Short Communication

Phylogenetic position of Placozoa based on large subunit (LSU) and small subunit (SSU) rRNA genes

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Abstract

The phylum Placozoa, represented by its single species *Trichoplax adhaerens*, has always been considered one of the most basal animal clades due to its low morphological complexity. However, despite its importance, the phylogenetic position of the Placozoa remains uncertain, as does the relationships between the basal metazoans. We sequenced the nearly complete large subunit (LSU) of the *T. adhaerens* rRNA gene together with the small subunit (SSU) rRNA and used several methods to infer the phylogenetic position of Placozoa and the relationships between the basal animal phyla. The phylogenetic trees obtained from the LSU, SSU and SSU + LSU datasets support monophyly of all basal metazoan phyla, except the Porifera. However, the relationships between all these phyla were not consistently resolved due to a strong conflict between the LSU and SSU phylogenetic signals. The hypotheses that Placozoa is a derived Cnidaria or the most basal metazoan were not supported by our data. Moreover, our analyses suggests that Placozoa is most likely the sister group of Cnidaria and/or Bilateria.

The phylogenetic relationships between the major groups of animals is one of the most challenging problems in evolutionary biology, mainly due to the difficulty in finding homologous characters for these groups (Adoutte *et al.*, 1999). In the last decade molecular studies have contributed greatly to addressing some of these questions, but these efforts have concentrated on the relationships among the Bilateria groups. Unfortunately, the initial diversification of Metazoa remains largely unknown (Bridge *et al.*, 1995; Kim *et al.*, 1999; Borchiellini *et al.*, 2001) but it is essential to elucidate the relationships among the basal metazoan phyla in order to understand the main evolutionary steps which gave rise to the major animal bodyplans. One of the most enigmatic and little known basal animals is the phylum Placozoa, currently represented by the single species *Trichoplax adhaerens* (but see Voigt *et al.*, 2004). Placozoans were firstly described by Schulze (1883), being one of the morphologically most simple multicellular animal groups (Grell and Ruthmann, 1991; Ender and Schierwater, 2003). They lack a muscular system and nerve cells (but may have presynaptic mediators, Shuchert, 1993) as well as any organ and are histologically very simple, with only four kinds of somatic cells (Grell and Ruthmann, 1991; but see also Martinelli and Spring, 2003). At approximately 40 Mb the Placozoa genome is the smallest animal genome yet known (Ruthman, 1977).

Despite the increasing interest in the Placozoa, its phylogenetic position within the Metazoa remains highly controversial (Halanych, 2004). Its overall simplicity suggests that it may be one of the most basal animal groups, or even the most basal group (Grell and Ruthmann, 1991). Several molecular studies tried to elucidate the evolutionary relationships of the Placozoa, most studies having used the small subunit (SSU) rRNA gene alone, or in conjunction with other data, such as morphology. The main hypotheses presented by these studies are that the Placozoa is a sister group of Ctenophora + Cnidaria (Bridge *et al.*, 1995), Porifera + Ctenophora (Hanelt *et al.*, 1996), Ctenophora (Odorico and Miller, 1997) or Cnidaria (Kim *et al.*, 1999; Borchiellini *et al.*, 2001; Glenner *et al.*, 2004). Bridge *et al.* (1995), also based on 18S rDNA sequence data, suggested that Placozoa is positioned within the Cnidaria, as a sister group of Cubozoa, an hypothesis which implies that the simplicity of this animal is a derived condition (Collins,

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1998). However, this phylogenetic position was not supported by Ender and Schierwater (2003) who analyzed the mitochondrial 16S gene and the mitochondrial genome structure of the Placozoa and four groups of Cnidaria and showed that Placozoa has a circular mitochondrial genome like the majority of other animals which is different from the linear genomes found in Cnidaria Medusozoa. A close relationship between Placozoa and Bilateria (and of both of these with Cnidaria) has also been suggested by some studies (Collins, 1998; Collins, 2002; Anderson and Swofford, 2004). Unfortunately, all these studies were very limited as they used a partial representation of important basal metazoan taxa and/or a single gene. Poor taxon sampling has also been a major limitation in all recent studies that used new alternative genes, such as elongation factor-1 alpha, actin and the homeobox genes, brachyury and Tbx/2/3 (Martinelli and Spring, 2003; 2004; 2005).

One of the most complete molecular studies of the basal diversification of the Metazoa was made by Medina *et al.* (2001) who compared the SSU and the large subunit (LSU) rRNA genes from 23 groups including Fungi, Choanoflagellida, Mesomycetozoa, Porifera, Ctenophora, Cnidaria and representative Bilateria, but not the Placozoa. These authors confirmed the monophyly of the Metazoa, Cnidaria, Ctenophora and Bilateria but not of the Porifera, which was split into two different clades. However, the relationships among most of these phyla were not resolved with a high level of confidence.

Here we present a nearly complete sequence of the *T. adhaerens* LSU gene (submitted to GenBank under accession number AY303975) and, including the LSU and SSU data from Medina *et al.* (2001) and data from the basal bilaterian acoelomorph flatworm *Paratomella rubra* (Telford *et al.* 2003), try to infer more accurately the phylogenetic position of Placozoa.

We cultured a lineage of the placozoan *Trichoplax* in glass Petri dishes in 33% salinity artificial seawater (RED SEA®) at a constant temperature of 19 °C and a light/dark cycle of 12:12 h. Every two weeks we replaced the seawater and fed the *T. adhaerens* cultures with the green algae *Pyrenomonas* (Cryptophyta) and *Dunaliella* (Chlorophyta). Seven individual *T. adhaerens* were collected and their DNA extracted using phenol-chloroform as described by Sambrook and Russel (2001). Amplifications were done by long PCR with the following conditions: 94 °C for 1 min, 35 X (94 °C for 30 s; 45 °C for 1 min; 68 °C for 6 min), 68 °C for 10 min, in a total volume of 25 µL containing Elongase Enzyme Mix (Invitrogen, Carlsbad, CA, USA). The nuclear LSU and SSU rRNA genes were amplified using the set of primers described by Medina *et al.* (2001) and Medlin *et al.* (1988), respectively. The LSU was sequenced almost completely but only about 500 bp from both extremities of the SSU gene was sequenced to compare with the Placozoa SSU sequences available in GenBank. As our partial sequences were identical with

GenBank sequence U65478, we used this sequence in all the analyses. The PCR products were sequenced with the ET-Terminator cycle sequencing kit (Amershan Biosciences, Piscataway, USA) and read in a MegaBACE 1000 sequencer. All chromatograms were manually checked. We used the Clustal X program (Thompson *et al.* 1997) with default options to align the additional sequences to the SSU and LSU alignments presented in Medina *et al.* (2001) as kindly provided by M. Medina (Joint Genome Institute, CA, USA). The same regions that could not be satisfactorily aligned and were excluded by Medina *et al.* (2001) were also excluded here. Our analyses were done considering three datasets, the SSU and LSU individual alignments and the SSU + LSU concatenated alignment.

Phylogenetic analyses of the three datasets were performed using maximum likelihood (ML) and maximum parsimony (MP) with the PAUP* 4.0b10 program (Swofford, 2002), using, when appropriate, the models of nucleotide substitution as suggested by the Modeltest V 3.06 program (Posada and Crandall, 1998). For the MP analyses both equally weighted and 2:1 weighting for transversion to transition were used, as recommended by Medina *et al.* (2001). Bootstrap statistical support was performed for both ML and MP with 200 replications of heuristic search and simple taxon addition and for MP Bremer support was also estimated using the SEPAL 1.1 program (Salisbury, 1999). Additional ML analyses were done using the PhyML V 2.4.3 program (Guindon and Gascuel, 2003) and the MetaPIGA V 1.02 program (Lemmon and Milinkovitch, 2002) with settings as suggested in the program manuals, the HKY85 model was used in the MetaPIGA program because this was the most general model implemented in this program. Bayesian inference (BI) phylogenetic analyses were performed with the MrBayes 3.0 program (Huelsenbeck and Ronquist, 2003) using four multiple runs for each gene from different starting seeds, the default settings and the General time-reversible model model (GTR) of DNA substitution with gamma-distributed rate variation across sites. For the Markov Chain Monte Carlo process, four chains were run simultaneously for 10,000,000 generations, with trees sampled every 100 generations for a total of 100,000 trees. Burn-in time was determined when visual inspection indicated that the log-likelihood values achieved an asymptote over a large number of generations. The posterior probability of each bipartition was estimated using the 50% majority-rule consensus tree constructed from the remaining trees using PAUP*. The PHASE package (Hudelot *et al.*, 2003), specifically designed for tree construction using RNA sequences and secondary structure, was also used to estimate Bayesian phylogenies, with appropriate settings following the program manual. The RNA model of evolution was estimated in the Simulate program, implemented in the PHASE package. All analyses described above were repeated with the exclusion of *Dugesia tigrina* or *D. tigrina*

and *Paratomella rubra* to determine the influence of the relatively more elevated rate of substitution of these genes in these species.

To test the key hypotheses for the phylogenetic placement of the Placozoa put forth in the literature, we used the Kishino-Hasegawa test as implemented in PAUP*. As these hypotheses were defined *a priori* it was unnecessary to use the modified Shimodaira-Hasegawa test. We also used the approximately unbiased test (au) p-values as estimated by the Consel V 0.1f program (Shimodaira and Hasegawa, 2001). The hypotheses tested were Placozoa as a sister group of Bilateria, Cnidaria, Bilateria + Cnidaria, Ctenophora, and Metazoa. After our LSU placozoans sequence had been submitted to GenBank, Voigt *et al.* (2004) published six additional almost complete *T. adhaerens* 28S sequences. Additional exploratory analyses with MP (with PAUP*) were done including the two most divergent of these sequences, H1 and H8, to test the influence of these extra sequences on our results (not shown).

We obtained a nearly complete (2,946 bp) sequence of the *T. adhaerens* LSU gene, the alignment of the primary sequence and the secondary structure predicted by comparison with known gene structures support the hypothesis that this is the functional LSU gene of this species. The main characteristics of the SSU, LSU, and SSU + LSU gene alignments used here (available on request), with the ambiguously aligned regions excluded, are presented in Table 1. Considering both genes together, there can be as many as about four thousand positions, of which about 47% may be variable. The best substitution model as estimated by Modeltest was Tamura-Nei with invariant sites and gamma parameter (TrN + I + G) for all three datasets. The Simulate program, in the PHASE package, chose the RNA7A + dG4 + I (see program manual) as the most likely evolution model.

As expected, since the placozoans sequences including H1 and H8 (Voigt *et al.*, 2004) were not very divergent considering the distances between phyla the resultant phylogenetic trees were identical to those estimated with a single placozoans sequence. Therefore, since most of our phylogenetic analyses were very time consuming and it was thus important to keep the number of sequences as small as possible, we presented the results with our LSU sequence only.

Table 2 shows the sister groups of Placozoa in all phylogenetic analyses carried out by us and Figure 1 presents the ML tree constructed from the SSU + LSU concatenated genes using PAUP* (all other trees can be obtained from the authors). The relationships among the metazoan phyla differ significantly in the SSU and LSU gene trees, while for each dataset the trees were usually similar for the different methods (Table 2). The SSU + LSU trees were usually more similar to the SSU than to the LSU tree, although the latter presented more informative characters (Table 1). In general, the SSU and SSU + LSU trees showed the Placozoa as the sister group of Cnidaria and/or Bilateria, and LSU as a sister group of Metazoa, *Leucosolenia* (Porifera), or Cnidaria + Ctenophora. However, in none of the phylogenetic methods the position of the Placozoa was the same for all three data sets (SSU, LSU and SSU + LSU) (Table 2).

Interestingly, this incongruence was true even for different implementations (programs) of the same basic methods (*e.g.*, mL in PAUP* and PhyML or BI in MrBayes and PHASE). Branch support varied widely among methods and datasets, from high (100) to very low (< 50) support, with low correlation among datasets or methods. Removing the relatively much faster evolving sequences of *D. tigrina*

Table 1 - Basic statistics of the data sets.

Dataset	Full length (bp)	Conserved sites	Variable characters	Parsimony informative sites	Gamma shape parameter
Small subunit (SSU)	1594	844	750	500	0.5702
Large subunit (LSU)	2408	1273	1135	790	0.5346
SSU + LSU	4002	2117	1885	1290	0.5410

Table 2 - Placozoa sister group in each analysis. Support values are shown in parentheses: posterior probability values for BI, bootstrap percent values for mL, and bootstrap percent values and Bremer support for MP.

| Analysis method (program)*	Small subunit (SSU)	Large subunit (LSU)	SSU + LSU
		Placozoa sister group to:	
Maximum likelihood (PAUP)	Cnidaria + Bilateria (94)	*Leucosolenia - Porifera* (< 50)	Cnidaria (< 50)
Maximum likelihood (PHYML)	Cnidaria (66)	Metazoa (92)	Cnidaria (66)
Maximum likelihood (metaPiga)	Cnidaria + Bilateria (100)	Metazoa (100)	Cnidaria (100)
Maximum parsimony	Bilateria (71/1)	Metazoa (< 50/10)	Bilateria (63/4)
Bayesian (PHASE)	Cnidaria (79)	Ctenophora + Cnidaria (59)	Cnidaria (88)
Bayesian (MrBayes)	Cnidaria + Bilateria (100)	*Leucosolenia-Porifera* (94)	Bilateria (62)

*See text for details.
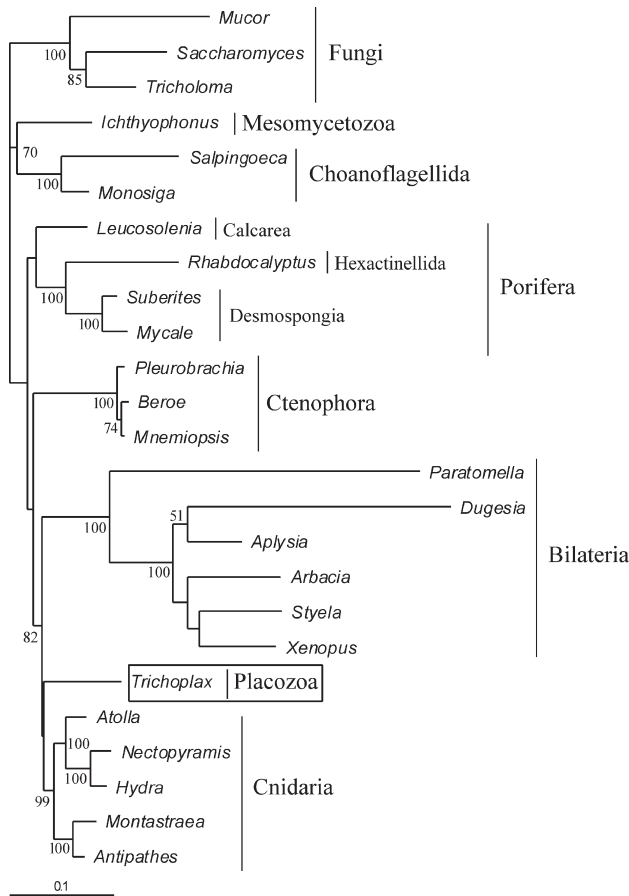
Figure 1 - Maximum likelihood large subunit (LSU) + small subunit (SSU) tree. Bootstrap values (%) are shown at the nodes. Nodes without numbers indicate values below 50.

and/or *P. rubra* had no substantive impact on the results of the phylogenetic analysis (results not shown).

The Kishino-Hasegawa and Consel tests (Table 3) only rejected a few of the *a priori* hypotheses for the phylogenetic position of Placozoa, such as its position as the most basal metazoan phyla or as a sister group to Ctenophora.

Overall, our results were similar to those of Medina *et al.* (2001), corroborating the monophyly of the Bilateria, Cnidaria, Ctenophora and Metazoa, and suggesting the paraphyly of the Porifera, due to the unstable position of the Calcarea sponge *Leucosolenia*.

The phylogenetic position of Placozoa varied considerably among the trees inferred from the different datasets and methods. The major conflict was between the SSU and LSU trees, which always disagreed in at least one relationship considering the same method. The basal nodes of the Metazoa usually presented low support values, despite the relatively high number of sites used. These results were similar to those of Rokas *et al.* (2003), who could not resolve the basal Metazoan tree using combined protein datasets (α-tubulin, β-tubulin, EF-2 and HSP90), probably due to conflicting phylogenetic signals from individual

Table 3 - Maximum likelihood tests of *a priori* phylogenetic hypotheses.

Hypotheses	Kishino-Hasegawa test/Consel test[1]		
Placozoa sister group to:	Small subunit (SSU)	Large subunit (LSU)	SSU + LSU
Bilateria	0.9316/0.515	0.3262/0.237	0.9028/0.559
Cnidaria	0.9416/0.532	0.4001/0.296	1.000/0.695
Ctenophora	0.0769/0.036*	0.4007/0.273	0.0721/0.036*
Cnidaria + Bilateria	1.000/0.576	0.1351/0.077	0.6182/0.343
Metazoa	0.0003*/0.002*	0.8278/0.544	0.0618/0.034*

[1]p-value of the approximately unbiased test.
*$p < 0.05$ (significant rejection).

genes. A likely cause for this difference could be the action of purifying selection on these highly conserved genes. A similar explanation could be postulated to explain the conflict between our SSU and LSU datasets.

Nevertheless, based on the frequency of occurrence of some relationships (Table 2), the Placozoa seemed more closely related to the Cnidaria and/or the Bilateria clade. The phylum Cnidaria was the most frequent sister group of the Placozoa, based mainly on the SSU and SSU + LSU trees but, interestingly, this relationship never occurred in the LSU trees (Table 2). We found that in several trees Cnidaria + Bilateria or Bilateria alone also emerged as sister groups of Placozoa. Previous studies support three relationships of the Placozoa described above (see introduction) and we suggest that the sister group of Placozoa is either Cnidaria or Bilateria. On the other hand, our results suggest the rejection of some hypotheses regarding the relationship of the Placozoa, although the Kishino-Hasegawa and the Consel tests significantly rejected only few of the *a priori* hypotheses (Table 3). For example, the suggestion by Grell and Ruthmann (1991) that the Placozoa may be the most "primitive" animal, *i.e.* a sister group to the rest of the Metazoa, received low support from our results, with only the LSU dataset supporting this hypothesis. Also, only very weak support was found for any close relationship between Placozoa and Porifera, although in two LSU trees the Placozoa grouped with the Calcarea sponge *Leucosolenia*. In addition, no support was found for Placozoa as the sister group of Ctenophora or as a secondarily reduced Cnidaria as suggested by Bridge *et al.*, 1995.

Our main results indicating that Placozoa and Cnidaria, or Placozoa and Cnidaria + Bilateria, are sister groups suggest that the very simple body architecture of Placozoa is misleading in relation to the phylogenetic position of Placozoa and that this group is likely to be the result of secondarily simplification from a more complex ancestor. The phylogenetic relationships described by us were very similar to those found by Collins *et al.*, (1998), which suggests that several similarities shared by Bilateria and Ctenophora should have evolved independently or were lost in Cnidaria and Placozoa. Placozoa and Cnidaria share

presynaptic mediators that react with antibodies against the neuropeptide RFamide (Shuchert, 1993), supporting their close relationship. Moreover, the relationship Placozoa + Bilateria with bilaterian *P. rubra* basal, as suggested by our data, may help to explain the histological similarity between Placozoa, which posses three cell layers with an interspace like a mesenchyme (Grell and Ruthmann, 1991) and the acoelomorph flatworm that possess a central syncytium (Collins *et al.*, 1998).

We suggest that the Placozoa is not the most basal Metazoa but is probably the sister group of Cnidaria and/or Bilateria. Although we have used a large dataset and a very diverse set of phylogenetic inference methods, as with other studies (*e.g.* Medina *et al.*2001, Rokas *et al.*, 2003), the relationships among most of the basal Metazoan phyla, including Placozoa, is still unresolved. Our results strengthen the scenario of a very rapid radiation for the initial diversification of the Metazoa and a genomic approach might be necessary to resolve this fascinating issue.

Acknowledgments

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Associate Editor: João S. Morgante