Correspondence

Placozoa — no longer a phylum of one

Oliver Voigt[1,2], Allen G. Collins[1,3*], Vicki Buchsbaum Pearse[4], John S. Pearse[4], Andrea Ender[1,5], Heike Hadrys[1] and Bernd Schierwater[1]

More than a century ago, the simplest of all metazoans was discovered and described as *Trichoplax adhaerens* [1]. These tiny, flattened animals lack symmetry, mouth, gut, nervous system, and extra-cellular matrix and constitute the apparently monotypic phylum Placozoa. Placozoans diverged early in metazoan history [2–7], making them important organisms for evolutionary research [2,3,8]. Placozoans can be found in warm, shallow, marine environments around the world [9] and all observed individuals fit the general morphological description of *T. adhaerens*. Our analyses, however, show that the phylum Placozoa is significantly more diverse than previously thought.

Extensive genetic variation in Placozoa is revealed (Figures 1 and 2) by four molecular markers: the small and large ribosomal subunits (SSU and LSU), the internal transcribed spacers 1 and 2 and ribosomal 5.8S (ITS), and the large subunit of the mitochondrial ribosome (16S). With SSU, for example, the genetic distances between individual placozoans are comparable to those documented between genera (within families) and even between families (within orders) of other diploblastic, early diverging metazoan phyla (Supplemental Data). Moreover, from only 31 placozoans sampled around the world (Table 1), we obtained eight different haplotypes of mitochondrial 16S (Figure 2), displaying length variation of up to 145 bp, a level far exceeding that documented for any metazoan species or genus. The inferred secondary structures for 16S are correspondingly diverse (Figure 2).

Tree topologies inferred from all four markers are largely congruent (Figures 1 and 2). Intra-individual variation in ITS is low relative to the divergences between the five deeper divisions indicated by both 16S and ITS (Figure 2). These data strongly reject the idea that the phylum Placozoa is represented by a single extant species. Thus, caution is warranted in interpreting comparative studies that use a single clonal lineage. In addition, the combined SSU and LSU data (Figure 1), as well as two other recent studies [7,10], convincingly refute the view of placozoans as degenerate medusozoan cnidarians [11].

We have advanced in our understanding of placozoan biodiversity, but much further investigation of these intriguing animals is needed. The species richness of Placozoa is still to be determined. More importantly, placozoans have been studied mostly in the laboratory and nearly all observations have been made on a single clonal lineage, the Grell culture-strain originating from the Red Sea (H1). The importance of studying animals living in their natural habitat is undeniable: a complete reproductive cycle has never been reported in all the decades placozoans have been kept in the laboratory.

Based on limited sampling to date, H6, 7 and 8 are exclusively Pacific, and H1, 2, 3 and 5 are not found in the Pacific (Table 1). Otherwise, our data have so far displayed little biogeographic signal. Several placozoan lineages show a widespread and overlapping geographic distribution while several locales have yielded multiple sympatric lineages. A general correlation between small body size and high abundance suggests that microbial eukaryotes (< 1 mm) may be less likely to exhibit biogeographic patterns because their large populations experience few dispersal barriers [12]. When captured in the field on settling

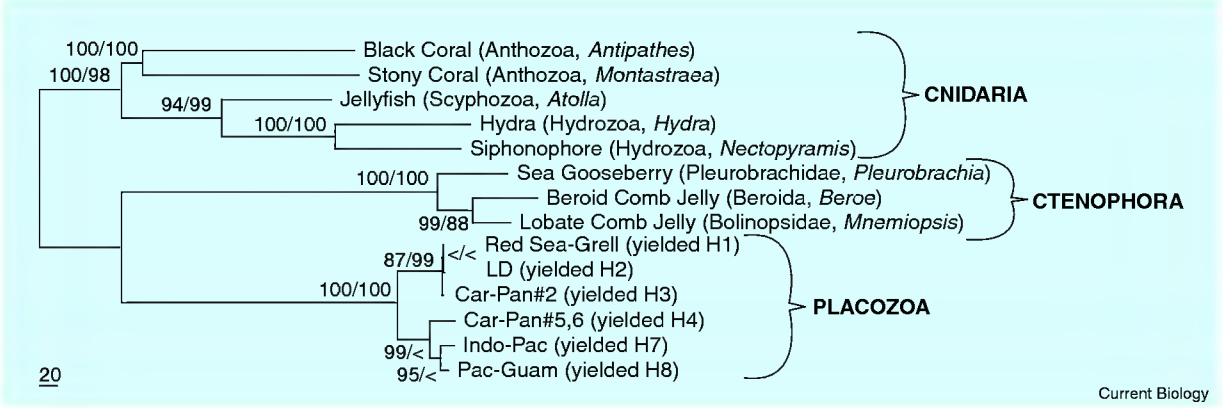

Figure 1. Parsimony phylogram of placozoans based on combined SSU (18S) and LSU (28S) data.

The tree is rooted with disparate cnidarians and ctenophores (Accession numbers: *Antipathes*, AF100943, AY026365; *Montastraea*, AY026382, AY026375; *Atolla*, AF100942, AY026368; *Hydra*, AF358080, AY026371; *Nectopyramis*, AF358068, AY026377; *Pleurobrachia*, AF293677, AY026378; *Beroe*, AF293694, AY026369; and *Mnemiopsis* AF293700, AY026373), and with bootstrap indices under parsimony (1000 replicates) and likelihood (100 replicates). '<' indicates a bootstrap value of less than 50. Assumed model of nucleotide evolution for likelihood analyses: one rate for transversions, two rates for transitions, proportion of invariant sites, and shape parameter (TrN+I+G). The 16S haplotype derived from each of the samples is shown in parentheses. Scalebar: 20 character changes.

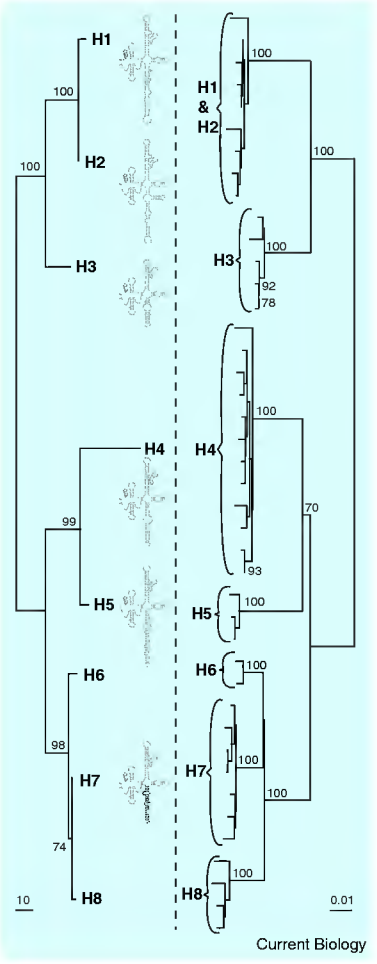

Figure 2. Correspondence of relationships based on 16S (left) and ITS (right).

Left: MP phylogram of eight 16S haplotypes obtained from 31 samples (Table 1), with bootstrap indices (1000 replicates). Icons representing differing secondary structures are shown for six of the haplotypes. Scalebar: 10 character changes. Right: ME phylogram of ITS, with bootstrap indices (200 replicates) exceeding 69. Assumed model of nucleotide evolution: four rates for transversions, one rate for transitions, and gamma shape parameter (TVM+G). Scalebar: 0.01 substitutions per site.

slides, placozoans are only ~100–200 µm across, so they could fit this model. Another possible explanation for weak biogeographic patterning are widespread anthropogenic introductions. Indeed, both natural dispersal and artificial transport may contribute to the distribution of placozoans.

Our data provide the beginnings of a systematic hierarchy for Placozoa. However, placozoan diversity is currently best classified simply by referring to the well-supported clades (Figure 2). When the full life cycle and the extent of morphological and ecological variation are documented, a systematic classification — free from historical bias — can be erected for this entire phylum.

Table 1. Origin of samples, listed by 16S haplotype.

H1:	Red Sea (Grell Clone, *n* = 1); Caribbean (Panama, *n* = 1)
H2:	Caribbean (Panama, *n* = 2); Mediterranean (Italy, *n* = 4); Unknown (Aquarium, *n* = 1)
H3:	Caribbean (Panama, *n* = 1)
H4:	Caribbean (Panama, *n* = 3; Venezuela, *n* = 1); Pacific (Panama, *n* = 5)
H5:	Mediterranean (Italy, *n* = 3)
H6:	Pacific (Panama, *n* = 2)
H7:	Pacific (Panama, *n* = 1); Indo-Pacific (Aquarium, *n* = 1); Unknown (Aquaria, *n* = 2)
H8:	Pacific (Guam, *n* = 1; Panama, *n* = 2)

Acknowledgments

We thank C. Asholt, N. Boero, G. Caccone, R. Collin, J. Collins, C. Diaz, J. Fromont, S. Hagemann, Y. Hirano, J. Hooper, H. Lessios, N. Knowlton, C. Meyer, G. Paulay, V. Scholey, J. Starmer, A. Terlizzi, and P. Tomassetti for assistance in obtaining samples. Deutsche Forschungsgemeinschaft (Schi277/10) and the Human Frontier Science Program (HFSP RGP0221/2001-M) are gratefully acknowledged. AGC thanks NSF Grant EAR-9814845 for support of this project at an early stage.

Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/14/22/R944/DC1/

References

1. Schulze, F.E. (1883). Über *Trichoplax adhaerens*. Physik. Abh. Kgl. Akad. Anz. *6*, 92–97.
2. Martinelli, C., and Spring, J. (2003). Distinct expression patterns of the two T-box homologues Brachyury and Tbx2/3 in the placozoan *Trichoplax adhaerens*. Dev. Genes Evol. *213*, 492–499.
3. Jakob, W., Sagasser, S., Dellaporta, S., Holland, P.W.H., Kuhn, K., and Schierwater, B. (2004). The Trox-2 Hox/ParaHox gene of *Trichoplax* (Placozoa) marks an epithelial boundary. Dev. Genes Evol. *214*, 170–175.
4. Collins, A.G. (1998). Evaluating multiple alternative hypotheses for the origin of Bilateria: An analysis of 18S molecular evidence. Proc. Natl. Acad. Sci. USA *95*,15458–15463.
5. Kim, J.H., Kim, W., and Cunningham, C.W. (1999). A new perspective on lower metazoan relationships from 18S rDNA sequences. Mol. Biol. Evol. *16*, 423–427.
6. Syed, T., and Schierwater, B. (2002). *Trichoplax adhaerens*: a phylum discovered, forgotten, and rediscovered. Vie Mil. *52*, 177–187.
7. Ender, A., and Schierwater, B. (2003). Placozoa are not derived cnidarians: Evidence from molecular morphology. Mol. Biol. Evol. *20*, 130–134.
8. Schierwater, B., and Kuhn, K. (1998). Homology of Hox genes and the zootype concept in early metazoan evolution. Mol. Phylogenet. Evol. *9*, 375–381.
9. Pearse, V.B. (1989). Growth and behavior of *Trichoplax adhaerens*: First record of the phylum Placozoa in Hawaii. Pacif. Sci. *43*, 117–121.
10. Collins, A.G. (2002). Phylogeny of Medusozoa and the evolution of cnidarian life cycles. J. Evol. Biol. *15*, 418–432.
11. Cavalier-Smith, T., and Chao, E.E.Y. (2003). Phylogeny of choanozoa, apusozoa, and other protozoa and early eukaryote megaevolution. J. Mol. Evol. *56*, 540–563.
12. Finlay, B.J. (2002). Global dispersal of free-living microbial eukaryote species. Science *296*, 1061–1063.

[1]Institute of Animal Ecology & Cell Biology, Division of Ecology & Evolution, Bünteweg 17d, 30559 Hannover, Germany. [2]Department of Molecular Plant Physiology, Friedrich-Alexander Universität Erlangen-Nürnberg, Staudtstr. 5, 91058 Erlangen, Germany. [3]NMFS, National Systematics Laboratory, National Museum of Natural History, MRC-153, Smithsonian Institution, P.O. Box 37012, Washington, District of Columbia 20013-7012 USA. [4]Long Marine Laboratory, University of California, Santa Cruz, 100 Shaffer Rd., Santa Cruz, California 95060, USA. [5]Katharinenhospital, Zentralinstitut für Transfusionsmedizin und Blutspendedienst, Keplerstrasse 32, 70174 Stuttgart, Germany.
*E-mail: CollinsA@SI.edu

Correspondence

Supplemental data: Placozoa — no longer a phylum of one

Oliver Voigt[1,2], Allen G. Collins[1,3*], Vicki Buchsbaum Pearse[4], John S. Pearse[4], Andrea Ender[1,5], Heike Hadrys[1] and Bernd Schierwater[1]

Experimental procedures

DNA was extracted from 31 individuals collected from seven localities, clonal cultures maintained in the laboratory, and local aquaria (Table S1). PCRs were carried out under the following parameters: 5 min/94°C, 30–38 cycles (94°C/30s; primer specific annealing temperature (AT)/30 s; 72°C/ for target specific elongation time (ET)), 5 min/72°C. Nearly complete sequences of the gene coding for the small subunit of the nuclear ribosome (SSU or 18S) were obtained using standard PCR and sequencing primers [S1] and (AT: 50°C to 53°C). Alternatively, a smaller fragment of SSU was obtained by PCR and sequenced with placozoan specific primers (fw: 5′GAAGTATGGTTGCAAAGCTG3 ; rv: 5′ AACCGTAAAGTCACGC-CATC3′; AT:52°C; ET:50 s).

Primer sets for PCR and sequencing from Medina *et al.* [S2] were used to obtain near complete sequences for the large subunit of the nuclear ribosome (LSU or 28S). After amplification of the whole fragment with F63mod and R3264 (AT:53°C; ET: 3:13 min), internal primers were combined (F63mod+R2077sq; F1379+R3264; AT:57°C; ET:2:15 min) in a secondary PCR to yield enough DNA template for sequencing reactions. In addition, new sequencing primers were designed (F2800: 5′GCAGGTGTCCTAAGGYRAGCT C3′; R2800:5′GAGCTYRCCTTAGGACA CCTGC3′).

We applied placozoan specific primers (fw: 5′GTTAATTGCTGGCCTGTATG3′; rv: 5′TTGATCGTTGTCTATCCCAC3′; AT: 52°C; ET:50s) for PCR and cycle sequencing reactions in order to obtain a fragment of the large subunit of the mitochondrial ribosome (16S).

Finally, universal primers located at the 3′ end of nuclear SSU and the 5′ end of nuclear LSU (fw: 5′GGTTTCCGTAGGTGAACCTGC GGAAGGATC3 ; rv: 5′GCATATCAATAAGCGGAGGA3′; AT:57°C, ET:50s) were used to obtain sequences spanning internal transcribed spacers 1 and 2 and ribosomal 5.8S (ITS). When sequencing of PCR product was not successful, products were ligated into pGEM-T-vector using the pGEM-T vector system (Promega) and were transformed into *E. coli* DH5α competent cells (Invitrogen). One to five clones from each sample were sequenced applying T7 and SP6 vector primers. We uncovered intra-individual variation in ITS, but the level appears to be low relative to the divergences between the five deeper divisions between groups, and may in part be due to errors introduced by

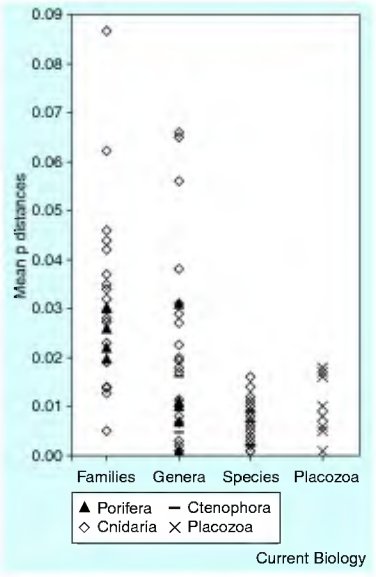

Figure S1. Mean uncorrected p distances of SSU between families (within orders), genera (within families), and species (within genera) of Porifera, Cnidaria, and Ctenophora compared to those between placozoan lineages.

PCR. Cycle sequencing reactions using DYEnamic™ E.T.-Terminator cycle sequencing kit (Amersham Biosciences) were visualized with a Megabace 500 Sequencer (Amersham Biosciences). All sequences are deposited in GenBank (accession numbers, SSU: AY652577–AY652582,

Table S1. Source and number of placozoan samples.

Source	Number
Clonal Cultures	
Red Sea (Grell-Red Sea)[a]	1
Indo-Pacific (Indo-Pac)[b]	1
Unknown Locality (LD)[c]	1
Unknown Locality (AQ)[c]	2
Environmental Samples	
Mediterranean Italy (Med), Orbatello Lagoon[d]	7
Caribbean Venezuela (Car-Ven), Isla Cubagua	1
Caribbean Panama (Car-Pan), Bocas del Toro, STRI	7
Pacific Panama (Pac-Pan-N), Naos Island Lab., STRI	2
Pacific Panama (Pac-Pan-II), Isla Iguana	5
Pacific Panama (Pac-Pan-AL), Achotines Lab.	3
Pacific Guam (Pac-Guam), U. of Guam Marine Lab.	1

[a]Derived from the well studied clonal lineage of K.G. Grell, which was cultured for many years at the University of Bochum, and which has been maintained in Hannover for the past 10 years.
[b]From aquarium with organisms from Indo-Pacific localities.
[c]From aquarium with organisms from multiple tropical localities.
[d]From aquarium with recently collected samples of *Polydora caeca*.

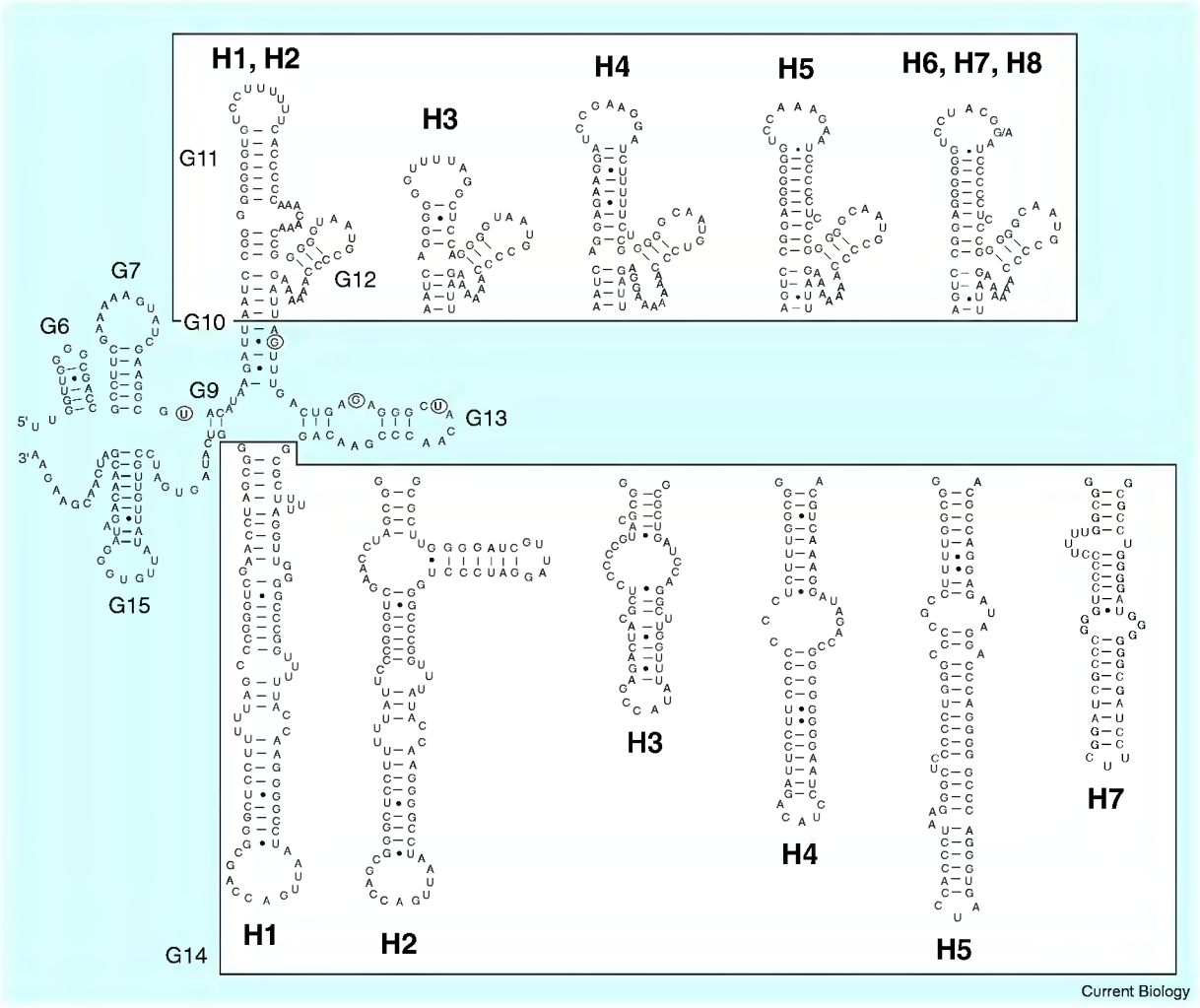

Figure S2. Comparison of inferred secondary structures for part of mitochondrial 16S. H1 to H8 refer to the eight 16S haplotypes present in our samples (Table S1). Note that our reverse primer is located within G15. Circled positions are variable and the displayed base represents the Grell-Red Sea lineage (GenBank Acc. AY169371).

LSU: AY652582–AY652587,
16S: AY652522–AY652529,
ITS: AY652530–AY652576).

Alignments were obtained by employing Clustal W and correcting by eye using Seaview [S3]. In order to compare SSU rDNA divergences within Placozoa to those observed within Porifera, Cnidaria, and Ctenophora, we aligned all publicly available SSU sequences from GenBank, along with additional cnidarian sequences (A.G. Collins, unpublished), and calculated uncorrected pair-wise p distances (Figure S1; http://ecolevol.de/archives/Voigt-etal-Tables-S2-3.xls). Using MEGA2 [S4], mean values between families within orders were calculated for those orders for which at least two different families were represented. Similarly, mean p distances between genera within families were calculated only for those families for which two or more genera were represented. See http://ecolevol.de/archives/Voigt-etal-Tables-S2-3.xls for taxonomy, accession numbers, and additional details of calculations. As expected, there is no strict relationship between Linnaean taxonomic rank and genetic divergence of SSU, as clearly indicated by Figure S1. That said, *p* distances between placozoan SSU samples fall within the range of those found between genera (within families) and between families (within orders) of other early diverging metazoan phyla. For example, the maximum placozoan divergence (0.018) exceeds all 29 of those that have been measured between species within a genus, is greater than or equal to 18 of 29 measured divergences between genera within families, and exceeds 4 of 23 average distances measured between families within orders. Thus, it seems likely that the ages of at least some placozoan divergences fall within the range of those that separated cnidarian and ctenophoran genera and families.

Phylogenetic analyses using the optimality criteria of maximum parsimony (MP), maximum likelihood (ML), and minimum evolution (ME) were carried out on three separate data sets (combined SSU and LSU, 16S, and ITS) using PAUP*4.0 [S5]. For ML and ME analyses, an assumed model of evolution was obtained

by using likelihood ratio tests as implemented in ModelTest [S6]. In order to assess the root divergence within our placozoan samples, optimal topologies based on 4,615 alignable characters from SSU and LSU rDNA sequences from six placozoans, three ctenophores, and five cnidarians were obtained. Rooting of the placozoan lineages is uncertain because MP and ML analyses converge on different optima, reflected in low ML bootstrap indices on some nodes.

Analyses of 16S and ITS data were done without outgroups in order to maximize the number of informative characters within Placozoa. 16S and ITS sequence regions that could not be reliably aligned across Placozoa were excluded from further analysis. One ITS GenBank sequence (U65478), derived from a placozoan of unknown origin, was included in our analyses. Because tree searches under the MP and ML criteria using all ITS sequences were too computationally demanding to complete, we conducted searches under these criteria using consensus sequences of clones for each sample. Bootstrap analyses under each criterion were conducted in order to assess node support.

The considerable length variation in 16S implied potential differences in secondary structure among our samples. Our structural predictions (Figure S2) are based upon published structures for a placozoan [S7], the anthozoan *Metridium senile* [S8], and the comparative RNA website [S9]. Predictions of secondary structures for large insertions not shared by *Metridium senile* were generated using a free-energy minimization method using Mfold [S10] with default settings and temperature set to 20°C. Note that the structures inferred here for the G13 and G14 regions differ from those presented in an earlier study [S7].

References

S1. Medlin, L., Elwood, H.J., Stickel, S., and Sogin, M.L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like ribosomal RNA-coding regions. Gene *71*, 491–500.

2S. Medina, M., Collins, A.G., Silberman, J.D., and Sogin, M.L. (2001). Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc. Natl. Acad. Sci. USA *98*, 9707–9712.

S3. Galtier, N., Gouy, M., and Gautier, C. (1996). SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput. Appl. Biosci. *12*, 543–548.

S4. Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics *17*, 1244–1245.

S5. Swofford, D.L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). (Sunderland, MA: Sinauer Associates).

S6. Posada, D., and Crandall, K.A. (1998). MODELTEST: Testing the model of DNA substitution. Bioinformatics *14*, 817–818.

S7. Ender, A., and Schierwater, B. (2003). Placozoa are not derived cnidarians: Evidence from molecular morphology. Mol. Biol. Evol. *20*, 130–134.

S8. Beagley, C.T., Okimoto, R., and Wolstenholme, D.R. (1998). The mitochondrial genome of the sea anemone Metridium senile (Cnidaria): introns, a paucity of tRNA genes, and a near standard genetic code. Genetics *148*, 1091–1108.

S9. Cannone, J.J., Subramanian, S., Schnare, M.N., Collett, J.R., D'Souza, L.M., Du, Y., Feng, B., Lin, N., Madabusi, L.V., Muller, K.M., et al. (2002). The Comparative RNA Web (CRW) Site: An Online Database of Comparative Sequence and Structure Information for Ribosomal, Intron, and other RNAs. 3.

S10. Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. *31*, 3406–3415.

[1]Institute of Animal Ecology & Cell Biology, Division of Ecology & Evolution, Bünteweg 17d, 30559 Hannover, Germany; [2]Department of Molecular Plant Physiology, Friedrich-Alexander Universität Erlangen-Nürnberg, Staudtstr. 5, 91058 Erlangen, Germany; [3]NMFS, National Systematics Laboratory, National Museum of Natural History, MRC-153, Smithsonian Institution, P.O. Box 37012, Washington, DC 20013-7012 USA; [4]Long Marine Laboratory, University of California, Santa Cruz, 100 Shaffer Rd., Santa Cruz, CA 95060 USA; [5]Katharinenhospital, Zentralinstitut für Transfusionsmedizin und Blutspendedienst, Keplerstrasse 32, 70174 Stuttgart, Germany
*E-mail: CollinsA@SI.edu