# Systematic Relationships of the Urochordates Based on Partial 18S rDNA Sequences

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**Urochordates, the most primitive group in phylum Chordata, are mostly sessile as adults although some are free living. Presently, the ancestral stock of urochordates as well as chordates has been the focus of interest and two conflicting hypotheses have been presented. A free swimming ancestor is one and a sessile, filter feeding ancestor is the other. To clarify the phylogenetic relationships within the urochordates, 22 urochordates and five others as outgroups were used. And we applied neighbor joining, maximum likelihood, and maximum parsimony methods to partial 18S rDNA sequences. The inferred phylogeny in all analyses indicates that order Aplousobranchia of class Ascidiacea appears to be the most ancestral group among urochordates. But it is not clear for the low bootstrap value. The remaining two orders of ascidians, Phlebobranchia and Stolidobranchia, form monophyletic groups respectively, which are well supported by high bootstrap values. These two orders are closer to classes of Thaliacea and Appendicularia than to the Aplousobranchia. While class Appendicularia is strongly supported by the monophyletic group, the phylogenetic position of class Thaliacea is unclear in this study.**

Since the early nineteenth century when the theory of organic evolution became the focal point for determining out relationships between groups of living organism, zoologists have debated the question of chordate origins. Due to the nearly total absence of fossil records of the earliest protochordates, nearly every major of the invertebrate group, namely arthropods, annelids, echinoderms, and so on, had at one time or another been advanced as a candidate for the chordate ancestral group. However there is no compelling reason to designate any of them as a chordate ancestral stock, zoologists are beginning to focus on groups within the chordate phylum itself. All members of the phylum Chordata share four anatomic features at some time in their life histories - a notochord, a dorsal tubular nerve cord, pharyngeal gill slits, and a postanal tail. The three subphyla which bear these characters are Urochordata, Cephalochordata, and Vertebrata. The vertebrates comprise by far the greatest number of the chordates and the back boneless members of the phylum, Urochordata and Cephalochordata, are usually inferred to as protochordate and have long been considered good candidates for the vertebrate ancestral stock. Cephalochordates, of which the lancelet *Amphioxus* is its famous representative, possess many vertebrate-like characters such as muscle somites and axial skeleton retained throughout life. So they have been known as the sister group of vertebrate and urochordates are thought to be the most primitive group within chordates (Hickman et al., 1979; Brusca and Brusca, 1990; Kozloff, 1990).

Most urochordates are sessile as adults, but some are free living. Urochordates are divided into three classes - Ascidiacea, Appendicularia, and Thaliacea, of which the members of ascidians are by far the most common and best known. All ascidians are sessile with no exception and may be solitary and colonial. But the remaining two classes of urochordates, Appendicularia and Thaliacea, are free swimming as a part of the plankton. All appendicularians are solitary and among the thaliaceans, some are entirely colonies, while the others can change from the colony to solitary alternately. Thus, the ancestral form of chordates, two different views have been presented. Free swiming ancestor is the one that considers free swimming tadpole larva of urochordate to be a relic of the chordate ancestor. Adult ascidians then come to be regarded as degenerate sessile descendants of the ancestral form. The appendicularians were accordingly considered to be the least changed from the ancestor (Barnes, 1989; Meglitsch and Schram, 1991; Wada and Satoh, 1994; Hickman and Roberts, 1995).

From the other view, the tadpole larva is not a relic of the chordate ancestor but is an urochordian creation. Urochordates are primitively sessile form with a capacity for budding, the solitary form and the tadpole has been

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evolved within the group (Hickman et al., 1979; Brusca and Brusca, 1990; Kozloff, 1990; Hickman and Roberts, 1995).

Recently, some studies with molecular technique imply that the appendicularians are the most primitive within urochordates, so the ancestor of urochordates as well as chordates may have been a free swimming form (Turbeville et al., 1994; Wada and Satoh, 1994; Wada, 1998). They used only a few urochordate species (Turbeville et al., 1994; Wada and Satoh, 1994) and did not include the ascidian order Aplousobranchia (Wada, 1998). All of the aplousobranchians are colonies and very simple in their anatomy, while the another ascidians are mostly solitary without the capacity of budding, and larger and more complex than aplousobranchians. To assess the ancestral form of urochordates, we think, aplousobranchians should be included.

In this study, to reexamine our knowledge of the phylogeny of urochordates, we used the 18S rDNA sequences of all urochordate orders and we also used a few outgroups to clarify the relationship between urochordates and other chordates.

## Materials and Methods

Specimens, collected from the southeastern parts of Korea between 1996 and 1998, were identified to be eight ascidians. They range over all orders of ascidians, one species of the order Aplousobranchia, three species of order Phlebobranchia, and four species of order Stolidobranchia. For the phylogenetic analysis of urochordates, previous sequences of another urochordate species were obtained from EMBL and other literature (Wada et al., 1992). Five non-urochordate species were also included in the analyses as outgroups.

The animals examined in the present study belonged to the three phyla, namely, 25 chordates, one echinoderm, and one hemichordate. Table 1 shows the taxonomic classification and their source for the 27 species used in this study. Eight species for which new sequence data were collected are indicated and their EMBL acession number are also presented. Total genomic DNA was isolated from liver or gonad by using the modification of the standard procedure (Sambrook et al., 1989).

A pair of primers were selected to permit amplification of the region of about 1000 bp from the central part of 18S rDNA (5′-ACGGGCGGTGTGTAC-3′, 5′-CAGCCG CGGTAATT-3′). Double stranded amplification products of the 18S rDNA gene were sequenced directly. DNA sequencing was conducted on both strands using T7 sequenase v2.0 (Amersham) and six forward primers (numbers correspond to positions on the human sequence: 802 - 816, 5′-TAATTTTTTCAAAGT-3′; 958 - 971, 5′-CCAAGAATTTCACC-3′; 1188 - 1207, 5′-CCGTC AATTCCTTTAAGTTT-3′; 1320 - 1335, 5′-CCATGCACC ACCACCC-3′; 1374 - 1388, 5′-TCTCGTTCGTTATCG-3′; and 1494 - 1508, 5′-TCTAAGGGCATCACA-3′) and the reverse of each primer.

Table 1. Taxa used in this study

| Classification | Species | Sequence source |
|---|---|---|
| Phylum Echinodermata | *Asterias amurensis* | D14358 |
| Phylum Hemichordata | *Balanoglossus carnosus* | D14359 |
| Phylum Chordata | | |
| Subphylum Urochordata | | |
| Class Ascidiacea | | |
| Order Aplousobranchia | *Didemnum* sp.* | AJ250779 |
| Order Phlebobranchia | *Ciona savignyi* | Wada et al. (1992) |
| | *Ciona intestinalis** | AJ250778 |
| | *Perophora japonica* | Wada et al. (1992) |
| | *Ascidia sydneiensis* | Wada et al. (1992) |
| | *Ascidia zara** | AJ250777 |
| | *Chelyosoma dofleini** | AJ250776 |
| Order Stolidobranchia | *Cnemidocarpa clara** | AJ250775 |
| | *Polyandrocarpa misakiensis* | Wada et al. (1992) |
| | *Symplegma reptans* | Wada et al. (1992) |
| | *Dendrodoa aggregata** | AJ250774 |
| | *Styela clava* | Wada et al. (1992) |
| | *Styela plicata* | L12444 |
| | *Halocynthia roretzi* | AB013016 |
| | *Herdmania mirabilis** | AJ250773 |
| | *Pyura vittata** | AJ250772 |
| Class Thaliacea | *Pyrosoma atlanticum* | AB013011 |
| | *Doliolum natinalis* | AB013012 |
| | *Thalia democratica* | D14366 |
| Class Appendicularia | *Oikopleura dioica* | AB013014 |
| | *Oikopleura* sp. 1 | D14360 |
| | *Oikopleura* sp. 2 | AB013015 |
| Subphylum Cephalochordata | *Branchiostoma floridae* | M97571 |
| Subphylum Vertebrate | *Homo sapiens* | X03205 |
| | *Xenopus laevis* | J00999 |

* 18S rDNAs were sequenced in the present study.

The nucleotide sequences for all species were aligned with Clustal W multiple alignment program (Tompson et al., 1994) and then it was refined by eye.

The phylogenetic tree was constructed by using the neighbor-joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) methods. We used NEIGHBOR and DNAML programs in PHYLIP 3.54 (Felsenstein, 1994) for NJ and ML analyses. The heuristic search option in PAUP 3.1.1. (Swafford, 1993) was used for MP analysis. In NJ analysis, we calculated the distance matrix using the DNADIST with Kimura's model. And in ML analysis, transition/transversion ratio was set as 2. One echinoderm species, *Asterias amurensis*, is used as an outgroup in all analyses. The degrees of support for internal nodes in the trees were assessed by 100 bootstrapping.

## Results

We obtained a common continuous sequence of 1005 bp of 18S rDNA gene for all 27 species. The sequence alignment had 396 variable sites, 278 of which are informative for parsimony analysis.

Fig. 1 shows the NJ tree based on Kimura's two parameter method (Kimura, 1980) with bootstrap values. Although the bootstrap value was under 50%, all chordate species form a monophyletic group and the hemichordate species, *Balanoglosus carnosus* joins with the echinoderm. Then, the clade of chordates divides again into two clades. In one clade, two vertebrate species, *Homo sapiens* and *Xenopus laevis*,

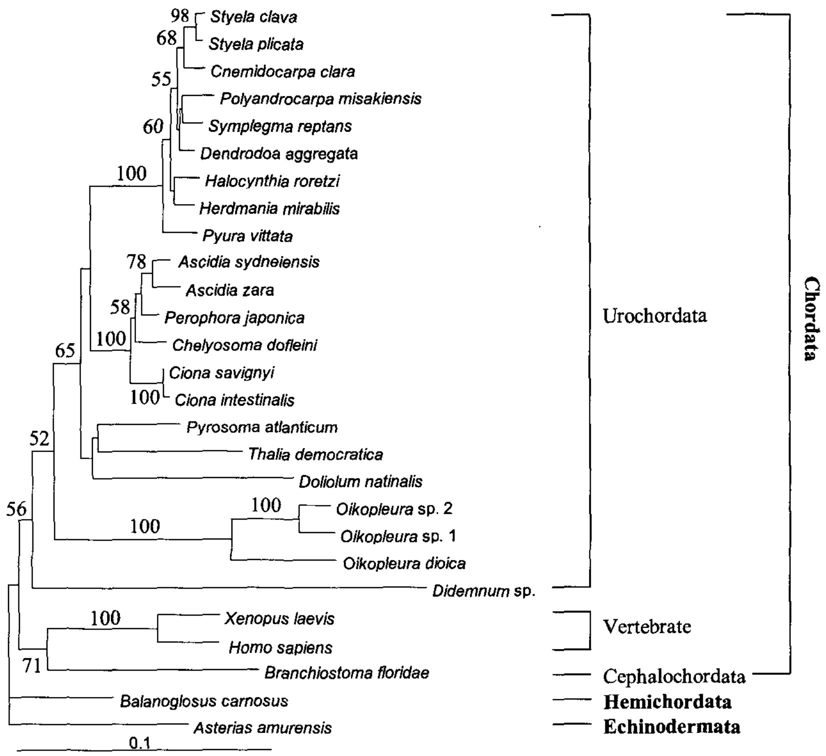


**Fig. 1.** Phylogenetic relationships of urochordates deduced from the neighbor-joining method. Number at each branch indicates the percentage of times a node was supported in 100 bootstrap replications, only those over 50% being represented. This is an unrooted tree, rooted defining the *Asterias amurensis* as the outgroup.

have a common ancestor with one cephalochordate species, *Branchiostoma floridae,* and in the other clade, all urochordate species are included. The bootstrap value of the clade with vertebrates and cephalochordate is relatively high at 71% but the value of the urochordate clade is just a little more than 50%.

*Didemnum* sp. of class Ascidiacea branches off at the first in the urochordates but the other ascidians get divided into two monophyletic groups corresponding to ascidian orders, Phlebobranchia and Stolitobranchia, which are strongly supported by high bootstrapping values respectively. These two orders are closer to the classes Thaliacea and Appendicularia than to *Didemnum* sp. The two urochordate classes form a clade respectively, but class Ascidiacea is paraphyletic including the former two classes.

The clade of appendicularians receives the complete support from bootstrap and forms a first clade in urochordates except *Didemnum* sp. The node including thaliaceans and ascidians is weakly supported.

The ML tree shows almost the same topology with the NJ tree but the clade of appendicularians is brought about from the common ancestor with thaliaceans (Fig. 2). However, for the low bootstrap values on the nodes with thaliaceans, monophyly of them and their phylogenetic position remains unclear. The clades of two ascidian orders, Phlebobranchia and Stolitobranchia, and class Appendicularia are also well supported in ML analysis.

MP analysis performed with all informative sites results in a single most parsimony tree, with 960 steps and a consistency index of 0.507 (the tree is not shown). The MP tree is almost coincident with the NJ tree in topology but vertebrates and cephalochordate are clusterd with the hemichordate, and thaliaceans are closely related with ascidian order Stolobranchia. These different parts are not reliable for their low bootstrap values.

## Discussion

In this study, the results of all phylogenetic analyses are mostly coincident with one another. But the difference among them can be summarized into two parts. In phylogenetic trees of ML and NJ analyses, all chordate species formed a monophyletic cluster, though it is weakly supported in NJ. The chordates then divide into two clades, one of which includes only urochordates and the other includes both of cephalochordate and vertebrates. And these two clades of chordates are well supported by high bootstrapping values. The result of parsimony analysis differs from the former in having the hemichordate connected to the clade of cephalochordate and vertebrates (the tree is not shown), but the urochordate clade is well supported in parsimony method, too. Though uncertainty of the monophyly of

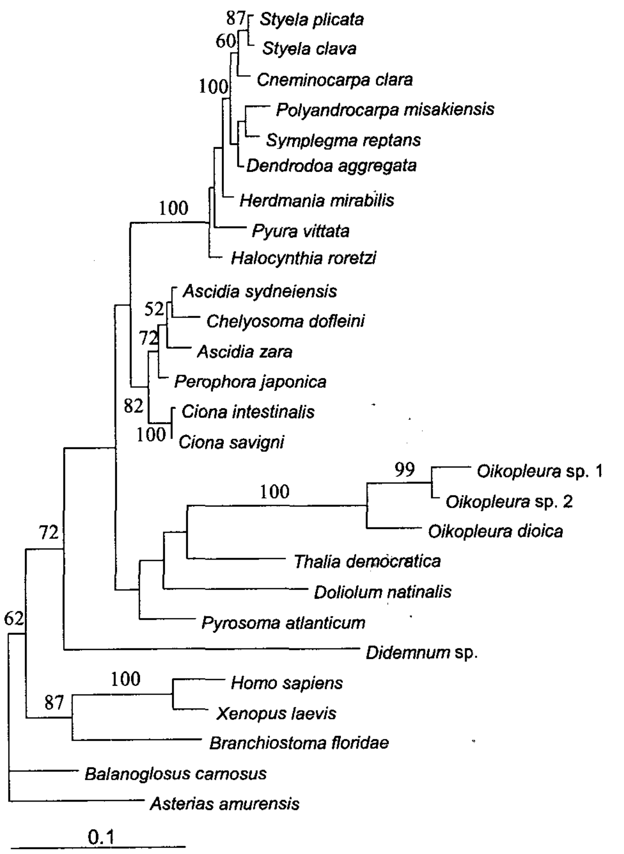


**Fig. 2.** Relationships within urochordates inferred from the maximum likelihood method. All branch lengths are drawn to scale. Numbers at nodes are percentages of 100 bootstrap replicates that support the branch, only those over 50% being represented.

chordates, our results strongly suggest the monophyly of urochodates.

The second difference among the results of three analyses is the positon of thaliaceans. In NJ and MP analyses, they are clustered. In NJ analysis, they are connected with a node including two ascidian orders, Plebobranchia and Stolidobranchia, while with stolidobranchians in MP analysis. In ML analysis, they include the clade of aplousobranchians. In general, morphological feature of zooid of thaliaceans is similar to those of ascidian, though they are always free living. Three thaliacean species used are representatives of three orders of thaliaceans, Pyrosomida, Doliolida, and Salpida, and each of them have very distinctive features. For a more definite phylogeny of thaliaceans, they must be conducted continuously.

All of the analytic methods which we employed identified the early node within ascidians as that joining the aplousobranchian, *Didemnum* sp., but this was not stable for the low bootstrap value. So our study is not sufficient to resolve the question of urochordate ancestor, but it presents some possibility that the ancestor of urochordates may not have been the free swimming type as reported by Wada and Satoh (1994), Turbeville et al. (1994), and Wada (1998).

Any view of ascidian phylogeny should take into account the origin of the tadpole larva and the frequent, but not universal occurrence of budding. Vertebrates are now widely believed to have originated as the tadpole-like larva of an ancient protochordate stock (Garstang, 1928; Berrill, 1955). The probable origin of the tadpole larva itself is therefore of interest, not only because it is a unique ascidian from, but also in relation to vertebrate ancestry.

There are two ways of regarding the origin of the ascidians (Millar, 1966). First is the view that the sessile adults are derived from the free swimming ancestor like tadpole larva. The second possibility was that a sessile adult is the ancestor and the adult may have been colonial as Van Name (1921) and Garstang (1928) believed, or solitary as Berrill (1950, 1955) and Kott (1972) mentioned. It is difficult to imagine that the colonial ascidians have been derived from solitary ascidians by a decrease in the size and complexity of structure of the individuals and by the acquirement of the power of budding.

The Ascidiacea is the highest form of animal life and budding found only in the lower phyla. We can also find out that in the closely allied ascidian pairs, one produces buds and forms colonies, the other does not. Such pairs of closely related simple and compound pairs would indicate, according to the Berrill's view, that the budding power has been very recently acquired and, since no other important morphological differentiation has taken place. So, it is more likely that budding was a function formerly possessed by all ascidians. Though the morphological structure has become more complex and more highly organized, certain ascidians have lost this function.

Another problem in the phylogeny of urochordates is the evolution of classes Appendicularia and Thaliacea. The Appendicularia are free swimming and solitary like the tadpole larva but their morphological characters are extremely specialized (Garstang, 1928; Berrill, 1936). They have a long tail and notochord in the adult and have no larval stage. Their pharynx are too small for filter feeding whereas the function of gill slits in another ascidians is carried out within their house. From the results of this study, the Appendicularia are presented as a monophyletic group which was well supported in all analyses. The phylogenetic position of the Thaliacea is not clear in this study. The Thaliacea as a whole evidently originated from a single stock, evidenced by their peculiar mode of budding and highly specialized branchial sac, atrium, and musculature. However, their viscera structure is similar to that of ascidians, especially, the position of the post-branchial gut and the heart resembles that in *Ciona* or *Ascidia*. For the resolution of the phylogenetic relation of the Thaliacea, we need further extensive investigations.

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