is limited). The representatives of the Cephalopoda and Scaphopoda were found to cluster together in all analyses, although this clade often included *Chaetoderma* branching with the Cephalopoda (Figures 6, 7, 10, and 11). The Aculifera , Conchifera, and Bivalvia were not recovered as monophyletic clades under any analysis. The Polyplacophora usually clustered with bivalves (e.g. Figures 8-11) contrary to both the Aculifera and Conchifera hypotheses. In the case of the Bivalvia, *Arctica,* and *Phaxas* consistently branched closest to one another but separate from the other bivalves. Interestingly, *Arctica* and *Phaxas* also have higher rates of nucleotide substitution than other bivalves.

To assess the impact of the relatively quickly evolving cephalopod sequences, ML analysis of the LSU + SSU dataset was conducted with *Nautilus* and *Histioteuthis* excluded. Branching order among the Polyplacophora + Bivalvia + Gastropoda was not affected, however representatives of the Scaphopoda, Neomeniomorpha, and Chaetodermomorpha branch most closely with outgroup taxa (not shown). Exclusion of outgroup taxa from the LSU + SSU dataset produced similar topologies, with the exception that *Helicoradomenia* branches with the Polyplacophora (not shown).

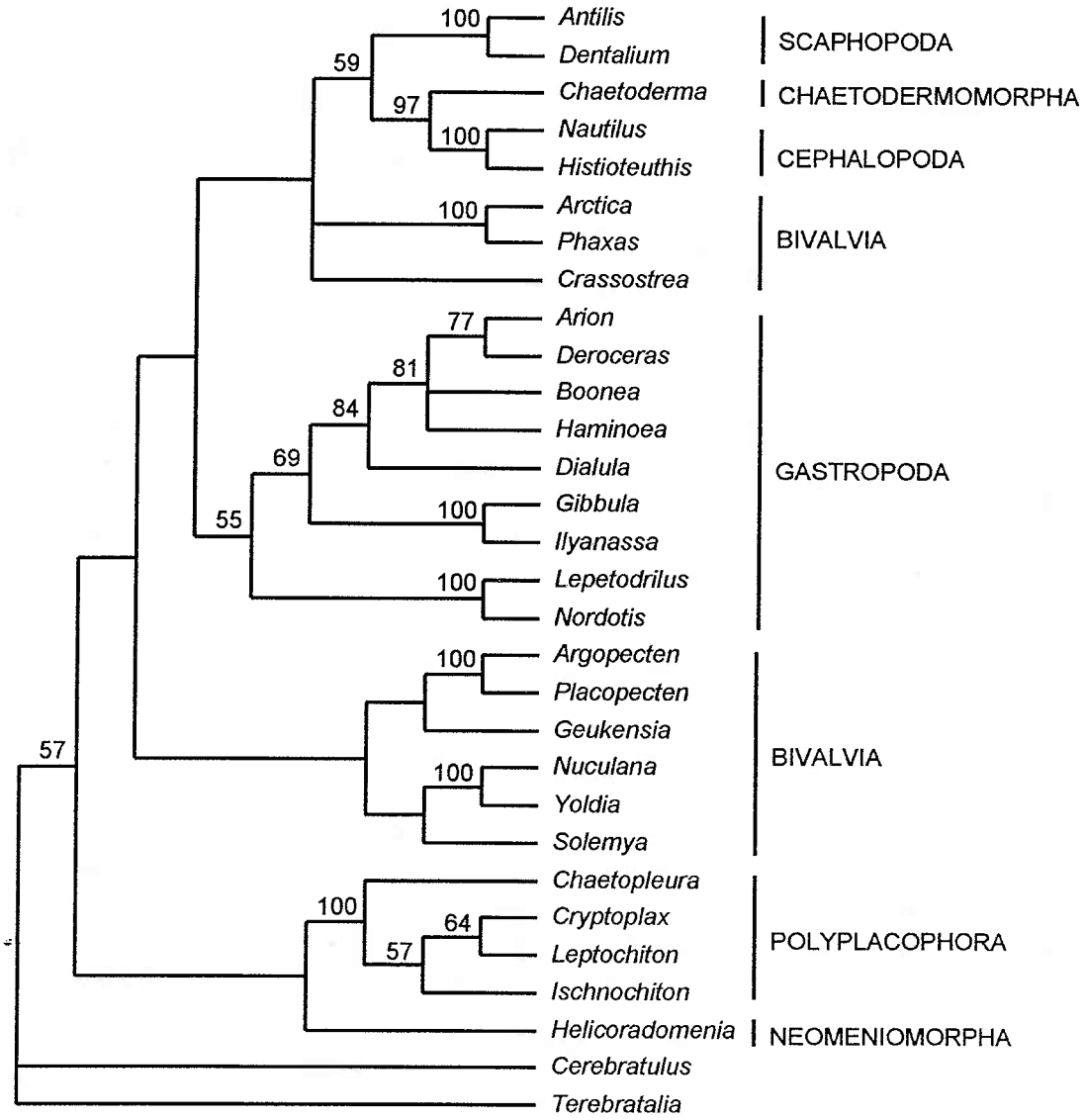

Figure 6: Maximum parsimony (MP) analysis of the SSU dataset, with *Nautilus* and *Histioteuthis* as representatives of the Cephalopoda. MP analysis using heuristic search with TBR of 1000 sequence additions replicates. Majority rule consensus of 9 best trees found. Score = 1578. Bootstrap values are shown above nodes where support was >50% from 1000 replicates.

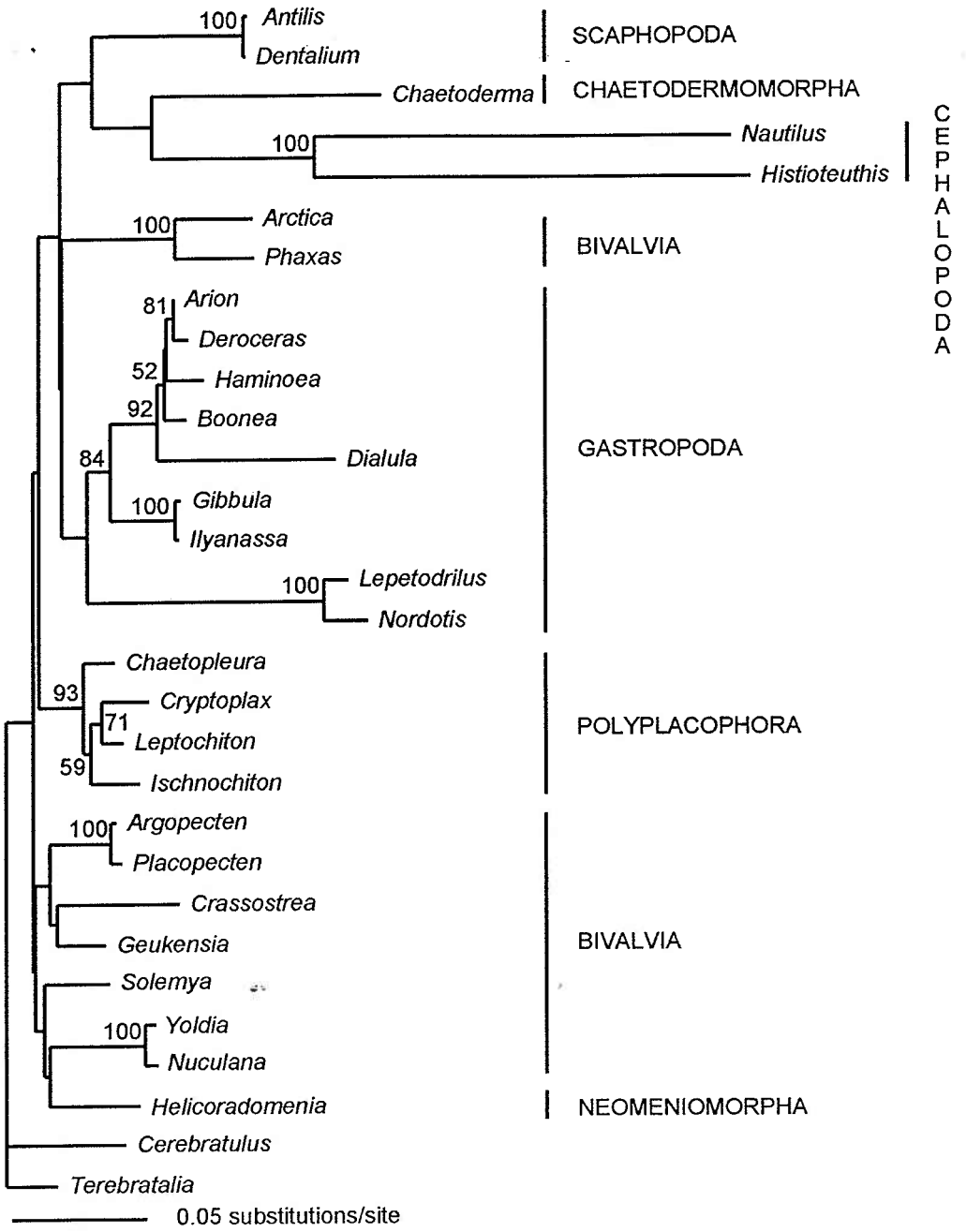

Figure 7: Maximum likelihood (ML) analysis of the SSU dataset, with *Nautilus* and *Histioteuthis* as representatives of the Cephalopoda. ML analysis using heuristic search with TBR of 100 replicates. Analysis performed under the Tamura-Nei (TrN) model with proportion of invariant sites (Pinv = 0.3487) and gamma distribution of among site rate variation (G = 0.5887) estimated from the data. Score –lnL = 9165.6521. Bootstrap percentages based on 100 replicates.

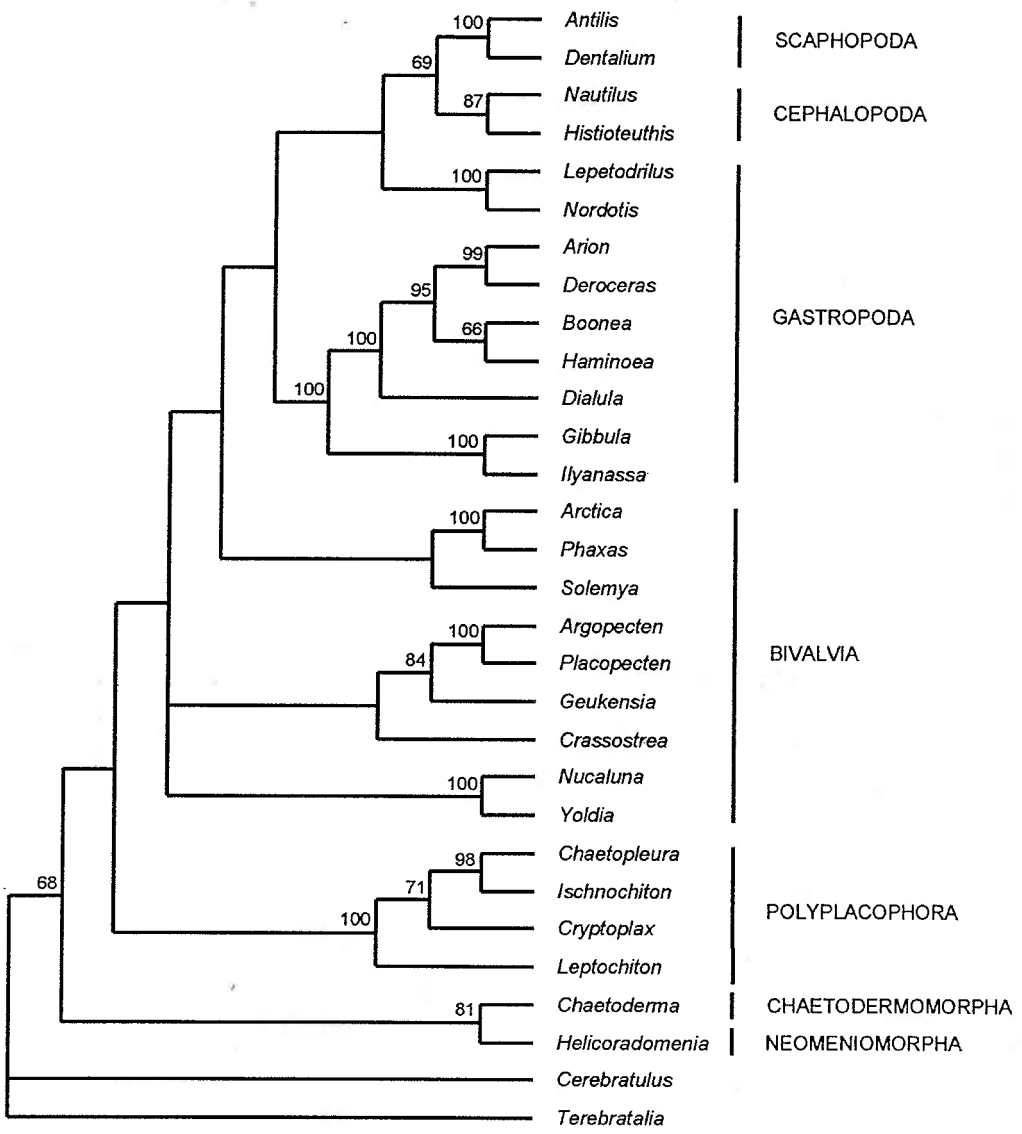

Figure 8: Maximum parsimony (MP) analysis of the LSU dataset, with *Nautilus* and *Histioteuthis* as representatives of the Cephalopoda. MP analysis using heuristic search with TBR of 1000 heuristic sequence additions replicates. Majority rule consensus of 3 best trees found. Score =2570. Bootstrap percentages based on 1000 replicates.

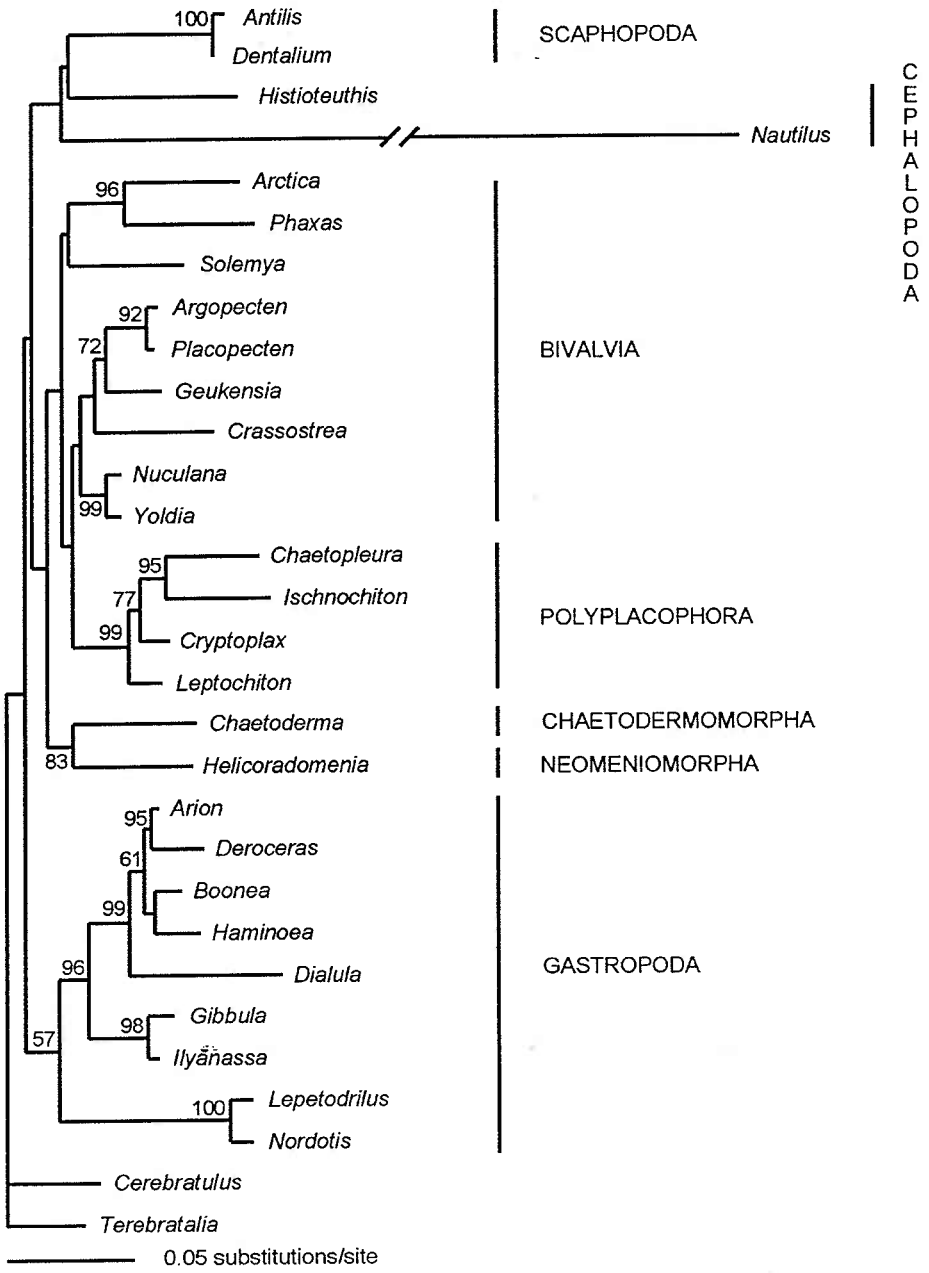

Figure 9: Maximum likelihood (ML) analysis of the LSU dataset, with *Nautilus* and *Histioteuthis* as representatives of the Cephalopoda. ML analysis using heuristic search with TBR of 100 heuristic replicates under the TrN + I + G model. Pinv = 0.3313, G = 0.4520. Score –lnL = 14825.2782. Bootstrap percentages based on 100 replicates.

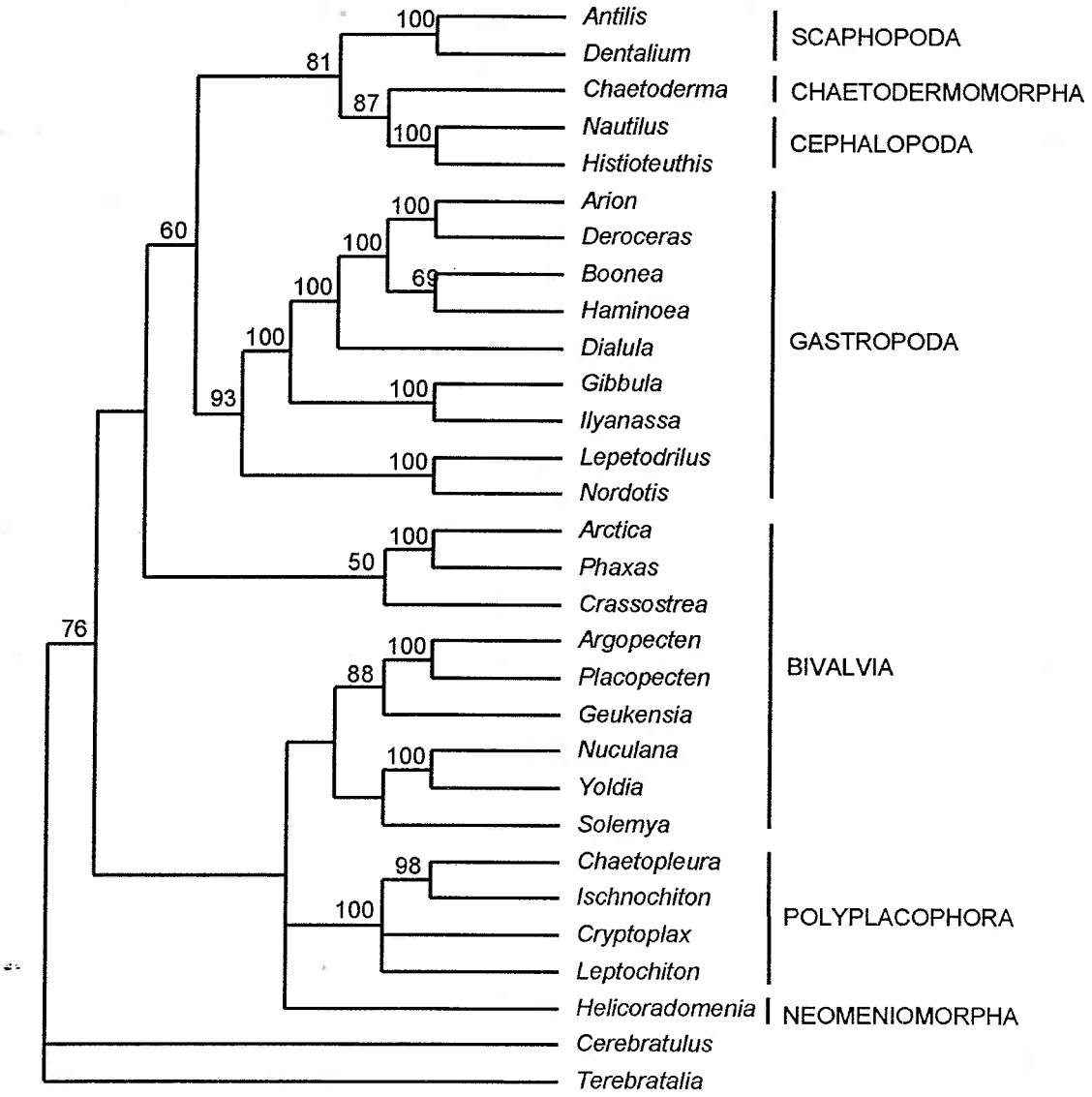

Figure 10: Maximum parsimony (MP) analysis of the combined LSU+SSU dataset, with *Nautilus* and *Histioteuthis* as representatives of the Cephalopoda. MP analysis using heuristic search with TBR of 1000 heuristic sequence additions replicates. Consensus of 2 best trees found. Score = 4181. Bootstrap percentages based on 1000 replicates.

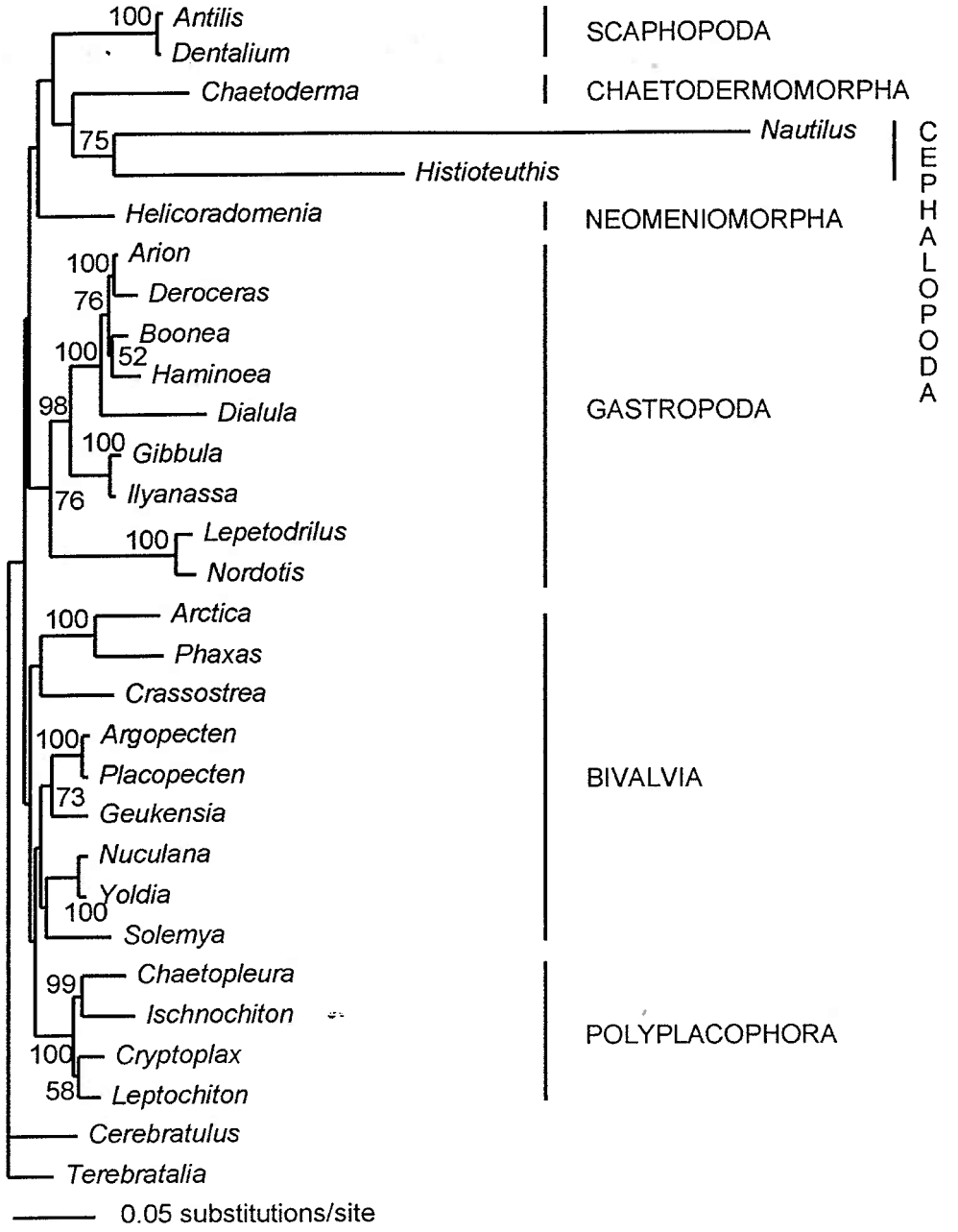

Figure 11: Maximum likelihood (ML) analysis of the combined LSU+SSU dataset, with *Nautilus* and *Histioteuthis* as representatives of the Cephalopoda. ML analysis using heuristic search with TBR of 100 heuristic replicates under the TrN + I + G model. Pinv = 0.3373, G = 0.4956. Score -lnL = 24200.9917. Bootstrap percentages based on 100 replicates.

Hypothesis testing

Even when internal branch lengths are short and bootstrap support for nodes is low, sufficient phylogenetic signal may still exist in the dataset to allow competing hypotheses to be evaluated. To this end, the Shimodaira-Hasegawa (SH) test was used to assess support for alternative hypotheses of molluscan evolution. SH tests did not reject any alternative *a priori* hypotheses regarding the basal radiation of the Mollusca (Table 5). Within the Conchifera, the hypothesis of the Diasoma (Bivalvia + Scaphopoda) (Runnegar and Pojeta, 1974) is rejected by the LSU data. The hypothesis of the Diasoma and Cyrtosoma (Gastropoda + Cephalopoda) as sister groups is also rejected by analyses of both the LSU and LSU + SSU datasets. The optimal LSU tree also differs significantly from one where Bivalvia branches basally among the Conchifera, with Scaphopoda more closely related to the Cyrtosoma. The LSU + SSU ML tree also differed significantly from a tree constrained to fit the tradition division of the Conchifera into Diasoma and Cyrtosoma clades. Trees constrained such that the Bivalvia formed a monophyletic clade did not differ significantly from unconstrained results for the three datasets (Table 5).

The SH test was used to evaluate consistency between trees recovered for the three SSU, LSU, and LSU + SSU datasets under ML analyses. Likelihood scores for the LSU and SSU ML trees differed significantly, when tested under the respective datasets and associated models (Table 5). However, LSU + SSU likelihood scores did not differ significantly from those of either the SSU or LSU ML trees.

Table 5: Shimodaira-Hasegawa test of support for alternative a priori hypotheses, P values

	SSU	LSU	LSU+SSU
Molluscan relationships:			
Molluscan monophyly	1.000	1.000	1.000
Basal molluscan relationships:			
Aculifera (Aplacophora + Polyplacophora)	0.107	0.112	0.190
Testaria (Chaetodermomorpha basal)	0.088	0.090	0.180
Testaria (Neomeniomorpha basal)	0.072	0.069	0.151
Conchiferan relationships			
Bivalve monophyly	0.254	0.251	0.366
Diasoma (Bivalvia + Scaphopoda)	0.084	**0.021***	0.109
(Bivalvia + Scaphopoda) + (Gastropoda + Cephalopoda)	0.090	**0.009***	**0.047***
(((Gastopoda + Cephalopoda) + Scaphopoda) + Bivalvia)	0.107	**0.029***	0.096
(((Scaphopoda+ Cephalopoda) + Gastropoda) + Bivalvia)	0.108	0.073	0.188
ML unconstrained analyses			
SSU ML tree	1.000	**0.000***	0.068
LSU ML tree	**0.000***	1.000	0.224
LSU + SSU ML tree	0.291	0.076	1.000

* $P < 0.05$ - Hypotheses in bold are rejected under the given dataset.
Note. – Analyses carried out using the dataset listed at the top of each column, using the appropriate likelihood model as calculated with Modeltest.

Discussion

The LSU + SSU data provided high bootstrap support for some relationships within the major molluscan clades, but showed limited ability to confidently recover relationships between these clades. Recent studies employing LSU + SSU datasets to investigate metazoan phylogenetics (Medina et al., 2001; Winchell et al., 2002; Mallatt and Winchell, 2002), have suggested the utility of LSU, particularly when combined with SSU, in elucidating major events in metazoan diversification. In each of these cases findings from the LSU + SSU data generally agreed with those from SSU alone, with LSU + SSU providing greater bootstrap support. In the case of the Mollusca however, we find the SSU trees to be significantly different from the LSU and LSU + SSU trees (Table 5).

In assessing relationships among the major molluscan groups, we observed a high level of variability in the resultant topologies. Variability in branching order among the major molluscan groups may be a function of 1) high levels of rate heterogeneity between lineages represented in the dataset, and/or 2) a rapid radiation of the major molluscan groups. A majority of the pairwise relationships between LSU + SSU sequences showed significant rate differences regardless of outgroup choice. For example, within the Bivalia, *Arctica* and *Phaxas* display unstable placement in the trees and have substitution rates significantly different from those of other bivalves sampled. Such rate heterogeneity has previously been found for SSU sequences from bivalves (Steiner and Müller, 1996; Steiner and Hammer, 2000) and is suggested to explain problems recovering the monophyly of the Bivalvia. Our findings show significant rate variation across the major molluscan lineages, as well within the recognized classes. The potential

for rate heterogeneity between lineages to produce artifacts is well known, particularly the case of long-branch attraction (Felsenstein, 1978).

Lack of the resolution in the relationships between the major lineages of the Mollusca may also be interpreted as evidence of a rapid radiation. Most of the major clades in the Mollusca first appear in the fossil record during the Cambrian (Runnegar and Pojeta, 1985), which has been viewed as a period of diversification and cladogenesis thoughout the Metazoa (Valentine, 1994). Under such a scenario the amount of change accumulated in rRNA gene sequences may have been insufficient to allow reliable reconstruction of the radiation and/or changes may have accumulated mainly at rapidly evolving sites in the gene and been subsequently masked by additional substitutions.

Rate heterogeneity may be a general characteristic of molluscan genomic evolution, rather than a phenomenon specific to the rRNA genes sequenced here. Studies of mitochondrial gene order show numerous transpositions and inversions of protein coding and tRNA genes between bivalves, gastropods, and cephalopods (Wilding et al., 1999; Kurabayashi and Ueshima, 2000; Tomita et al., 2002). Within mollusks, and within some clades of mollusks, such as gastropods (Kurabayashi and Ueshima, 2000), greater variation in mitochondrial gene rearrangements has been observed than between the polyplacophoran *Katharina tunicata* and the brachiopod *Terebratulina retusa* (Stechmann and Schlegel, 1999). In some cases these rearrangements appear to have occurred between closely related species over relatively short time scales (Rawlings et al., 2001). Rate heterogeneity in gene sequence evolution will need to be a careful consideration for future studies of molecular phylogenetics within the Mollusca.

Despite variability between reconstructions, several relationships between the major lineages of the Mollusca were consistently found in the analyses. A close relationship between the Scaphopoda and Cephalopoda was recovered in nearly all reconstructions, with likelihood scores under the LSU and LSU + SSU datasets being significantly better than those for placing the Diasoma, grouping the Bivalvia + Scaphopoda, as sister group to the Cyrtosoma, containing Gastropoda and Cephalopoda. These findings suggest a reassessment of the view that scaphopods and bivalves are closely related to one another, as in the Diasoma hypothesis (Runnegar and Pojeta, 1974). Although these results may be questioned because of the high substitution rates within the cephalopod sequences sampled, they are supported by recent analyses of molluscan morphological characters. Waller (1998) has suggested the Bivalvia diverged prior to the common ancestor of the Gastropoda, Scaphopoda, and Cephalopoda, with scaphopods and cephalopods being most closely related to one another. Alternatively, cladistic analysis by Haszprunar (2000) also support the monophyly of Gastropoda + Scaphopoda + Cephalopoda, with the scaphopods as sistergroup to the Gastropoda + Cephalopoda.

The polyplacophorans and aplacophorans are widely viewed as being the most basal molluscan lineages, although the relationship between these groups has been variously interpreted (Salvini-Plawen, 1972; 1980; Salvini-Plawen and Steiner, 1996; Scheltema, 1993). In the results presented here, a basal position for the Polyplacophora was recovered only under MP analyses of the SSU and LSU datasets. In ML and MP analyses of the LSU+SSU dataset, and ML analysis of the LSU dataset, reconstructions placed the polyplacophorans close to bivalves. While likelihood scores for ML trees did not differ significantly from those of trees where the Polyplacophora branches basally to

the Conchifera, the results presented here bear further investigation. Corroboration of this relationship with other molecular markers would require a reinterpretation of morphological evolution in the Mollusca (e.g. the homology of sclerites in polyplacophorans and aplacophorans). The close relationship recovered for LSU sequences of *Helicoradomenia* and *Chaetoderma* suggests the monophyly of Aplacophora, though this finding is not recovered with the SSU or LSU + SSU data. The branching of *Chaetoderma* with scaphopods and cephalopods under analyses of the SSU and SSU + LSU datasets deserves further scrutiny given the accelerated rates of evolution in these lineages. Aplacophorans have previously been suggested to be secondarily simplified through a process of progenesis (Scheltema, 1993). Yochelson (1978) likewise suggested aplacophorans to be derived, rather than direct descendents of primitive molluscs.

This study represents the most comprehensive molecular sampling of the Mollusca to date, including taxa from all the major molluscan lineages except the monoplacophorans. Given the short length of deep internal and the instability of nodes connecting the major lineages, it is expected that additional taxon sampling of ribosomal genes will provided limited additional resolution. Investigations of protein coding genes and genomic organization may provide valuable future directions improving our understanding of molluscan relationships.

Chapter 3

Assessing Lophotrochozoan phylogeny with combined LSU and SSU ribosomal RNA gene sequences

Abstract

The clade Lophotrochozoa, which includes mollusks, annelids, brachiopods, flatworms and their allies, encompasses the greatest body plan diversity of the three major bilaterian lineages. Lophotrochozoan interphyletic relationships are not well understood in part because analyses on the topic have been limited to morphology and/or small ribosomal subunit (SSU) data. To further elucidate the clade's phylogenetic history, we have analyzed DNA sequences of the large-subunit ribosomal RNA (LSU) gene from a diversity of lophotrochozoans. Unlike SSU data alone, the LSU and combined LSU + SSU datasets recover the monophyly of most recognized lophotrochozoan phyla, a prerequisite of evaluating interphyletic relationships. The data show Bryozoa diverged prior to the diversification of other lophotrochozoans, suggesting a cryptic early evolution of the lineage leading to bryozoans. Lophophorata, an exclusive Bryozoa/Brachiopoda/Phoronida clade, is significantly rejected as is a Bryozoa/Entoprocta clade. Contrary to previous reports, Platyzoa (including platyhelminthes, rotifers, and acanthocephalans) appears to be derived within lophotrochoazoans rather than a sister group to the Lophotrochozoa. In the LSU and LSU + SSU data, entoprocts and cycliophorans form a clade sister to Platyzoa. The monophyly of taxa possessing "trochophore" larvae was not recovered.

Introduction

The Lophotrochozoa encompasses the greatest body-plan diversity of the three major Bilaterian clades, however, relationships within the clade are poorly resolved hindering our understanding of metazoan evolution. The clade, initially identified with small nuclear ribosomal subunit (SSU) sequences (Halanych et al., 1995), comprises all descendents of the common ancestor of the lophophorates (Brachiopoda, Phoronida and Bryozoa), mollusks and annelids. Subsequent studies (e.g., Mackey et al., 1996; Balavoine, 1997; De Rosa et al., 1999; Mallatt and Winchell, 2002; Peterson and Eernisse, 2001) have supported the clade and included additional protostomes (e.g., platyhelminthes, sipunculans, nemerteans, and entoprocts). The present study aimed to more thoroughly resolve lophotrochozoan phylogeny providing a comparative framework.

Previous studies of lophotrochozoan relationships have relied heavily on SSU data, morphological cladistic analyses, or a combination of the two (e.g., Eernisse, 1997; Zrzavry et al., 1998; Giribet et al., 2000). Unfortunately, SSU data do not even cluster taxa into well-recognized monophyletic units (e.g., Mollusca, Nemertea, Brachiopoda). Utilizing morphological characters to recover relationships between phyla is inherently problematic. Organisms were separated into distinct "phyla" primarily because features grouping organisms together were lacking. More importantly, choice and definition of morphological characters that are applicable across phyla can be subjective (Jenner, 2001). For example, both spiral cleavage pattern and trochophore larvae are still used as important phylogenetic characters, yet they have subjective definitions that group

different taxa. Nonetheless, some progress has been made in understanding lophotrochozoan relationships.

Herein, we build on previous data by examining combined SSU and large nuclear ribosomal subunit (LSU) data to address three hypothesized lophotrochozoan taxa that shape our overall understanding of the group's evolution: Lophophorata, Platyzoa, and Trochozoa. Hyman (1959) grouped the bryozoans, brachiopods, and phoronids together as the "Lophophorata" based on inferred homology of the ciliated feeding structure. Although the monophyly of this group has not been demonstrated and evidence suggests that not all "lophophores" are homologous (Halanych, 1996; Nielsen, 2001), the "Lophophorata" has been perpetuated in invertebrate textbooks and is commonly accepted. Molecular sequences support protostome affinities (Field et al., 1988; Halanych et al., 1995; Schtemann and Schlegel, 1998; de Rosa et al., 1999), but the exact placement of Bryozoa (a.k.a., Ectoprocta) has been contentious. To date, molecular analyses of bryozoan affinities have relied upon SSU sequences, which do not recover bryozoan monophyly and place them as basal members of the Lophotrochozoa (e.g., Halanych et al., 1995; Giribet et al., 2000; Peterson and Eernisse, 2001). Nielsen (1985) has suggested bryozoans to be most closely related to entoprocts, but this has not been evidenced by molecular data.

Platyzoa was originally diagnosed as ciliated non-segmented acoelomates or pseudocoelomates lacking a vascular system (i.e., Platyhelminthes, Rotifera, Acanthocephala, Gastrotricha, and Ganthostomulida; Cavalier-Smith, 1998). Although traditionally viewed as basal lineages within Bilateria, interpretations of platyhelminth

and rotifer cleveage as spiral or "modified spiral" suggest an evolutionary relationship with spiralian lophotrochozoans such as mollusks, annelids, echiurans, sipunculans, and entoprocts (Boyer et al 1998; Gilbert 1989). Recent analyses of SSU sequences and combined SSU + morphological datasets have suggested Platyzoa represents a sister clade to the Lophotrochozoa (Giribet et al., 2000), or a grade which diversified basal to the last common ancestor of the Lophotrochozoa (Peterson and Eernisse, 2001). Our understanding of Platyzoa has been altered by recent analyses that place the acoelomorph playhelminthes outside Platyzoa at the base of Bilateria (Ruiz-Trillo et al., 2002). Although Cycliophora were initially hypothesized to have evolutionary affinities to the Entoprocta (Funch and Kristensen, 1995; 1997), SSU analyses (Winnepenninckx et al., 1998) suggest a close relationship with the Syndermata (acanthocephalans and rotifers, Ahrlichs, 1995; Garey et al., 1996). Lastly, the hypothesized grouping Nemertea and Platyhelminthes (a.k.a. Parenchyma; Nielsen, 2001), based up simplicity of body organization, is of interest with respect to the Platyzoa concept.

The term "Trochozoa" refers to taxa that have a certain type of ciliated larvae, a trochophore. The term was originally applied specifically to annelids (Hatschek, 1878), but it has been loosely applied to several other protostome lineages causing confusion in the literature. Recognizing this problem, Peterson and Eernisse (2001) use several different terms to define nested clades with trochophore or trochophore-like larvae. The Neotrochozoa (i.e., annelids including echiurids, mollusks, and sipunculans) is the most restrictive clade recognized, whereas the Eutrochozoa (Nemertea & Neotrochozoa) and Trochozoa (Entoprocta & Eutrochozoa) are more inclusive. Whether these form natural

(i.e., monophyletic) units, influences our understanding of 1) the early history of larval forms and 2) the evolutionary plasticity of characters considered important to phylogeny (e.g., metatroch and apical tuft).

Deciphering the relationships within the Lophotrochozoa requires critical evaluation of phylogenetic hypotheses such as the Lophophorata, Platyzoa, and Trochozoa, among others. However, recovering the monophyly of individual lophotrochozoan phyla is prerequisite to evaluating interphyletic relationships - on this point SSU data have failed. Previous simulation study (Halanych, 1998) and recent phylogenetic analyses (Medina et al., 2001; Mallatt and Winchell, 2002; Winchell et al., 2002) suggested that combined LSU + SSU data offer more resolution than SSU data alone. To this end, we examined approximately 5Kb of nuclear rRNA gene sequence for 36 lophotrochozoan taxa. Compared to SSU data, both LSU + SSU data and LSU data alone more consistently recover recognized phyla as monophyletic, allowing us to begin elucidating interphyetic relationships. The Lophophorata is significantly rejected, but data are more equivocal on "Trochozoa" hypotheses. The monophyly of the Platyzoa is not rejected, but LSU + SSU data suggest this clade is derived within the Lophotrochozoa rather than a basal sister-lineage. This placement has profound repercussions for our interpretation of metazoan morphological evolution.

Materials and Methods

Taxon sampling

Thirty-six taxa were chosen to provide broad representation of extant lophotrochozoan lineages (Table 6). Two deuterostomes and three ecdysozoans with low rates of nucleotide substitution were chosen as outgroups (de Rosa et al., 1999; Giribet et al., 2000; Peterson and Eernisse 2001; Mallatt and Winchell, 2002). LSU data were collected from 20 taxa. SSU data were also collected for taxa not in GenBank.

Data Collection

Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen). Primer sequences utilized for PCR and sequencing are provided in Chapter 2. Both genes were amplified using a long PCR protocol. PCR reactions contained 15µl 3.3x rTth buffer, 2.5µl 10 µM primer, 5µl 2mM dNTPS, 0.4 µl rTth (PE Applied Biosystems), 1µl Vent polymerase (New England BioLabs) (diluted 1:100 in a buffer composed of 50% glycerol, 20mM HEPES, 10mM KCL, 1mM DTT, 0.1mM Na_2EDTA, 0.0025% Tween-20, and 0.0025% NP-40), with genomic DNA and water to a final volume of 45µl. Following a 5 minute denaturation, 5µl of 25mM $Mg(OAc)_2$ was added to each reaction. PCR involved 30 cycles of denaturation at 94°C for 30 sec, annealing at 45-55°C for 1 min, and extension at 65°C for 12 min LSU or 8 min for SSU. A final extension was carried out at 72°C for 10 min. PCR products were cleaned with QIAquick PCR Purification Kit (Qiagen) and incubated 10 minutes at 70°C with *Taq* polymerase (Promega) and 0.4mM dATP to create adenine overhangs. Fragments were cleaned a second time and cloned using the pGEM-T Vector System (Promega).

Sequencing used BigDye Terminator v2.0 Sequencing Reaction chemistry (Applied Biosystems) on an ABI 377 automated sequencer (Applied Biosystems). For each taxon, each gene was sequenced in both directions.

Phylogenetic analyses

Sequences were aligned by the profile alignment function of ClustalW (Thompson et al., 1994), using existing alignments from the Ribosomal Database Project II (Maidak et al., 2001) as guides. Alignments were checked manually with MacClade 4 (Maddison and Maddison, 2000), and regions of questionable alignment were excluded.

To better understand relative contributions of each rDNA gene, analyses were carried out on SSU data alone, LSU data alone, and combined LSU + SSU data. Due to the need for brevity, we mainly focus on the combined analyses. Maximum likelihood (ML) analyses were conducted in PAUP* version 4.0 b10 (Swofford, 2002), with appropriate models determined by Modeltest (Posada and Crandall, 1998). Details of phylogenetic reconstructions are given in the figure legends. Support for previously published lophotrochozoan hypotheses was evaluated using the Shimodaira-Hasegawa (1999) test implemented in PAUP*4.0b10.

Table 6. Species and GenBank accession numbers

Species	LSU	SSU
Mollusca		
Arion silvaticus	AY145392	AY145365
Chaetopluera aplicata	AY145398	AY145370
Ilyanassa obsoleta	AY145511	AY145379
Leptochiton acellus	AY145414	AY145382
Nucalana pernula	AY145419	AY145385
Placopecten magellanicus	AF342798	X53899
Nemertea		
Amphiporus sp.	AF342786	AF119077
Cerebratulus lacteus	AY145396	AY145368
Oerstedia dorsalis	AY210465*	AY210448*
Tubulanus annulatus	AY210473*	AY210452*
Sipuncula		
Apionsoma misakianum	AY210454*	AY210440*
Phascolion strombi	AY210468*	AY210449*
Phascolopsis gouldii	AF342795	AF342796
Bryozoa		
Alcyonidium diaphanum	AY21045*	
Alcyonidium gelatinosum		X91403
Bugula turrita	AY210457*	AY210443*
Crisia sp.	AY210458*	AY210443*
Entoprocta		
Barentsia gracilis	AY210456*	AY210442*
Brachiopoda		
Glottidia pyramidata	AY210459*	U12647
Laqueus californianus	AY210460*	U08323
Neocrania anomola	AY210463*	U08328
Terebratalia transversa	AF342802	AF025945
Phoronida		
Phoronis vanvouverensis	AF342797	AY210450*
Echiura		
Arhynchite pugettensis	AY210455*	AY210441*
Urechis caupo	AF342804	AF342805
Annelida		
Eisenia fetida	AF212166	X79872
Nereis succinea	AY210464*	AY210447*
Proceraea cornuta	AF212165	AF212179
Riftia pachyptila	AY210470*	AF168745
Platyhelminthes		
Dugesia tigrina	U78718	AF013157
Sytlochus zebra	AF342800	AF342801

Acanthocephala		
Oligacanthorhynchus tortuosa	AY210466*	AF064817
Oncicola sp.	AY210467*	AF064818
Rotifera		
Philodona roseola	AY210469*	AF154567
Sinantherina socialis	AY210471*	AY210451*
Cycliophora		
Symbion sp. (from *Homarus americanus*)	AY210472*	
Symbion pandora		Y14811
Myzostomida		
Myzostoma polycyclus	AY210462*	AY210446*
Ecdysozoa		
Limulus polyphemus	AF212167	U91490
Misumenops asperatus	AY210461*	AY210445*
Halicryptus spinulosus	AF342789	AF342790
Deuterostomia		
Antedon serrata		D14357
Florometra serratissima	AF212168	
Ptychodera flava	AF278681	AF278681

* New sequences

Results

The number of aligned, unambiguously aligned, variable, and informative characters for each dataset are given in Table 7. ML trees for the LSU + SSU, LSU and SSU datasets are presented in Figures 12-14, respectively. Phylogenetic reconstructions from the LSU and LSU + SSU datasets recover the monophyly of the nearly all lophotrochozoan phyla. Although the bootstrap support for these nodes is weak, this result is a substantial improvement over the situation with SSU data alone (compare Figures 12 and 14). This boost in signal is clearly due to the LSU data, which recovered a tree (Figure 13) much more consistent with our current understanding of animal relationships than the SSU topology. SSU reconstructions have also been maligned because of the potential for long-branch attraction (e.g., Maley and Marshall, 1998). Interestingly, all the long branches clump together in the SSU tree, but not in the LSU or LSU + SSU tree suggesting that rate effects may be less severe in these datasets. Table 8 gives the results of the Shimodaira-Hasegawa tests for LSU + SSU data, LSU, and SSU data sets. The most striking result, and consistent with the recovered tree topologies, the monophyly of the Lophophorata was not supported in either the LSU or LSU + SSU datasets (Table 8). In all analyses, Bryozoa consistently fell out basal to other lophotrochozoans, including brachiopods and phoronids. The resultant non-monophyly of Brachiopoda in the LSU + SSU analysis bears further investigation. Additionally, the hypothesis that Bryozoa is sister to Entoprocta was rejected for both the LSU and LSU + SSU data sets. Neither result appeared to be affected by the presence of *Myzostoma*

within the Bryozoa, as bryozoan monophyly was not significantly rejected under either data set.

In both the LSU and LSU + SSU analyses a clade was recovered which included the Entoprocta, Cycliopohora, Platyhelminthes, Syndermata (Rotifera + Acanthocephala), and Nemertea. Within this clade the Entoprocta + Cycliophora appear as each others closest relatives and form a sister group to the Platyzoa. Although the nemertean *Tubulanus* branches within the Brachiopoda in the LSU tree, the Nemertea are recovered as monophyletic in the LSU + SSU analysis. An SH test found the LSU + SSU analysis uniting Platyhelminthes + Syndermata had a likelihood score significantly better than that of a tree where the Platyhelminthes and Nemertea are sister taxa.

The data are more equivocal about the reality of various "trochozoan" hypotheses. LSU data place sipunculans as the sister to annelids, which includes echiurids and siboglinids (a.k.a. pogonophorans). However, the placement of mollusks relative to this clade still is not clear.

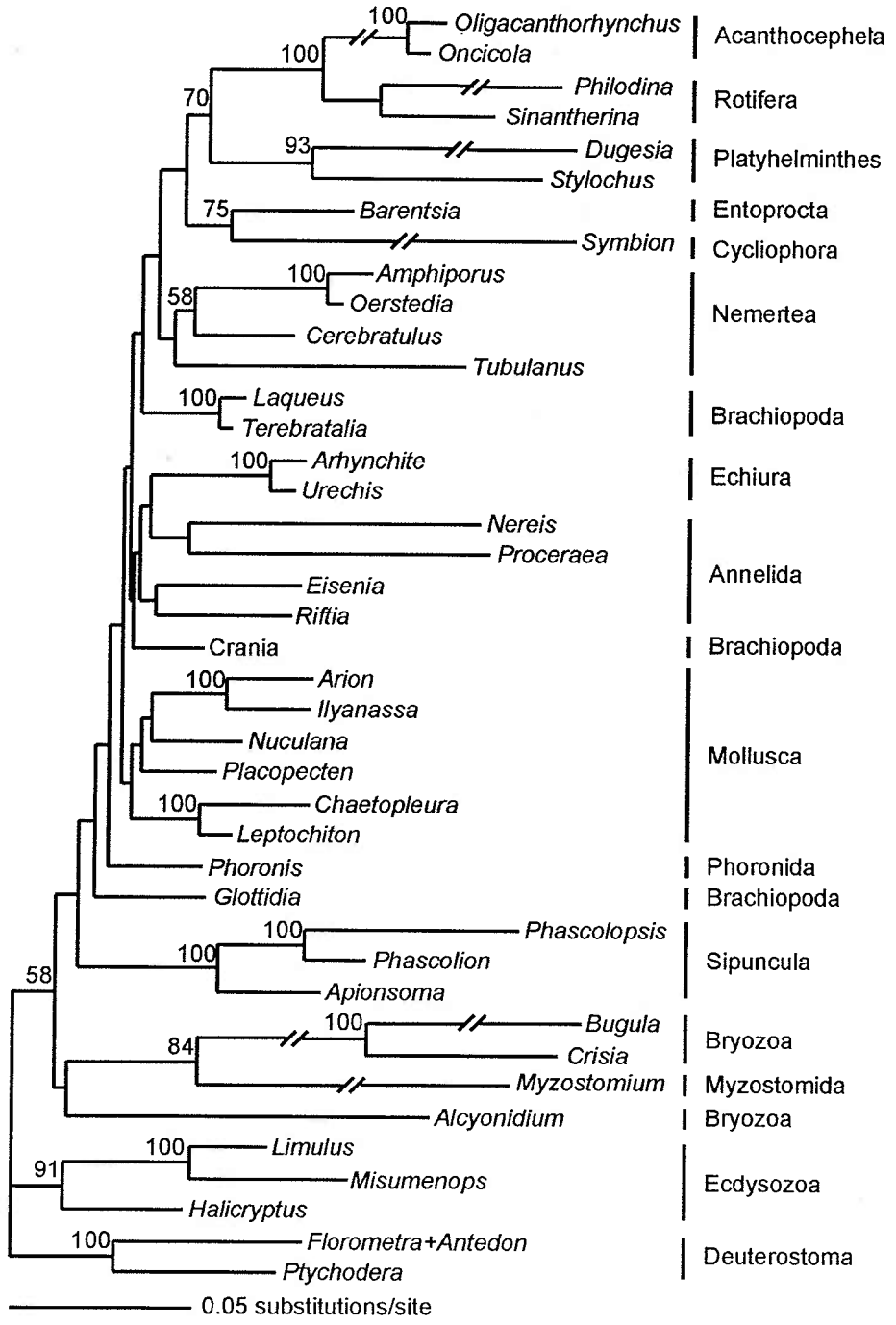

Figure 9: ML tree for the combined LSU + SSU dataset. 100 hueristic replicates were performed using the Symmertical Model (Zharkikh, 1994) with equal base frequencies and estimation of gamma parameter shape distribution (G = 0.5750) and proportion of invariant sites (I = 0.3234). ML bootstrap (100 replicates) values are shown above nodes with values > 50%.

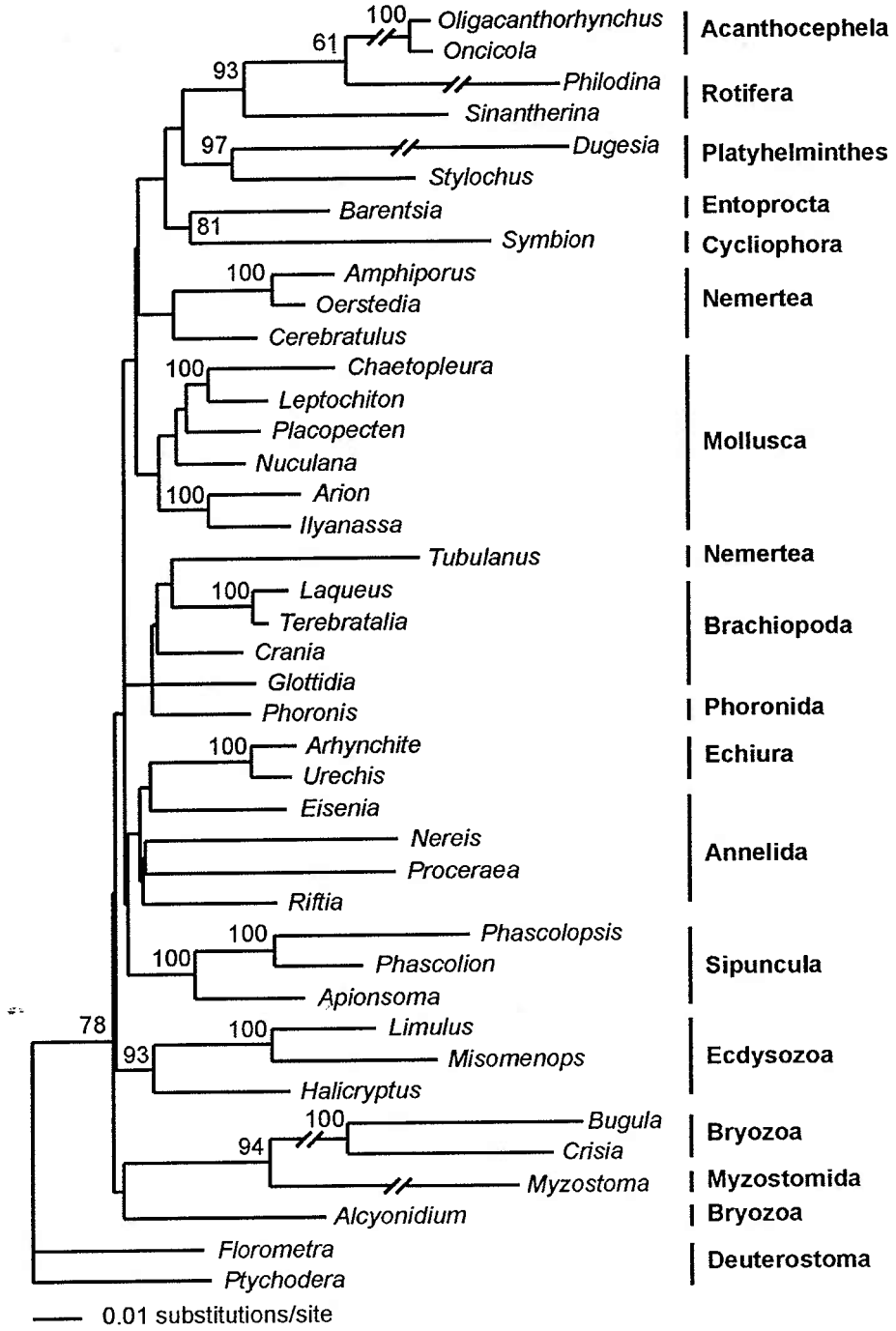

Figure 10: ML tree for the LSU dataset. 100 hueristic replicates were performed using the Transition Model with equal base frequencies and estimation of gamma parameter shape distribution (G = 0.5229) and proportion of invariant sites (I = 0.3228). ML bootstrap (100 replicates) values are shown above nodes with values > 50%.

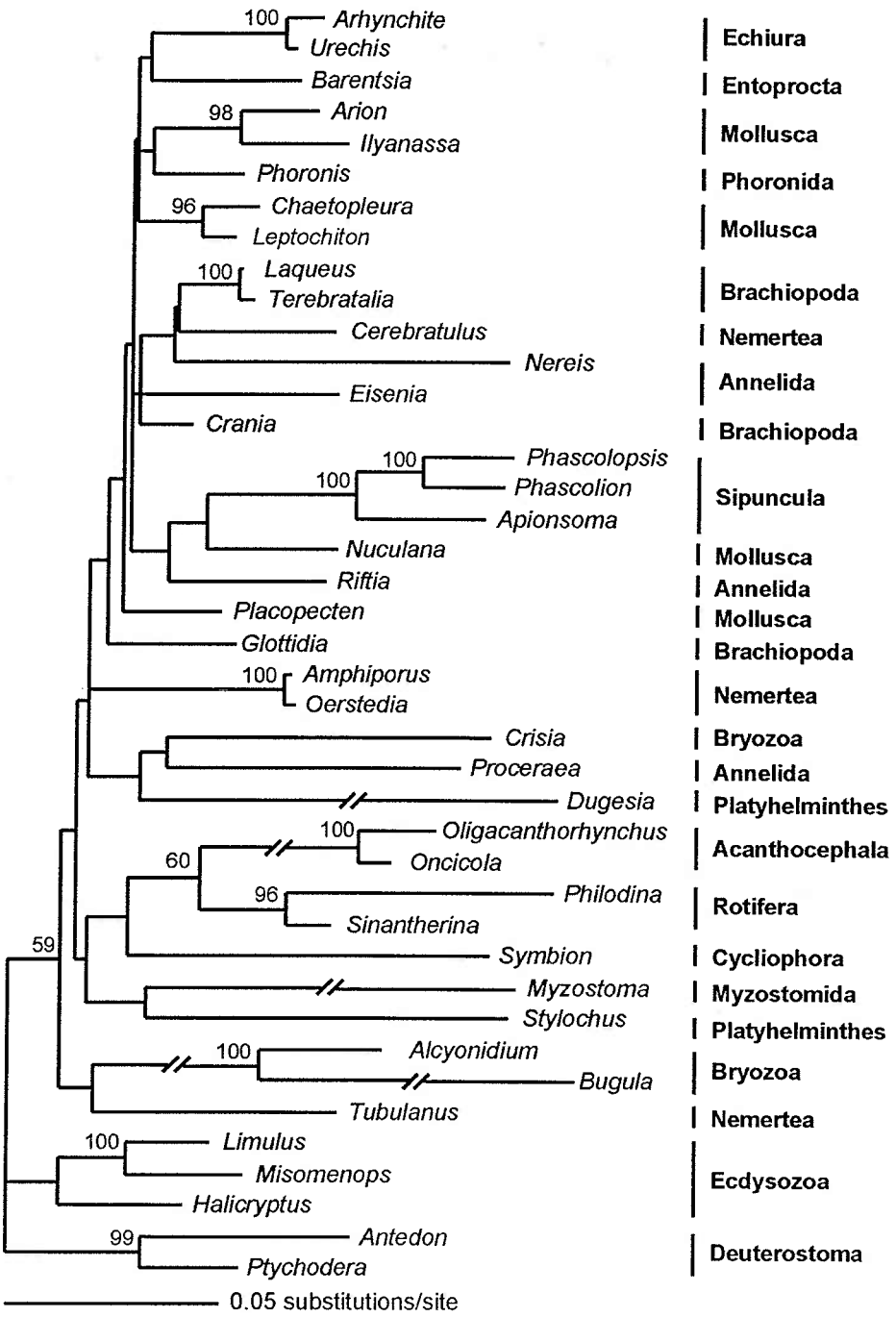

Figure 11: ML tree for the SSU dataset. 100 hueristic replicates were performed using the Tamura-Nei (Tamura and Nei 1993) model with equal base frequencies and estimation of gamma parameter shape distribution (G = 0.6199) and proportion of invariant sites (I = 0.3112). ML bootstrap (100 replicates) values are shown above nodes with values > 50%.

Table 7: Total, unambigouosly aligned, variable and parsimony informative characters

	Total	Unambiguous	Variable	Informative
SSU	2048	1508	783	499
LSU	4611	2370	1183	804
LSU+SSU	6659	3878	1966	1303

Table 8: Shimodaira-Hasegawa test results

	SSU	LSU	LSU +SSU
Lophophorata monophyly	0.128	**0.005***	**0.041***
Bryozoa + Entoprocta monophyly	0.173	**0.013***	**0.017***
Bryozoa monophyly	0.312	0.052	0.275
Platzoa sister group of Trochozoa	0.212	0.059	0.133
Parenchyma monophyly	0.050	0.362	**0.011***
Neotrochozoa monophyly	0.164	0.443	0.269
Eutrochozoa monophyly	0.056	0.220	0.165
Trochozoa monophyly	0.066	0.114	0.269

* $P < 0.05$ – Hypotheses in bold are rejected under the given dataset.

Note. – Analyses carried out using the dataset listed at the top of each column, using the appropriate likelihood model as calculated with Modeltest.

Discussion

The LSU data greatly improve the phylogenetic signal recovered for lophotrochozoan interphyletic relationships over SSU data alone. LSU sequences recover monophyly of nearly all recognized phyla sampled, including mollusks and annelids which have consistently appeared as polyphyletic in studies using SSU alone (e.g. Giribet et al., 2000; Eernisse, 1997; Peterson and Eernisse, 2001). This increase in resolution provides a tool by which we can begin to decipher deep-level relationships within Lophotrochozoa.

This study provides the most conclusive evidence to date that Lophophorata is not monophyletic. While the position of the Bryozoa differs between the LSU and LSU + SSU trees, both reconstructions place bryozoans basal to other lophotrochozoans. Alternative hypotheses regarding the origin of the Bryozoa are not supported by ML reconstructions and SH tests of the LSU and LSU + SSU datasets. The "Lophophorata" hypothesis which unites bryozoans with brachiopods and phoronids (Hyman, 1959), is rejected under SH tests of both the LSU and LSU + SSU datastets. Likewise, grouping of the Bryozoa and Entoprocta as sister taxa (Nielsen, 2001) is not supported. These results confirm previous arguments (Halanych, 1996; Nielsen, 1987 – among others) that the similarities in feeding mechanics, ciliation patterns, and gross morphology in bryozoans, brachiopods, phoronids, and other tentacular suspension feeders (e.g. pterobranch hemichordates) are the product of convergent evolution rather than common ancestry. This recognition renders the term "lophophorates" descriptive of function rather than history.

Moreover, given the results herein, Bryozoa diverged by at least the early Cambrian period. Such an early divergence is at odds with the fossil record, as the Bryozoa have not been found from before the Ordovician, despite being well preserved in later sediments (Lehmann and Hillermer, 1983). Apparently, Bryozoa went through an extended period of cryptic evolution, unrecorded in the fossil record. A late evolution of a calcified skeleton is one possible explanation for this discontinuity between the molecular data and the fossil record.

Analyses of both the LSU and LSU + SSU datasets supports the monophyly of the Platyzoa, and places the group well within the Lophotrochozoa. Despite the placement of the Nemertea near the Platyhelminthes, the rejection of the Parenchyma hypothesis under the SH test of the LSU + SSU dataset strengthens support for the monophyly of the Platyzoa. SSU datasets have found the Platyzoa to branch basally to the Lophotrochozoa (Giribet et al., 2000; Peterson and Eernisse, 2001) supporting, in a general sense, that bilaterians evolved from simple to complex. In contrast, LSU and LSU + SSU data suggest that the morphology of Platyzoans represent secondary simplification of body form. Drawing on recent studies that show platyhelminthes are polyphyletic (with acoelmorphs as basal bilaterians), we favor that both possibilities of bilaterian evolution are correct. However in the specific case of platyzoans, it will be critical to sample gnathostomulids and gastrotrichs to test Cavalier-Smith's (1998) ideas. Although a basal divergence is not rejected by the SH test of the LSU or LSU + SSU trees, the placement of the Platyzoa as a derived clade within the Lophotrochozoa provides a markedly different interpretation of bilaterian evolution which warrants further investigation.

One putative member of the Platyzoa whose evolutionary affinities are drawn into question is the cycliophoran *Symbion*. Analyses of SSU data, including those presented here, have suggested that cycliophorans are closely related to rotifers and acanthocephelans. In contrast, the recovery of Cycliophora and Entoprocta as sister taxa in the LSU and LSU + SSU analyses is consistent with the evolutionary relationship hypothesized when this enigmatic taxon was first described (Funch and Kristensen, 1995; 1997), as well as with the results of morphological cladistic analysis (Zrzavy et al., 1998).

The recovered LSU and LSU + SSU topologies suggest that trochozoans represent an evolutionary grade rather than a distinct clade, although hypotheses supporting the monophyly of trochozoan taxa are not rejected under SH tests. If the trochophore larva is a plesiomorphy of the Lophotrochozoa (excepting the Bryozoa) it appears to have been lost or highly modified in some descendent lineages, such as phoronids, brachiopods, and platyzoans. The sister relationship of the Annelida and Sipuncula in the LSU tree supports the presence of the trochophore in the common ancestor of these two groups. A close relationship between annelids and sipunculans has also been suggested based upon similarities in mitochondrial gene arrangement (Boore and Staton, 2002).

LSU sequence data presented here provide improved resolution of lophotrochozoan relationships. Unlike SSU data, LSU and LSU + SSU sequences recover the monophyly of most recognized lophotrochozoan phyla, a prerequisite to evaluating interphyletic relationships. Several findings have important implications for our understanding of developmental and morphological evolution. In particular, the finding of a derived Platyzoa closely related to entoprocts provides support for secondary simplification of the

group, perhaps due to a neotonic origin. Increased attention on the evolutionary origin of the Bryozoa will also be of particular interest given their possible early divergence during protostome diversification.

Chapter 4

A Survey of Hox genes in the bryozoan *Bugula turrita*

Abstract

The present study surveys the complement of Hox genes present in the genome of the bryozoan *Bugula turrita*. Although the clade Lophotrochozoa was defined as including bryozoans, recent studies have not reliably recovered the position of the Bryozoa among metazoans. Hox genes sequences have the potential to provide an additional set of evidence for the phylogenetic position of bryozoans. Hox genes appear to have undergone independent duplication events in each of the three major bilaterian clades: lophotrochozoans, ecdysozoans, and deuterosotmes. Two Hox gene paralogs, *Post1* and *Post2*, appear to have arisen subsequent to the divergence of the Lophotrochozoa and can therefore serve as a synapomorphy for members of the clade. Six Hox genes were identified from *Bugula turrita*, including an ortholog of *Post2*. The identification of a bryozoan *Post2* ortholog provides novel evidence for a close evolutionary relationships between bryozoans and other lophotrochozoans.

Introduction

The Bryozoa remain among the most enigmatic of metazoan phyla with respect to their phylogenetic position (Giribet 2002). Bryozoans have traditionally been viewed as closely related to brachiopods and phoronids. Together these three groups are referred to as lophophorates, based upon the inferred homology of their ciliated tentacular feeding structures (Hyman, 1959; Willmer, 1990). Inference of the phylogenetic position of lophophorates based upon morphological and embryological characters has been complicated by the fact that they display a mosaic of archetypal protostome and deuterostome conditions. Differing interpretations of developmental and morphological traits has lead to the assignment of lophophorates as protostomes (Gutmann et al., 1978), deuterostomes (Zimmer, 1973), intermediates between the two groups (Salvini-Plawen, 1982; Seiwing, 1976), or an independent radiation (Willmer, 1990).

However, detailed structural and functional analyses of bryozoan tentacles suggest that they are not homologous to the lophophores of phoronids and brachiopods (Nielsen and Riisgard, 1998), as widely believed (e.g. Brusca and Brusca, 1990; Willmer, 1990; Knoll and Carroll, 1999). As the lophophore is the primary feature uniting bryozoans with brachiopods and phoronids, failure to establish the homology of this structure undermines the validity of the Lophophorata hypothesis (Halanych, 1996; de Rosa et al., 2001). Nielsen has suggested that bryozoans may be most closely related to entoprocts, on the basis of developmental similarities between the two groups (Nielsen, 1971; 2001).

Several recent studies have utilized cladistic methods to reconstruct metazoan phylogenies from explicit matrices of morphological and developmental character states

(e.g. Zrzavy et al., 1998; Peterson and Eernissee, 2001). The placement of bryozoans within these studies varies based the characters chosen and the way these characters were chosen (Jenner, 2001). Zrzavy et al. (1998) coded bryozoans as possessing a lophophore, and recovered the bryozoans as an outgroup to Phoronida + Brachiopoda + Deuterostomia. In a recent study, Peterson and Eernissee (2001) did not code bryozoans as having a lophophore, and found bryozoans to be closely related to spiralian protostomes such as mollusks and annelids and entoprocts.

With the advent of molecular phylogenetics there arose the potential for an independent set of characters for analyzing the relationship between bryozoans and other metazoan phyla. Using small-subunit ribosomal gene (SSU rDNA) sequence, Halanych et al., (1995) found, bryozoans, brachiopods and phoronids to be more closely related to the protostome annelids and mollusks than to deuterostomes. Based upon these results, the clade Lophotrochozoa was defined as "the last common ancestor of the three traditional lophophorate taxa, the mollusks, and the annelids, and all of the descendents of that common ancestor." Halanych et al., (1995) did not recover lophophorates as monophyletic, instead finding that the bryozoan sequence branched basally to the other lophotrochozoans sequenced. Although this study utlilized only a single bryozoan, analysis of SSU sequnces from additional bryozoan species has also failed to recover lophophorate monophyly (Giribet et al., 2001).

Subsequent sampling has suggested that the Lophotrochozoa encompasses a broad assemblage of invertebrates, such as nemerteans, sipunculans, and entoprocts (e.g. Mackey et al., 1996). Platyhelminthes and rotifers may also be members of the clade, or