Placozoa Are Not Derived Cnidarians: Evidence from Molecular Morphology

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The phylum Placozoa is represented by a single known species, *Trichoplax adhaerens*, a tiny marine organism that represents the most simple metazoan bauplan. Because of the latter, placozoans were originally considered the most basal metazoan phylum. A misinterpretation of the life cycle at the turn of the century and some more recent molecular phylogenetic analyses have placed *Trichoplax* as a derived species within the Cnidaria. The latter hypothesis assumes that the primitive organization of the Placozoa is the result of secondary reduction. Here we compare the molecular morphology of the predicted 16S rDNA structure and the mitochondrial genome between *Trichoplax* and representatives of all four cnidarian classes. *Trichoplax* shares a circular mtDNA molecule as a plesiomorphy with all other metazoans except for the derived cnidarian classes Hydrozoa, Scyphozoa, and Cubozoa. The predicted secondary structure of the 16S rRNA molecule differs substantially between *Trichoplax* and cnidarians, particularly with respect to the number and length of stem and loop regions. The new molecular morphological characters provide compelling evidence that *Trichoplax* is not a derived (medusozoan) cnidarian. Furthermore, it was found that the mitochondrial genome in Cubozoa consists of four linear molecules instead of a single circular molecule or two linear molecules, suggesting that the cubozoans may represent the most derived cnidarian group.

Introduction

Molecular phylogenetics has not yet resolved evolutionary relationships at the base of metazoan radiation. Instead 18S rDNA sequence data added more confusion than clarity to the evolution of the basal diploblast phyla. In this context the phylogenetic position of the phylum Placozoa, which is represented by a single species, *Trichoplax adhaerens*, has always been a key issue. Traditionally a basal position for the Placozoa has been proposed on the grounds of morphological and developmental simplicity (Grell 1971; Ivanov 1973, see also Syed and Schierwater 2002*a*, 2002*b*). *Trichoplax adhaerens* F. E. Schulze 1883 represents the morphologically most simply organized multicellular animal known (fig. 1), and thus has often been seen as the "living ancestor" of all metazoans. This tiny marine organism is unique in many respects, shows little similarity to other diploblastic animals, and thus has been given its own phylum, the Placozoa. *Trichoplax* is built upon a highly simple bauplan, consisting of a functional lower and upper side (often misleadingly named "dorsal" and "ventral" epithelia, despite the fact that dorsoventrality is an invention of the triploblastic animals), a few cells in between (fibric cells), and altogether only four different somatic cell types (e.g., Grell and Ruthmann 1991). All other diploblast phyla, i.e., the Porifera, Ctenophora, and Cnidaria, are substantially more complex in their organization, and none of the other phyla share any obvious synapomorphies with the Placozoa that could suggest a closer relationship (cf. Syed and Schierwater 2002*a*). Other views, however, place placozoans somewhere between Porifera and Cnidaria (e.g., Ax 1996; Peterson and Eernisse 2001). Molecular data have become a *conditio sine qua non* to resolve the controversies.

Key words: *Trichoplax adhaerens*, Placozoa, Cnidaria, molecular systematics, mtDNA genome structure, 16S rDNA, molecular morphology.

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Based on ribosomal genetic markers, 18S rDNA and 28S rDNA, a number of molecular phylogenetic analyses and reanalyses have shaken the phylogenetic position of *Trichoplax adhaerens*, and all possible phylogenetic scenarios have been suggested for the Placozoa (Christen et al. 1991; Lafay et al. 1992; Wainright et al. 1993; Smothers et al. 1994; Aleshin et al. 1995; Bridge et al. 1995; Sidall et al. 1995; Vladychenskaya et al. 1995; Cavalier-Smith et al. 1996; Pawlowski et al. 1996; Odorico and Miller 1997; Collins 1998; Kim, Kim, and Cunningham 1999; Podar et al. 2001). Most of these studies are in conflict with one another and with the traditional morphological view. The most cited views place the placozoan *Trichoplax adhaerens* in a clade within the Cnidaria (Bridge et al. 1995; Sidall et al. 1995) or in close relation to the Cnidaria forming one clade with nearly any cnidarian species used (Kim, Kim, and Cunningham 1999 and references therein). We here test the hypothesis of that *T. adhaerens* is a secondarily reduced cnidarian.

Materials and Methods

We apply two new mtDNA markers, the molecular morphology of (a) the mtDNA molecule (circular versus linear) and (b) the 16S rRNA molecule (stem and loop morphology). The first marker can rigorously decide whether Trichoplax is a derived (and secondarily reduced) cnidarian, because all derived cnidarians (Scyphozoa, Hydrozoa, and Cubozoa) share a linear (instead of the normal circular) mtDNA molecule as a unique synapomorphy within the Metazoa (Bridge et al. 1992). The second marker, the 16S rRNA molecular morphology adds further and higher resolution evidence. For the comparative analyses, *T. adhaerens* and the following representatives from the four cnidarian classes were investigated: *Nematostella vectensis* (Anthozoa), *Aurelia aurita* (Scyphozoa), *Carybdea marsupialis* and *Tripedalia cystophora* (Cubozoa), and *Eleutheria dichotoma* (Hydrozoa). Cnidarians were maintained in the laboratory in artificial

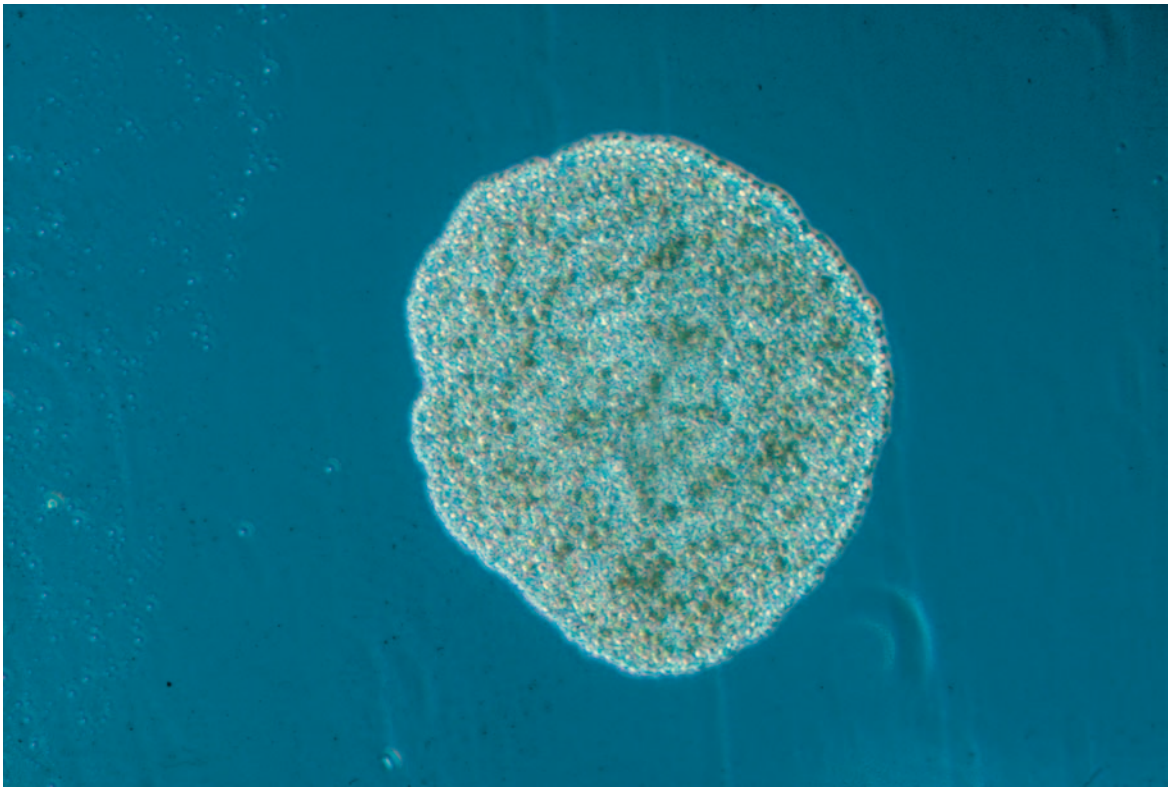

FIG. 1.—Life picture of *Trichoplax adhaerens*, the most simply organized metazoan. In sharp contrast to the Cnidaria, only four somatic cell types make up the bauplan, which does not show any sign of a defined symmetry or constant body axis. The only polarity relates to the functional lower (facing the substrate) and upper (facing the water) epithelium (see also Syed and Schierwater, 2000*a*, 2002*b*).

seawater (35‰ salinity) at 18°C and fed two times a week on 3–4-day-old brine shrimp larvae, *Artemia salina* (Schierwater 1989). Trichoplax was cultured as a clonal lineage and fed ad libitum. with the algae *Cryptomonas* (Schierwater and Kuhn 1998). Prior to DNA extraction, animals were starved for 48 h to avoid contamination by food. Each 10 to 20 individuals were homogenized in HOM buffer (100 mM Tris-HCL, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 8.0), and DNA was extracted once with phenol-chloroform-isoamylalcohol followed by an ethanol precipitation. DNA quantities were estimated in comparison to digested lambda DNA of known concentrations on 0.8% agarose gels. For polymerase chain reaction (PCR) amplification of the 16S rDNA fragment, a variety of primer sets were tried, but only the following sets worked: 16S-L: GAC TGT TTA CCA AAA ACA TA (Cunningham and Buss 1993) and 16S-H: CAT AAT TCA ACA TCG AGG (Werner Schroth, personal communication). Polymerase chain reactions were carried out with 5–20 ng of total genomic DNA in a volume of 25 µl using a 9600 thermocycler (PerkinElmer) and a temperature profile of 5 cycles (92°C/50 s, 45°C/50 s, ramp 3 s/1°C, 72°C/1 min) followed by 30 cycles (92°C/50 s, 50°C/1 min, ramp 3 s/1°C, 72°C/1 min); finally, fragments were elongated at 72°C for 5 min. The PCR products from *Carybdea marsupialis* and *Nematostella vectensis* were directly sequenced on an ABI 310 automated DNA sequencer using BigDyeTerminator chemistry (Applied Biosystems), and the remaining products were cloned and sequenced as described in Ender et al. (1996). The sequences have been deposited to GenBank (accession numbers AF360118 *Carybdea marsupialis*, AY169373 *Aurelia aurita*, AY169372 *Eleutheria dichotoma*, AY169370 *Nematostella vectensis*, AY169371 *Trichoplax adhaerens*). DNA sequences were first aligned with the aid of Clustal (Thompson, Higgins, and Gibson 1994) and subsequently controlled by eye (cf. Schierwater and Ender 2000). Secondary structure diagrams for the given taxa were created using information from Gutell (1996) and the Comparative RNA Web site (http://www.rna.icmb.utexas.edu). Based on a reduced alignment (elimination of ambiguous nucleotide positions), phylogenetic reconstruction analyses were done using maximum parsimony and Neighbor-Joining methods (PAUP*, Swofford 2001). For Southern blot analyses of the mtDNA genome structure (Bridge et al. 1992), total genomic DNA was electrophoresed on 0.8% agarose gels and subsequently blotted onto a nylon membrane (Roche Diagnostics). Species-specific 16S rDNA probes from *N. vectensis*, *T. adhaerens*, *E. dichotoma*, *A. aurita*, *C. marsupialis*, and *T. cystophora* were nonradioactively labeled by incorporation of digoxygenin during PCR (1:5 DIG-11-dUTP:dTTP ratio). Gel-purified DIG-labeled 16S fragments were hybridized to the mtDNA overnight at 42°C in a formamide-containing hybridization buffer. The membrane was washed twice for 5 min at RT (2× SSC; 0.1% SDS), followed by two stringent washing steps in 0.5× SSC and 0.1× SSC (15 min at 68°C). Signals were detected by

chemiluminescence using CSPD (Roche Diagnostics) and exposure to X-ray film for 1–2 h according to the manufacturer's protocol.

Results and Discussion

Southern blot analyses of the mtDNA molecules revealed a single circular mtDNA molecule for both the anthozoan *Nematostella vectensis* and the placozoan *Trichoplax adhaerens* (fig. 2). The migration pattern of the mtDNA of both species is identical in that both signals slightly smear and slowly migrate below the chromosomal fraction. This is typical for circular mtDNA molecules (Bridge et al. 1992; Nosek et al. 1998). In sharp contrast, the mtDNA of the hydrozoan *Eleutheria dichotoma* and the scyphozoan *Aurelia aurita* shows a quite different migration pattern (fig. 2*A*), revealing discrete and sharp bands. This is expected for linear mtDNA molecules of around 16 kb and 18 kb (cf. Nosek et al. 1998) and agrees with the report of Bridge et al. (1992) that all derived (nonanthozoan) cnidarians harbor linear mtDNA molecules. It should be noted here that Bridge et al. (1992) confirmed the differential migration patterns of circular and linear mtDNA molecules by comparison to known circular and linear controls and also by transmission electron microscopy analyses. Because it is unquestioned that a circular mtDNA molecule represents the plesiomorphic state for metazoans, the shared occurrence of this feature in *T. adhaerens* provides strong evidence that the Placozoa are not a derived cnidarian group within in the Hydrozoa/Scyphozoa/Cubozoa clade.

A quite remarkable feature was found in the cubozoan mtDNA. Here, Southern analyses revealed linear mtDNA molecules of close to 4 kb in size (fig. 2*B*). To verify this surprising observation and to exclude degradation artifacts (as this might have occurred during DNA preparation), we examined another species and another five independent DNA isolates of *Carybdea marsupialis*. All of the latter confirmed the original observation (data not shown). The other cubozoan species examined, *Tripedalia cystophora*, also harbors linear mtDNA molecules of slightly below 4 kb in size (fig. 2*B*, lane 4). Two separate linear mtDNA molecules of about 8 kb each have been known from *Hydra* species (Warrior and Gall 1985; Bridge et al. 1992), and linear molecules have been reported from the cubozoan *Carybdea marsupialis* before (Bridge et al. 1992, Table 1; *C. marsupialis*, 16 kb). According to the known linear doublets in *Hydra*, the smaller cubozoan molecules must be interpreted as quartets; i.e. we assume the presence of four separate linear molecules of around 4 kb each in the cubozoan mitochondrion. This observation would make cubozoans an excellent model system for study of the replication process of short linear DNA molecules, for which several modes are discussed (Nosek et al. 1998 and ref. therein).

The amplified 16S rDNA region corresponds to the 3′ half of the gene and showed significant length variation among taxa. Sequence analyses revealed a remarkable size difference between the Placozoa and the Cnidaria. The representatives of the four cnidarian classes harbor

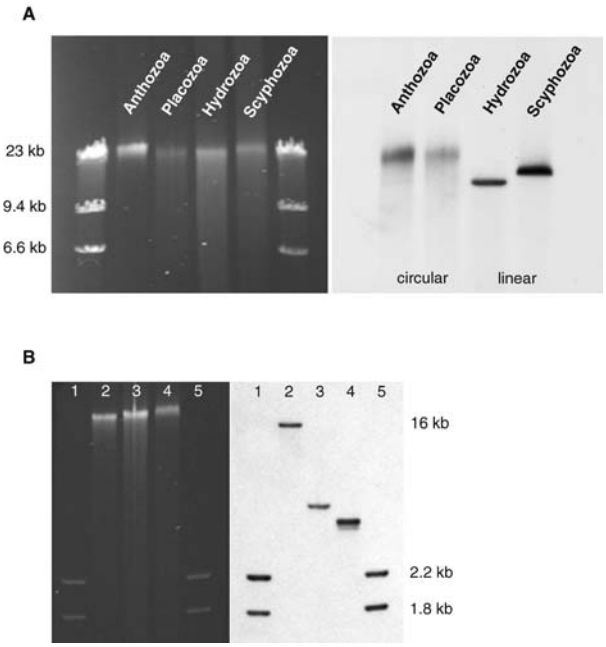

FIG. 2.—Southern analyses reveal a circular mtDNA molecule in Placozoa and short linear mtDNA molecules in Cubozoa. Total genomic DNA was separated on 0.8% agarose gels and hybridized to species-specific DIG-labeled mtDNA probes. *A*, Placozoa mtDNA runs at 16 kb circular. From left to right: *Nematostella vectensis* (Anthozoa), *Trichoplax adhaerens* (Placozoa), *Eleutheria dichotoma* (Hydrozoa), and *Aurelia aurita* (Scyphozoa). Lanes 1 and 6 show lambda/*Hin*dIII markers. As described by Bridge et al. (1992), anthozoans possess a circular mtDNA molecule and hydrozoans and scyphozoans a linear mtDNA molecule. The linear scyphozoan molecule runs a little above the linear hydrozoan mtDNA, because of its slightly bigger size (cf. Bridge et al. 1992). The mtDNA of the placozoan *Trichoplax adhaerens* runs together with the circular mtDNA of the anthozoan *Nematostella vectensis*. *B*, Cubozoa mtDNA runs at around 4 kb. Lane 2 *Eleutheria dichotoma* (Hydrozoa), lane 3 *Carybdea marsupialis* (Cubozoa), lane 4 *Tripedalia cystophora* (Cubozoa). Lanes 1 and 5 show a DIG-labeled size marker (Roche Diagnostics). The linear mtDNA of *E. dichotoma* (lane 2) runs at 16 kb (cf. fig. 2*A*). The cubozoan molecules run slightly above 4 kb (*C. marsupialis*, lane 3) and slightly below 4 kb (*T. cystophora*, lane 4).

comparatively short fragments—*Carybdea marsupialis* 482 bp, *Tripedalia cystophora* 480 bp (estimated from gel electrophoresis), *Eleutheria dichotoma* 518 bp, *Aurelia aurita* 521 bp, *Nematostella vectensis* 604 bp-whereas the homologous region in Placozoa reads 933 bp. This observation by itself supports a more distant rather than a close phylogenetic relationship between the Placozoa and Cnidaria. Further evidence for the latter hypothesis derives from structural information of the 16S rRNA molecule (fig. 3). The uncorrected alignment of 16S sequences comprises 903 nucleotide positions. Ambiguous regions, which in most cases surround variable loops introduced by the Trichoplax sequence, were removed (alignment is available upon request). The DNA sequences of the reduced alignment of 357 bp did not harbor sufficient phylogenetic information to unequivocally resolve phylogenetic relationships at the given taxonomic level of phyla and classes (phylograms not shown).

Quite noteworthy, the 16S molecular *morphology* harbors valuable phylogenetic information. Based on a secondary structure model of the 16S rRNA molecule

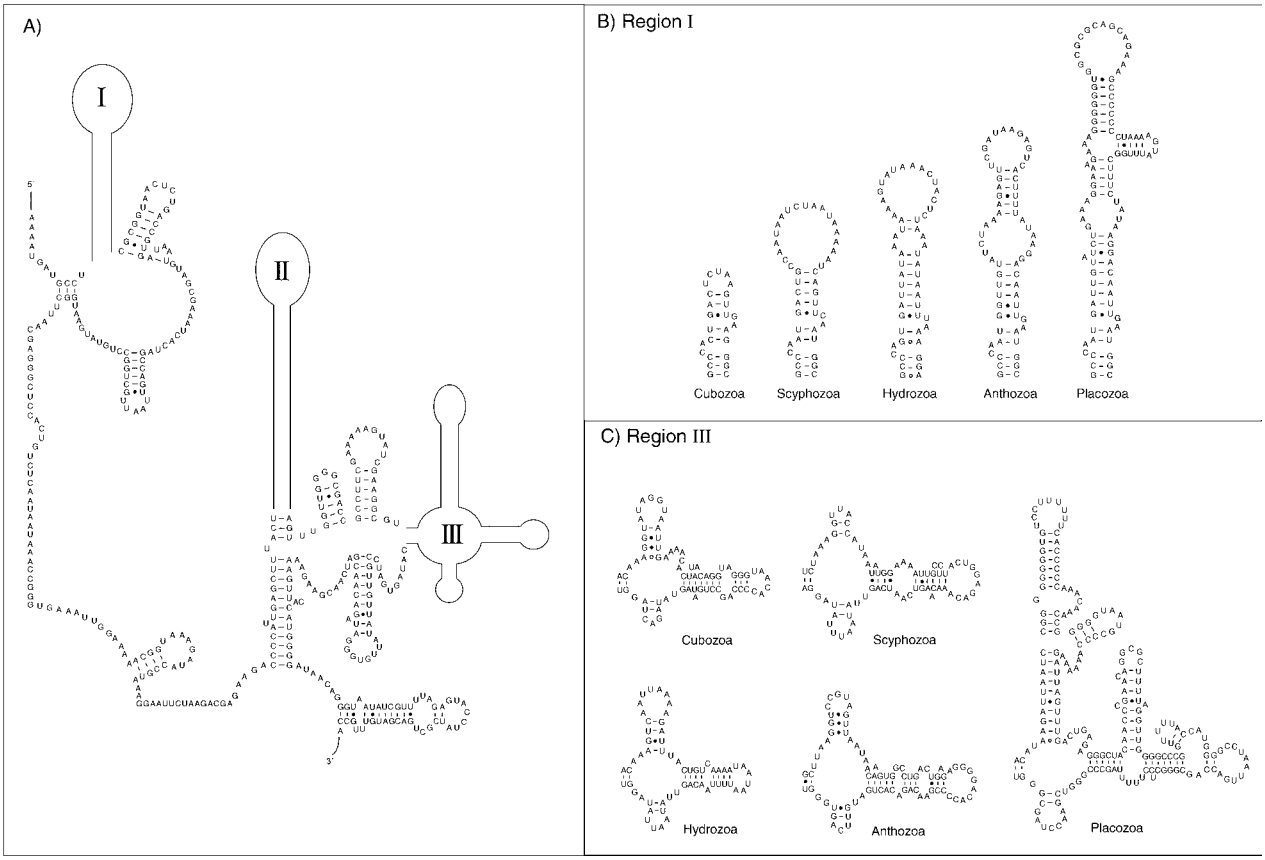

FIG. 3.—Secondary structure models for 3′ region mitochondrial large subunit ribosomal RNA molecule (cf. Gutell 1996). *A*, The 16S RNA molecule of *Trichoplax adhaerens* shows long regions of inserted nucleotide positions (I–III, drawn as continuous lines). *B*, In region I the placozoan molecule shows a longer stem and at least an extra loop compared to any of the cnidarian classes. *C*, In region III the number of nucleotides, stems, and loops is at least three times that of any cnidarian.

from *T. adhaerens*, ambiguous regions (I–III) are illustrated in figure 3*A*. Both well-conserved and highly derived regions were found between cnidarians and the placozoan. All four cnidarian classes show a phenotypically very similar pattern in region I, while the placozoan 16S secondary structure in this region is obviously different (fig. 3*B*). The basal stem of this region is significantly extended in Placozoa and looks more similar to secondary structures known from protists than from other metazoan taxa (Comparative RNA Web Site, http://www.rna.icmb.utexas.edu). Given that the Anthozoa are basal within the Cnidaria, region I suggests an interesting hypothesis, a shortening of the stem region during the course of anagenetic evolution, i.e., from more basal to more derived taxa. Another striking feature is found in the predicted secondary structure of region III, which is highly different in cnidarians and Placozoa (fig. 3*C*). None of the complexity found in the Trichoplax region III is found in any of the four cnidarian classes. Although we think that it is premature to assign character states to stem and loop morphology of the 16S rRNA, we nonetheless would like to note that the molecular morphology data seem to provide better supported tree topologies than the available sequence data. When we assigned seven characters (circular vs. linear, number and size of stems and loops) in different ways, we always found that the relevant nodes were best supported (highest bootstrap values) in the seven-character molecular morphology tree. 18S rDNA data showed the lowest support, and combined 18S and molecular morphology data gave intermediated support values. The topologies of the trees remained unchanged independent of which data set and which algorithm (Neighbor-Joining or maximum parsimony) was used (phylograms not shown; data sets are available from the authors upon request).

Both the mitochondrial genome (linear vs. circular) and 16S secondary structure morphology provide compelling evidence that (1) the placozoan *T. adhaerens* is not a derived (medusozoan) cnidarian and (2) the Cubozoa are the most derived taxon within the Cnidaria with respect to these characters. Furthermore the data suggest a distant rather than any close relationship between the Placozoa and Cnidaria. Complete mtDNA genome sequences of the Porifera (Werner Mueller, work in progress) and Placozoa (The Trichoplax Consortium, work in progress) will finally decide on the phylogenetic placement of *T. adhaerens* within the diploblasts.

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