Correspondence

Supplemental data: Placozoa — no longer a phylum of one

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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

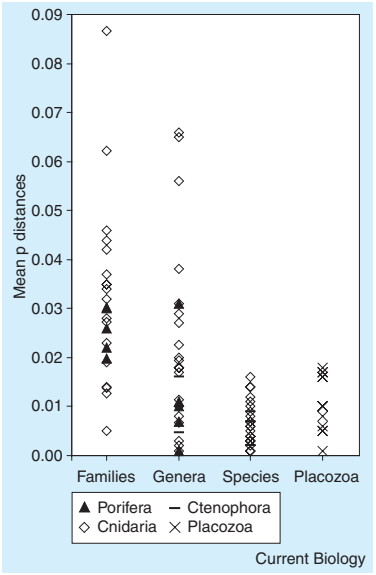

Figure S1. Mean uncorrected p distances of SSU between families (within orders), genera (within families), and species (within genera) of Porifera, Cnidaria, and Ctenophora compared to those between placozoan lineages.

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.*

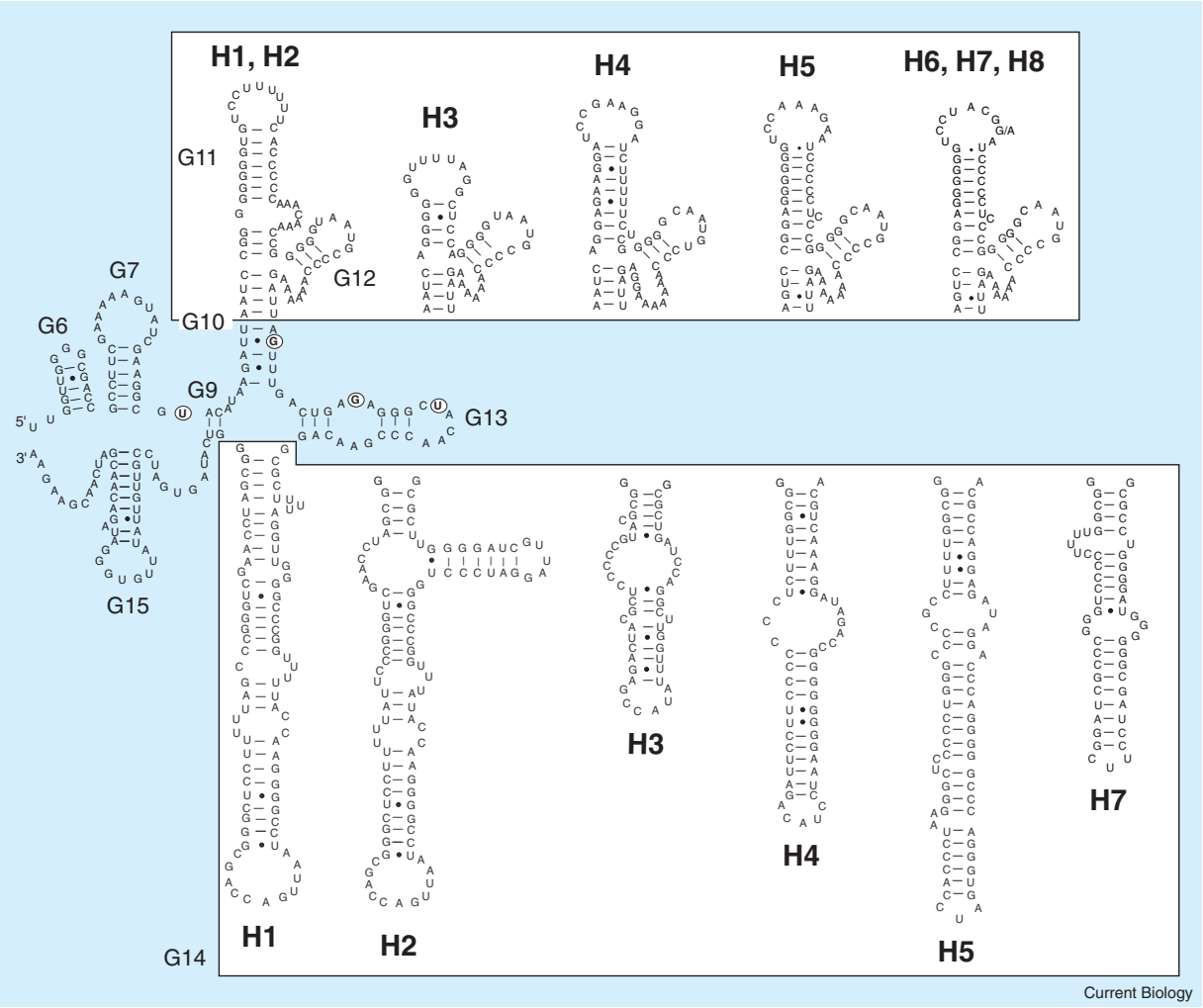

Figure S2. Comparison of inferred secondary structures for part of mitochondrial 16S. H1 to H8 refer to the eight 16S haplotypes present in our samples (Table S1). Note that our reverse primer is located within G15. Circled positions are variable and the displayed base represents the Grell-Red Sea lineage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

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