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A Molecular Phylogenetic Framework for the Phylum Ctenophora Using 18S rRNA Genes

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This paper presents the first molecular phylogenetic analysis of the phylum Ctenophora, by use of 18S ribosomal RNA sequences from most of the major taxa. The ctenophores form a distinct monophyletic group that, based on this gene phylogeny, is most closely related to the cnidarians. Our results suggest that the ancestral ctenophore was tentaculate and cydippid-like and that the presently recognized order Cydippida forms a polyphyletic group. The other ctenophore orders that we studied (Lobata, Beroida, and Platyctenida) are secondarily derived from cydippid-like ancestors, a conclusion that is also supported by developmental and morphological data. The very short evolutionary distances between characterized ctenophore 18S rRNA gene sequences suggests that extant ctenophores are derived from a recent common ancestor. This has important consequences for future studies and for an understanding of the evolution of the metazoans. © 2001 Academic Press

Ctenophores (comb jellies) represent a distinct phylum of gelatinous invertebrates that are found in virtually all marine environments (coastal and oceanic, from the surface to the deep sea, and from the tropics to the poles). Most species are free swimming, although one order (Platyctenida) is represented by species that crawl on substrates. Of the known species of ctenophores, most were described in the 19th century and during the early 1900s (Chun, 1880, 1898; Mayer, 1912). It is currently believed that many of those species are synonyms or represent developmental stages of others. The number of valid described ctenophores species is between 100 and 150 (Mills, 2001), and it is believed that there are many deep-sea species still to be discovered. Ctenophores are very poorly known, primarily because they are extremely fragile and difficult to collect and identify, they cannot be preserved, and many species inhabit hard-to-reach locations (Harbison *et al.*, 1978). Some species reach high densities in coastal blooms and one lobate ctenophore (*Mnemiopsis leidyi*) caused major ecological disturbances after being introduced into the Black Sea, presumably through discharges of ballast water from Western Atlantic ports in the 1970s (GESAMP, 1997). Molecular markers (DNA sequences) could greatly improve the accuracy of species identification and will be invaluable for taxonomic and ecological studies.

Although morphologically quite diverse, ctenophores are unified by a number of unique derived characters (synapomorphies) present in adult individuals or, in some species, during specific developmental stages: biradial symmetry, eight rows of combs or ctenes (fused macrocilia) controlled by an apical organ, two retractable tentacles, specialized adhesive cells (colloblasts), and a characteristic developmental stage ("cydippid larva").

The phylogenetic position of Ctenophora among the Metazoa and the direction of evolution within the group remain controversial yet are very important for an understanding of the origins of triploblastic metazoans. Whereas many investigators considered ctenophores to be diploblastic metazoans, closely allied to the Cnidaria (Hyman, 1940), certain developmental and morphologic characters have led other investigators to consider them an intermediate step in the evolution of bilaterality (Harbison, 1985; Martindale and Henry, 1998) or even degenerate Deuterostomes (Nielsen, 1995). The scarce molecular data available have further complicated this matter. Based on 18S rRNA sequences from two species, a recent study (Collins, 1998) concluded that ctenophores are closer to the sponges (phylum Porifera) than to the Cnidaria/Placozoa group or to the Bilateria. The virtual absence of ctenophores from the fossil record has seriously hampered attempts for a phylogenetic classification of the different groups within this phylum. The characteristic "cydippid larva," which is present in the ontogeny of all ctenophores except the beroids, has led to the general idea that the ancestral ctenophore resembled modern

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cydippids (globular body, with two retractable tentacles and eight comb rows). In this scenario, other body plans would have been the result of adaptation to different environments and life styles. Platyctenids adapted to crawling on substrates, lost their comb rows, and superficially resemble flatworms. Other ctenophores (lobates and cestids) adapted to active swimming by reducing the tentacular apparatus and developing large auricles and oral lobes or an elongated ribbon-like shape. Finally, beroid ctenophores lost the tentacles entirely, adopted a sac-shaped body and a large mouth, and became active swimmers and predators.

In the most recent work on the classification of Ctenophora (Harbison, 1985), it was proposed, based entirely on morphological characters, that the traditional order Cydippida is not monophyletic. Harbison concluded that the five families that he recognized in this order (Haeckeliidae, Bathyctenidae, Lampeidae, Mertensiidae, and Pleurobrachiidae) were more closely related to members of other orders than to each other. Two alternative evolutionary scenarios were proposed, hypothesizing that either a cydippid or a beroid was the last common ancestor of recent ctenophores. Harbison (1985), however, pointed out that, regardless of which of the two phylogenetic scenarios was preferable or whether another scenario might be chosen, there was still strong morphological evidence that the Cydippida was not monophyletic. He also stressed that a drastic alteration of the presently recognized taxa should not be undertaken until a great deal of new information became available.

The Ctenophora is the only major metazoan phylum that has not been extensively studied at the molecular level in phylogenetic, developmental, or ecological investigations. Here we present a phylogenetic study using 18S rDNA sequences from 26 species of ctenophores. For the first time, we can compare the "traditional" phylogenies with those derived from sequence data. This is an important step in the revision of the major subgroups of Ctenophora, defined many decades ago based on characters that may have limited phylogenetic significance. We also analyze the phylogenetic relationship between the ctenophores and other basal metazoan phyla.

MATERIALS AND METHODS

For this study we determined the complete nucleotide sequence of 18S rDNA genes from 26 species of ctenophores and internally transcribed spacer (ITS) from 18 of these species. We collected animal specimens from different locations (Table 1) using plankton nets or hand-held containers, by scuba diving or submersibles. For two of the species (*Beroe forskalii* and *B. cucumis*), we obtained specimens from both the Atlantic and the Pacific oceans. Four specimens represent species that have only recently been discovered and are yet to be formally described and named (Fig. 1). When the animals could not be kept alive until DNA isolation, they were frozen at −80°C or preserved in 70% ethanol at −20°C. Live specimens of *Vallicula multiformis* were donated by Morgan Lidster from Inland Aquatics (Terre Haute, IN). Ethanol-preserved specimens of *Mertensia ovum* were donated by Jonathan Martin (Dauphin Island Sea Lab, AL). A DNA sample of *Coeloplana banworthii* was donated by Dr. Igor Eeckhaut (Laboratoire de Biologie Marine, Mons, Belgium).

For DNA isolation, we used the entire animal (in the case of tiny specimens, such as *Haeckelia, Vallicula*) or 100- to 200-mg tissue fragments, following a CTAB extraction protocol previously described (France *et al.*, 1996). 18S rDNA genes were amplified with *Taq* DNA polymerase and universal eukaryotic primers (Medlin *et al.*, 1988). The primers 1400F (5′TGYACACACCGCCCGTC3′) and 5′28Sr (5′CTTAAGTTCAGCGGGTAGTCTCG3′) were used to amplify ITS sequences. The products were cloned into a pGEM T vector (Promega).

Plasmid DNAs from 4–10 individual clones were sequenced individually or as a pool with the SequiTherm EXCEL II Long-Read DNA sequencing kit LC (Epicentre Technologies) and analyzed on a LI-COR 4200 IR2 instrument. Sequences were deposited in GenBank under Accession Nos. AF293673 through AF293700. In the cases in which both 18S and ITS sequences have been determined, one GenBank record contains the entire contig.

Conserved regions in 18S rDNA sequences were aligned using the computer program SeqEdit (Gary Olsen). Conserved helices in a secondary structure model of ctenophore 18S rRNA (Fig. 2) were used to refine the sequence alignment in regions where length variations occurred. The 18S rDNA were very well conserved among the sampled ctenophore species, with lengths between 1801 and 1809 nucleotides (nt) and with a maximum divergence between two species of 87 nt, i.e. less than 5%. This allowed us to unambiguously align all of the ctenophore sequences with each other over their entire length. In data sets that included outgroup sequences, there were short regions that could not be reliably aligned, and those portions of the alignment were excluded from the phylogenetic analysis. The final alignment files have been deposited in the EMBL–Align database (ALIGN_000141 and ALIGN_000142).

The conserved secondary structure of the 5.8S rRNA (158 nt long) contained within ITS served to identify the junctions to the flanking ITS1 and ITS2. Whereas ITS1 (174–282 nt) and ITS2 (200–322 nt) are unusually short in ctenophores compared to most other metazoa, they contained several repetitive sequences, which prevented us from confidently ascribing homologies. Therefore, we decided to exclude ITS sequences from our analysis, although they are likely to be valuable in

TABLE 1

List of Species and Sequences Used in This Study

Taxon, species	Source	Sequence length 18S	Sequence length ITS	Accession no.
Phylum Ctenophora (sequences determined in this study)				
Order Cydippida				
Fam. Haeckeliidae				
Haeckelia beehleri	Santa Barbara, CA (USA)	1803	647	AF293673
Haeckelia rubra	Santa Barbara, CA (USA)	1802	613	AF293674
Fam. Pleurobrachiidae				
Pleurobrachia pileus	Woods Hole, MA (USA)	1801	668	AF293678
Pleurobrachia bachei	Santa Barbara, CA (USA)	1801	675	AF293677
Hormiphora plumosa	Tortugas, FL (USA)	1802	543	AF293676
Undescribed sp. 1	Bahamas		ND	AF293675
Fam. Mertensiidae				
Mertensia ovum	Newfoundland, Canada	1807	ND	AF293679
Charistephane fugiens	Pt. Conception, CA (USA)	1809	ND	AF293682
Undescribed sp. 2	Bahamas	1807	532	AF293680
Undescribed sp. 3	Santa Barbara, CA (USA)	1802	ND	AF293681
Order Platyctenida				
Fam. Coeloplanidae				
Coeloplana bannworthi	Hansa Bay, Madagascar	1809	679	AF293683
Vallicula multiformis	Caribbean	1806	762	AF293684
Order Thalassocalycida				
Fam. Thalassocalycidae				
Thalassocalyce inconstans	Pt. Conception, CA (USA)	1803	ND	AF293685
Order Lobata				
Undescribed sp. 4	Bahamas	1802	604	AF293686
Fam. Bolinopsidae				
Bolinopsis infundibulum	Woods Hole, MA (USA)	1803	ND	AF293687
Mnemiopsis leidyi	Woods Hole, MA (USA)	1803	678	AF293700
Fam. Ocyropsidae				
Ocyropsis maculata maculata	Tortugas, FL (USA)	1802	646	AF293689
O. crystallina crystallina	Tortugas, FL (USA)	1802	653	AF293690
O. crystallina guttata	Tortugas, FL (USA)	1802	ND	AF293691
Fam. Leucotheidae				
Leucothea pulchra	Santa Barbara, CA (USA)	1802	671	AF293688
Order Cestida				
Fam. Cestidae				
Cestum veneris	Santa Barbara, CA (USA)	1802	ND	AF293692
Velamen parallelum	Santa Barbara, CA (USA)	1803	656	AF293693
Order Beroida				
Fam. Beroidae				
Beroe ovata	Woods Hole, MA (USA)	1801	697	AF293694
Beroe gracilis	Santa Barbara, CA (USA)	1802	ND	AF293696
Beroe cucumis (Atlantic)	Gulf Stream, Florida	1802	652	AF293695
Beroe cucumis (Pacific)	Santa Barbara, CA (USA)	1802	654	AF293699
Beroe forskalii (Atlantic)	Gulf Stream, Florida	1802	ND	AF293697
Beroe forskalii (Pacific)	Santa Barbara, CA (USA)	1802	610	AF293698
Outgroups (sequences retrieved from GenBank)				
Fungi				
Saccharomyces cerevisiae	GenBank	1798		M27607
Choanoflagellida				
Rosette sp.	GenBank	1809		L29455
Diaphanoeca grandis	GenBank	1794		L10824
Porifera				
Scypha ciliata	GenBank	1807		L10827
Placozoa				
Trichoplax adhaerens	GenBank	1787		L10828
Cnidaria,				
Anthozoa				
Anthopleura kurogane	GenBank	1796		Z21671
Astrangia danae	M. Sogin	1785		AY039209
Scyphozoa				
Aurelia aurita	M. Sogin	1808		AY039208
Platyhelminthes,				
Turbellaria				
Stenostomum sp.	GenBank	1815		U95947
Microstomum lineare	GenBank	1788		U70082
Mollusca				
Acanthopleura japonica	GenBank	1817		X70210

Note. ND, not determined.

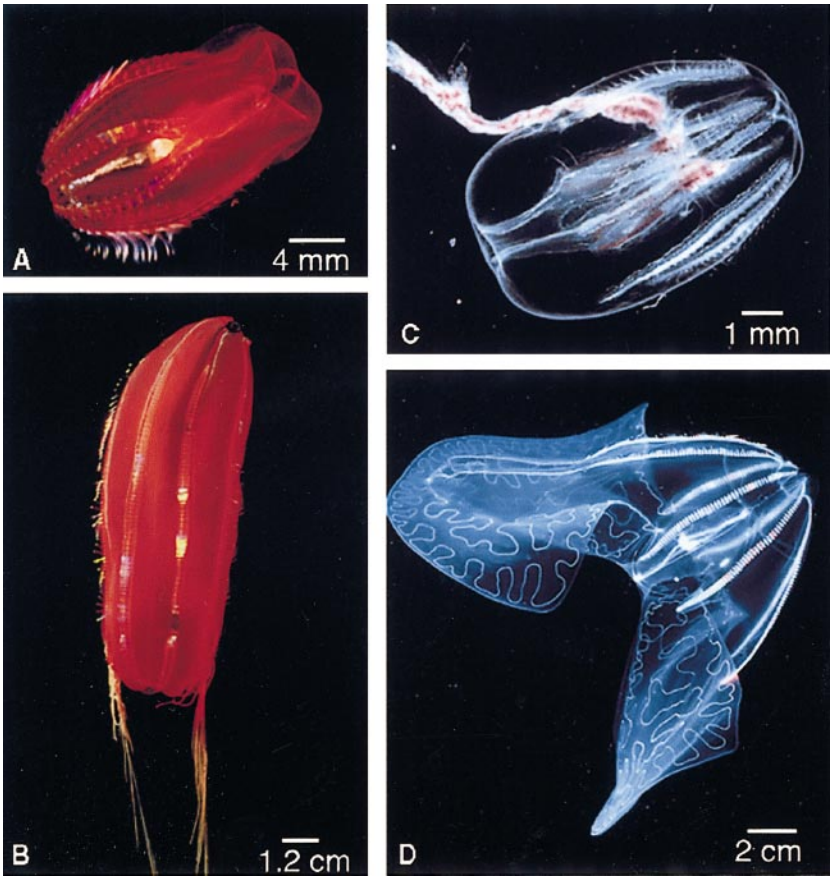

FIG. 1. Undescribed ctenophores analyzed in this study. (A) Cydippid, undescribed sp. 1; (B) cydippid, undescribed sp. 2; (C) cydippid, undescribed sp. 3; (D) lobate, undescribed sp. 4.

future studies of ctenophores at subspecies or population levels. A number of successful such studies have already been done with anthozoan ITS sequences, which also are extremely short (for example, McFadden *et al.*, 2001).

The phylogenetic analyses were performed with the computer program PAUP* (version 4.0b3 for Macintosh) (Swofford, 1996). Two separate sets of data were used. The first data set consisted of 18S sequences from all the ctenophores but included no outgroup taxa. The second data set included 18S sequences from 11 species of ctenophores and 10 outgroup species (1 fungus, 2 choanoflagellates, 1 sponge, 1 placozoan, 2 cnidarians, 2 flat worms, and 1 mollusk).

As a starting point in the analysis, a parsimony search was conducted on the ctenophore-only data set with the branch and bound method, which guarantees the shortest trees. Sequence ambiguities and gaps were treated as missing data.

For likelihood analyses, we first determined the maximum-likelihood scores and performed a Kishino–Hasegawa test (Kishino and Hasegawa, 1989) on the best trees obtained in the parsimony search. The best tree was then evaluated under 24 different models of evolution, which take into account combinations of equal or unequal base frequencies, invariant sites, types of possible substitutions, and rate heterogeneity. To determine the simplest model of evolution that was appropriate for the data, the likelihood scores (L*) of all models were compared to the score of the most complex model (general time reversible with invariant sites and among-site substitution heterogeneity, GTR + I + Γ) by use of the likelihood ratio test (LRT) (Huelsenbeck and Crandall, 1997; Sullivan and Swofford, 1997). This test indicated that for all the data sets, the simpler models had a significantly poorer fit to the data and, therefore, the GTR + I + Γ model was used in the maximum-likelihood searches. The model parameters were reevaluated by several successive likelihood searches until their values stabilized. A final unrestricted heuristic search was then conducted with those parameters, with starting trees obtained by 20 random stepwise addition of sequences and TBR branch swapping, and this resulted in a single best tree. Bootstrap analyses with 100 replicates and character resampling were conducted under both parsimony and maximum-likelihood.

The same strategy was used in the analysis of the data set that included ctenophores and outgroup taxa, with the exception that the starting trees for maxi-

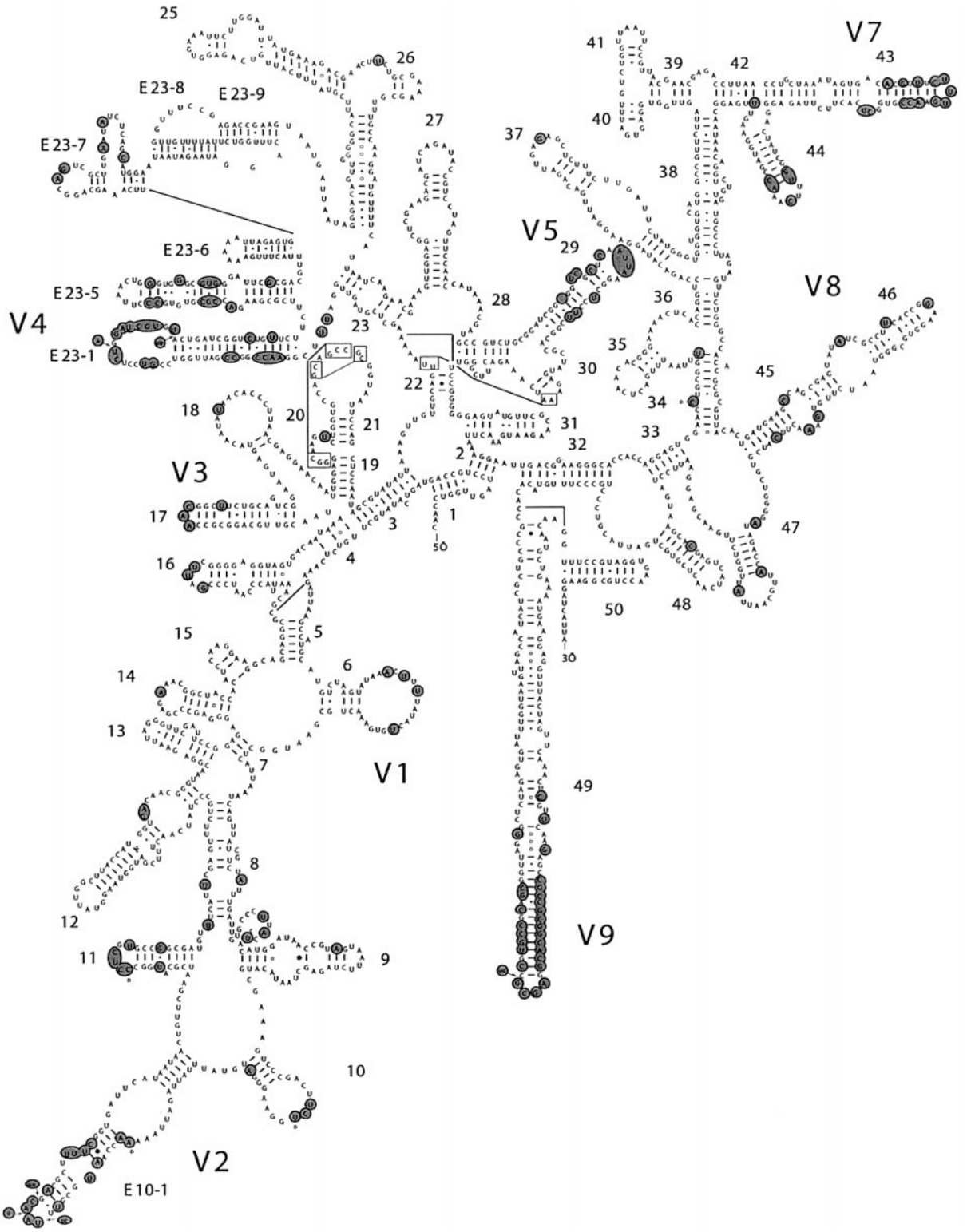

FIG. 2. Secondary structure diagram of ctenophoral 18S rRNA, based on *Mnemiopsis leidyi* sequence. Individual helices and variable domains are numbered according to the eukaryotic SSU rRNA consensus (Gutel (1994) and http://www.rna.icmb.utexas.edu/). Shaded residues indicate sites that vary within phylum Ctenophora.

mum-likelihood were derived from neighbor-joining searches with GTR + I + Γ distances.

To compare the among-site rate variation among ctenophores, sponges, cnidarians, and platyhelminths, we analyzed separate data sets for each of the four phyla. For the outgroup phyla, 18S rDNA sequence

alignments included a larger number of sequences: 6 species of sponge (*Sycon calcaravis, Scypha ciliata, Tetilla japonica, Mycale fibrexilis, Rhabdocalyptus dawsoni,* and *Microciona prolifera*), 6 species of cnidarians (*Anthopleura kurogane, Astrangia danae, Aurelia aurita, Bellonella rigida, Polypodium hydriforme,* and *Tripedalia cystophora*), and 15 species of flat worms (*Crenobia alpina, Dendrocoelum lacteum, Monocelis lineata, Archiloa rivularis, Nemertinoides elongatus, Urastoma* sp., *Macrostomum tuba, Microstomum lineare, Discocelis tigrina, Planocera multitentaculata, Stenostomum leucops, Echinococcus granulosus, Lobatostoma manteri,* and *Schistosoma mansoni*) (sequences from GenBank). For each of the data sets we performed maximum-likelihood searches using GTR + I + Γ evolutionary models. Based on the parameters of the most likely trees, we then determined the maximum-likelihood pairwise distances and calculated the average values within each phylum. We also calculated the average nucleotide differences based on the absolute pairwise distances, which do not take into consideration an evolutionary model.

RESULTS

We obtained and analyzed rRNA-coding regions from 26 (including 4 as yet undescribed) ctenophore species that spanned essentially all the orders of the phylum. By sequencing pools of clones or multiple individual recombinant clones, we have not only minimized the risk of introducing PCR errors in the final sequence, but we also assessed the degree of sequence heterogeneity within the same individual/population. Since rRNA genes exist in multiple copies per genome and sometimes display microheterogeneity (Gunderson *et al.*, 1987), sequencing of multiple clones reduced the risk of missing such sequence information.

The length of the 18S rDNA genes in our set of ctenophores varied between 1801 and 1809 nucleotides. With the conserved secondary structure of eukaryotic rRNA as a scaffold, 26 ctenophore sequences were manually aligned over their entire length. The alignment contained 1813 characters, of which 1638 were constant. Of the 175 variable characters, 125 were parsimony informative. This low level of variability at the 18S rDNA gene was also observed at the intraspecific level. Comparison of sequences from specimens of *B. cucumis* and *B. forskalii* collected from the Pacific and the Atlantic oceans revealed a single nucleotide difference in both species in the two geographically isolated populations. In the case of *Ocyropsis crystallina,* a two-nucleotide difference separates the two subspecies, *O. crystallina crystallina* and *O. crystallina guttata.*

A branch and bound search with the 18S data set under maximum-parsimony criteria has resulted in three best trees with a length of 336 steps. Since the topology of the strict consensus tree is virtually identical to that of the maximum-likelihood tree shown in Fig. 3, that parsimony tree is not shown. The maximum-likelihood search, conducted as described under Materials and Methods, resulted in a single most likely tree with the score of 4342. Several subgroups consisting of traditional ctenophore taxa can be distinguished. First, the order Beroida displays a strong sistership relationship (99/100 bootstrap support) with the family Haeckeliidae (order Cydippida). Another traditional family of cydippids (Pleurobrachiidae) appears to be closely related to the Beroidae–Haeckeliidae group. There is also strong support (90/90) for a cluster that includes the Lobata, Cestida, and Thalassocalycida plus the Beroida–Haeckeliidae and the Pleurobrachiidae. The statistical support in the Lobata–Cestida–Thalassocalycida region of the tree is weak. To better illustrate the relationships within this subgroup, we performed a branch and bound search on a restricted data set consisting of lobates, cestids, and the thalassocalycid, plus a beroid and a platyctenid species as outgroups. The resulting single most parsimonious tree (length of 116 steps) is shown in Fig. 4, its topology being virtually identical to that of the subgroup in the full data set tree. The same tree was also obtained under maximum-likelihood criterion (lnL = −3237.4; not shown). There is strong support for the Ocyropsidae forming a clade separate from the other two lobate families (Leucotheidae and Bolinopsidae). Interestingly, the orders Cestida and Thalassocalycida appear closely related to the leucotheid and the bolinopsid lobates, although there is no bootstrap support for a particular tree topology. This is most likely caused by the extremely short branches (low phylogenetic signal) within this subgroup.

A distinct and well-supported subgroup consists of families Coeloplanidae (order Platyctenida) and Mertensiidae (order Cydippida). One undescribed species (undescribed sp. 3, a red-tentacled cydippid) connects at the base of this subgroup, but at this stage its affiliation with a traditional higher taxon has to be further analyzed based on morphological characters.

In the next step of the analysis, we used a data set that contained a number of outgroup taxa. These included species that belong to the basal metazoan phyla Porifera, Placozoa, and Cnidaria. To minimize the effects of long branches, common to most bilaterians, we also selected several sequences representing Platyhelminthes and Mollusca, which have been previously shown to be relatively slow evolving (Aguinaldo *et al.*, 1997; Campos *et al.*, 1998). As outgroups to metazoan animals, we used sequences from two choanoflagellates and from the fungus *Saccharomyces cerevisiae.* The use of outgroups allowed us to root the ctenophoran tree in an effort to infer the direction of evolution within this phylum and allowed its placement relative to the other basal metazoan phyla. To reduce the computational complexity of the analysis, we used the sequences of only 11 species of ctenophores, representing all the

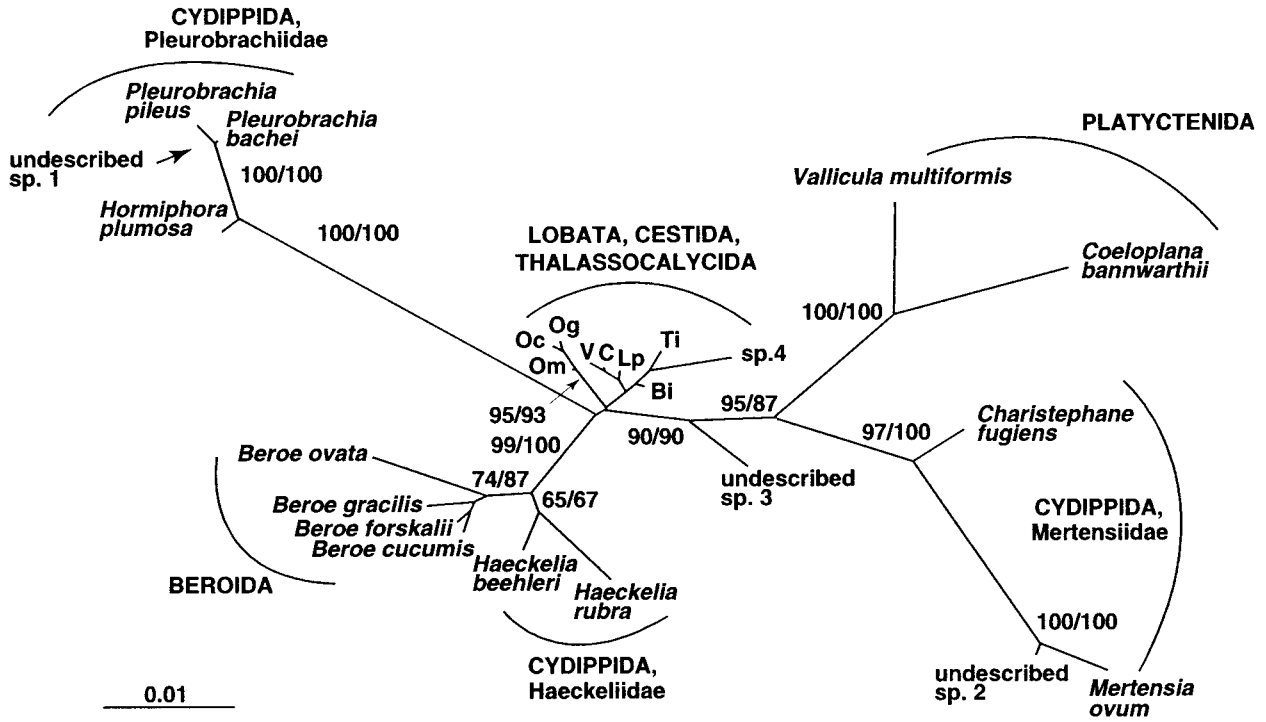

FIG. 3. Unrooted topology for Ctenophora found under maximum-likelihood (GTR + P_{invar} + Γ, ln L = −4342.7). The bootstrap values (parsimony/maximum-likelihood) less than 50 are not shown. Branch lengths reflect genetic distances among taxa. Abbreviations: Om, *Ocyropsis maculata;* Occ, *O. crystallina crystallina;* Ocg, *O. c. guttata* (Lobata, Ocyropsidae); Bi, *Bolinopsis infundibulum* (Lobata, Bolinopsidae); Lp, *Leucothea pulchra* (Lobata, Leucotheidae); C, *Cestum veneris;* V, *Velamen parallelum* (Cestida); Ti, *Thalassocalyce inconstans* (Thalassocaycida); sp. 4, undescribed species 4.

major subgroups. The maximum-likelihood search identified a single most likely tree, with the score of 8471 (Fig. 5). Bootstrapping provides support for the monophyly of Metazoa (bootstrap value of 88%) and for a sistership relationship between the Cnidaria and the bilaterian metazoans (represented here by flatworms and mollusks), Placozoa being immediately basal. With regard to the Ctenophora, although there was no support identifying a preferred branching pattern, we consistently observed a sister group relationship between that phylum and the other animals exclusive of the sponges.

The analysis reveals the monophyly of Ctenophora (100%) and suggests that the root of the ctenophore subtree occurs within the Mertensiidae (Cydippida)–Platyctenida group. Because of the weak bootstrap support (53%) we cannot strongly conclude whether the mertensiid cydippids or the platyctenids are closest to the root of the Ctenophora, although the most likely tree points to the mertensiids. All the other ctenophore taxa form a relatively well-supported cluster, composed of the same taxa recognized in the outgroup-free analysis: Pleurobrachiidae, Haeckeliidae–Beroida, and Lobata–Cestida.

An unexpected observation was that the genetic variability of 18S rDNA within Ctenophora was extremely low (i.e., very short branches) compared to that of the other related outgroup taxa (Porifera, Cnidaria, and Platyhelminthes). To obtain a quantitative estimate of these differences, we calculated the average genetic distances between species within each of the four phyla. As listed in Table 2A, the average distances (both the absolute distance and the maximum-likelihood evolutionary distance) between two ctenophore species are approx four to six times smaller than those between species of sponges, cnidarians, or flat worms. This difference is in fact underestimated, given that the alignments for outgroup phyla were significantly shorter, due to highly variable regions for which homology could not be unambiguously determined. To provide a more complete picture of the degree of genetic differences within Ctenophora, Table 2B shows the absolute distance matrix for 17 species of ctenophores representing all the major taxa sampled in this study.

DISCUSSION

Analysis of the 18S rDNA genes from 26 species revealed a highly conserved length (1801–1809 nt) and a low level of sequence divergence (<5%) of the small subunit rRNA in ctenophores. We are not aware of any other major metazoan phylum having such conserved ribosomal RNA genes. In most cases, 5–10% of the 18S rDNA nucleotides cannot even be reliably aligned in phylum-level phylogenies and are excluded from anal-

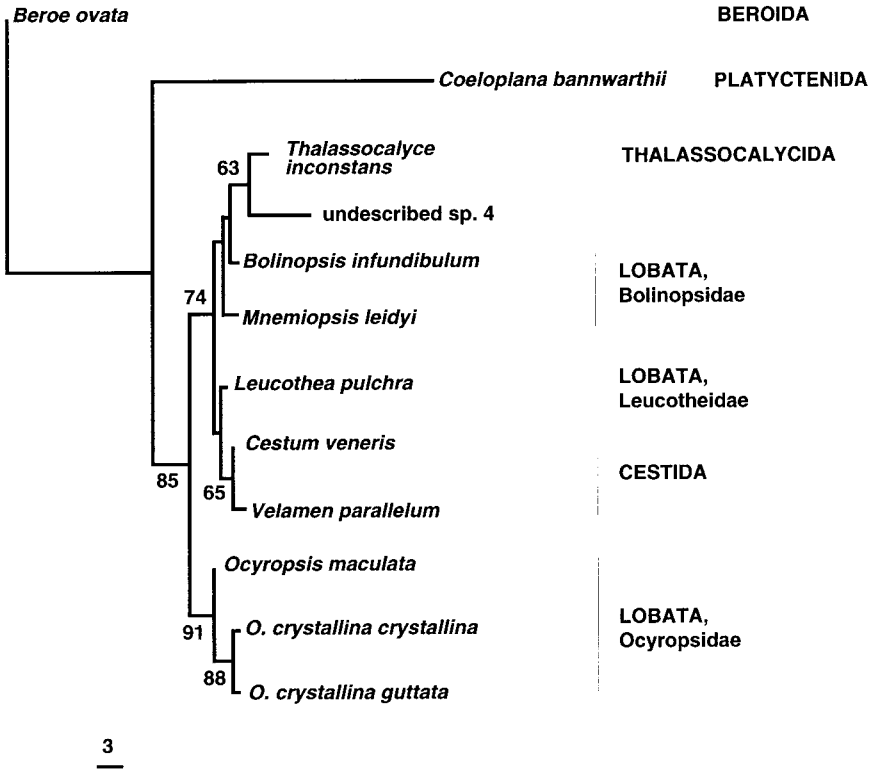

FIG. 4. Most parsimonious tree (length = 116 steps) for the Lobata–Cestida–Thalassocalycida group. The bootstrap values less than 50 are not shown. Horizontal branch lengths reflect genetic distances among taxa.

yses. This low level of divergence (which translates into short tree branches) prompted us to first analyze the phylogenetic relationships within Ctenophora in the absence of any long-branched outgroup taxa. As confidence in the reconstruction of evolutionary scenarios is strengthened by the obtaining of similar results with independent approaches, we used parsimony and maximum-likelihood methods side-by-side in data analysis. Whereas maximum-parsimony has been widely used to successfully infer phylogenies in the absence of assumptions about the evolutionary processes (i.e., without an evolutionary model), there are cases in which it has proven inconsistent due to the "long-branch attraction" phenomenon. By being able to accommodate complex evolutionary models, tailored for individual data sets, maximum-likelihood is far more consistent and robust than parsimony and allows for statistical comparisons of the resulting phylogenetic hypotheses (Swofford *et al.*, 1996).

Both parsimony and maximum-likelihood resulted in virtually identical tree topologies for Ctenophora. In addition, assessment of the reliability of individual internal branches by bootstrapping generated very similar values under both methods. These corroborating results strengthen our confidence in the evolutionary relationships within Ctenophora suggested by the 18S rDNA phylogeny.

One major result of the analysis is that the families of the traditional order Cydippida do not form a monophyletic group. Most authors have considered cydippids to be the ancestral ctenophore group, primarily because they resemble the developmental stages ("cydippid larva") of all the other groups except the Beroida. In fact, developmental stages of lobate ctenophores have probably been mistakenly described as "new" cydippid species (e.g., Dawydoff, 1946). The presence of a cydippid larva in the ontogeny of other orders strongly supports the ancestral position of some cydippids. In fact, "ontogeny recapitulates phylogeny" extremely clearly in many groups within the Ctenophora. However, some authors argue that the term "larva" should in fact be abandoned, since in ctenophores the transition from cydippid stage to adult is gradual, without an abrupt metamorphosis (Martindale and Henry, 1997).

Harbison (1985), ignoring a number of poorly known species, tentatively divided the order Cydippida into five families (Haeckeliidae, Lampeidae, Bathyctenidae, Pleurobrachiidae, and Mertensiidae), sharing the same overall morphology: globular or ovoid body with one pair of retractable tentacles, exiting through tentacle sheaths, well-developed comb rows, with paragastric and meridional canals ending blindly at the oral pole. Harbison (1985) challenged the monophyly of Cydippida, based on specific morphological characters that appeared to relate individual cydippid families

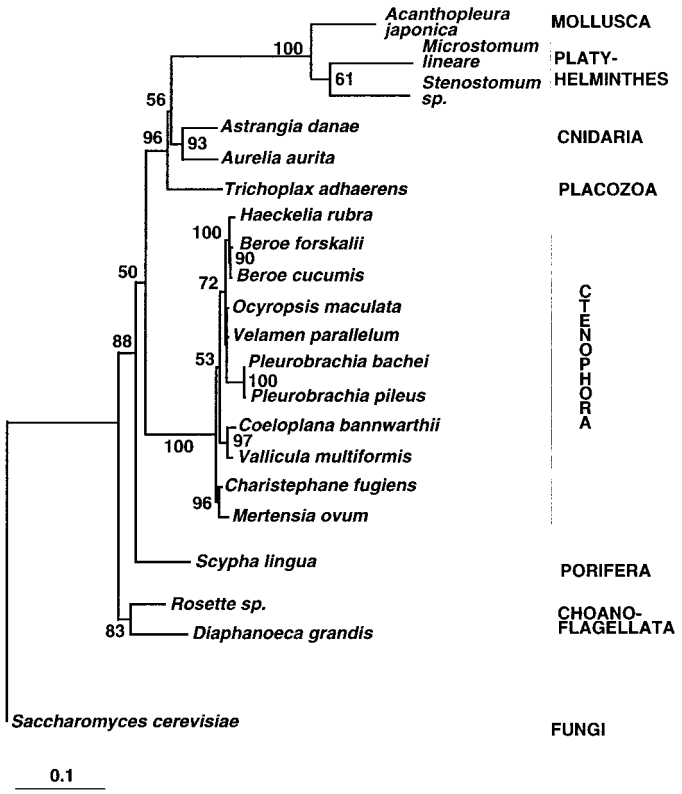

FIG. 5. Topology of the basal Metazoan phyla including Ctenophora under maximum-likelihood (GTR, $P_{invar} = 0.38$, $\Gamma = 0.753$, $\ln L = -8471.057$). The tree was rooted with fungi as outgroup. Bootstrap values lower than 50 are not shown. Horizontal branch lengths reflect genetic distances among taxa.

more closely to members of other orders than to each other. Although we have sampled representatives of three cydippid families only (Haeckeliidae, Pleurobrachiidae, and Mertensiidae), our analysis fully supports that view and definitively rejects the monophyly of the Cydippida. The haeckelids show a close relationship with the order Beroida (99/100 bootstrap support). Harbison suggested that both groups were closely related, based on morphology. Species of both taxa share the same overall body shape, the presence of aboral papillae, great reduction of the infundibular canal, and adradial canals emerging directly from the infundibulum.

A second cydippid family, Pleurobrachiidae, represented in our analysis by species of the genera *Hormiphora* and *Pleurobrachia* and the undescribed species 1 (not shown), displays a long radiation branch from its divergence node. Due to the short branch and lack of bootstrap support, we cannot clarify the relationship between the pleurobrachiids and the cluster of ctenophores consisting of the orders Lobata, Cestida, and Thalassocalycida. A close relationship between these taxa was proposed by Harbison (1985), based on similar characters (adradial canals united by an interradial canal, and tentacles exiting aborally in adult pleurobrachiids or during early development in lobates and cestids).

The third cydippid family included in our study, the Mertensiidae, shows a sistership relationship with representatives of order Platyctenida. One interesting finding is the close sequence similarity between the prototyical mertensiid *Mertensia ovum,* an arctic species, and a yet undescribed mertensiid species (species 2, in Fig. 1B) which inhabits the tropics. The two species differ by only 2 nucleotides at the level of the 18S rDNA genes, although anatomically they are quite distinct. Short genetic distances at the level of 18S rDNA genes have also been reported for sibling species of anthozoans, but they were on the order of 16–18 nucleotides (Berntson *et al.,* 1999).

The order Platyctenida is composed of creeping or sessile ctenophores that are strongly compressed in the oral–aboral axis and have lost the comb rows as adults in all but one genera. We were able to sample one of the four families of this order (Coeloplanidae), represented by *Vallicula multiformis,* a creeping species from the Carribean, and by *Coeloplana banwarthii,* an ectocommensal on the echinoid *Diadema setosum* from Madagascar. It should be noted that Harbison (1985) proposed a close relationship between the Mertensiidae and the Platyctenida based on the connections of the adradial and tentacular canals with the infundibulum. The fact that both the molecular and the morphological evidence produce similar results provides strong support to the hypothesis that the two groups are closely related. In conclusion, our analysis fully supports the previous challenge to the monophyly of Cydippida and suggests that revisions are indeed warranted.

The Lobata–Cestida–Thalassocalycida represents a region of the tree for which we could not ascribe a high-confidence topology. We believe that the reason for this resides in the low degree of variation at the level of the 18S rDNA genes in these taxa. For example, there is only a three-nucleotide difference between *Leucothea pulchra* (order Lobata) and *Cestum veneris* (order Cestida) and a seven-nucleotide difference between *Bolinopsis infundibulum* (order Lobata) and *Thalassocalyce inconstans* (order Thalassocalycida). These similarities translate into extremely short branches and strong destabilizing effects resulting from imperfections in the evolutionary models being used. To minimize the destabilizing effects of other long-branched taxa, we investigated the topology of the Lobata–Cestida–Thalassocalycida in the presence of different other ctenophore orders used as outgroups (Fig. 4). Both the most parsimonious and the maximum-likelihood trees suggest that the group is monophyletic. The lobate family Ocyropsidae appears to descend directly from a common ancestor that also gave rise to a diverse group that includes two other lobate families (Bolinopsidae and Leucotheidae) in addition to the cestids and the thalassocalycids. The morphologi-

TABLE 2

(A) Average Absolute Distances (Nucleotide Differences) and Evolutionary Distances (Substitutions/Site) Based on Maximum-Likelihood (ML) (GTR + P_{invar} + Γ) Model within Lower Metazoan Phyla; (B) Absolute Pairwise Distance Matrix (Nucleotide Differences) for Phylum Ctenophora (Representative Species)

(A)

Phylum	No. of species	No. of aligned nucleotides	Average distances	
			Absolute	ML
Ctenophora	24	1813	42 ± 20	0.032 ± 0.018
Porifera	6	1703	202 ± 63	0.245 ± 0.098
Cnidaria	6	1709	178 ± 92	0.181 ± 0.133
Platyhelminthes	15	1776	299 ± 58	0.344 ± 0.095

(B)

	Ctenophores						
	Hb.	Pp	Cf	Vm	Bi	Vp	Bg
Hr	17	69	55	65	29	31	25
Pb	61	3	74	74	52	53	61
Mo	68	87	40	70	60	63	66
Cb	65	79	55	34	58	58	67
Ti	25	55	41	51	7	9	33
Lp	23	53	39	51	5	5	31
Om	25	55	42	45	11	11	31
Cv	24	56	40	52	6	2	32
Bo	23	67	53	61	32	34	19
Bf	17	62	51	61	25	27	9

Note. Hb, *Haeckelia beehleri;* Hr, *H. rubra;* Pb, *Pleurobrachia bachei;* Pp, *P. pileus;* Cf, *Charistephane fugiens;* Mo, *Mertensia ovum;* Vm, *Vallicula multiformis;* Cb, *Coeloplana banwarthii;* Bi, *Bolinopsis infundibulum;* Lp, *Leucothea pulchra;* Om, *Ocyropsis maculata;* Ti, *Thalassocalyce inconstans;* Vp, *Velamen parallelum;* Cv, *Cestum veneris;* Bg, *Beroe gracilis;* Bo, *B. ovata;* Bf, *B. forskalii.*

cal evidence for the monophyly of the lobates is very strong, and ocyropsids have only one distinctive morphological character that separates them from the other lobates: the absence of tentacular apparatus in adults. This difference is due, however, to secondary loss during development, since both species of *Ocyropsis* have functional tentacles as larvae (Harbison and Miller, 1986) and adult *O. maculata* still retain the tentacular canal (Harbison, 1985). Three other families of lobates (Eurhamphaeidae, Bathocyroidae, and Kiyohimeidae) are not represented in our study and, therefore, the detailed phylogeny of the lobates will have to be addressed in the future.

The use of outgroup taxa allowed us to root the ctenophore tree, to establish a direction of evolution within Ctenophora, and to test its relationship with other metazoan phyla. The maximum-likelihood analysis fully supports the monophyly of Ctenophora and tentatively places the root for the phylum closest to the cydippid family Mertensiidae (Fig. 5). We can therefore, for the first time, compare hypotheses of phylogenetic transitions within Ctenophora obtained using molecular and morphological data.

Traditionally, the tentaculate cydippid body plan is considered primitive, with all the others having been secondarily derived as a result of adaptation to different environments and life styles. The primary reason for this view is the existence of the cydippid larva in the ontogeny of all ctenophoran groups. Unfortunately, the fossil record of Ctenophora is extremely sparse. The first fossil specimen purported to be ctenophore (*Paleocydippida brasseli*) was reported from the lower Devonian (Stanley and Stürmer, 1983). Since then, a number of specimens purported to be ctenophores have been reported from the Cambrian, including some which appear to lack tentacles (e.g., Chen *et al.*, 1991; Conway Morris and Collins, 1996). If those beroid-like remains are indeed ctenophores, then these early fossil records might indicate that the phylum arose from an atentaculate ancestor.

Given the great morphological diversity of modern cydippids and the recent view that the individual cydippid families are related more closely to other orders than to each other, the "ancestral cydippid" hypothesis becomes quite ambiguous. An alternative evolutionary scenario was proposed by Harbison (1985), suggesting that the first ctenophores could have had an atentaculate sack-like body, resembling modern beroids. The results of our analysis are contrary to the ancestral beroid hypothesis (but see below) and point to the mertensiid cydippids as being closest to the last common ancestor of modern ctenophores. The cydippid body plan was preserved and further diversified, resulting in today's cydippid families, which can be characterized as paraphyletic at most, thereby rejecting the traditional order Cydippida. Due to a weak bootstrap

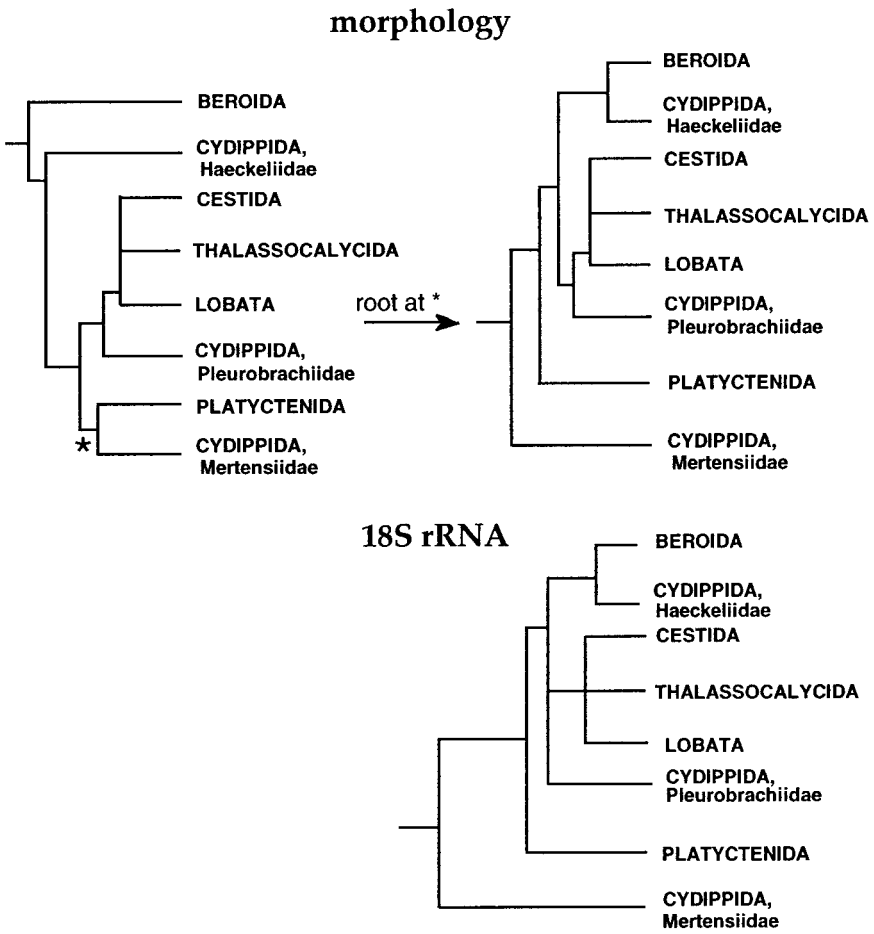

FIG. 6. Comparison between a hypothesis of ctenophore phylogeny based on morphology (Harbison, 1985) and the 18S rRNA phylogeny.

support the platyctenids may also appear to be candidates for ancestral status, but we consider that unlikely. Platyctenids are specialized, sessile or creeping ctenophores, with a body strongly compressed in the oral–aboral axis. All platyctenes, whether or not they have comb rows as adults, have functional comb rows as larvae. In many species, these larvae are not released, but are retained in brood pouches and are not released until the comb rows are regressed. Although it is not impossible that most extant ctenophores are descended from the larvae of platyctenes, since there are examples of such evolution in other groups (the Appendicularia in the Urochordata, for example (Lohmann, 1933)), it appears more likely that the Platyctenida evolved from a mertensiid-like pelagic ancestor. The other ctenophore body plans (lobate, cestid, beroid) are also secondarily derived and there is a surprisingly good agreement between the molecular and the anatomical data (Fig. 6).

The phylogenetic placement of Ctenophora within the metazoans continues to be unresolved. The traditional view, which considered ctenophores to be closely related to cnidarians and grouped with the Coelenterata, has been abandoned (Harbison, 1985). Now the challenge is to distinguish between two new and radically different hypotheses. The first, based on morphological, physiological, and developmental characters, considers ctenophores more closely related to Bilateria than are the cnidarians (Martindale and Henry, 1998). Nielsen (1995) tentatively treated ctenophores as a sister group to bilaterian deuterostomes, although he has since modified this position and now considers them basal to Bilateria (Nielsen, 2001). Certain characters common with bilaterians can be identified in ctenophores: a cellular mesenchyme with true muscle cells, synapses with acetylcholine, and a specialized nervous system. Ctenophores have been for decades one of the favorite subjects of study in embryology (Chun, 1880; Freeman, 1977; Martindale and Henry, 1998). They have evolved a unique and highly determinate cleavage pattern in early embryogenesis (unipolar, with diagonal determination), which is indicative of the important developmental pathways that occur during the first several cell divisions. The mesodermal lineage is generated by endodermal precursors (oral micromeres) and not by ectodermal precursors, which could relate ctenophores to bilaterians. The biradial symmetry found in the adult is unique among

metazoans, and its phylogenetic significance is unknown.

The second view, based upon 18S analyses with only one or two ctenophore species and summarized recently by Collins (1998), considers ctenophores to be the most primitive of metazoans at the tissue level of organization. Hence, they are basal to Cnidaria and Placozoa. According to this view, the apparent shared derived characters in Ctenophora and Bilateria either arose independently or were secondarily lost in Cnidaria and Placozoa. It is difficult, however, to explain how highly specialized subcellular structures such as synapses with acetylcholine could have appeared independently twice, and why the more complex muscular and nervous systems of ctenophores would have been simplified in cnidarians, of which many are actively swimming predators. What the molecular studies have usually neglected is the weak statistical support (bootstrap values or Bremmer indexes) for the position of Ctenophora, and in all cases they included only one or two ctenophore species. It is also possible that the 18S rDNA gene lacks adequate signal to resolve the evolutionary history of the lower metazoans. Some studies claim limited utility of rRNA for the inference of deep phylogenies related to the Cambrian "explosion" (Abouheif *et al.*, 1998; Philippe *et al.*, 1994). It is believed that corroborating results from the use of multiple gene phylogenies (including genes of the Hox cluster, elongation factor a, tubulins) will improve the reliability and resolution of the metazoan rDNA trees, and such studies are beginning to emerge (Baldauf *et al.*, 2000).

Our study is the first molecular phylogenetic analysis to provide an extensive coverage of the phylum Ctenophora. We expected that any inconsistencies in the positioning of this phylum relative to other metazoans that might have been previously caused by limited taxon sampling would be now eliminated. Instead, our results are similar to those described in previous studies (e.g., Bridge *et al.*, 1995; Kim *et al.*, 1999). Whereas the use of maximum-likelihood models of evolution, which incorporate variations in the substitution rates across the gene, allowed us to obtain a most likely tree in which ctenophores branch off before cnidarians, the statistical support is weak. We therefore must conclude that the placement of Ctenophora within Metazoa remains an open subject that must be addressed in future anatomical, developmental, and molecular studies. We believe, however, that our analysis has uncovered the lowest level of sequence variability at the level of the 18S rDNA gene in any metazoan phylum. One possible explanation for this phenomenon could be an unusually low substitution rate for this gene in ctenophores. Although we cannot explain why the 18S rDNA genes would have such a characteristic in this phylum, if true it may in part explain the debatable position of Ctenophora within Metazoa resulting from molecular phylogenies. It is therefore imperative that protein-encoding genes be sequenced and their phylogenies compared to that of the 18S rDNA. If a low level of genetic variability exists in these genes also, it could suggest that the extant ctenophores are all derived from a relatively recent common ancestor, a possibility that has never been addressed before. It has always been assumed that Ctenophora represents an ancient metazoan phylum, based on their simplicity. Unfortunately, the inadequate fossil record does not provide solid clues with regard to the antiquity of this phylum. The oldest purported ctenophore fossils are from the middle Cambrian (Conway Morris and Collins, 1996). Our genetic data could also be construed to suggest that the Ctenophora recently passed through a bottleneck (perhaps at the K–T boundary), and thus all extant taxa are relatively young. Thus, whereas the phylum may be so ancient that no clear relationship with other metazoans can be discerned with the 18S rRNA gene, all the extant taxa may have evolved from a relatively recent common ancestor. Figure 5 seems to support this hypothesis, since there is a long branch leading to the phylum Ctenophora and very short branches within the phylum. This would explain the 75-year old puzzle (Krumbach, 1925) that has not yet been answered by either morphologists or molecular biologists: "Although it is easy in a given case to determine whether or not a particular animal is a ctenophore, it is equally difficult to establish how closely or distantly ctenophores are related to other forms of animals." Harbison (1985) essentially confirmed Krumbach's (1925) enigma, finding no morphological evidence that allied the Ctenophora with any other extant phylum. Numerous molecular studies using the 18S rRNA gene (including this one) have come to the same conclusion. Perhaps some other gene can be used to establish such a relationship, or perhaps molecular biogeographic data could provide helpful information with regard to the timing of the last major radiation event within the phylum. Thus far, perhaps more than is the case for most other phyla, the evolution of ctenophores continues to hold many questions but relatively few answers.

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