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Ascidian molecular phylogeny inferred from mtDNA data with emphasis on the Aplousobranchiata

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Abstract

We explored the usefulness of mtDNA data in assessing phylogenetic relationships within the Ascidiacea. Although ascidians are a crucial group in studies of deuterostome evolution and the origin of chordates, little molecular work has been done to ascertain the evolutionary relationships within the class, and in the studies performed to date the key group Aplousobranchiata has not been adequately represented. We present a phylogenetic analysis based on mitochondrial cytochrome *c* oxidase subunit I (COI) sequences of 37 ascidian species, mainly Aplousobranchiata (26 species). Our data retrieve the main groups of ascidians, although Phlebobranchiata appeared paraphyletic in some analyses. Aplousobranch ascidians consistently appeared as a derived group, suggesting that their simple branchial structure is not a plesiomorphic feature. Relationships between the main groups of ascidians were not conclusively determined, the sister group of Aplousobranchiata was the Stolidobranchiata or the Phlebobranchiata, depending on the analysis. Therefore, our data could not confirm an Enterogona clade (Aplousobranchiata + Phlebobranchiata). All of the tree topologies confirmed previous ideas, based on morphological and biochemical characters, suggesting that Cionidae and Diazonidae are members of the clade Aplousobranchiata, with Cionidae occupying a basal position within them in our analyses. Within the Aplousobranchiata, we found some stable clades that provide new data on the evolutionary relationships within this large group of ascidians, and that may prompt a re-evaluation of some morphological characters.

Keywords: Ascidians; Phylogeny; COI; Maximum likelihood; Parsimony; Bayesian inference

1. Introduction

Tunicates are a key group in studies of deuterostome evolution, particularly those addressing the origin of chordates. Recently, molecular tools have been applied to the study of topics such as deuterostome phylogeny (Cameron et al., 2000; Holland, 1991; Turbeville et al., 1994; Wada and Satoh, 1994; Winchell et al., 2002), ancestral chordate lifestyle (Wada, 2000), the origin of ascidian coloniality (Jacobs et al., 2000; Wada et al., 1992), and the origin of anural development (Hadfield et al., 1995; Jeffery et al., 1999). Molecular data have also been used to shed light on relationships between major Tunicate groups (Christen and Braconnot, 1998; Stach and Turbeville, 2002; Swalla et al., 2000). In general, these studies have supported the monophyly of the Tunicata, but not of the class Ascidiacea, as the Thaliacea appear related to the Phlebobranchiata. The position of the Appendicularia remains controversial (Swalla et al., 2000), although a recent study suggests that they are the sister group to the aplousobranch ascidians (Stach and Turbeville, 2002). Phylogenetic relationships within the ascidians, on the other hand, have received little attention in molecular studies.

Since the seminal works of Seeliger (1885) and Lahille (1890), and other studies in the last century (e.g., Berrill, 1936, 1955; Garstang, 1928; Millar, 1966; Tokioka, 1971)

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the debate on the characteristics of the ascidian ancestor and the main evolutionary lines within the group has relied on non-cladistic schemes. Taxonomically oriented studies currently classify the ascidians according to two main characters, one considering the structure of the branchial basket and the other the position of the gonads. Lahille (1886, 1887, 1890) established the groups Aplousobranchiata, Phlebobranchiata, and Stolidobranchiata on the basis of a progressive structural complication of the branchial sac. Aplousobranch ascidians have simple branchial walls with only transverse vessels between rows of stigmata; phlebobranch pharynges have distinct papillae projecting from the transverse vessels, and these papillae are in many cases connected by longitudinal vessels. In stolidobranch species the branchial wall features longitudinal vessels, but it is plicated, forming internal longitudinal folds. In addition, Perrier (1898) and later Garstang (1928), developed a classification scheme based on gonad position, either attached to the body wall (Pleurogona) or associated with the digestive system (Enterogona). In fact, it is common to find a combination of the two schemes, in which ascidians are classified into the orders Enterogona and Pleurogona, the former comprising the suborders Aplousobranchiata and Phlebobranchiata and the latter the suborder Stolidobranchiata (Berrill, 1950; Kott, 1985; Millar, 1970). However, other authors use only Lahille's classification, and Aplousobranchiata, Phlebobranchiata, and Stolidobranchiata are ranked as orders (Harant and Vernières, 1933; Monniot et al., 1991; Van Name, 1945).

The relationships between the members of Aplousobranchiata are unclear (Kott, 1990), a fact that is reflected in the poor agreement in its internal classification. Some recent, taxonomically oriented studies have tended to accept a reduced number of large groupings; for instance, the Aplousobranchiata would comprise only the families Polycitoridae, Polyclinidae, and Didemnidae (Monniot et al., 1991; Nishikawa, 1990), or the families Clavelinidae, Holozoidae, Polycitoridae, Polyclinidae, and Didemnidae (Monniot and Monniot, 2001). In contrast, Kott (1985, 1990, 1992, 2001), in her detailed monographs on the Australian ascidians, considered up to 14 families within the Aplousobranchiata on the basis of careful morphological observations. The placement of the Cionidae and Diazonidae within the Phlebobranchiata (the traditional view) or the Aplousobranchiata (Kott, 1969, 1990) is another point on which no general agreement has been reached (see Stach and Turbeville, 2002).

New, independent sets of data such as sequence information, in combination with formal evolutionary analyses, may be particularly useful in ascertaining ascidian evolution and establishing a sound classification scheme. To date, however, molecular studies have been applied only to a small fraction of ascidian species. The main groups (at the order/suborder level) have generally been recovered in molecular analyses (Stach and Turbeville, 2002; Swalla et al., 2000), although many traditional families appeared as paraphyletic or polyphyletic. In addition, limited data are available on aplousobranch ascidians. In a thorough study combining molecular data and morphology, Stach and Turbeville (2002) were the first to generate aplousobranch sequences of the genes 18S rRNA (three species) and cytochrome *c* oxidase subunit I (COI, five species). In addition, Kakuda (2001) and Kurabayashi et al. (2003) utilized mitochondrial DNA data to address some phylogenetic problems in ascidians. The mitochondrial COI gene, due to its high variability, has been the molecule of choice in studies of population genetics and phylogeography (Avise, 2000), and has been used in ascidians to address cryptic speciation and invasions (López-Legentil and Turon, 2004; Tarjuelo et al., 2001; Turon et al., 2003). However, the COI gene may provide useful information at higher taxonomic levels (Hebert et al., 2003; Remigio and Hebert, 2003). Problems such as incomplete lineage sorting and introgression that hinder analyses at the species level using mitochondrial data (Ballard and Whitlock, 2004) may not be relevant over the larger timescales of evolutionary processes.

In this study, we present novel partial COI sequences for 28 ascidian species, of which 21 belong to the Aplousobranchiata sensu Kott (1990). We added nine sequences (five of them from aplousobranchs) published by other authors to obtain a representative database of 11 families sensu Kott (1985, 1990, 1992, 2001). Although our study is limited to a single gene, thus necessitating more data sources before definitive conclusions can be reached, our aim was to explore the contribution of COI sequence data to the phylogeny of ascidians, particularly the aplousobranchs. Given the paucity of formal phylogenetic studies (molecular or morphological) in ascidians, we believe that this new database will represent a step forward in the unravelling of the evolutionary pattern in this group.

2. Materials and methods

2.1. Ascidian samples

Twenty-eight species of ascidians, 21 of the Aplousobranchiata (following the assignment of Kott, 1990), three Phlebobranchiata and four Stolidobranchiata were collected from sites in the Balearic Islands and NE Spain (Western Mediterranean) by SCUBA diving (Table 1). Two samples were from the Eastern Atlantic (*Clavelina oblonga* from Azores and *Archidistoma aggregatum* from Galicia, NW Spain). Taxonomic identification was performed following Turon (1987), with the exception of *Pycnoclavella* sp., previously identified in Turon (1987) as *Clavelina nana*. This sample was not identified at the species level since the taxonomy of the genus *Pycno-*

Table 1
Species whose sequences were used in this study, and their assignment to families and to the groups of Aplousobranchiata (Ap), Phlebobranchiata (Ph), and Stolidobranchiata (St) after Monniot et al. (1991) (family 1), and after Kott (1985, 1990, 1992, 2001) (family 2)

Species	Family 1	Family 2	GenBank No.
Aplidium pseudolobatum (Pérès, 1956)	Polyclinidae (Ap)	Polyclinidae (Ap)	AY600967[a]
Aplidium elegans (Giard 1872)	Polyclinidae (Ap)	Polyclinidae (Ap)	AY600971[a]
Aplidium fuscum (Drasche, 1883)	Polyclinidae (Ap)	Polyclinidae (Ap)	AY600975[a]
Aplidium conicum (Olivi, 1792)	Polyclinidae (Ap)	Polyclinidae (Ap)	AY600969[a]
Pseudodistoma cyrnusense (Pérès, 1952)	Polyclinidae (Ap)	Pseudodistomidae (Ap)	AY600970[a]
Pseudodistoma crucigaster (Gaill, 1972)	Polyclinidae (Ap)	Pseudodistomidae (Ap)	AY600979[a]
Polysyncraton lacazei (Giard, 1872)	Didemnidae (Ap)	Didemnidae (Ap)	AY600986[a]
Diplosoma spongiforme (Giard, 1872)	Didemnidae (Ap)	Didemnidae (Ap)	AY600972[a]
Polycitor adriaticum (Drasche, 1883)	Polycitoridae (Ap)	Polycitoridae (Ap)	AY600982[a]
Eudistoma banyulensis (Brément, 1912)	Polycitoridae (Ap)	Polycitoridae (Ap)	AY600973[a]
Eudistoma posidoniarum (Daumézon, 1908)	Polycitoridae (Ap)	Polycitoridae (Ap)	AY600974[a]
Eudistoma planum (Pérès, 1948)	Polycitoridae (Ap)	Polycitoridae (Ap)	AY600977[a]
Eudistoma plumbeum (Della Valle, 1877)	Polycitoridae (Ap)	Polycitoridae (Ap)	AY600978[a]
Cystodytes dellechiajei (Della Valle, 1877)	Polycitoridae (Ap)	Polycitoridae (Ap)	AY523068[b]
Cystodytes aucklandicus (Nott, 1982)	Polycitoridae (Ap)	Polycitoridae (Ap)	AY523059[b]
Archidistoma aggregatum (Garstang, 1891))	Polycitoridae (Ap)	Polycitoridae (Ap)	AY600966[a]
Clavelina lepadiformis (Müller, 1773)	Polycitoridae (Ap)	Clavelinidae (Ap)	AY603104[a]
Clavelina oblonga (Herdman, 1880)	Polycitoridae (Ap)	Clavelinidae (Ap)	AY603106[a]
Clavelina dellavallei (Zirpolo, 1925)	Polycitoridae (Ap)	Clavelinidae (Ap)	AY603105[a]
Pycnoclavella sp.	Polycitoridae (Ap)	Pycnoclavellidae (Ap)	AY600988[a]
Rhopalaea neapolitana (Philippi, 1843)	Cionidae (Ph)	Diazonidae (Ap)	AY600983[a]
Ecteinascidia herdmanni (Lahille, 1870)	Perophoridae (Ph)	Perophoridae (Ph)	AY600968[a]
Phallusia ingeria (Traustedt, 1883)	Ascidiidae (Ph)	Ascidiidae (Ph)	AY600976[a]
Phallusia mammillata (Cuvier, 1815)	Ascidiidae (Ph)	Ascidiidae (Ph)	AY600980[a]
Halocynthia papillosa (Linnaeus, 1767)	Pyuridae (St)	Pyuridae (St)	AY600981[a]
Polycarpa pomaria (Savigny, 1816)	Styelidae (St)	Styelidae (St)	AY600984[a]
Botryllus schlosseri (Pallas, 1766)	Styelidae (St)	Styelidae (St)	AY600987[a]
Styela partita (Stimpson, 1852)	Styelidae (St)	Styelidae (St)	AY600985[a]
Aplidium nordmanni (Milne Edwards, 1841)	Polyclinidae (Ap)	Polyclinidae (Ap)	AY116596[c]
Aplidium stellatum (Verrill, 1871)	Polyclinidae (Ap)	Polyclinidae (Ap)	AY116595[c]
Clavelina picta (Hartmeyer, 1909)	Polycitoridae (Ap)	Clavelinidae (Ap)	AY116598[c]
Perophora viridis (Verrill, 1871)	Perophoridae (Ph)	Perophoridae (Ph)	AY116604[c]
Ascidiella aspersa (Müller, 1776)	Ascidiidae (Ph)	Ascidiidae (Ph)	AY116600[c]
Styela clava (Herdman, 1881)	Styelidae (St)	Styelidae (St)	AY116607[c]
Ciona intestinalis (Roule, 1886)	Cionidae (Ph)	Cionidae (Ap)	AK116803[d]
Ciona savignyi (Herdman, 1882)	Cionidae (Ph)	Cionidae (Ap)	AB079784[e]
Halocynthia roretzi (Drasche, 1884)	Pyuridae (St)	Pyuridae (St)	AB024528[f]

GenBank accession numbers are also indicated.
[a] This study.
[b] López-Legentil and Turon (2004).
[c] Stach and Turbeville (2002).
[d] Satou et al. (2002).
[e] Yokobori et al. (2003).
[f] Yokobori et al. (1999).

clavella in the Mediterranean is not well established at present. One sample (*Cystodytes aucklandicus*) came from Mayotte (Western Indian Ocean) and was identified following Monniot (1988). We added sequences from nine other species from GenBank (also listed in Table 1). Of these, six were from the study by Stach and Turbeville (2002). Some of the sequences reported there may correspond to pseudogenes (Stach, pers. commu.), so we used only those that could be unambiguously aligned with our own and had more than 70% base concordance. We included the remaining sequences of that study in preliminary analyses; they featured long branches and fell outside the remaining ascidians in our cladograms. We therefore excluded them from further analyses. The same happened with the appendicularian sequence of *Oikopleura dioica* published by Stach and Turbeville (2002). The other three sequences obtained from GenBank were those of *Ciona intestinalis*, *Ciona savignyi*, and *Halocynthia roretzi*. Table 1 lists the species names with authorities, their taxonomic assignment according to two contrasting criteria and GenBank accession numbers. Sequences of the cephalochordate *Branchiostoma floridae* (GenBank Accession No. 5881414; Boore et al., 1999) and the echinoderm *Cucumaria curata* (GenBank Accession No. U31901; Arndt et al., 1996) were used as outgroups.

2.2. DNA extraction, amplification, and sequencing

Zooids (colonial species) or mantle (solitary species) were separated from the tunic, fixed in absolute ethanol, and stored at −20 °C until used. Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). We used the universal primers LCO1490, 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and HCO2198, 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′, described in Folmer et al. (1994), for the amplification of a fragment (ca. 680 bp) of the mitochondrial gene cytochrome *c* oxidase subunit I (COI).

PCR amplification was performed in a 20 μl total reaction volume with 0.4 μl of each primer (25 μM), 0.5 μl dNTP's (10 mM), 2 μl 10× buffer containing 15 mM $MgCl_2$ (Promega), 1 U *Taq* Polymerase (Promega) and 0.5 or 1 μl template DNA. A single soak at 94 °C for 2 min was followed by 35 cycles (94 °C for 1 min, 39 °C for 1 min, and 72 °C for 1 min 30 s) and a final extension step at 72 °C for 7 min, in a Perkin–Elmer 840 PCR machine. The amplified DNA was directly sequenced. The sequencing reaction was carried out on a Perkin–Elmer PCR system 9700 with the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer). The PCR products were sequenced in an ABI Prism 377XL automated sequencer.

2.3. Alignment

Sequences were edited and aligned with BioEdit Sequence Alignment Editor (Hall, 1999). Alignments were confirmed visually. No gap was needed in the alignment step of our sequences and all of them could be translated into amino acids without stop codons. The final sequence length after alignment and trimming was 617 bp. Addition of two of the sequences (potential pseudogenes) published by Stach and Turbeville (2002) resulted in a one-base gap each in our sequences and in two one-base and two two-base gaps when adding a third species. Analyses of nucleotides were performed with these gaps, but for analysis of the amino acid translation the areas that required gaps were excluded from the study as they resulted in stop codons.

Amplification of certain DNA fragments with the aid of *Taq* DNA polymerase may result in a few mismatched bases per kilobase of DNA. To rule out the existence of sequencing errors, we repeated the amplification and sequencing anew from the extracted DNA for most species.

2.4. Phylogenetic analysis

Given that the gene used is known to have high variability, we performed a preliminary analysis of the degree of gene saturation by examining the relationships of uncorrected *p* distances with the number of substitutions (transitions and transversions separately) between species pairs. We did this separately for first, second, and third codon positions. Codon position assignment and distance data for this analysis were obtained using MEGA v. 2.1 (Kumar et al., 2001).

The data matrix was analyzed using parsimony, maximum likelihood, and Bayesian inference methods. We analyzed the nucleotide sequence data and the translated amino acid sequence to compare the resulting topologies.

For the parsimony approach (MP), we used PAUP* v. 4.0b 10 (Swofford, 2002). Data were analyzed using a heuristic search strategy with random stepwise addition (1000 replicates) and TBR branch swapping. Bootstrap values were obtained after 1000 repetitions of the same search strategy defined above with 10 random addition sequence replicates each.

For maximum likelihood (ML) analyses, the best-fit model of nucleotide substitution for our data was selected by statistical comparisons of 56 different models of evolution with the program Modeltest 3.0 (Posada and Crandall, 1998) using the Akaike Information Criterion (AIC). The model selected was then input in the Treefinder (as of December 2003) program (Jobb et al., 2004) for analysis of the nucleotide sequences. We used four rate categories of among-site variation and activated the Treefinder option of allowing different relative rates according to codon position in protein-coding sequences such as ours. Nodal support was assessed with 100 bootstrap replicates.

Analyses of the amino acid data were conducted using the Bayesian inference (BI) method. We used the MrBayes 3.0b4 program (Huelsenbeck and Ronquist, 2001) with a model of amino acid substitution specially developed for mtDNA-encoded proteins, the mtREV model (Adachi and Hasegawa, 1996). Runs of 500,000 generations were executed, with a sampling frequency of 100 and a burnin parameter of 200, therefore retaining 4800 trees. Stability of the likelihood scores was assessed in preliminary trials before setting the burnin parameter. Bayesian posterior probabilities were given by the percentage of runs that produced each branch. We repeated the analysis five times to confirm that the results converged to the same topology.

3. Results

3.1. Sequence saturation

A fragment of 617 bp from the mitochondrial COI gene was compared for 37 ascidian species. Four hundred and thirteen variable sites were found in the data set, of which 361 were parsimony informative. Of the nucleotide substitutions, 31.4% occurred at first codon positions, 11.6% at second codon positions, and 56.9%

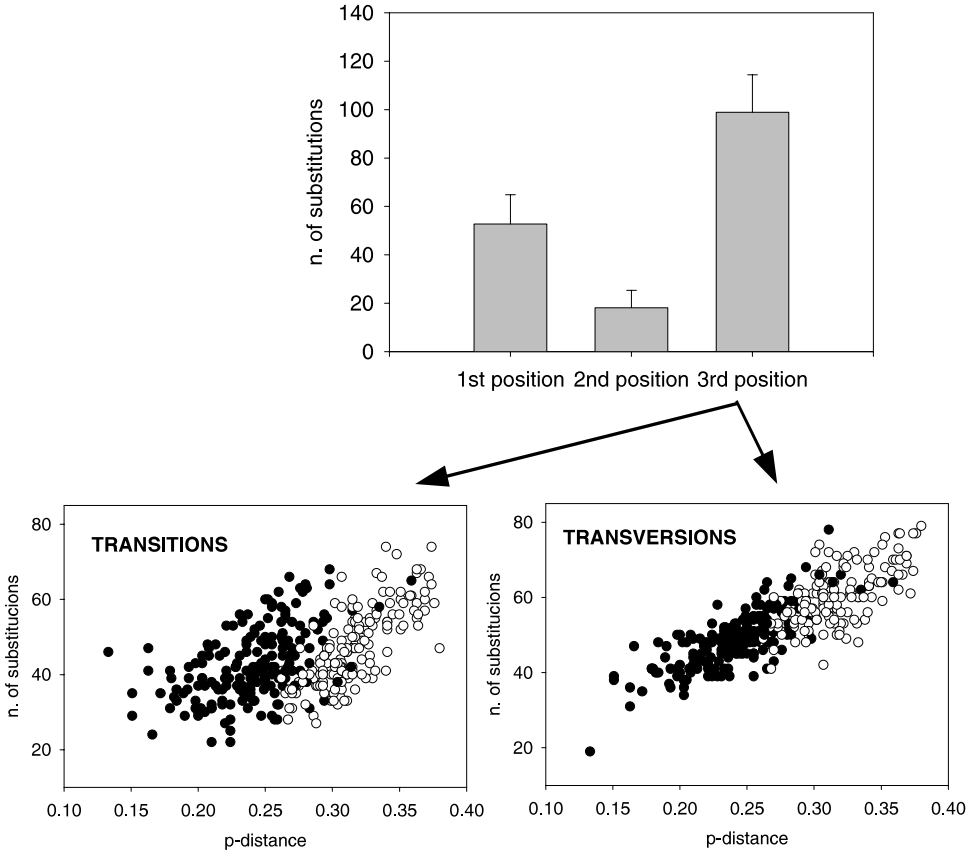

Fig. 1. Means (±SD) number of substitutions in pairwise comparisons among ascidian species for first, second, and third codon positions. For the latter, number of transitions and transversions in pairwise comparisons are plotted against p distances. Solid circles correspond to comparisons within the groups of Aplousobranchiata, Phlebobranchiata, and Stolidobranchiata; open circles to inter-group pairs.

occurred at third positions (Fig. 1), with an overall transition/transversion ratio of 0.91. To check the possibility of this gene being saturated at the phylogenetic level considered, we plotted uncorrected p distances against the number of substitutions in third codon positions (the most likely candidates for saturation in a coding gene) for all species pairs. Visual inspection of the plots (Fig. 1) reveals high dispersion of the number of substitutions (transitions in particular) for a given distance value, but there was a steady increase in the number of substitutions with genetic distance, so no clear sign of saturation was present.

3.2. Phylogenetic analysis

Figs. 2–5 show the trees obtained with the different methods, together with the corresponding bootstrap values (or Bayesian posterior probabilities) of the nodes. For the MP analysis of DNA sequence data, a single most parsimonious tree was found (Fig. 2). When the analysis was performed on deduced amino acid sequences, 247 trees of equal length were obtained; the strict consensus is presented in Fig. 3.

In the Modeltest procedure, the Akaike Information Criterion (AIC) showed that the GTR+I+G model (Rodriguez et al., 1990) was the best-fit model among those evaluated. The parameters of the model were as follows: base frequencies, $A = 0.2660$, $C = 0.0831$, $G = 0.1566$, $T = 0.4943$; substitution rate matrix, $A-C = 0.7202$, $A-G = 18.3498$, $A-T = 1.1331$, $C-G = 7.3702$, $C-T = 18.3498$, $G-T = 1.0000$; proportion of invariable sites 0.3046; gamma shape parameter 0.5367. These parameters were input in Treefinder, except the proportion of invariable sites, not available in this program. In addition, we used different change rates according to codon position. The rates estimated were: first position = 0.3801, second position = 0.1008, third position = 2.5189. The tree obtained with Treefinder is shown in Fig. 4. Lastly, Fig. 5 depicts the 50% majority rule consensus tree of the 4800 trees retained from the Bayesian inference (BI) analysis of the deduced amino acid sequences.

The orders Aplousobranchiata sensu Kott (1990) and Stolidobranchiata appeared in all trees, generally with high support values. In contrast, the clade Phlebobranchiata sensu (Kott, 1985) was paraphyletic in the MP tree derived from nucleotides, and found support below 50% in the MP tree from amino acid data and in the ML tree. Only the BI tree supported clearly a Phlebobranchiata group (100% posterior probability). In all cases,

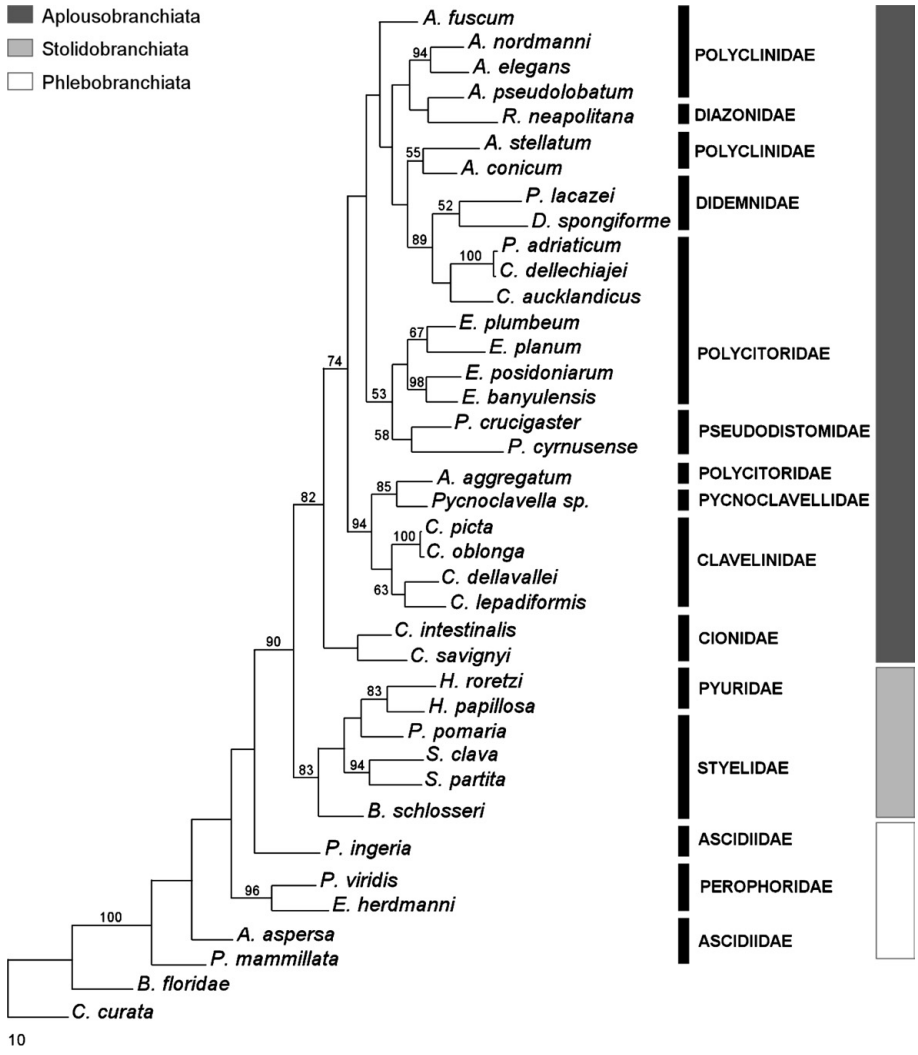

Fig. 2. Parsimony tree (MP) derived from the nucleotide sequences analyzed (tree length = 3,278 steps, CI = 0.257, and RI = 0.416). Numbers indicate bootstrap support percentages (only when $\geqslant 50\%$). Scale bars indicate number of changes. Family assignment as in family 2 (Table 1) is indicated.

Aplousobranchiata appeared as the most derived order with respect to the outgroups, but the relationships with the other ascidian orders were not consistent. When nucleotide sequences were used, the Stolidobranchiata appeared as the sister group of the Aplousobranchiata, whereas in trees derived from amino acid data the Phlebobranchiata and Aplousobranchiata formed a monophyletic clade. Bootstrap/posterior probabilities for these groupings were also very variable.

Within Phlebobranchiata and Stolidobranchiata, and in spite of the low number of taxa included in the analyses, most trees supported traditional family-level groups. Thus, within Stolidobranchiata, the Pyuridae were retrieved in all trees, with high support. The Styelidae were retrieved in ML and BI trees, but were paraphyletic, with Pyuridae branching within them, in MP trees. In the Phlebobranchiata we had representatives of two traditionally recognized families, Ascidiidae and Perophoridae. The Perophoridae were retrieved with high support values in all trees, whilst the Ascidiidae appeared in ML (72% bootstrap support) and BI trees (80% posterior probability), but were polyphyletic in the MP tree derived from nucleotides and were not resolved in the consensus MP tree from amino acids.

As for the Aplousobranchiata—the main focus of this study—in all cases the sequences of the two *Ciona* species (family Cionidae) and of *Rhopalaea neapolitana* (family Diazonidae) grouped with the Aplousobranchiata. However, whereas the *Ciona* clade always appeared as the sister group of the remaining Aplousobranchiata, the position of *R. neapolitana* was unstable. The Didemnidae sequences (*Polysyncraton lacazei* and *Diplosoma spongiforme*) formed in all trees a monophyletic clade generally well supported, and they branched next to the Cionidae at the base of Aplousobranchiata in all cases except in the MP tree obtained from nucleotides. As for the remaining aplousobranch groups, there was considerable variation, but some clades did appear

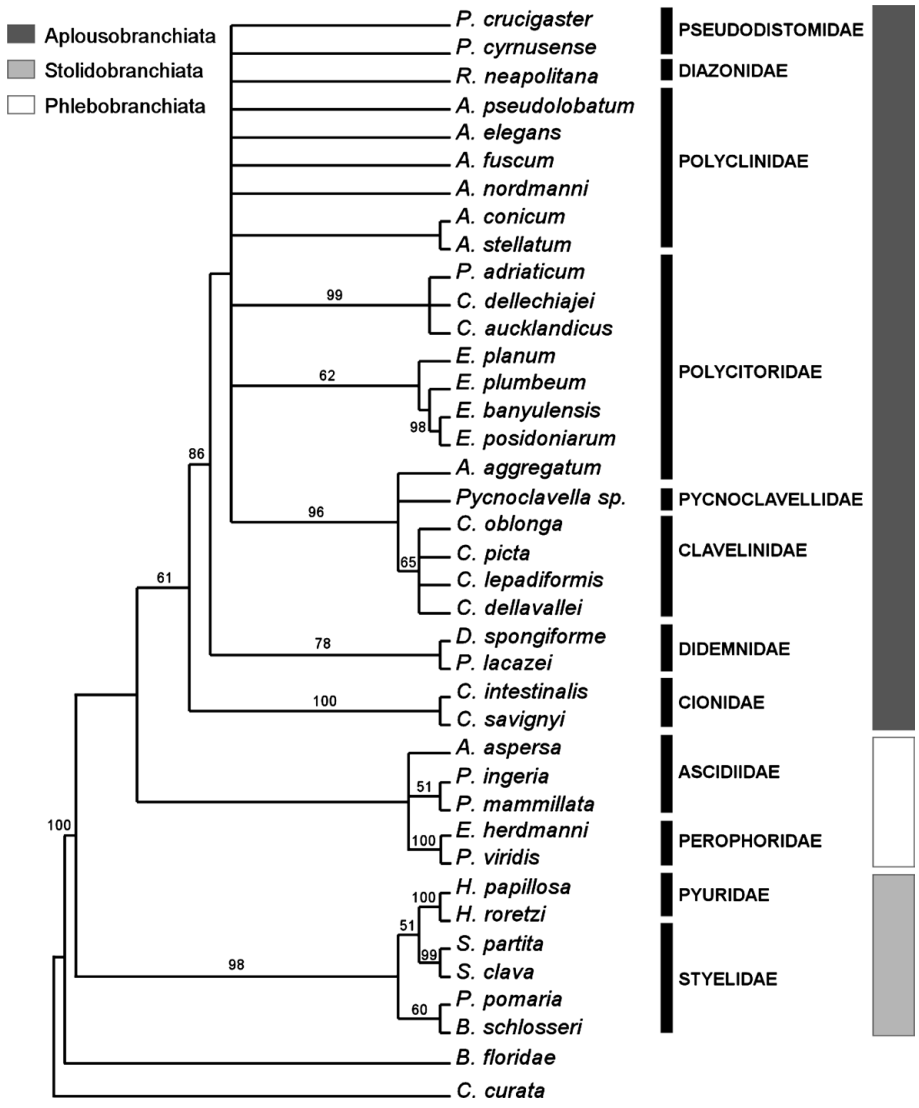

Fig. 3. Strict consensus of 247 most parsimonious trees derived from the deduced amino acid sequences analyzed (tree length = 634 steps, CI = 0.536, and RI = 0.693). Numbers indicate bootstrap support percentages (only when $\geqslant 50\%$). Family assignment as in family 2 (Table 1) is indicated.

consistently. For instance, all analyses retrieved a clade comprising the *Clavelina* species, *Pycnoclavella* sp. and *Archidistoma aggregatum* with bootstrap values or posterior probabilities higher than 94%. The *Eudistoma* and the *Pseudodistoma* species also formed a well-supported monophyletic clade in all trees except the strict consensus MP tree derived from amino acids, where the two *Pseudodistoma* species did not form a resolved clade. Moreover, *Polycitor adriaticum* and the two *Cystodytes* species formed a monophyletic clade in all trees, usually with high support values. The six species of the genus *Aplidium* included in the analysis did not form a monophyletic group in any of the trees except in the ML tree, and with a weak (57%) bootstrap support value. Except for the basal position of Cionidae (and possibly the Didemnidae), the sister group relationships between the aplousobranch clades were not well resolved in our trees and the topologies obtained were variable.

4. Discussion

The combination of diverse analytical approaches, each with a different underlying philosophy, may be particularly useful for testing the robustness of the phylogenetic signal recovered from the data. The results obtained using MP, ML, and BI methods differed in some topological aspects, but the main groups obtained were similar. Stach and Turbeville (2002) reported a lack of congruence between results obtained with COI and 18S rRNA data in ascidians, and attributed this either to a high mutation rate of COI, leading to saturation, or to the presence of pseudogenes. In our case, there

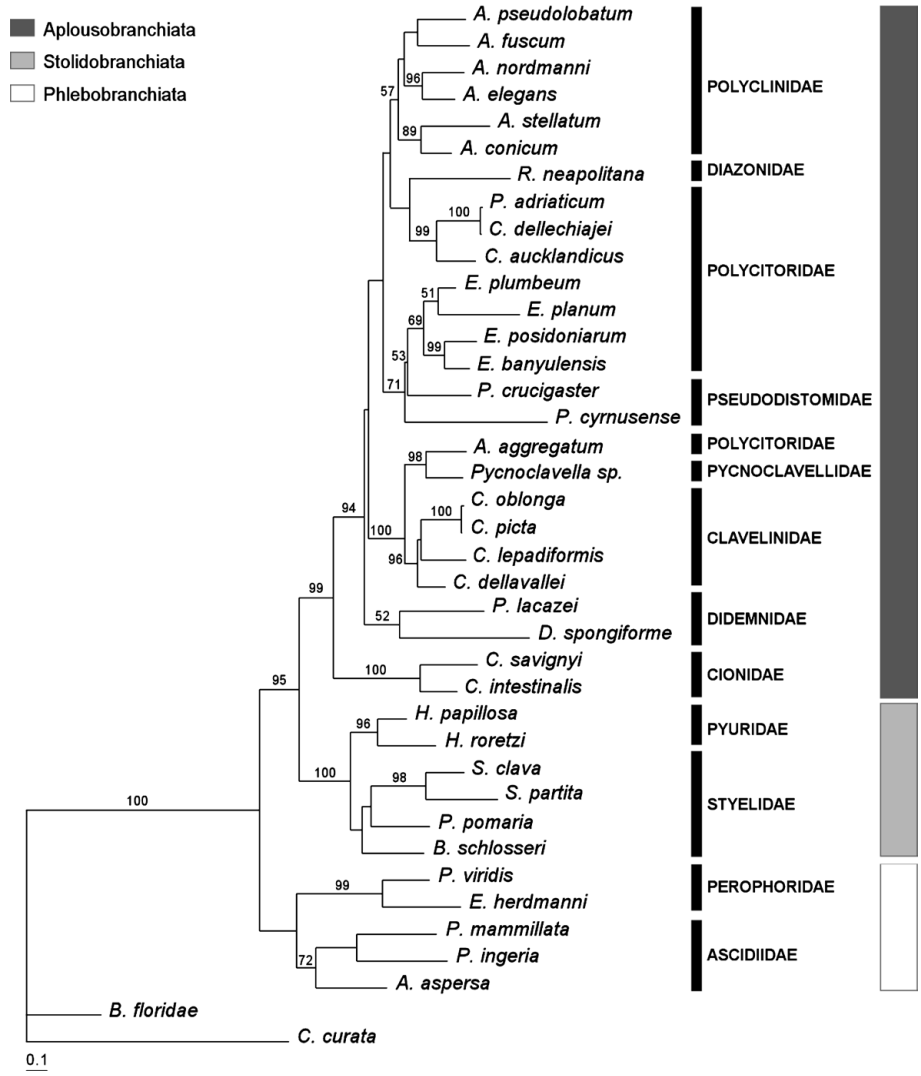

Fig. 4. Maximum likelihood tree (ML) derived from the nucleotide sequences analyzed (−ln $L = 12791.60$). Numbers above branches indicate bootstrap support percentages (only when $\geqslant 50\%$). Scale bars indicate number of substitutions per site. Family assignment as in family 2 (Table 1) is indicated.

was no clear sign of saturation, in spite of the high genetic variability, and analyses of the nucleotide and deduced amino acid sequences (which should remove most of the saturation effects) showed little difference. Furthermore, a relationship between the lack of phylogenetic structure and rapidly evolving sites (i.e., third codon positions) is not always warranted (Källersjö et al., 1999). Finally, given the lack of indel events and stop codons in the sequences generated, we are confident that no pseudogenes were present (Bensasson et al., 2001).

The clades Aplousobranchiata sensu Kott (1990) and Stolidobranchiata were retrieved in all our analyses, but our results did not conclusively confirm the Phlebobranchiata clade. In this context, Stach and Turbeville (2002) found the phlebobranchs to be paraphyletic using morphological data, but the 18S rRNA data set, as well as the combined analysis of morphology and sequence data, showed a monophyletic Phlebobranchiata. Further studies with more species are needed before a sound evolutionary scheme can be established for phlebobranch ascidians. The relationships of Thaliacea with this clade (Stach and Turbeville, 2002; Swalla et al., 2000) should also be taken into account. Unfortunately, we could not include thaliaceans in our studies. Inclusion of Appendicularia in our analyses would also be of great interest, especially in view of their reported relationships with Aplousobranchiata (Stach and Turbeville, 2002), but the published *Oikopleura dioica* sequences were difficult to align with ours and resulted in a long branch outside Ascidiacea (not shown). More research including representatives of thaliaceans, appendicularians and ascidians will be necessary before a complete picture of tunicate relationships can be drawn.

Aplousobranch ascidians always appeared as a derived group, indicating that their simple branchial structure is not plesiomorphic. However, the relative position

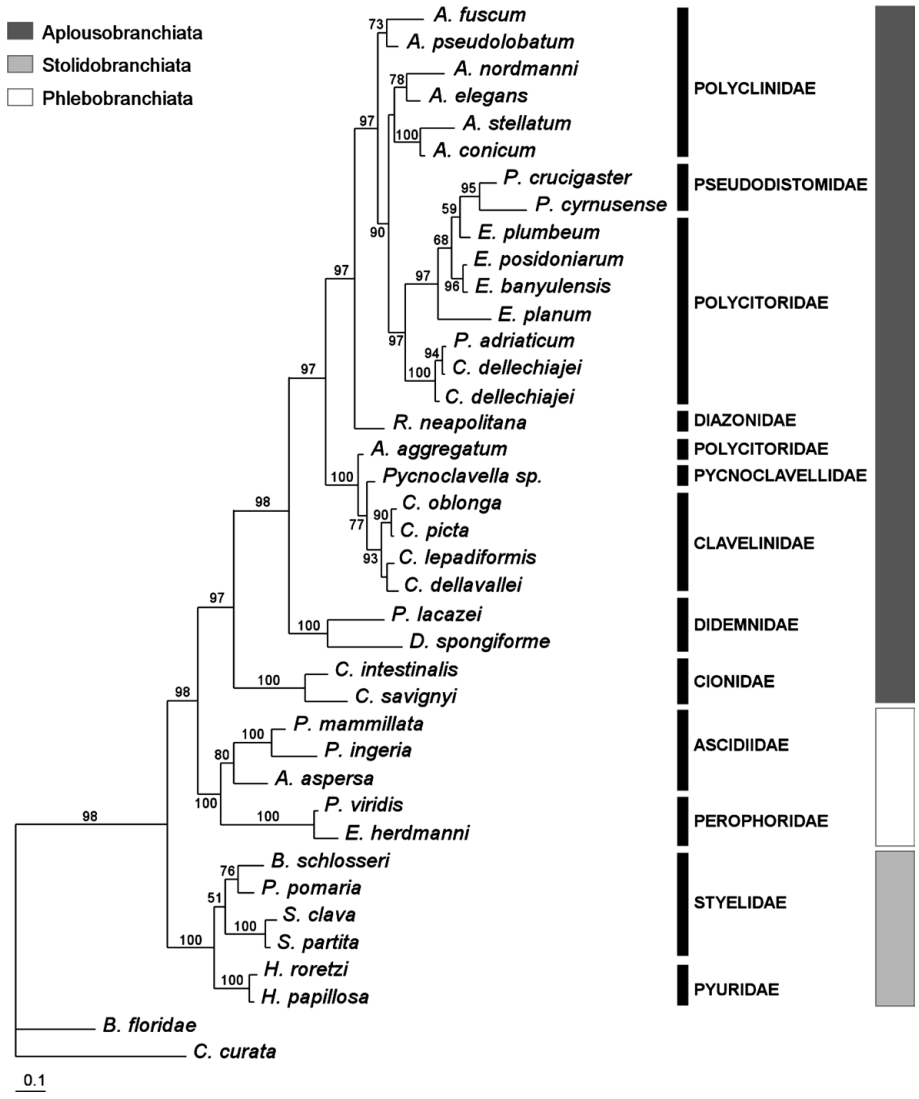

Fig. 5. Fifty percentage majority rule consensus of 4800 trees retained in the Bayesian inference (BI) analysis of the deduced amino acid sequences. Numbers on nodes indicate posterior probabilities. Scale bars indicate number of substitutions per site. Family assignment as in family 2 (Table 1) is indicated.

of the main clades within Ascidiacea varied in our analyses, since a sister group relationship was found between aplousobranchs and stolidobranchs in some cases, and between aplousobranchs and phlebobranchs in others. Therefore, we could not confirm the Enterogona clade (in the sense of Garstang, 1928; grouping Aplousobranchiata and Phlebobranchiata). Aside from the gonad position (which may be plesiomorphic in tunicates; Stach and Turbeville, 2002), the Enterogona are characterized by the origin of the atrial cavity from a pair of dorsal invaginations (as opposed to a single invagination in Pleurogona) but, again, this is possibly the plesiomorphic condition (Kott, 1985).

In all analyses, Cionidae and Diazonidae appeared grouped with the aplousobranch ascidians. This fact confirms Kott's (1969, 1990) view, also supported by evidence from vanadium oxidation state (Hawkins et al., 1983), that these families belong within the Aplousobranchiata and not within the Phlebobranchiata, as traditionally recognized. In the only molecular analyses that included some aplousobranch species, Stach and Turbeville (2002) did not find *Ciona* or *Diazona* to be close to the aplousobranch species in analyses of 18S rRNA, but *Ciona* did cluster with high bootstrap value with an aplousobranch and an appendicularian in analyses of COI sequences. These authors also performed a parsimony analysis of a matrix incorporating 24 morphological, biochemical, and life history characters (including vanadium oxidation state), in which Aplousobranchiata appeared as monophyletic without Cionidae or Diazonidae in it. Some remarks, however, can be made about the morphological data used in that work. The synapomorphies found for Aplousobranchiata (without Cionidae or Diazonidae) were: (a) body divided in regions, (b) ever-

sible larval papillae, (c) arrangement of larval papillae in a row, and (d) vanadium state. The latter is shared with Cionidae and Diazonidae, whereas the three former characters are compatible with the view of a progressive reduction in body size and increased larval complexity associated with brooding. Diazonidae have the body divided in regions, and Cionidae have at least the gut posterior to the branchial wall. Big larvae tend to have complex papillae arranged in a vertical row. However, relatively small larvae within the Aplousobranchiata (e.g., those of *Clavelina lepadiformis*; see Turon, 1991) have simple, non-everting papillae, and a triangular arrangement of the adhesive papillae is not uncommon in aplousobranch larvae (Kott, 1990).

Most morphological characters fit into a scheme of reduction and simplification of branchial structures and zooid size, concomitant with brooding and increase in larval size, as having occurred along an evolutionary line from a common ancestor of Cionidae, Diazonidae, and the remaining aplousobranch families. Characteristics of the branchial basket link Cionidae and Diazonidae with other phlebobranchs in Stach and Turbeville's (2002) data matrix, but these characters are plesiomorphic if the branchiae of aplousobranchs are secondarily reduced.

Aplousobranchiata, including Cionidae and Diazonidae, are better defined by characteristics other than branchial structure per se: for instance, in all their members the epicardium has a regenerative/blastogenetic role, and all have gut, heart and gonads located posterior to the branchial basket (Kott, 1990). Cionidae (oviparous solitary species with simple larvae) appears to be the sister group of the remaining forms. These are composed of brooding colonial species with generally complex larvae, except for the Diazonidae, a family with both solitary and colonial forms that feature an oviparous mode of reproduction and simple larvae. This arrangement suggests that coloniality in the aplousobranch ascidians—as well as brooding, complex larval types (Svane and Young, 1989) and simple branchial sac—are secondary characters derived from an ancestral oviparous solitary form with phlebobranch pharynx and simple larvae. The Diazonidae display transitional characteristics towards coloniality, probably inherited from an ancestor that had already acquired the potential for a colonial lifestyle. Coloniality also appears in unrelated families of Phelobranchiata (Perophoridae) and Stolidobranchiata (Styelidae). Our trees, therefore, favor the view that the ascidian ancestor was a solitary form (Berrill, 1955), and that coloniality has arisen several times (Wada et al., 1992).

Several species relationships within the Aplousobranchiata appeared consistently in our trees, suggesting monophyletic groups that can be used to arrange the family-level layout of this order. The sequences of *Clavelina*, *Pycnoclavella*, and *Archidistoma* appeared in all trees as a monophyletic group with high support values. Kott (1990) defined the family Pycnoclavellidae, removing its members from Clavelinidae on the basis of important differences in larval structure and budding pattern: abdominal division in Pycnoclavellidae and terminal stolonic budding in Clavelinidae (Kott, 1990; Trason, 1963; but see Monniot and Monniot, 1996; for a different point of view). On the other hand, although *Archidistoma* is commonly placed in Polycitoridae, it has an unusual colony form for this Family: almost independent zooids arising from a common sheet of tunic material traversed by stolons. This colonial arrangement is the same as that found in many Clavelinidae and Pycnoclavellidae. Our results indicate that Clavelinidae and Pycnoclavellidae are in fact closely related, and that some species previously placed within Polycitoridae also belong to this clade.

One of the striking findings of this study is the close relationship between *Eudistoma* and *Pseudodistoma* species. Millar (1966) already noted that the two genera may be closely related, but this view did not gain acceptance and *Eudistoma* has been placed in the family Polycitoridae, whilst *Pseudodistoma* was included in the Polyclinidae. Recently, Kott (1992) described the new family Pseudodistomidae to include *Pseudodistoma* and the newly defined genus *Anadistoma.* Both *Eudistoma* and *Pseudodistoma* have three rows of branchial stigmata, with the first row deflected anteriorly, but *Eudistoma* species have strong transverse muscle bands in the thorax and long gut loops, whilst *Pseudodistoma* exhibits weak transverse thoracic muscles, shorter gut loops and a developed post-abdomen. However, a shortening of the gut loop may well have resulted in the gonads and heart occupying a post-abdominal position, and some Pseudodistomidae (e.g., *Anadistoma*) have a long oesophageal region, a relatively short post-abdomen and well developed transverse thoracic musculature, which may suggest a relationship with *Eudistoma* (Kott, 1992). The Family Polycitoridae, as redefined by Kott (1990), appeared to be polyphyletic, as *Eudistoma* and *Archidistoma* did not group in our analyses with the other members of the family, represented by the consistent grouping of *Polycitor adriaticum* and the two *Cystodytes* species.

The family Didemnidae, represented here by the genera *Diplosoma* and *Polysyncraton*, appeared in all trees. Although members of this family have gone a long way in following the tendency of reduction and simplification of structures (Lafargue and Wahl, 1987), in all but one of our trees they branched after the Cionidae, close to the base of the aplousobranchs. Hawkins et al. (1983) also postulated a deep branching origin of the Didemnidae within Aplousobranchiata based on high levels of vanadium in the genus *Leptoclinides*.

The family Polyclinidae is represented in our data set by six species of *Aplidium*. Kott (1992) split the old fam-

ily Polyclinidae into six families, of which five were new. The genus *Aplidium* remained within the Polyclinidae sensu (Kott, 1992). The six sequences included here often appeared as a paraphyletic assemblage, but we clearly need more sequences of other genera, together with rigorous morphological analysis, to ascertain the validity and relationships of this family.

In conclusion, our results show some stable groupings within the Aplousobranchiata, some compatible with current taxonomic (family-level) layouts and some not. Further work will be necessary before a definite arrangement can be formalized, and this arrangement should include the families Cionidae and Diazonidae. This work, based on a single gene, was intended to provide a new data set to study phylogenetic relationships in ascidians, and as a touchstone for current taxonomic thinking that may lead to the re-evaluation of some morphological characters. Undoubtedly, further testing will refine this scheme, and we hope that our study will stimulate further research. Crucial to this issue is the inclusion of sequences from non-ascidian tunicates. There is little doubt that continued investigation will soon lead to a sound reconstruction of ascidian and tunicate phylogeny.

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