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# Urochordates Are Monophyletic Within the Deuterostomes

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*Abstract*.—Understanding the phylogenetic relationships of the three major urochordate groups within the deuterostomes is central to understanding the evolution of the chordates. We have prepared a detailed phylogenetic analysis of urochordates based on comparisons of 10 new urochordate 18S ribosomal DNA sequences with other urochordate sequences in GenBank. Maximum parsimony, neighbor-joining, minimum evolution, and maximum likelihood analyses of this large urochordate data set are consistent with a topology in which the urochordates are monophyletic within the deuterostomes and there are four separate clades of urochordates. These four distinct clades—styelid + pyurid ascidians, molgulid ascidians, phlebobranch ascidians + thaliaceans, and larvaceans—are mostly consistent with traditional morphological hypotheses and classifications. However, we find that the ascidians may not be a monophyletic group (as they have been considered traditionally) but instead appear paraphyletic. Another disparity with traditional classification is that the thaliaceans do not form a separate urochordate clade but rather cluster with the phlebobranch ascidians. Larvaceans have long branch lengths, which can be problematic for molecular phylogenetic methods, and their position within the urochordates cannot be unequivocally determined with 18S rDNA. This is important because the tadpole morphology of larvacean and ascidian larvae is the key trait of interest that distinguishes urochordates as chordates. Nevertheless, the present data set resolves at least three clades of urochordates and suggests strongly that urochordates form a monophyletic clade within the deuterostomes. [Ascidians; chordate evolution; deuterostome evolution; larvaceans; thaliaceans; urochordate evolution; urochordate phylogeny.]

It is important to understand the phylogenetic relationships between the various deuterostome phyla and the chordate subphyla to study possible mechanisms of chordate evolution. The phylum Chordata contains three subphyla: Urochordata (tunicates), Cephalochordata (amphioxus), and Vertebrata (vertebrates). The urochordates are further divided into three classes: the ascidians (sea squirts), larvaceans (appendicularians), and thaliaceans (doliolids, salps, and pyrosomids). The chordate characters of a dorsal hollow nerve chord, notochord, and postanal muscular tail are found in the urochordate tadpole in the larvaceans and most ascidians (Jeffery and Swalla, 1997). However, ascidians undergo metamorphosis into a sessile adult with an endostyle and pharyngeal gill slits, whereas the larvaceans retain the tadpole structures and the tadpole body plan after metamorphosis into a planktonic adult. Salps and pyrosomids lack a tadpole stage and have a body plan similar to adult ascidians except that it is modified for planktotrophy. Recent molecular evidence suggests that larvaceans may be the sister group of the rest of the urochordates, and the phlebobranch ascidians and thaliaceans may be closely related (Wada, 1998).

Morphological evidence suggests many taxa have deuterostome affinities (Kirsteuer, 1969), but recent molecular phylogenies suggest that lophophorates (Halanych et. al., 1995; Halanych, 1996a; Mackey et al., 1996), chaetognaths (Telford and Holland, 1993; Halanych, 1996b), and the pogonophoron worms (Young et al., 1996; McHugh, 1997) are likely to be protostomes. Current

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phylogenies using both morphological and molecular evidence agree that the echinoderms, hemichordates, and chordates are unequivocally deuterostomes (Turbeville et al., 1994; Wada and Satoh, 1994; Zrzavy et al., 1998). Recently, 18S ribosomal DNA (rDNA) has been used by several researchers to construct molecular phylogenies of the urochordates within the deuterostome phyla (Field et al., 1988; Wada et al., 1992; Turbeville et al., 1994; Wada and Satoh, 1994; Hadfield et al., 1995; Cohen et al., 1998; Wada, 1998). Ribosomal DNA sequence data are used to reconstruct phylogenies because they offer many informative characters for inferring relationships, are highly conserved and ubiquitous among metazoans, and provide an independent test of hypotheses that are based on morphology (Olsen and Woese, 1993; Turbeville et al., 1994). Some of the branches of deuterostome phylogeny are not well supported by 18S rDNA, in part because of unequal evolutionary rates among taxa and light sampling of taxa within the urochordates (Turbeville et al., 1994; Wada and Satoh, 1994).

In this study, we include 18S rDNA sequences of individuals from 7 of the 14 families of ascidians, three larvaceans, and three thaliaceans (a salp, a pyrosomid, and a doliolid), in an effort to resolve relationships within the urochordates and examine whether urochordates are monophyletic within the deuterostomes. More than 2,500 ascidian species have been described (Satoh, 1994), but only about a hundred species of larvaceans and even fewer species of salps, pyrosomids, and doliolids (Berrill, 1950; Bone, 1998). We have included 26 urochordate 18S rRNA sequences found in the GenBank database and have added 10 new sequences in the present analysis. Besides including more taxa, we also introduce an alignment guided by secondary structure (Neefs et al., 1993), which differs from the 18S rDNA alignments used in previous studies of chordate phylogeny (Turbeville et al., 1994; Wada and Satoh, 1994). Our results suggest that there are at least three distinct clades of urochordates—one that includes the Pyuridae and Styelidae families, a second representing the Molgulidae family (Hadfield et al., 1995), a third containing all of the Phlebobranch families and the thaliaceans—and larvaceans may constitute a fourth clade. Our phylogenetic analyses show clearly that urochordates are monophyletic within the deuterostomes, a result strongly supported by each of the phylogenetic methods used to analyze this dataset.

## Materials and Methods

### *Biological Materials, DNA Isolation, and DNA Sequencing*

Urochordate DNA was isolated according to Hadfield et al. (1995) and resuspended in a buffer of 10 mM Tris-Cl, 1 mM EDTA, pH 8 at 1 mg/ml. DNA was then diluted in sterile distilled water to an optimal concentration for polymerase chain reaction (PCR), which was used to amplify a region of ~1,000 bp of the 18S rDNA. PCR reactions were performed with standard conditions (10 mM Tris-HCl, 1.5 mM MgCl, primer, and 50–100 ng of DNA) in a Stratagene Robocycler (Stratagene, La Jolla, CA). Annealing temperature varied slightly from 40°C to 50°C, depending on the primers. PCR products were separated with a 1% agarose gel and photographed with a Polaroid camera. The desired band was excised and purified with Sephaglas beads (Pharmacia Biotech, Piscataway, NJ). Sequencing was performed on an ABI Automatic Sequencing Machine (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ) after sequencing reactions had been performed with an ABI Cycle Sequencing Kit.

The primers used for PCR amplification and subsequent DNA sequencing were 18S-A 5'-CAGCAGCGCGGTAATTCCAGC TC-3' and 18S-B 5'-AAAGGGCAGGGACG TAATCAACG-3' (Wada et al., 1992; Hadfield et al., 1995). Additional primers used for sequencing were 18S-C 5'-TTAGAGT GTTCAAAGCAGGC-3', 18S-F 5'-GCCTGCT TTGAACACTCTAA-3', 18S-G 5'-GCGATCA GATACCGCCCTAGTT-3', and 18S-H 5'-C GTTCTTAGTTGGTGGAGCGAT-3'. In some cases, nearly full-length 18S sequences were obtained by using additional primers:

18S-BS5'-CCTGGTTGATCCTGCCAG-3' (5' end) and 18S-PH 5'-TAATGATCCATCT GCAGGTTCACCT-3' (3' end).

The regions of the 18S gene in which each of the primers is located are as follows, according to the eukaryotic secondary structure determined by De Rijk et al. (1992) and Neefs and De Wachter (1990): 18S-A (region 19), 18SB (region 30), 18S-C and 18S-F (region E21-6 in V4, primers are complementary), 18S-G (region 25), and 18S-H (region 37-38).

### *Sequences*

The 18S rDNA sequences used for this study are shown in Table 1. Unpublished sequences were generated for this study as indicated; available accession numbers for these sequences from GenBank are listed in Table 1. The following sequences were obtained from Dr. Hiroshi Wada and Noriyuki Satoh and deposited into GenBank (Wada et al., 1992; Satoh, 1994): *Ascidia sydneiensis*, *A. zara*, *Ciona intestinalis*, *C. savignyi*, *Chelyosoma siboja*, *Corella japonica*, *Halocynthia roretzi*, *Perophora japonicus*, *Polyandrocarpa misakiensis*, *Pyura mirabilis*, and *Symplegma reptans*.

### *Alignment and Phylogenetic Analyses*

Urochordate sequences were aligned with those of other deuterostomes and protostomes (see Table 1) according to a secondary structure model (Neefs et al., 1993) using the DCSE program (De Rijk and De Wachter, 1993). The five protostome sequences were used as an outgroup to the deuterostomes so we could examine the relationships of the urochordates within the deuterostomes. When either vertebrates or echinoderms were used as outgroups, the urochordates showed the same phylogenetic relationships (Swalla and Garey, personal obs.; Wada, 1998). Sites containing gaps were excluded from phylogenetic analyses to reduce systematic errors. Alignments were analyzed with the MEGA program (Kumar et al., 1994) and PAUP*4.0b2 (Swofford, 1999) to produce Neighbor-Joining (NJ) trees using Jukes and Cantor (Jukes and Cantor, 1969), Kimura two-parameter (Kimura, 1980), and LogDet (Lake, 1994) models of nucleotide substitution to correct for multiple substitutions at the same site. For the gamma distribution model, three different values were used for the shape parameter alpha. The value $\alpha = 0.24$ was calculated from the data set by using maximum likelihood with PAUP*, whereas $\alpha = 0.5$ was used as the PAUP* default value, and $\alpha = 0.72$ was used because it has been used in numerous rDNA studies of metazoan evolution (e.g., Winnepenninckx et al., 1995). Minimum evolution (ME) trees were produced by heuristic searches in PAUP* under the same models of nucleotide substitution described above for NJ trees. Bootstrapped maximum parsimony (MP) trees were produced by using PAUP*. Maximum likelihood (ML) trees were calculated with the DNAML component of PHYLIP (Felsenstein, 1993) from a subset of the data containing 24 taxa that used substitution models with various multiple rate categories. Confidence in NJ, ME, MP, and ML trees was determined by analyzing either 1000 or 100 bootstrap replicates (see figure legends). An additional parameter used to determine tree reliability was the congruence of trees produced with different tree-making algorithms (NJ, ME, MP, and ML).

### *Results*

The alignment was 966 positions long with gaps, containing a total of 821 sites when gaps and missing data were excluded; and there were 238 informative sites for parsimony analysis. The alignment is available at http: //chuma.cas.usf.edu/ ~garey/alignments/ alignment.html. Figures 1, 2, 3, and 4 show the phylogenetic trees obtained with NJ, ME, MP, and ML methods, respectively. The NJ tree calculated with Kimura two-parameter evolutionary distances is shown in Figure 1 with branches drawn to scale. In general, this analysis agrees with traditional classification of urochordates at the family level, but there are major discrepancies at higher tax-

TABLE 1. 18S rDNA sequences and common names of the taxa used in these analyses.

| Taxonomic designation | Accession number | Common name |
|---|---|---|
| Phylum Chordata | | |
| Subphylum Vertebrata | | |
| *Homo sapiens* | M10098[a] | human |
| *Rattus norvegicus* | K01593[b] | rat |
| *Petromyzon marinus* | M97575[c] | hagfish |
| Subphylum Cephalochordata | | |
| *Branchiostoma floridae* | M97571[c,d] | amphioxus |
| Subphylum Urochordata | | |
| Class Larvacea | | |
| *Oikopleura dioica* | AB013014[e] | larvacean |
| *Oikopleura species* 1 | D14360[d,e] | larvacean |
| *Oikopleura species* 2 | AB013015[e] | larvacean |
| Class Thaliacea | | |
| Order Salpida | | |
| *Thalia democratica* | D14366[d,e] | salpid (colonial) |
| Order Pyrosomida | | |
| *Pyrosoma atlanticum* | AB013011[e] | pyrosomid (colonial) |
| Order Doliolida | | |
| *Doliolum nationalis* | AB013012[e] | doliolid (colonial) |
| Class Ascidiacea | | |
| Order Enterogona | | |
| Suborder Phlebobranchiata | | |
| Family Ascidiidae | | |
| *Ascidia ceratodes* | L12378[f] | ascidian |
| *Ascidia sydneiensis* | AF165819[g,m] | ascidian |
| *Ascidia zara* | AF165820[m] | ascidian |
| Family Cionidae | | |
| *Ciona intestinalis* | AB013017[e] | ascidian |
| *Ciona savignyi* | AF165823[g,m] | ascidian |
| Family Perophoridae | | |
| *Perophora japonica* | AF165824[g,m] | ascidian (colonial) |
| Family Corellidae | | |
| *Chelysoma siboja* | AF165821[m] | ascidian |
| *Corella japonica* | AF165822[m] | ascidian |
| Order Pleurogona | | |
| Suborder Stolidobranchiata | | |
| Family Molgulidae | | |
| *Bostrichobranchus digonas* | L12379[f] | ascidian |
| *Eugyra arenosa* | L12414[f] | ascidian |
| *Molgula bleizi* | L12418[f] | ascidian |
| *Molgula citrina* | L12420[f] | ascidian |
| *Molgula complanata* | L12422[f] | ascidian |
| *Molgula manhattensis* | L12426[f] | ascidian |
| *Molgula occidentalis* | L12428[f] | ascidian |
| *Molgula occulta* | L12430[f] | ascidian |
| *Molgula oculata* | L12432[f] | ascidian |
| *Molgula provisionalis* | L12434[f] | ascidian |
| *Molgula socialis* | L12436[f] | ascidian |
| *Molgula tectiformis* | L12438[f] | ascidian |
| Family Styelidae | | |
| *Cnemidocarpa finmarkiensis* | L12413[f] | ascidian |
| *Dendrodoa grossularia* | L12416[f] | ascidian |
| *Pelonaia corrugata* | L12440[f] | ascidian |
| *Polyandrocarpa misakiensis* | AF165825[g,m] | ascidian (colonial) |
| *Polycarpa pomaria* | L12441[f] | ascidian |
| *Styela clava* | L12442[f] | ascidian |
| *Styela plicata* | L12444[f] | ascidian |
| *Symplegma reptans* | AF165826[g,m] | ascidian (colonial) |

TABLE 1. Continued.

| Taxonomic designation | Accession number | Common name |
|---|---|---|
| Family Pyuridae | | |
| *Halocynthia roretzi* | AB013016[d,e] | ascidian |
| *Herdmania curvata (momus)* | AF165827[m] | ascidian |
| *Pyura mirabilis* | AF165828[g,m] | ascidian |
| Phylum Hemichordata | | |
| *Balanoglossus carnosus* | D14359[d,e] | acorn worm |
| Phylum Echinodermata | | |
| *Ophioplocus japonicus* | D14361[d] | brittle star |
| Phylum Arthropoda | | |
| *Artemia salina* | X01723[h] | brine shrimp |
| *Tenebrio molitor* | X07801[i] | meal worm |
| *Eurypelma californica* | X13457[j] | bird spider |
| Phylum Annelida | | |
| *Nephtys hombergii* | U50970[k] | polychaete worm |
| *Glycera americana* | U19519[l] | bloodworm |

[a]Torczynski et al., 1985.
[b]Chan et al., 1984.
[c]Stock and Whitt, 1992.
[d]Wada and Satoh, 1994.
[e]Wada, 1998.
[f]Hadfield et al., 1995.
[g]Wada et al., 1992.
[h]Nelles et al., 1984.
[i]Hendriks et al., 1988a.
[j]Hendriks et al., 1988b.
[k]Nadot and Grant, 1996; direct submission to GenBank.
[l]Halanych et al., 1995.
[m]Results from this paper.

onomic levels. The most significant result is that urochordates consistently fell into four clades, with high bootstrap support. Clade I contains styelid and pyurid ascidians; Clade II consists solely of molgulid ascidians; Clade III contains a number of different families within the phlelbobranch ascidians plus the thaliaceans; and the larvaceans make up Clade IV. The ascidian order Pleurogona appears polyphyletic, with some families appearing in Clade I (Styelidae and Pyuridae) and some in Clade II (Molgulidae).

NJ trees calculated with other distance methods were nearly identical with respect to Clades I, II, III, and IV (see Table 2). ME trees were congruent with the NJ trees. Figure 2 shows the ME tree found by PAUP* with Kimura two-parameter distances as the optimality criterion. Similar results were found with other distance methods (Table 2).

Two MP trees of 1135 steps were recovered by using heuristic searches with PAUP* with default parameters. A bootstrapped MP (Fig. 3) is consistent with the NJ and ME trees, although taxa that form the distinct Clade III with NJ or ME analyses instead form a mixed polytomy in the bootstrapped MP tree.

ML analyses of 100 bootstrap replicates (Fig. 4) with a reduced data set (complete alignment, but only 24 taxa) revealed results similar to the MP analysis; that is, the taxa that formed the distinct Clade III with NJ or ME analyses instead formed a mixed polytomy in the bootstrapped ML tree. The ML trees were recovered by using DNAML and associated programs from PHYLIP with use of four rate categories: 100 (0.13 of sites), 10 (0.13 of sites), 1 (0.14 of sites), and 0 (0.6 of sites). Initial rate categories were determined by examining the alignment (for example, 60% of the sites are invariant) and then modified empirically to maximize the likelihood of the recovered tree. The best ML tree had a ln likelihood of −5335.

Figure 5 summarizes the hypothetical relationships of the urochordates, showing the morphological changes that have occurred in adult and larval body plans. Our results clearly indicate that colonial adults have evolved several times independently within the urochordates. Figure 2 shows the species in which adults are colonial. Within the thaliaceans, the pyrosomids and salps seem to have secondarily lost a tadpole larval stage, whereas the doliolids retain the tadpole larvae (Bone, 1998).

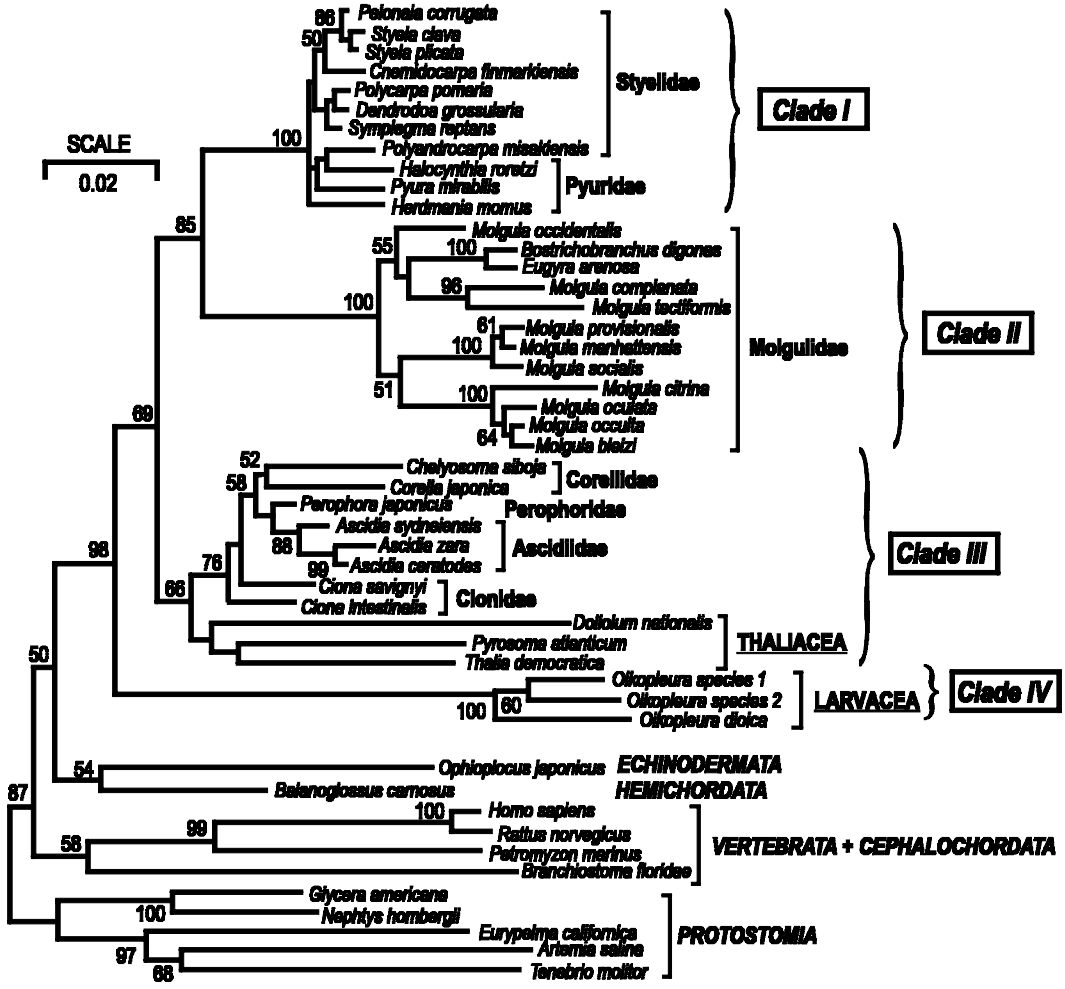


FIGURE 1. The neighbor-joining tree was calculated by using Kimura two-parameter evolutionary distances with branches drawn to scale. The major urochordate groups are labeled as Clades I, II, III, and IV and do not completely agree with morphology-based urochordate phylogenetic hypotheses. Bootstrap values are shown as percentages of 1000 replicates at each node only when ≥50%. Urochordate families are marked with square brackets, and urochordate order names are underlined. NJ trees calculated with a number of other evolutionary distance methods were consistent with the tree shown above (see Table 2).

## DISCUSSION

Increasing the number of urochordate taxa and sampled using secondary structure features as a guide provided a relatively unambiguous alignment. Although our data set does not resolve the deep deuterostome branches as well as some previous studies that used the entire 18S rDNA (Turbeville et al., 1994; Wada and Satoh, 1994), there is good resolution of relationships within the urochordates.

Relationships among the three classes of urochordates based on earlier morphological studies have been unclear (cf. Ihle, 1913; Garstang, 1928; Berrill, 1936; Holland, 1988). Molecular studies have agreed with Ihle (1913) and Berrill (1936), showing support for the larvaceans as the sister group to the rest of the urochordates (Wada and Satoh, 1994; Wada, 1998). Our NJ and ME results agree with previous studies, but MP and ML analysis show an unresolved polytomy

TABLE 2. Bootstrap values obtained through different phylogenetic methods, supporting the monophyly of the urochordates and Clades I, II, and III within the urochordates.

| Bootstrap values | Urochordates | Clade I | Clade II | Clade III |
|---|---|---|---|---|
| NJ LogDet | 99 | 100 | 100 | 54 |
| NJ Jukes–Cantor | 100 | 100 | 100 | 61 |
| NJ Kimura | 98 | 100 | 100 | 66 |
| NJ Kimura $\gamma = 0.24$ | 99 | 98 | 100 | 65[a] |
| NJ Kimura $\gamma = 0.50$ | 99 | 100 | 100 | 67[a] |
| NJ Kimura $\gamma = 0.72$ | 100 | 99 | 100 | 72 |
| ME LogDet | 99 | 100 | 100 | 78 |
| ME Jukes–Cantor | 99 | 100 | 100 | 90 |
| ME Kimura | 100 | 100 | 100 | 86 |
| ME Kimura $\gamma = 0.24$ | 99 | 100 | 100 | 69[a] |
| ME Kimura $\gamma = 0.50$ | 99 | 100 | 100 | 87 |
| ME Kimura $\gamma = 0.72$ | 100 | 100 | 100 | 85 |
| MP | 95 | 93 | 100 | [b] |
| ML | 97 | 76 | 100 | [b] |

[a] In this tree Clade III appeared as a distinct clade, but the branch leading to Clade III formed a polytomy with the branch leading to Clade IV and with the branch leading to Clades I and II.
[b] Clade III was not a distinct clade (see Figs. 3 and 4).

of Clade III and Clade IV. Morphological similiarities between thaliaceans and colonial ascidians have been well documented (Berrill, 1936, 1975), but it has not been obvious to which group of ascidians the thaliaceans might be most closely related. Berrill (1936), early in his career, discussed thaliacean morphology and concluded, "Thus, of the various types of ascidians, the Thaliacea can be related only with *Ciona*, and the evidence in this case is merely negative, suggesting that the Thaliacea may have evolved at almost any stage during the evolution of the stock that culminated in *Ciona*." However, despite this uncanny insight, the phylogenetic tree he draws in the same paper (Berrill, 1936) puts the Thaliacea as an outgroup to the ascidians. In later papers Berrill never again mentions the idea that the Thaliacea may be related to *Ciona*, a phlebobranch. Our results and those of Wada (1998) suggest that all three orders of thaliaceans (salps, pyrosomids, and doliolids) may be related to phlebobranch ascidians, which include the family *Ciona*.

There appears to be a great deal of variation in evolutionary rates within and between families of the ascidians. For example, in Figure 1, the branches leading to Clade I (styelids + pyurids) are shorter than those leading to Clade II (molgulids), suggesting that urochordates have unequal evolutionary rates in comparison with the other large chordate subphyla, the vertebrates. A relative rates test using a subset of this data showed that the molgulid ascidian 18S rRNA genes are evolving significantly faster than the rest of the ascidian clades (Huber et. al., 2000). It remains to be seen if these unequal rates reflect the evolution of urochordates in general or are limited only to the 18S rRNA gene. In the nematode *C. elegans*, the rapid rate of 18S rRNA gene is also observed in about two-thirds of 36 protein-coding genes examined (Mushegian et al., 1998). If unequal evolutionary rate variation is high among urochordates, then the faster-evolving urochordates such as molgulid ascidians may represent a more recent expansion than, for example, the styelid ascidians. Unfortunately, urochordates are soft-bodied and the fossil record is extremely sparse for this subphylum (Valentine et al., 1996), unless the ancestral forms are similar to calcichordates, as some palentologists have suggested (Jeffries et al., 1996). However, after obtaining and examining calcichordate casts, we are not convinced that they represent urochordates (B.J. Swalla, unpub.).

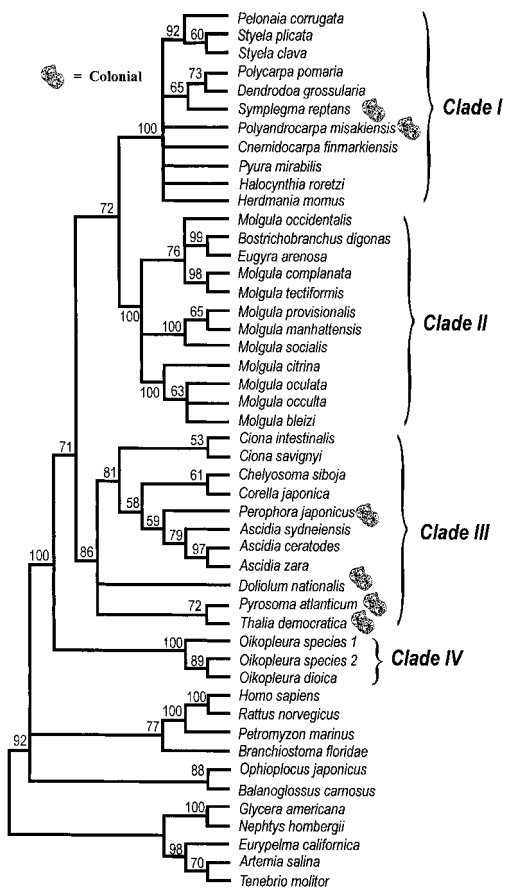


FIGURE 2. Majority rule consensus of 100 bootstrap replicates of minimum evolution trees recovered by using Kimura two-parameter evolutionary distances. Nodes with <50% bootstrap support are shown collapsed. The distribution of species with a colonial life-cycle are marked to demonstrate the polyphyly of colonialism among urochordates. Clades I, II, III, and IV include the same taxa as in Figure 1 and are marked with braces. ME trees recovered by using a number of other evolutionary distance methods were consistent with the tree shown above (see Table 2).

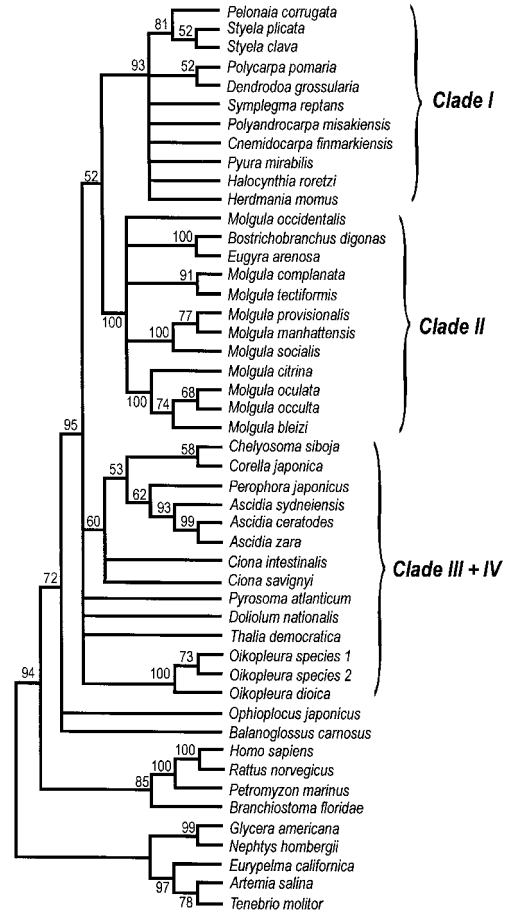


FIGURE 3. Majority rule consensus of 100 bootstrap replicates of maximum parsimony trees recovered by using PAUP*. Two equally parsimonius trees of 1135 steps were recovered in the initial analysis. Nodes <50% bootstrap support are shown collapsed. Taxa within Clades I, II, III, and IV as defined in Figures 1 and 2 are marked with braces.

Our analyses reveal several new clades within the urochordates that do not correspond to traditional urochordate classification schemes based on morphological features (Van Name, 1945; Berrill, 1950). At the ordinal level, the looping of the gut is the main diagnostic character. In the order Enterogona, which contains molgulid (Clade II), and styelid and pyurid (Clade I) ascidans, gonads occur only on one side of the body and remain a single unit within the primary loop of the gut (Berrill, 1950, 1975; Van Name, 1945). In contrast, in Pleurogonid ascidians (Clade III), the gonads and the gut become associated with the mantle wall, and multiple gonads lie either across the gut or within a secondary loop (Berrill, 1950, 1975; Van Name, 1945). Folds and whorls in the branchial sac are a major character used to distinguish separate fami-

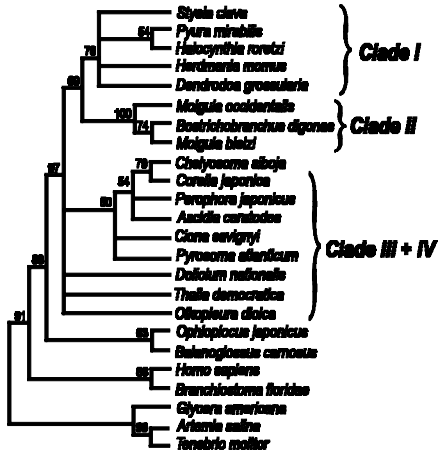


FIGURE 4. Majority rule consensus of 100 bootstrap replicates of maximum likelihood trees recovered by using DNAML and associated programs from PHYLIP. A subset of the data (24 of 48 taxa) used for Figures 1–3 was analyzed. Nodes with <50% bootstrap support are shown collapsed. Taxa within Clades I, II, and III+IV as defined in Figures 1 and 2 are marked with braces.

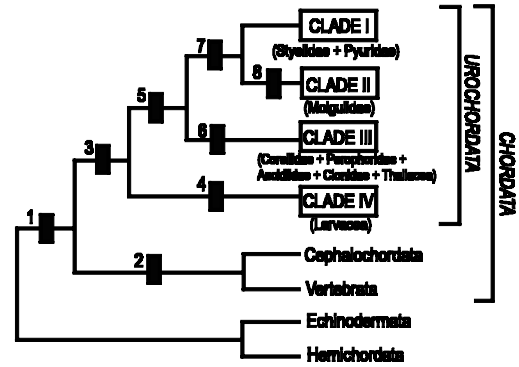


FIGURE 5. One possible tree showing the evolution of the chordates and urochordates as based on molecular and morphological analyses. Possible morphological characters defining the nodes are shown. Characters are represented as black bars at each node and stand for the following traits: (1) Chordata: notochord, pharyngeal gill slits, muscular postanal tail, dorsal nerve cord. (2) Vertebrata + Cephalochordata: presence of somites. (3) Urochordata: endostyle, tunic. (4) Larvacea: planktonic adults that secrete a special mucus house. (5) Urochordates that lose their tail after metamorphosis. (6) Branchial sac without longitudinal folds. (7) Branchial sac with longitudinal vessels having a discrete number of folds. (8) Solitary adults, single gonads, presence of symbiotic cyanobacteria.

lies within orders. The families Styelidae, Molgulidae, and Pyuridae are all considered part of the order Pleurogona, suborder Stolidobranchiata, because in these families the branchial structure has many elaborate folds that are specific to each family (Berrill, 1950, 1975). The present study suggests that these three families form a robust monophyletic clade (Fig. 1, Clades I and II), with Molgulidae a separate clade (Clade II; Fig. 5) within the group.

Clade I, which was well resolved by all of the phylogenetic methods (Table 2), includes Styelidae and Pyuridae, which were known to be closely related by morphological analysis (Van Name, 1945; Berrill, 1975). The species we analyzed from the family Pyuridae were *Halocynthia roretzi*, *Herdmania curvata,* and *Pyura mirabilis*. Originally our analysis also included *Herdmania momus* (now called *Herdmania curvata*; Degnan and Lavin, 1995; GenBank no. X53538; Degnan et al., 1990), which had a very long branch length compared with other species of Pyuridae and Styelidae within Pleurogona, causing it to fall as a sister group to all of the other taxa in Clade I. This sequence was one of the first ascidian 18S rRNA genes to be sequenced and was obtained from a genomic library (Degnan et al., 1990), not from PCR, now the method of choice for 18S rDNA isolation from genomic DNA. We obtained a new 18S rDNA sequence from *Herdmania curvata* (DNA sent by Bernie Degnan) from PCR and found its position in the phylogenetic trees differed dramatically from the earlier published genomic sequence. Possibly the genomic clone sequenced earlier is not usually transcribed or is rare in copy number. Therefore, we included only the new *H. curvata* sequence in our analysis, because we believe it better represents the Herdmania genus.

Molgulidae appear well separated from the rest of the ascidians (Fig. 1, Clade II, and Table 2). The relationships within Molgulidae have been reported previously, by Hadfield et al. (1995), who showed that tailless

ascidian larvae are likely to have evolved four times independently within this one ascidian family. All of the Molgulidae species are solitary, all have a single hermaphroditic gonad above the intestinal loop on the left side and above a well-developed renal gland containing cyanobacteria symbionts on the right side (Saffo, 1991). The results reported here suggest that the ascidian family Molgulidae forms a natural clade that might be best described as a separate order within the Ascidiacea. Further phylogenetic analysis of the molgulid ascidians has shown that the original tailed ancestors must have been circumpolar because two tailless species in the northern Pacific Ocean fall into European clades, which contain both tailed and tailless species (Huber et al., 2000).

Clade III is problematic; its membership varied according to the phylogenetic method used. The clade was well supported in NJ and ME trees (Figs. 1 and 2) but not in MP or ML trees (Figs. 3 and 4). Clade III was made up of phlebobranch ascidians and also included the salps, pyrosomes, and doliolids from the class Thaliacea. The relationships within this clade were not well resolved with 18S rDNA (Table 2). In this study the inclusion of all three orders of ascidians suggests that the thaliaceans may have been derived from the ascidian suborder Phlebobranchiata, a result consistent with another recent study (Wada, 1998). Wada mentioned that the ML tree with his data was the same topology as the NJ trees, but the tree itself was not shown. The Phlebobranch ascidians are united by a single gonad that lies some distance from the atrial cavity and exhalant siphon and thus requires rather long sperm ducts and oviducts. Many of the eggs of these species have elaborate follicles and float when spawned, unlike the Stolidobranch or Aplousiobranch eggs (Berrill, 1975). In addition, cross-fertilization studies with dechorionated eggs have shown that individuals from the family Cionidae can form viable hybrids within the family Ascidiidae, suggesting a close affinity between these families (Reverberi, 1971).

The evolution of life histories within the urochordates suggests that the switch from a solitary adult to a colonial lifestyle can be either gained or lost within a given clade. Earlier studies within the suborder Stolidiobranchiata, order Pleurogona, had suggested this to be true for ascidians (Wada et al., 1992; Cohen et al., 1998), but the results presented here suggest that this can also occur between orders. It had been traditionally believed that the colonial lifestyle exhibited by most pyrosomids was a trait allying the pyrosomids with the colonial ascidians. However, colony formation in both groups is a postmetamorphic phenonemon of growth into a multiple zooid organism that then reaches sexual maturity and undergoes sexual reproduction as a colony. We suggest that, given the evolution of colonial life histories in both Clade I and Clade III, the colonial lifestyle is not a trait that can conclusively link the thaliaceans to the ascidians. It is interesting that none of the species in Clade II are colonial, suggesting that a colonial life history is not always selectively advantageous. The colonial urochordates are identified by icons in Figure 2.

The three larvacean sequences used for our analysis all showed long branch lengths by NJ analysis. When there are unequal rates of evolution in a phylogenetic analysis, the branch lengths can confound results. Although distance-based methods showed a high bootstrap support for the larvaceans forming a separate clade, ML and MP trees did not. Clearly, this issue will be resolved only by sequencing urochordate genes with more equal rates of evolution. Larvaceans have a nonfeeding tadpole larva that develops quickly, metamorphosing within ~6 h into a planktonic adult that retains the tadpole tail with the chordate notochord throughout its life. The cleavage pattern and development of the larvacean nonfeeding tadpole larva are unmistakably characteristic of the urochordates. Larvacean adults are elongate with a locomotory tail, a hollow dorsal nerve cord, a notochord lying between the nerve chord and alimentary canal, and lateral skeletal muscles in the tail (Jeffery and Swalla, 1997).

The adult secretes a mucus house and beats its tail continuously to move seawater through the house to trap food particles. There is a typical chordate endostyle, and the gill spiracles open into atrial pouches. The larvaceans have no features that suggest having had ascidian adult-like features in their ancestry (Berrill, 1950; 1975). Rather, all of their adult features indicate a strictly pelagic history, particularly their secreted house, a novel structure for achieving planktotrophy (Bone, 1998).

The phylogenetic analysis provided here suggests that the ancestral urochordate had a nonfeeding larva and an adult that may have either been planktonic or bottom-dwelling but probably retained the tadpole tail, including the dorsal nerve cord, the notochord, and flanking muscle cells through the adult life. The rest of the urochordates diverged into a variety of adult forms—some undergoing dramatic larval to adult metamorphosis, and others having reduced or eliminated the larval forms. A colonial lifestyle has evolved several times independently, which suggests that the ancestral urochordate could be either solitary or colonial. The most successful present day life history appears to be that of the ascidians, which are cosmopoliton in the world's oceans. Further analysis will be necessary to elucidate the evolution of the dramatically different adult morphologies exhibited by the various clades found in the urochordates.

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