Internal and external relationships of the Cnidaria: implications of primary and predicted secondary structure of the 5′-end of the 23S-like rDNA

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SUMMARY

Since both internal (class-level) and external relationships of the Cnidaria remain unclear on the basis of analyses of 18S and (partial) 16S rDNA sequence data, we examined the informativeness of the 5′-end of the 23S-like rDNA. Here we describe analyses of both primary and predicted secondary structure data for this region from the ctenophore *Bolinopsis* sp., the placozoan *Trichoplax adhaerens*, the sponge *Hymeniacidon heliophila*, and representatives of all four cnidarian classes. Primary sequence analyses clearly resolved the Cnidaria from other lower Metazoa, supported sister group relationships between the Scyphozoa and Cubozoa and between the Ctenophora and the Placozoa, and confirmed the basal status of the Anthozoa within the Cnidaria. Additionally, in the ctenophore, placozoan and sponge, non-canonical base pairing is required to maintain the secondary structure of the B12 region, whereas amongst the Cnidaria this is not the case. Although the phylogenetic significance of this molecular character is unclear, our analyses do not support the close relationship between Cnidaria and Placozoa suggested by previous studies.

1. INTRODUCTION

The phylum Cnidaria comprises a diverse group (~ 9000 living species; Ruppert & Barnes 1994) of mostly marine organisms, which are characterized by the possession of cnidae. Cnidarians are amongst the simplest animals at the tissue level of organization. With the Ctenophora and a number of minor phyla, the Cnidaria are often referred to as diploblastic animals (having two tissue layers; c.f. triploblastic animals), although the validity of this distinction is questionable. Within the Cnidaria, two distinct types of life histories are seen: three of the four cnidarian classes (Hydrozoa, Cubozoa and Scyphozoa) display an alternation of generations (sessile polyp and mobile medusa), whereas the Anthozoa are distinguished by the absence of a medusoid stage. There is no consensus as to which of these two types of life history is ancestral. One view (figure 1) is that the medusa-only life cycle, seen today only amongst trachyline hydrozoans, represents the primitive condition which gave rise to the medusa–polyp alternation of generations characteristic of all other hydrozoans, scyphozoans and cubozoans (see, for example, Hyman 1940; Hand 1959; Brusca & Brusca 1990). Whilst this view regards the Anthozoa as having undergone secondary loss of the medusoid stage, another opinion is that this class is most representative of ancestral character states (i.e. that the polyp-only life cycle is ancestral), and that the medusa-only life cycle seen in the trachymedusae represents secondary loss of the polypoid stage (Hadzi 1953; Willmer 1990; Schuchert 1993; Nielsen 1995). External relationships of the Cnidaria are also unclear. Traditionally, the Ctenophora were grouped with the Cnidaria into the phylum Coelenterata, however, there is compelling morphological support for these being regarded as distinct phyla (Harbison 1985).

There have been relatively few attempts to apply molecular techniques to address either class-level relationships within the Cnidaria, or their external relationships. Bridge's (Bridge *et al*. 1992) demonstration that amongst cnidarians, only the Anthozoa have typical animal (i.e. small and circular) mitochondrial genomes implies ancestral status for this class. A preliminary distance analysis of 5S rDNA data (Hori & Satow 1991) for a restricted range of cnidarians (i.e. no cubozoans were used) is also consistent with this, but neither study was informative with respect to the positions of the other classes. Bridge's later parsimony analyses of combinations of (nuclear) 18S and partial (mitochondrial) 16S rDNA sequences with morphological data (Bridge *et al*. 1995) resulted in a number of unresolved polychotomies, and add little to the understanding of cnidarian relationships. The Placozoa, represented by the single genus *Trichoplax* are small marine animals with very restricted cell complements (Grell & Ruthmann 1991), whose phylogenetic position is equivocal (c.f. Ruppert

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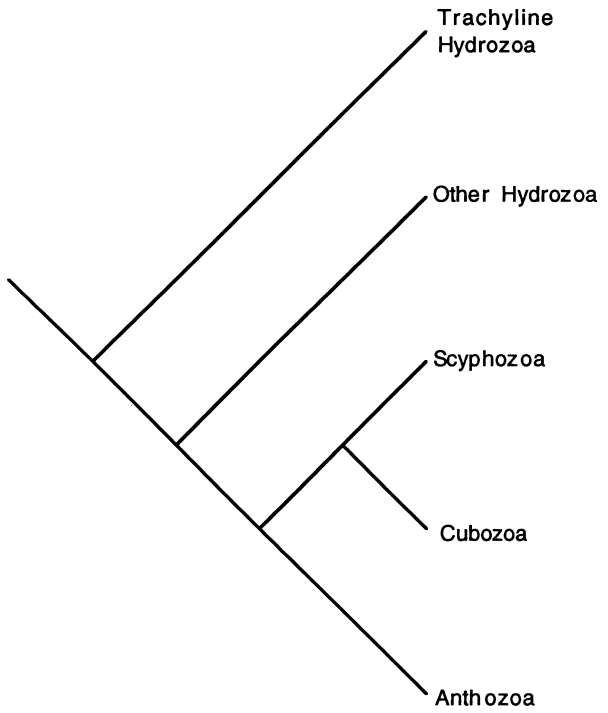

Figure 1. Phylogenetic relationships of the Cnidaria based on Brusca & Brusca (1990), supporting Hyman's (1940) view of hydrozoan ancestry.

& Barnes 1994). Two studies based on complete 18S rDNA sequences support a sister group relationship between Cnidaria and Placozoa (Wainwright *et al*. 1993; Philippe *et al*. 1994), whereas Bridge's analysis of 'stable' 18S rDNA characters puts *T. adhaerens* into the cnidarian clade (see Bridge *et al*. 1995, figure 2).

Because both internal (class-level) and external relationships of the Cnidaria remain unclear on the basis of analyses of 18S and (partial) 16S rDNA sequence data, we examined the informativeness of a combined 5.8S and partial 28S rDNA dataset. Several phylogenetic analyses have been based on sequence data for the 5′-end of the 28S rDNA (see, for example, Viscogliosi *et al*. 1993; Littlewood 1994; Chen *et al*. 1995; Sorhannus *et al*. 1995). However, in none of these cases has combined 5.8S and 28S rDNA data been used. The eukaryotic 5.8S rRNA corresponds to the 5′ terminal region of the 23S rRNA of prokaryotes (Nazar 1980; Jacq 1981), and there is extensive secondary structure complementarity between the 5′-end of the 28S rRNA and the 5.8S rRNA molecules. Schnare *et al*. (1996) demonstrated that group-specific structural features of the 23S-like (5.8S + 28S) rRNA can be phylogenetically informative. The conserved nature of the 5′-end of this region of the rRNA transcription unit at both the primary and secondary structure levels, and the availability of a substantial body of comparative data, imply that it may be helpful in resolving relatively deep phylogenetic relationships. Below, we describe preliminary analyses of both primary and predicted secondary structure data for the 5′-end of the 23S-like rDNA from representatives of all four Cnidarian classes and a range of other 'lower' metazoans. Whereas previous attempts to apply this approach to kingdom-level relationships (De Rijk *et al*. 1995) suffered the limitations imposed by the need to align highly divergent sequences, no comparable difficulties were encountered in the present case.

2. MATERIALS AND METHODS

(*a*) *Material*

Species used in the present study were: *Hymeniacidon heliophila* (Porifera; Class Demospongia), *Trichoplax adhaerens* (Placozoa), *Bolinopsis* sp. (Ctenophora; Class Tentaculata; Order Lobata), *Chironex fleckerii* (Cnidaria; Class Cubozoa), *Dendronephthya* sp. (Cnidaria; Class Anthozoa; Subclass Alcyonaria), *Scapophyllia cylindrica* (Cnidaria; Class Anthozoa; Subclass Zoantharia), *Cyanea capillata* (Cnidaria; Class Scyphozoa), *Millepora exaesa* (Cnidaria; Class Hydrozoa; Order Milleporina) and *Distichopora* sp. (Cnidaria; Class Hydrozoa; Order Stylasterina).

(*b*) *DNA extraction*

DNA from *T. adhaerens*, *H. heliophila* and *C. capillata* was kindly provided by K. G. Grell, J. Holden and P. A. Anderson, respectively. In all other cases DNA was extracted essentially as described in Chen *et al*. (1995).

(*c*) *Polymerase chain reaction, cloning and DNA sequencing*

Two forward primers were used in conjunction with the reverse primer (5′-GCTTTGGGCTGCAGTCCCAAGCA-ACCCGACTC-3′; *Pst*I site underlined) described in Chen *et al*. (1995), which anneals at positions 278–312 in the mouse 28S rDNA. Forward primer 1 (5′-GGTACCCTTTGTAC-ACACCGCCCGTCGCT-3′) anneals at positions 1621–1645 in the 18S rDNA of *Anemonia sulcata* (GenBank accession number X53498); forward primer 2 (5′- GAATTCCGTA-GGTGAACCTGCGGAAGGAT-3′) anneals at positions 1769–1794. *Kpn*I and *Eco*RI restriction sites (underlined in the respective sequences) were included on the ends of the forward primers to facilitate cloning the PCR products. PCR, cloning, and sequencing conditions were as previously described (Chen *et al*.1995; Veron *et al*. 1996).

(*d*) *Sequence analyses*

The DNA sequences were submitted to GenBank (accession numbers U65477–U65485). The analyses were based on combined data for the 5.8S rDNA and approximately 240 bases at the 5′-end of the 28S rDNA. The pre-aligned eukaryotic sequences of the LSU RNA database located at the rRNA WWW Server (http://rrna.uia.ac.be/) (De Rijk *et al*. 1994), were utilized to aid alignment. This database contains secondary structure information in addition to primary sequence alignments. CLUSTAL W 1.5 (Thompson *et al*. 1994) was used to align sequences, which were then edited manually using SeqApp 1.9 (Gilbert 1992*a*). Nucleotide substitution patterns were assessed by pairwise comparisons using MEGA 1.02 (Kumar *et al*. 1993). Not all of the data shown were used in the phylogenetic analyses; regions which could not be aligned with a high degree of confidence were excluded. Exhaustive parsimony analyses were conducted using PAUP 3.1.1 (Swofford 1991). Distance analyses were carried out using DNADIST (with Kimura correction) to calculate the distance matrices after 1000 bootstraps (SEQBOOT), followed by the NEIGHBOR routine (PHYLIP 3.5c; Felsenstein 1993). Maximum likelihood analyses made use of DNAML (100 bootstraps with global

rearrangements and ten random additions) in PHYLIP 3.5c (Felsenstein 1993). The permutation tail probability (PTP) test (Archie 1989; Faith & Cranston 1991) was used to determine if the data set contained phylogenetically informative characters. The RANDOMISER program package 1.0 (Trueman 1994) was used to generate 1000 randomized datasets which were analysed in PAUP 3.1.1 to obtain minimum tree lengths. If a significant proportion (as defined by Q' (Farris 1991); $Q' \geqslant 1.3$ at the 95% confidence level) of the minimum tree lengths obtained from the randomized data are larger than the length of minimum-length trees obtained from the actual data, then the null hypothesis that the data are phylogenetically uninformative can be rejected.

(*e*) *Secondary structures*

Secondary structure analyses were initially compared with data in the LSU RNA alignment database (De Rijk *et al.* 1994), which allowed identification of secondary structure features of the lower metazoan sequences. The final structures were drawn with the aid of the programs MulFold (Gilbert 1990) and LoopDloop (Gilbert 1992*b*), but modified according to Gutell *et al.* (1993) and Schnare *et al.* (1996).

3. RESULTS

Little size variation was observed between taxa in the regions encoding the 5.8S rDNA and 5′-end of the 28S rDNA (size range 425 to 428 base pairs). Nucleotide composition data show the (G + C) content for the LSU ranging from 46.1% in *T. adhaerens* to 52.2% in *H. heliophila* , with the mean composition of the four bases across all nine taxa being 27.3% A, 24.2% T, 22.2% C and 26.3% G. Neither the partial 28S nor the 5.8S sequences showed significant bias in base composition between taxa. The mean transition/transversion ratio (ts/tv ratio; table 1) for the complete dataset was 1.44 (range 0.88–3.39), and for the 5.8S and partial 28S sequences 1.80 and 1.39 respectively. These high ts/tv ratios indicate that saturation has not occurred, and therefore that parsimony analyses are appropriate. Use of the permutation test with 1000 randomizations of the dataset yielded a Q' value (Farris 1991) of 3.0 ($p < 0.001$), which again indicates that the data are likely to be informative for cladistic analyses.

Of the total 433 bases of aligned sequence, 399 were used in phylogenetic analyses. Of these, 147 sites were variable and 89 (20.5% of positions) informative for parsimony analysis. Parsimony, maximum likelihood, and distance analyses produced trees in which the Cnidaria were monophyletic and which were congruent with respect to relationships within the cnidarian clade, but differed in the relative positions of the ctenophore and placozoan (figure 2). In each case, the Anthozoa were clearly resolved from the rest of the Cnidaria (i.e. the Medusozoa (Hydrozoa, Scyphozoa and Cubozoa) were monophyletic), and the Hydrozoa were well separated from a clade comprising the cubozoan and the scyphozoan. Both maximum likelihood and distance analyses supported a sister group relationship between the placozoan and the ctenophore (figure 2) not seen in the shortest parsimony tree (figure 2).

In an attempt to resolve external relationships of the Cnidaria, we compared predicted secondary structures for this region. For all taxa used in the present study almost all substitutions in this region were compensatory, resulting in no change at the level of predicted secondary structure. However, one exception to this was observed in the B12 region *sensu* De Rijk *et al.* (1994). Figure 3*a* shows the predicted folding pattern of the region sequenced in this study for the hydrozoan *Distichopora* sp. In the ctenophore, placozoan and sponge, non-canonical base pairing is required to maintain the secondary structure of the B12 region, whereas amongst the Cnidaria this is not the case (see figure 3*a*, *b*).

4. DISCUSSION

All three methods of phylogenetic analysis strongly support the basal position of the anthozoan branch within the Cnidaria, which is consistent with 5S rDNA sequence (Hori & Satow 1991) and mitochondrial genome structure (Bridge *et al.* 1992) comparisons. The consensus is therefore that within the Cnidaria, the polyp-only life cycle is ancestral and alternation of generations secondarily derived. The most convincing candidate cnidarians in the Ediacaran fauna are those which resemble pennatulacean anthozoans (but see Seilacher 1989), hence the fossil record may be interpreted as being consistent with the primitive status of the Anthozoa within the Cnidaria (Conway-Morris 1993). Our analyses also support a sister group

Table 1. *Number of nucleotide differences (transitions/transversions) in pairwise comparisons*

taxon	1	2	3	4	5	6	7	8	9
1 *H. heliophila*		57/37	57/44	52/50	53/47	65/52	63/47	63/48	58/48
2 *S. cylindrica*			39/41	37/19	26/24	39/22	38/27	44/28	51/30
3 *Bolinopsis* sp.				35/40	39/42	42/43	44/44	41/43	45/40
4 *C. capillata*					31/28	32/15	34/17	44/13	47/34
5 *Dendronephthya* sp.						38/30	34/34	41/33	46/45
6 *Distichopora* sp.							23/8	42/20	46/29
7 *M. exaesa*								44/25	47/32
8 *C. fleckerii*									46/38
9 *T. adhaerens*									

Overall transition/transversion ratios ranged from 0.88 to 3.39 (mean = 1.44); transition/transversion ratios for the 5.8S gene only ranged from 0.71 to 3.50 (mean = 1.80); transition/transversion ratios for the 28S region only ranged from 0.76 to 3.78 (mean = 1.39).

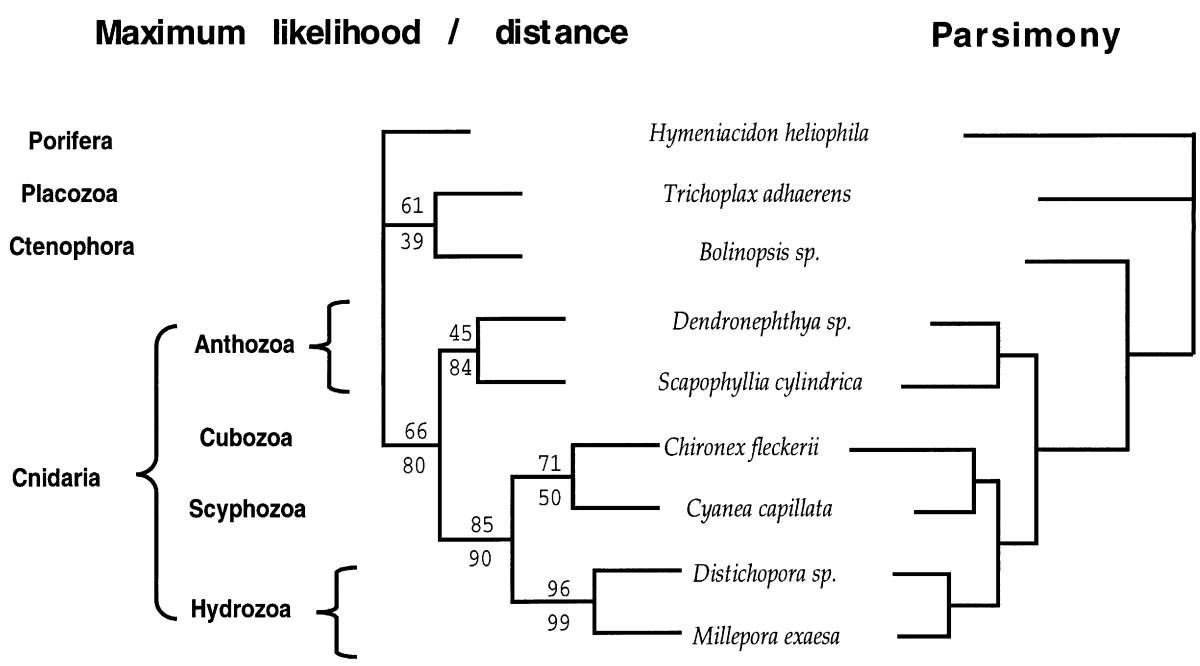

Figure 2. Phylogenetic analyses of the 23S-like rDNA dataset. The phylogenetic tree shown on the left resulted from maximum likelihood analysis. Distance analysis produced the same topology, except that the ctenophore and placozoan clade was the sister group of the Cnidaria. Numbers above the branches indicate the percentages of 100 bootstrap replicates for the maximum likelihood analysis supporting the topology shown; those below the branches are the corresponding values from distance analysis (1000 bootstrap replicates). The tree shown on the right resulted from exhaustive parsimony analysis, and is drawn as a phylogram. The most parsimonious tree had a length of 297 (and consistency index of 0.690), compared with an average of 337 for 1 000 000 random trees generated from the same dataset (PAUP 3.1.1). The g_1 value of -0.525 was below the critical value ($g_1 = -0.3$, $p = 0.01$) for nine taxa, indicating that a significant phylogenetic signal exists in the data (Hillis & Huelsenbeck 1992).

relationship within the Medusozoa between the Scyphozoa and the Cubozoa. Whilst there is substantial morphological support for this (see, for example, Schuchert 1993), all previous molecular analyses have failed to resolve relationships within the Medusozoa (Bridge *et al*. 1995).

Although the Cnidaria were always clearly resolved from the other phyla, aspects of their external relationships remain equivocal. Whereas previous analyses, based on 18S rDNA sequences, suggest either a sister group relationship between *Trichoplax* and cnidarians (Wainright *et al*. 1993; Philippe *et al*. 1994), or have *Trichoplax* falling within the Cnidaria (see Bridge *et al*. 1995, figure 2), our analyses (figure 2) imply a closer relationship between *Trichoplax* and the ctenophore *Bolinopsis*. In distance and maximum likelihood analyses, the ctenophore and placozoan formed a sister group to the Cnidaria. While the topology of the most parsimonious tree disagrees with respect to this point, phylum-level relationships were not robust in the corresponding analyses. Forcing a sister group relationship between the ctenophore and placozoan (by means of the tree tool options in MacClade, see Maddison & Maddison 1992) required an increase in the total length of the parsimony tree of only a single unit. Conversely, the relationships implied within the Cnidaria from the parsimony analyses were more robust; for example, forcing basal branching of the Hydrozoa within the Cnidaria required at least four additional steps.

Since relationships between the phyla were not unequivocally resolved by phylogenetic analyses, we examined aspects of the predicted secondary structure of this region of the rRNA transcription unit. Again, these analyses resolved the Cnidaria from the other 'lower' Metazoa; the B12 region in all of the cnidarian sequences closely resembled that of 'higher' metazoans, whereas maintaining the corresponding structure required introduction of a non-canonical bond in the cases of the sponge, placozoan and ctenophore sequences (figure 3). To exclude the possibility that this apparent difference was due to taxon choice, we examined the corresponding region in the range of Cnidaria, Ctenophora and Porifera used in a previous study (Christen *et al*. 1991), which corroborated our finding. An extensive search of the databases revealed that, although the nucleotide sequence of the B12 region is highly conserved across eukaryotes, only in the ctenophores, placozoans, sponges and plants are non-canonical H-bonds required to generate the presumed secondary structure. Cnidarians resemble all other metazoans, fungi and protists in their predicted secondary structures for this region. The infrequency with which departures from this general rule arise (the exceptions are a polychaete, an apicomplexan, a fish and two ascomycetes, accession numbers X80649, L28037, X07623, U40096 and U40098, respectively) suggests the possibility of sequencing errors. Although the phylogenetic significance of this molecular character is unclear, our analyses do not support the close relationship between Cnidaria and Placozoa suggested by previous studies. A primitive position for the

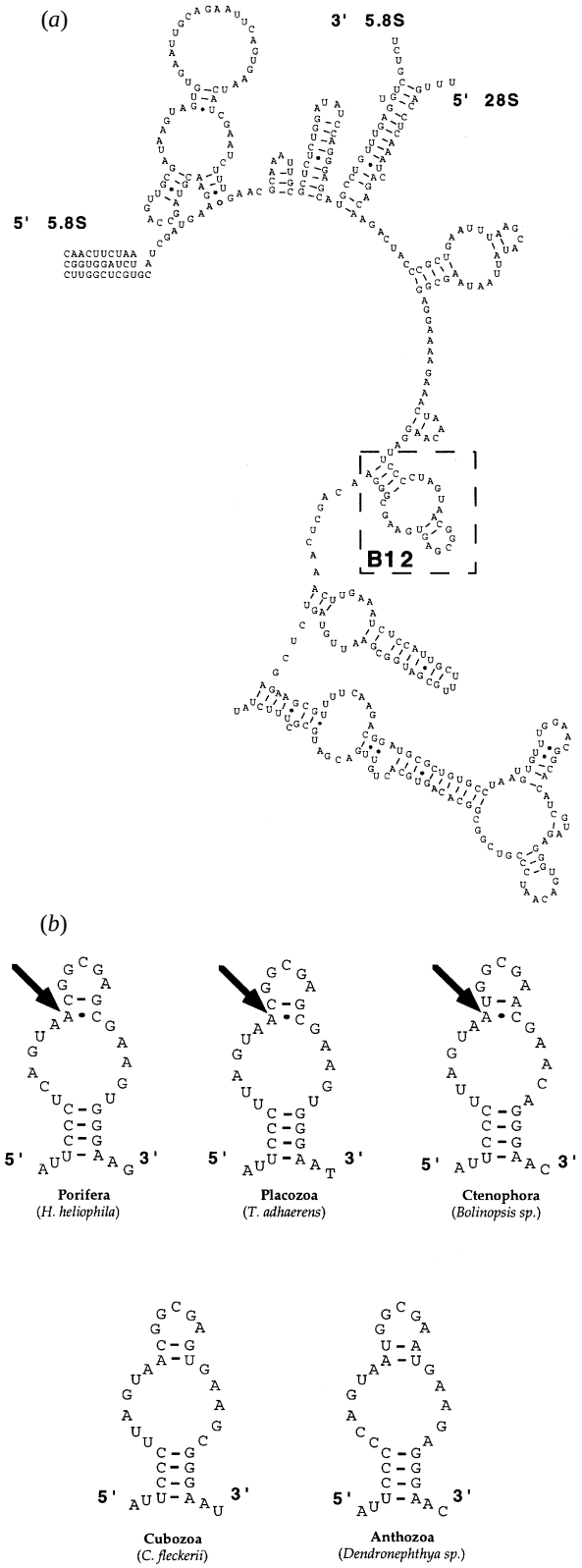

Figure 3. (*a*) Secondary structure model of the 5′-end of the 23S-like rRNA of *Distichopora* sp. (Class Hydrozoa). The B12 region is indicated, showing the characteristic canonical base pairing. (*b*) Predicted secondary structures of the B12 region. For representatives of three lower metazoan phyla, the Porifera, Placozoa and Ctenophora, a non-canonical A–C bond is required at the position indicated to maintain the secondary structure in the B12 region. Also illustrated are two cnidarian sequences, which show the more typical canonical pairing.

placozoan is consistent with the views of Grell (Grell & Ruthmann 1991) and others, who view its simple structure as original rather than derived. However, the relationship between the Ctenophora and Placozoa implied by our analyses appears to be without precedent in the literature. Although there is little evidence to link these phyla, some authors consider that the ancestral ctenophore may have been little more than a bag of ciliated cells (Conway-Morris & Collins 1996), resembling *Trichoplax*.

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