Mitochondrial genome of *Trichoplax adhaerens* supports Placozoa as the basal lower metazoan phylum

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Mitochondrial genomes of multicellular animals are typically 15- to 24-kb circular molecules that encode a nearly identical set of 12–14 proteins for oxidative phosphorylation and 24–25 structural RNAs (16S rRNA, 12S rRNA, and tRNAs). These genomes lack significant intragenic spacers and are generally without introns. Here, we report the complete mitochondrial genome sequence of the placozoan *Trichoplax adhaerens*, a metazoan with the simplest known body plan of any animal, possessing no organs, no basal membrane, and only four different somatic cell types. Our analysis shows that the *Trichoplax* mitochondrion contains the largest known metazoan mtDNA genome at 43,079 bp, more than twice the size of the typical metazoan mtDNA. The mitochondrion's size is due to numerous intragenic spacers, several introns and ORFs of unknown function, and protein-coding regions that are generally larger than those found in other animals. Not only does the *Trichoplax* mtDNA have characteristics of the mitochondrial genomes of known metazoan outgroups, such as chytrid fungi and choanoflagellates, but, more importantly, it shares derived features unique to the Metazoa. Phylogenetic analyses of mitochondrial proteins provide strong support for the placement of the phylum Placozoa at the root of the Metazoa.

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Trichoplax adhaerens [Shulze 1883] is a marine invertebrate distributed in tropical waters worldwide (1–3). It is the simplest known free-living animal, displaying no axis of symmetry, lacking a basal membrane, possessing only four somatic cell types (4–6), and having one of the smallest known animal genomes (7–9). Until recently, *T. adhaerens* was the sole representative of the phylum Placozoa, but recent field studies and molecular analyses indicate genetic diversity underlying apparent morphological uniformity within the Placozoa (3, 10). In the laboratory, placozoans reproduce asexually by either binary fission or budding dispersive propagules called swarmers. Eggs have been observed, and recent DNA polymorphism analysis has provided evidence for sexual reproduction within the Placozoa (10).

The phylogenetic placement of Placozoa among the metazoans, i.e., the animals, remains unresolved. In particular, its placement among lower metazoans, that is, the phyla Cnidaria, Ctenophora, and Porifera, has been controversial. Most studies place Porifera at the base of the metazoan tree of life (11–15), but others support placozoans as one of the earliest branching lineages of Metazoa (16–20). Conflicting data, including 18S, 28S, and 16S analysis, have suggested that Placozoa form a sister clade to all bilaterians or a sister clade to both cnidarians and bilaterians (14, 21–27).

Comparative mitochondrial genomics is becoming an effective tool to resolve phylogenetic placements because of several unique properties of mitochondrial genomes, including uniparental inheritance, orthologous genes, and lack of substantial intermolecular recombination (reviewed in refs. 28–30). Although some have questioned the utility of comparative mitochondrial genomics based on problems of convergence (31), in many cases, mitochondrial data have provided robust phylogenetic trees capable of resolving evolutionary relationships among fungi (32), protists (33), diploblasts (34), and bilaterians (35–42).

The closest living relatives of animals, the choanoflagellates and fungi, possess large mitochondrial genomes with extensive intragenic spacers, introns, and several ORFs of unknown function. The unicellular choanoflagellate, *Monosiga brevicollis*, has mtDNA that is nearly four times larger (76,568 bp) than the typical animal mtDNA genome and encodes 55 different genes, often separated by large intragenic spacer regions, including two genes interrupted by introns (43). Metazoans, on the other hand, have compacted 15- to 20-kb circular mitochondrial genomes that encode a nearly identical set of 12–14 proteins for oxidative phosphorylation and 24–25 structural RNAs (16S rRNA, 12S rRNA, and tRNAs) without significant intragenic spacers and, generally, without introns. Mitochondrial DNA variants exist in metazoans, such as the presence of type I introns and linear mtDNA molecules found in cnidarians (34, 44, 45), the presence of the *atp9* gene in sponges (15, 46), and the secondary expansion of mtDNA found in some mollusks (47, 48) and insects (49).

Our analysis shows that the *Trichoplax* mitochondrion possesses the largest known metazoan mtDNA genome, at 43,079 bp, more than twice the size of the typical metazoan mtDNA. Its large size is due not to secondary expansion but to features shared with metazoan outgroups, such as intragenic spacers, several introns, ORFs of unknown function, and protein-coding regions that are generally larger than that found in animals. The large *Trichoplax* mtDNA is the least derived mitochondrial genome of any animal. Moreover, the *Trichoplax* mitochondrion shares unique derived features with other lower metazoans, notably the loss of all ribosomal protein genes. These structural features of the *Trichoplax* mitochondrial genome, along with Bayesian and maximum-likelihood (ML) analyses of mitochondrial proteins from metazoans and outgroups, provide robust support for the phylogenetic placement of the phylum Placozoa at the root of the Metazoa.

Results and Discussion

We cloned the full-length mitochondrial genome from the placozoan *T. adhaerens*, determined its complete sequence and

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Abbreviation: ML, maximum likelihood.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ112541).

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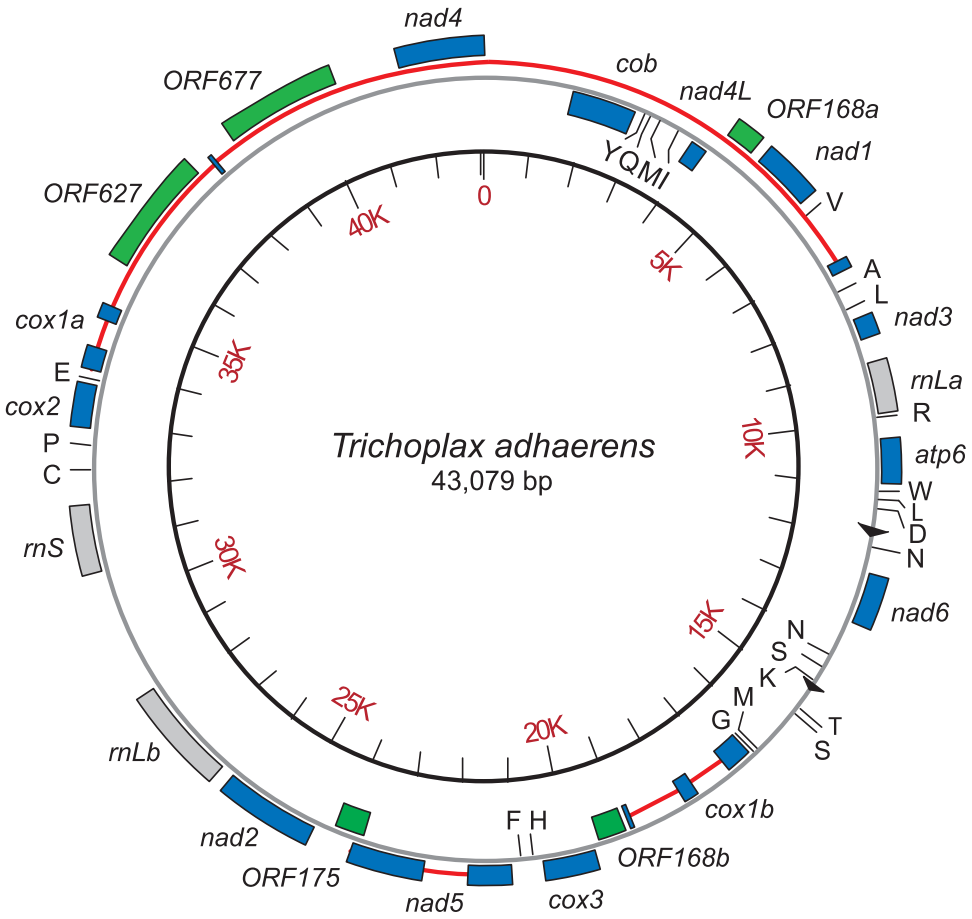

Fig. 1. Scale drawing of the mitochondrial genome of *T. adhaerens.* The complete sequence of the 43,079-bp mitochondrial genome from a Red Sea isolate of *T. adhaerens* (59) was determined and annotated by identifying ORFs with the National Center for Biotechnology Information's ORF FINDER using genetic code 4. Known mitochondrial proteins (blue rectangles) were identified by BLAST and by alignment to corresponding proteins found in poriferans (NC_006894, NC_006990, and NC_006991), cnidarians (NC_000933 and NC_003522), and the choanoflagellate *Monosiga* (NC_004309) to infer the start of translation. Genes transcribed in the clockwise direction are shown on the outer circumference; genes transcribed in the counterclockwise direction are shown on the inner circumference. Large (*rnLa* and *rnLb*) and small (*rnS*) ribosomal genes are represented as gray rectangles. The tRNAs (black lines) were identified by using TRNASCAN-SE (62) and are annotated by their International Union of Pure and Applied Chemistry (IUPAC) amino acid codes. ORFs encoding unknown proteins >100 aa in length are identified by their amino acid coding capacity (green rectangles). Introns in the *cox1* and *nad5* genes are shown as red lines connecting exons (blue rectangles). A 103-bp imperfect direct repeat is shown as black triangles. Note that the carboxy-terminal region of *cox1* (exons 5–7) is inverted with respect to *cox1* exons 1–4 because of the presence of a large 16-kb inversion encompassing the region from *trnP* to *trnV*. This inversion has been confirmed experimentally in the Red Sea isolate but does not exist in another placozoan taxon (A. Signorovitch, L. Buss, and S.L.D., unpublished data).

organization (Fig. 1), and compared it with the mtDNA genomes of other diploblasts (15, 44, 45) and the choanoflagellate *Monosiga brevicollis* (43). The 43,079-bp circular mtDNA genome of *Trichoplax* is more than twice the size of the mtDNA found in most metazoans (17–25 kb), including those of poriferans (15, 46) and cnidarians (44, 45) (Table 1), making it the largest known animal mtDNA genome. The size and composition of the *Trichoplax* mtDNA genome resembles that of the choanoflagellates and is in striking contrast to the streamlined genomes found in virtually all metazoans. In rare cases of relatively large mtDNAs (up to 42 kb) found in animals, these instances are known to be a result of secondary duplications, repeat expansions, or extensive A + T-rich regions (47–49).

The *Trichoplax* mtDNA genome encodes a typical complement of animal mtDNA genes, including ATP synthase subunits (*atp6*), cytochrome oxidase subunits (*cox1*, *cox2*, and *cox3*), apocytochrome b (*cob*), reduced nicotinamide adenine dinucleotide ubiquinone oxireductase subunits (*nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, and *nad6*), a full complement of tRNAs (24 in all), and small and large ribosomal RNAs (*rrnS* and *rrnL*) (Fig. 1 and Table 1). Additional features of the *Trichoplax* mtDNA include extra introns in the *cox1* gene and the physical separation of the *rrnL* and *cox1* genes into two discrete domains (Fig. 1). Several unknown ORFs are also found in the *Trichoplax* mitochondrial genome, including one that encodes a 627-aa protein containing reverse transcriptase (pfam00078) and Type II intron maturase (pfam01348) domains, located just downstream of the second exon of *cox1*. We have not identified the *atp8* gene in *Trichoplax* mtDNA.

Notably, *Trichoplax* mtDNA shares characteristics found in the mitochondrial genomes of known metazoan outgroups. The large size of the *Trichoplax* genome is due to the presence of large intragenic spacers, several ORFs of unknown function, and additional *cox1* introns, two of which share identical positions within the choanoflagellate *Monosiga* and the fungus *Monoblepharella cox1* genes (32, 43). Furthermore, most mtDNA genes in *Trichoplax* encode larger proteins than those found in other animals. On average, protein-coding regions are 10% larger in *Trichoplax* than in other diploblasts, which is comparable to the difference between choanoflagellates and diploblasts (43). Similar to *Monosiga* (43), only about half of the *Trichoplax* mtDNA contain coding regions, whereas other dip-

Table 1. Comparison of representative mitochondrial genomes

Organism	Size, bp	Coding, %	tRNAs	rRNA	ORFs	Introns	RPs	RC subunits
Choanoflagellata								
Monosiga	76,568	≈47	25	*rrnL, rrnS*	6	4	*rps 3, 4, 8, 12–14, 19*	*atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6*
Placozoa								
Trichoplax	43,079	≈50	24	*rrnLa, rrnLb, rrnS*	5	>3	0	*atp 6, cob, cox 1–3, nad1–4, 4L, 5, 6*
Porifera								
Axinella	25,610	≈76	25	*rrnL, rrnS*	0	0	0	*atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6*
Geodia	18,020	≈98	24	*rrnL, rrnS*	0	0	0	*atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6*
Tethya	19,*565*	≈92	25					*atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6*
Cnidaria								
Metridium	17,443	≈86	2	*rrnL, rrnS*	1	2	0	*atp 6, 8, cob, cox 1–3, nad1–4, 4L, 5, 6*
Acropora	18,338	≈85	2	*rrnL, rrnS*	0	1	0	*atp 6, 8, cob, cox 1–3, nad1–4, 4L, 5, 6*

Data compiled from mitochondrial genomes taken from GenBank: *Monosiga brevicollis* (NC_004309), *T. adhaerens* (this study) the poriferans *Axinella corrugata* (NC_006894), *Geodia neptuni* (NC_006990), and *Tethya actini* (NC_006991), and the onidarians *Metridium senile* (NC_000933) and *Acropora tenuis* (NC_003522). Coding percentage calculated from the proportion of sequence having protein coding (known mitochondrial proteins or ORF >100 aa) tRNA genes, and rRNA coding sequences; ORFs, number of reading frames encoding unknown proteins >100 aa; RPs, ribosomal protein genes; RC subunits, respiratory chain subunit genes.

loblast mtDNA genomes range from 76% to 98% coding capacity in the 25.6- and 18-kb mtDNAs of the poriferans *Axinella* and *Geodia* (15, 46), respectively (Table 1).

The *Trichoplax* mitochondrial genome shares metazoan features lacking in choanoflagellates and fungi. Notably, we find no evidence of the presence of ribosomal protein genes in *Trichoplax*, a property shared with other metazoan mtDNAs, suggesting that loss of ribosomal protein genes may be a synapomorphy for the animal kingdom. Mitochondrial DNA features that had heretofore been thought to be restricted to either sponges or cnidarians are all found in *Trichoplax*. Specifically, the mitochondrial genomes of both cnidarians and *Trichoplax* mtDNA have conserved introns in the *nad5* and *cox1* genes as well as unknown ORFs.

To further examine the phylogenetic position of *Trichoplax* among the lower metazoans, we performed Bayesian and ML analyses on 2,730 amino acid positions derived from 12 well conserved protein sequences (*cox1–3*, *cob*, *atp6*, *nad1–4*, *4L*, and *5–6*) common to the mitochondrial genomes of *T. adhaerens*; the cnidarians *Metridium senile* (NC_000933), *Acropora tenuis* (NC_003522), *Anacropora matthai* (NC_006898), and *Montipora cactus* (NC_006902); the poriferans *Geodia neptuni* (NC_006990), *Axinella corrugata* (NC_006894), and *Tethya actinia* (NC_006991); and the choanoflagellate *Monosiga brevicollis* (NC_004624). *Monoblepharella sp. JEL15* (NC_004624) was included as an outgroup taxon for this analysis because chytrids are regarded as the basal fungal taxon (32). The predicted amino acid sequences for each of the 12 genes were aligned by using CLUSTALW (50) and edited, manually and computationally, by using GBLOCKS (51), to remove ambiguous sites. These alignments were concatenated to produce a final data set of 2,730 aa (see Data Set 1, which is published as supporting information on the PNAS web site).

Partitioned Bayesian analysis, implemented in MRBAYES 3.1.1 (52), was performed by using the mtREV amino acid substitution model, with substitution-rate variation among sites modeled by a discrete approximation of the γ-distribution with a proportion of invariable sites ($I + \Gamma$). This analysis produced the phylogeny depicted in Fig. 2*A*. The posterior probabilities exceeded 99% for each node, with overwhelming support for *Trichoplax* being basal to both poriferans and cnidarians. ML analysis, implemented in PAML 3.14 (53), using star decomposition tree search and the mtREV amino acid substitution model, produced an identical tree topology with the bootstrap values shown in Fig. 2. Using site-wise log-likelihoods generated by PAML, statistical tests, implemented in CONSEL 0.1i (54), were conducted to test all possible placements of *Trichoplax* among lower metazoans (Table 2). The *P* values of the Approximately Unbiased (55), the weighted and unweighted Kishino–Hasegawa (56), and the weighted and unweighted Shimodaira–Hasegawa (55) tests all exceeded 0.999 for the tree shown in Fig. 2*A*, with no other topology supported by *P* values >0.002.

Inclusion of bilaterian mtDNA data from the deuterostomes *Strongylocentrotus purpuratus* (NC_001453) and *Saccoglossus kowalevskii* (NC_007438) and the protostomes *Artemia franciscana* (NC_001620) and *Katharina tunicata* (NC_001636) in the phylogenetic analyses yielded a bifurcation at the base of metazoans between two clades (Fig. 2*B*), one comprising all bilaterians and the other comprising all diploblasts. Inclusion of additional bilaterian taxa in the analysis did not change this topology (data not shown). This result is consistent with that reported by Lavrov *et al.* (15) and may be due to long branch attraction that is known to affect analyses of fast evolving metazoan sequences (57). A relative-rates test comparing bilaterians to diploblasts using *Monosiga* and *Monoblepharella* as outgroups was performed by using RRTREE (58). The *P* value for bilaterians evolving at the same rate as diploblasts was 10^{-7}, indicating that the conditions for long branch attraction are present. Most importantly, regardless of whether bilaterian sequences are included or not, the basal phylogenetic position of Placozoa within the lower metazoans is robust, with *P* values between 0.924 and 1.000 for the various statistical tests (see Table 3, which is published as supporting information on the PNAS web site). Finally, the "placozoan-basal" topology was robust to the choice of outgroups, including the addition or substitution of chytrid fungi (*Allomyces macrogynus* NC_001715 and *Rhizophydium* sp. 136 NC_003053) as outgroups (see Fig. 3, which is published as supporting information on the PNAS web site).

Our results demonstrate that the placozoan *Trichoplax* possesses an unusual and unique mitochondrial genome, with structural and compositional features characteristic of both choanoflagellate mtDNAs, the closest relatives to animals, and typical lower metazoan mtDNAs. Like choanoflagellates, the *Trichoplax* mtDNA is much larger than the typical metazoan mtDNA, with substantial noncoding regions, genes generally larger than those found in other metazoans, several unknown ORFs, and conserved introns in both *nad5* and *cox1* genes. The large mtDNA genome found in *Trichoplax*, although consistent with the idea that marked gene loss and mtDNA compaction occurred during the emergence of multicellular animals, none-

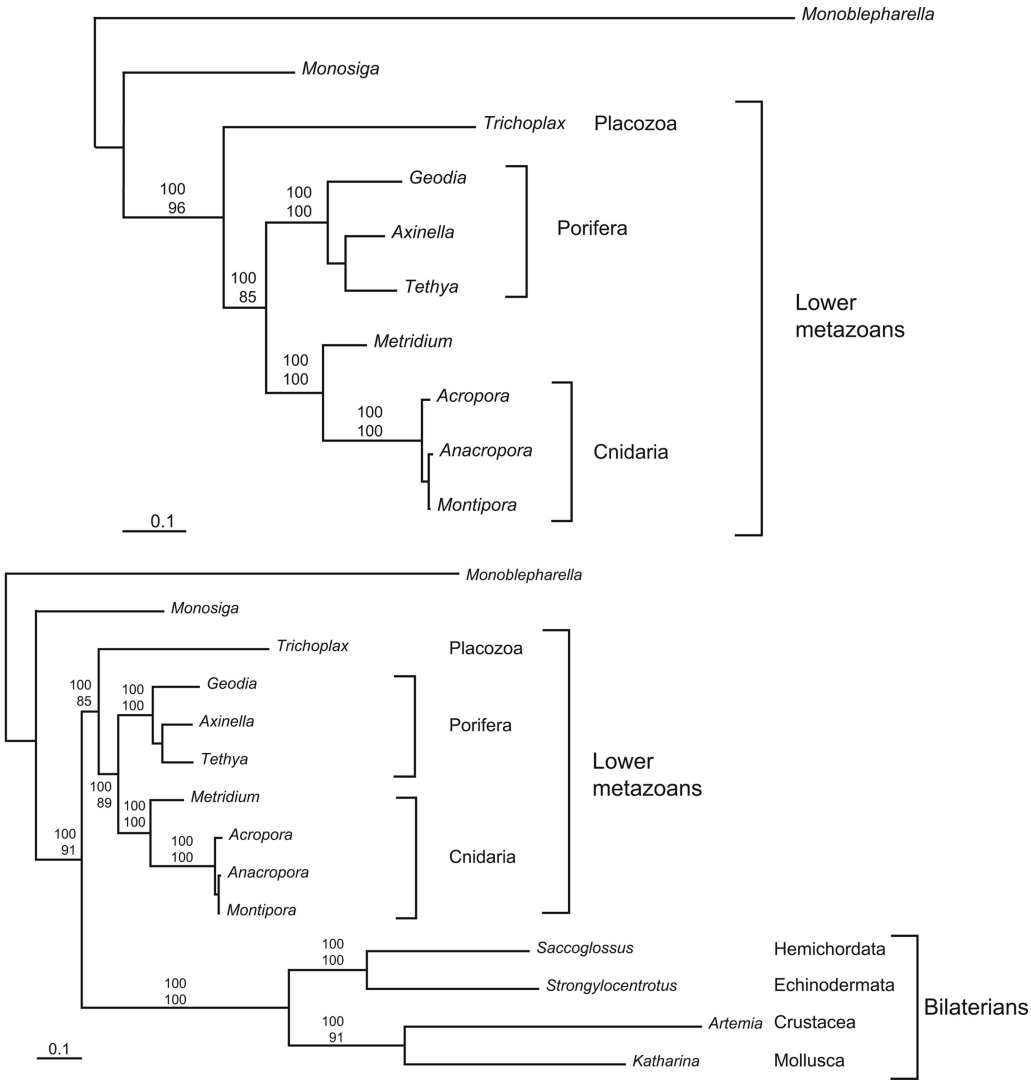

Fig. 2. Phylogenetic analysis of concatenated mitochondrial proteins. The data set consisted of a total of 2,730 amino acid positions concatenated from 12 mitochondrial protein sequences (atp6 cob, cox1–3, nad1–4, and 4L, 5, and 6). Partitioned Bayesian analysis was performed with MRBAYES for 500,000 generations by using four chains and the mtREV amino acid substitution model. Substitution rate variation among sites was modeled by a discrete approximation of the γ-distribution with a proportion of invariable sites (I + Γ). ML analysis performed with PAML using the mtREV amino acid substitution model and star decomposition tree search gave an identical tree topology. Posterior probability (*Upper*) and bootstrap (*Lower*) values are shown for each node. In these analyses, the output trees were rooted by using the chytrid fungus *Monoblepharella*.

theless indicates that pronounced compaction was not coincident with the origin of the Metazoa.

Texts of invertebrate zoology have long been unanimous in placing the Porifera as the basal metazoan phylum, based largely on the striking resemblance of the collared cells of choanoflagellates to the choanocytes of sponges. Choanocytes, however, are adaptations for filter-feeding in these two taxa, and their absence is expected in placozoans, which have a different mode of

Table 2. CONSEL statistical tests of the tree topology obtained by Bayesian and ML analysis

	P					
Hypothesis	au	kh	wkh	sh	wsh	pp
Trichoplax basal to Porifera + Cnidaria	1.000	0.999	0.999	1.000	1.000	1.000
Trichoplax within Porifera	10^{-44}–10^{-4}	0–0.001	0–0.001	0–0.113	0–0.002	10^{-111}–10^{-29}
Porifera basal to *Trichoplax* + Cnidaria	10^{-104}–10^{-4}	0–10^{-4}	0–10^{-4}	0–0.073	0–10^{-4}	10^{-291}–10^{-33}

Summary of statistical tests on all possible phylogenetic positions of *Trichoplax* within lower metazoans by using the Approximately Unbiased (54), the weighted and unweighted Kishino–Hasegawa (55); and the weighted and unweighted Shimodaira–Hasegawa (54) tests, as implemented in CONSEL. In addition, posterior probabilities pp are also displayed.

feeding. The data and analyses presented here provide strong support for the phylogenetic placement of Placozoa as the basal extant lower metazoan phylum.

Materials and Methods

Cloning and Sequencing of *T. adhaerens* Mitochondrial DNA. Total genomic DNA was isolated from a cultured Red Sea isolate (59). Approximately 20 μg of genomic DNA was resuspended in 0.5 ml of Tris-EDTA buffer, sheared by two quick passages through a 20-gauge needle attached to a 1-ml syringe, end-repaired by using the DNA Terminator Kit (Lucigen, Middleton, WI) and size fractionated by pulse-field electrophoresis. The 30- to 40-kb DNA fraction was gel purified, ligated into the pCC1FOS vector, packaged *in vitro*, and plated on EPI300 *Escherichia coli* cells according to manufacturer's instructions (EPICENTRE Biotechnologies, Madison, WI). Several independent, overlapping fosmid clones containing near-full-length (36- to 40-kb) mitochondrial DNA inserts were identified by colony hybridization using a 16S rRNA probe (27). Purified fosmid DNA was isolated and sheared by sonication and end-repaired and fractionated by gel electrophoresis. The 2- to 4-kb fraction was gel purified and ligated to pSMART LC-Kan vector and transformed into *E. coli 10G*-competent cells according to manufacturer's instructions (Lucigen). Approximately 384 random subclones were chosen for sequencing. Template DNA was prepared by using TempliPhi amplification (GE Healthcare) and sequenced by BigDye Terminator version 3.1 cycle sequencing on ABI PRISM 3700 DNA analyzers (Applied Biosystems) with both forward and reverse vector primers (Lucigen). Selected regions of poor quality or low coverage were resequenced by using fosmid DNA template and custom DNA primers designed by the Autofinish feature of CONSED (60).

Sequence Assembly and Annotation. DNA sequence chromatograms generated from random subclones and custom primer sequencing were processed and assembled by using the PHRED–PHRAP–CONSED software suite release 13.0 (www.phrap.org). The assembled mitochondrial genome sequence was analyzed with the National Center for Biotechnology Information's ORF FINDER using genetic code 4. Predicted ORFs were subjected to a similarity search using BLASTP. A custom-made Perl script, available upon request, automated this process. Each identified mitochondrial protein sequence was aligned to the corresponding sequences from related taxa, including poriferans (NC_006894, NC_006990, and NC_006991), cnidarians (NC_000933, NC_003522, NC_006898, and NC_006902), and the choanoflagellate *Monosiga* (NC_004309) to infer translational start sites, intron–exon boundaries, and estimated boundaries of ribosomal RNA genes. The transfer RNAs were identified with TRNASCAN-SE 1.21 (www.genetics.wustl.edu/eddy/tRNAscan-SE). Twelve conserved mitochondrial proteins (atp6, cox1–3, cob, nad1–4, and 4L, 5, and 6) from *Trichoplax*, and other species were individually aligned by using CLUSTALW (50), edited manually and computationally by using GBLOCKS (51) to remove ambiguous sites, and concatenated to give a final data set of 2,730 aa for phylogenetic analysis (Data Set 1).

Phylogenetic Analysis. Partitioned Bayesian analysis, as implemented in MRBAYES 3.1.1 (52), was performed for 500,000 generations by using four independent chains and the mtREV amino acid substitution model. Substitution-rate variation among sites was modeled by a discrete approximation of the γ-distribution with a proportion of invariable sites ($I + \Gamma$). The first 1,250 samples (25%) were discarded as burn-in. ML analysis, implemented in PAML 3.14 (53), was performed by using the mtREV amino acid substitution model and star decomposition tree search. For bootstrap analysis, 100 resampling replicates were generated by using SEQBOOT (61) and analyzed by ML analysis using PAML. The topology given by MRBAYES and PAML was statistically tested for robustness against other possible tree topologies with CONSEL 0.1i (54) using site-wise log-likelihood outputs from PAML.

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