Concatenated Analysis Sheds Light on Early Metazoan Evolution and Fuels a Modern "Urmetazoon" Hypothesis

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For more than a century, the origin of metazoan animals has been debated. One aspect of this debate has been centered on what the hypothetical "urmetazoon" bauplan might have been. The morphologically most simply organized metazoan animal, the placozoan *Trichoplax adhaerens*, resembles an intriguing model for one of several "urmetazoon" hypotheses: the placula hypothesis. Clear support for a basal position of Placozoa would aid in resolving several key issues of metazoan-specific inventions (including, for example, head–foot axis, symmetry, and coelom) and would determine a root for unraveling their evolution. Unfortunately, the phylogenetic relationships at the base of Metazoa have been controversial because of conflicting phylogenetic scenarios generated while addressing the question. Here, we analyze the sum of morphological evidence, the secondary structure of mitochondrial ribosomal genes, and molecular sequence data from mitochondrial and nuclear genes that amass over 9,400 phylogenetically informative characters from 24 to 73 taxa. Together with mitochondrial DNA genome structure and sequence analyses and Hox-like gene expression patterns, these data (1) provide evidence that Placozoa are basal relative to all other diploblast phyla and (2) spark a modernized "urmetazoon" hypothesis.

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Introduction

Attempts to explain the origin of metazoan life seek to unravel both the transition from (1) single-celled to multicellular organisms and (2) diploblastic to triploblastic body plans. The most favored scenarios are based on five well-known hypotheses on the "urmetazoon" bauplan: Haeckel's gastraea, Jägersten's bilaterogastraea, Metschnikoff's phagocytella, Lankester's planula, and Bütschli's placula [1–5]. Attempts to unravel the urmetazoon bauplan and to provide support for any of the five hypotheses depends on identifying the most basal extant diploblast group. Two phylogenetic alternatives have remained under discussion; one sees the sponges (Porifera) and the other the placozoans (Placozoa) as basal relative to all other diploblast groups [6–10]. The latter view was accepted for the most part of the last century. The presence of only four somatic cell types, the smallest metazoan genome, and the lack of any foot or head structures, any anterior–posterior organization, or any kind of organs, and both a basal lamina and an extracellular matrix (ECM) places *Trichoplax* in a basal and isolated position relative to all other metazoan phyla [11–16] (cf. [17], however).

Tangled Roots at the Base of the Metazoan Tree of Life

Mainly because of misinterpretation of life cycle stages between *Trichoplax adhaerens* and the hydrozoan *Eleutheria dichotoma*, Placozoa lost their predominant role as the key model system for studying the origin of metazoan life [5,17]. This outcome was nourished by molecular studies based on a variety of character sources, which created a series of conflicting phylogenetic scenarios in which most often Porifera came out basal [18–24]. Figure 1 shows six plausible scenarios for the relationships of five taxonomic groups (Bilateria, Cnidaria, Ctenophora, Porifera, and Placozoa) and two plausible arrangements for four taxa when Placozoa are left out that are critical in assessing the early relationships of metazoans. For five taxa and one outgroup, there are 105 ways to arrange these taxa in dichotomous branching trees. Nearly 95% of these possible trees can be eliminated as not plausible based on existing data. All six of the hypotheses in Figure 1 have been suggested as viable in the literature over the past two decades (see Table S1 for a summary of papers in the last decade addressing the phylogenetics of these taxa).

All six hypotheses have been suggested in publications in the last year alone. For instance, Srivastava et al. (2008) [23] hypothesize Placozoa as the sister group to both Cnidaria and Bilateria, with sponges branching off earlier (arrow b in Figure 1). Another recent study, which suggests a basal position for Ctenophora and Anthozoa (arrow E in Figure 1), unfortunately does not add to the issue, since it does not include Placozoa in the analysis [25]. However, this study does

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Abbreviations: ML, Maximum Likelihood

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Author Summary

Following one of the basic principles in evolutionary biology that complex life forms derive from more primitive ancestors, it has long been believed that the higher animals, the Bilateria, arose from simpler (diploblastic) organisms such as the cnidarians (corals, polyps, and jellyfishes). A large number of studies, using different datasets and different methods, have tried to determine the most ancestral animal group as well as the ancestor of the higher animals. Here, we use "total evidence" analysis, which incorporates all available data (including morphology, genome, and gene expression data) and come to a surprising conclusion. The Bilateria and Cnidaria (together with the other diploblastic animals) are in fact sister groups: that is, they evolved in parallel from a very simple common ancestor. We conclude that the higher animals (Bilateria) and lower animals (diploblasts), probably separated very early, at the very beginning of metazoan animal evolution and independently evolved their complex body plans, including body axes, nervous system, sensory organs, and other characteristics. The striking similarities in several complex characters (such as the eyes) resulted from both lineages using the same basic genetic tool kit, which was already present in the common ancestor. The study identifies Placozoa as the most basal diploblast group and thus a living fossil genome that nicely demonstrates, not only that complex genetic tool kits arise before morphological complexity, but also that these kits may form similar morphological structures in parallel.

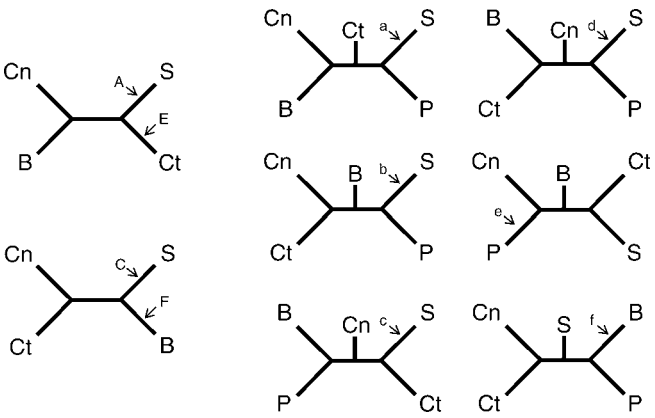

Figure 1. Discussed Relationships at the Base of the Metazoan Tree

Potential arrangements of five critical taxa (B, Bilateria; Cn, Cnidaria; Ct, Ctenophora; P, Placozoa; and S, Porifera) are shown on the right, and some hypotheses in the literature with only four taxa (Placozoa omitted) on the left. Arrows indicate the root of the networks. The letters at the arrows are for reference to Table S1. The uppercase letters refer to publications in Table S1 that support the indicated root for trees without Placozoa. The lowercase letters refer to publications in Table S1 that support the root for trees with all five taxa.

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suggest that Cnidaria are not sister to Bilateria, but rather to Porifera [25]. A study that does include Placozoa [26] also suggests that Bilateria and Placozoa are basal metazoans (arrow a in Figure 1). Striking examples of the diversity of hypotheses generated on these taxa are recent analyses of mitochondrial genome sequence data [27–29] that place Bilateria as sister to all non-Bilateria, with Placozoa as the most basal diploblast (arrow e in Figure 1). In the following, we use the term "diploblasts" for all nonbilaterian metazoans; we do not intend to contribute to the discussion of whether diploblastic animals may have a mesoderm, however [1,30–33].

Results and Discussion

A Concatenated Dataset for Metazoa

Given that both nonphylogenetic interpretation of morphological data as well as molecular analyses of sequence data have failed to resolve the issue, a more comprehensive, systematic analysis of morphological data and new molecular markers are now a requisite for identifying the root of the metazoan tree of life. To approach this goal, we conducted concatenated analyses for 24 metazoan taxa from all of the major organismal lineages in this part of the tree of life that included morphological characters (17 characters), both mitochondrial and nuclear ribosomal gene sequences (five gene partitions for 6,111 nucleotide positions) and molecular morphology [8] (ten characters), as well as nuclear coding genes (16 gene partitions derived from our database searches and another 18 gene partitions derived from the Dunn et al. (2008) study [25]; see Materials and Methods) for 8,307 amino acid positions and protein coding genes (16 gene partitions for 3,004 amino acid characters) to resolve phylogenetic relationships between recent diploblast groups. The total number of characters included was 17,664 from 51 partitions, giving 7,822 phylogenetically informative characters. We also constructed a matrix with a larger number of taxa based on the Dunn et al. (2008) [25] study with 73 taxa for the same gene partitions (see Materials and Methods and Tables S2 and S4). This matrix had 17,637 total characters and 9,421 phylogenetically informative characters. In addition, Hox gene expression was compared for a placozoan and a cnidarian bauplan to test predictions from the placula hypothesis [5].

Clarity and Confusion at the Root of the Metazoan Tree

Parsimony, likelihood (with morphological characters removed), and mixed Bayesian analysis of the smaller concatenated matrix using a variety of approaches, weighting schemes, and models is generally consistent with the view that Bilateria and diploblasts (Porifera, Ctenophora, Placozoa, and Cnidaria) are sister groups. In addition, Placozoa are robustly observed as the most basal diploblast group (Figure 2 and Figure 3). Figure 3 shows the support for several hypotheses of monophyly obtained from diverse methods of analysis. Porifera, Bilateria, and Fungi all form strong monophyletic groups (Figure 3). The four cnidarian classes (Anthozoa, Hydrozoa, Scyphozoa, and Cubozoa) together with the Ctenophora form a monophyletic group, the "Coelenterata." Within the Cnidaria, the generally accepted basal position of the anthozoans is also recovered by this analysis [34,35]. Both choanoflagellates and Placozoa are strongly excluded from a Porifera–Coelenterata monophyletic group. The basal position of Placozoa is also strongly supported by comparing the phylogeny in Figure 2 with hypotheses that place it more derived, using the statistical approach of Shimodaira and Hasegawa [36,37]. This battery of tests (Table 1) demonstrates that the basal position of the Placozoa is significantly better than other hypotheses. The 95% confidence tree includes the Maximum Likelihood (ML) and Bayesian trees (both with Placozoa as basal in the diploblasts) with a cumulative expected likelihood weight (ELW) of 0.960763.

The tree topology shown in Figure 2 summarizes the best supported phylogenetic hypothesis obtained by using Max-

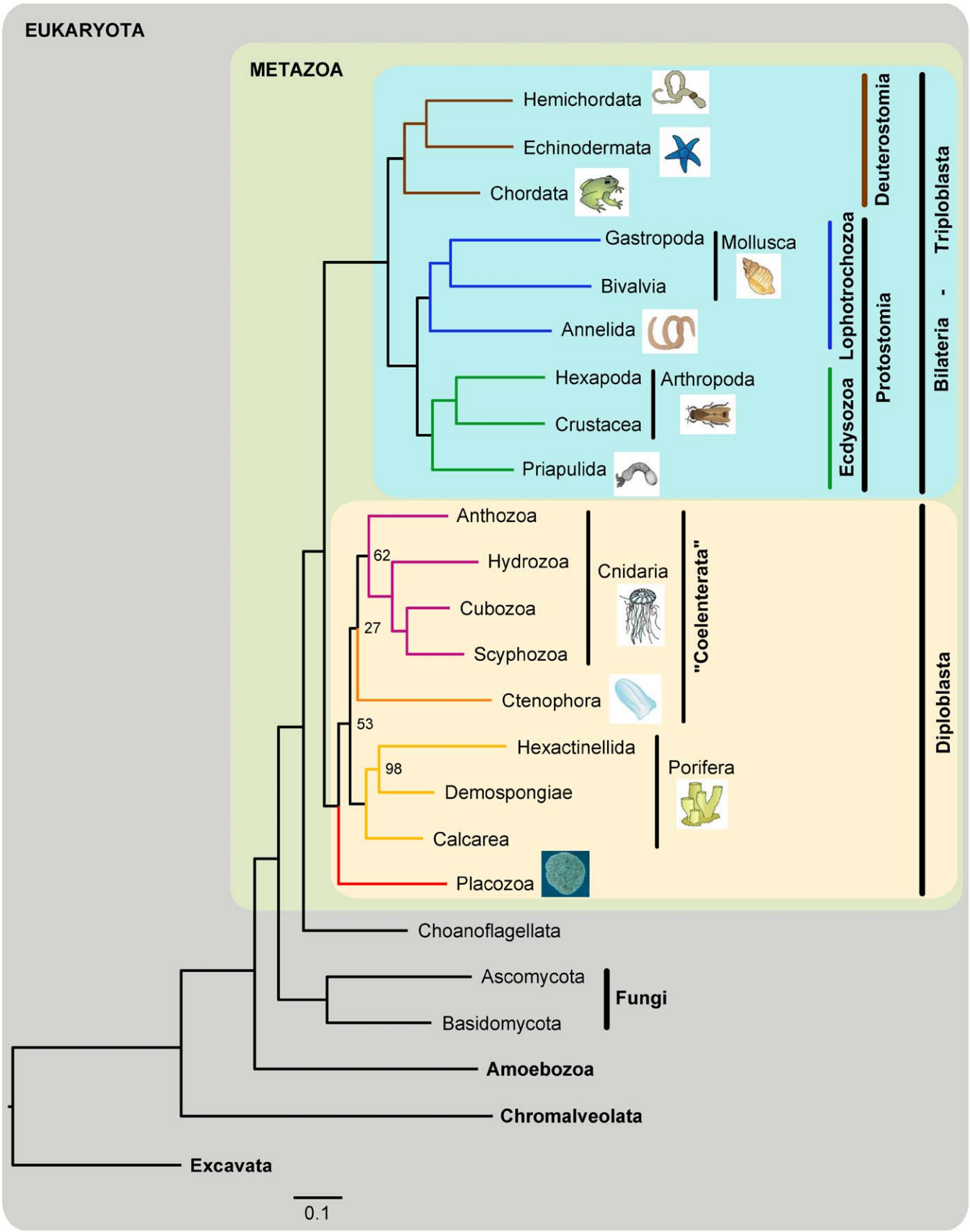
EUKARYOTA
METAZOA
Hemichordata
Echinodermata
Chordata
Gastropoda
Bivalvia
Mollusca
Annelida
Hexapoda
Crustacea
Arthropoda
Priapulida
Deuterostomia
Lophotrochozoa
Ecdysozoa
Protostomia
Bilateria - Triploblasta
Anthozoa
Hydrozoa
Cubozoa
Scyphozoa
Cnidaria
62
27
Ctenophora
"Coelenterata"
53
Hexactinellida
98
Demospongiae
Calcarea
Porifera
Placozoa
Diploblasta
Choanoflagellata
Ascomycota
Basidomycota
Fungi
Amoebozoa
Chromalveolata
Excavata
0.1

Figure 2. Maximum Likelihood Phylogenetic Tree of Metazoan Relationships Using the Concatenated Data Matrix

Node support is based on the best ML tree filtered through 1,000 rapid bootstrap replicates. Only support values below 100% are shown. Bayesian inference supported strongly (posterior probability = 1.0) all nodes with the exception of monophyly of Cnidaria. The maximum a posteriori and the Bayesian 50% majority-rule consensus trees disagreed with the best ML tree in supporting a Ctenophora–Anthozoa clade with posterior probability of 0.98. Please note that "Coelenterata" is not a taxonomic unit, but rather it is a traditional grouping for reasons of convenience. The alpha shape parameters of the Gamma distribution were 0.507454 and 0.651659 for the nucleotide and amino acid partitions, respectively. Log-likelihood = −261429.821426.

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imum Parsimony, ML, and Bayesian analyses of the concatenated dataset. Analysis of the larger matrix (Figure S2) was less well resolved within the Bilateria, but showed the same general topology as the smaller analysis. Specifically, Bilateria are monophyletic and sister to the diploblasts, with the choanoflagellate *Monosiga* basal to these taxa with high jackknife values and Bayesian posteriors. Diploblasts are also monophyletic, and Placozoa are the most basal taxon in the diploblasts. In addition, within the diploblasts, Porifera and Coelenterata are monophyletic, and within Bilateria, Ecdysozoa and Deuterostomia are monophyletic; all groupings with high node support.

The topology within the diploblasts is also robust when Bilateria are removed from the analysis. The full analysis seemingly misplaces the Bilateria clade as the sister to all diploblasts. The classical position of the Bilateria is in a highly derived position from within the diploblasts and usually sister to the Cnidaria. The seemingly "weird" prediction of a basal Bilateria from the present analysis has been observed before in other studies (see Table S1). Several studies have addressed phylogenetic problems specific to this region of the tree of life and have suggested that this region of the tree will be inherently difficult to resolve. These studies suggest that the compression of splitting events in this region renders the resolution of these nodes with high support difficult, if not impossible [38–42]. These studies have suggested that even large amounts of data might not resolve the problem. Other studies have pointed to taxon sampling and modeling as a potential problem in resolving this part of the tree of life [25,38–40]. Another problem is that the large number of molecular phylogenetic approaches creates multiple and possibly the most short-lived hypotheses in biology. The large repertoire of algorithms, models, and assumptions sometimes produces a forest of trees from the same dataset (cf. [43]). Thus, tree-building procedures are highly crucial and deserve particular attention if this region of the tree of life is to be resolved [38].

Possible Swamping by Mitochondrial Data?

Our analyses provide strong evidence for a basal position of Placozoa relative to other diploblasts, and thus agrees with

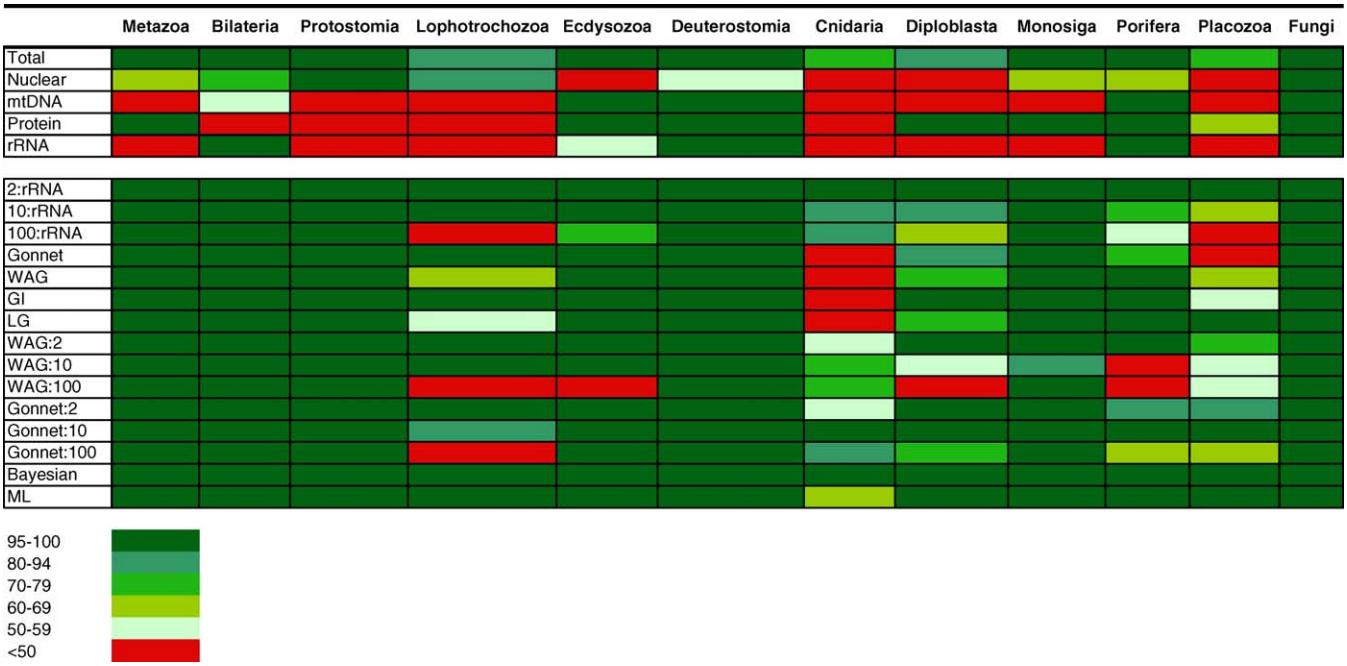

Figure 3. Phylogeny of Animals and Weighting Schemes

The impact of several weighting schemes on the phylogenetic hypothesis in Figure 2. The values in the table are jackknife values for maximum parsimony, rapid bootstrap for ML, and posterior clade probabilities for Bayesian inference. The color coding for the values is shown at the bottom of the table. The major monophyletic groups examined for jackknife support in Figure 2 are indicated in the top row. See Figure 2 for nodes defined by these groups. Monosiga refers to placing *Monosiga* as basal to Metazoa, and Placozoa refers to placing Placozoa as basal to diploblasts. Total in the first row refers to the entire dataset analyzed with equal weighting of all characters. The next four rows show results for analyses of partitioned datasets: mtDNA, mitochondrial partition; Nuclear, nuclear; Protein, protein; and rRNA, ribosomal RNAs from both nuclear and mitochondrial genomes. The bottom rows show results for various weighting schemes; 2:rRNA, 10:rRNA, and 100:rRNA refer to weighting schemes in which transversions are weighted 2, 10, and 100 times more than transitions, respectively. Protein weighting schemes are Gonnet weighting matrix, Whelan and Goldman (WAG) matrix, Le and Gascuel (LG) matrix, and genetic identity (GI). For details on weighting matrices, see Figure S4.

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Table 1. Comparison of Competing Phylogenetic Hypotheses

Phylogenetic Hypothesis	Tree Length (Steps)	Homoplasy Index	Log-Likelihood	SH Test	ELW
ML tree	49,076	0.3579	−261429.821426	Best	0.576167
Bayesian tree	49,103	0.3582	−261441.636024	NS	0.384596
Bilateria sister to Cnidaria	49,175	0.3591	−261620.290035	Significant	—
Bilateria sister to Porifera	49,193	0.3594	−261633.754060	Significant	—
Trichoplax sister to Cnidaria	49,134	0.3586	−261503.704225	Significant	—
Trichoplax within Porifera	49,129	0.3585	−261480.357306	NS	0.015007
Trichoplax within Cnidaria	49,196	0.3594	−261624.775575	Significant	—
Ctenophora basal	49,117	0.3584	−261473.944734	NS	0.024230

Tree length and homoplasy index are maximum parsimony measures, whereas log-likelihood, Shimodaira-Hasegawa (SH) test, and expected likelihood weights (ELW) are based on a likelihood framework. The 95% confidence tree set includes the ML and Bayesian trees with cumulative ELW of 0.960763 and was assessed with 100 bootstrap replicates.
NS, not significantly worse than the best topology; significant, $p < 0.05$.
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the mitochondrial genome data analyses (as indicated by arrow f in Figure 1; [27,28]). It is therefore important to examine whether the mitochondrial signal swamps out the nuclear data, to rule out the possibility that the topology we present in Figure 2 is biased by mitochondrial information. Figure S1 addresses this problem and demonstrates that nuclear information contributes positive support to 16 of the 21 nodes in the tree. Mitochondrial information contributes positive support to only 15 out of 21 nodes. In addition, examination of the amount of hidden support contributed by nuclear versus mitochondrial data (not shown) shows that the majority of the hidden support comes from nuclear information. Both of these results using partitioned support measures indicate that the addition of nuclear data does not conflict with mitochondrial information and is indeed contributing positively to the overall phylogenetic hypotheses

Resurrecting the "Placula"

Although the hypothesis in Figure 2 is in conflict with a recent analysis of coding genes from whole genomes [23] as well as is in conflict with other studies (Table S1), the scenario presented here is consistent with another set of studies and also with one of the major urmetazoon hypotheses, the placula hypothesis (Figure 4). This hypothesis fuels intriguing scenarios for the mechanisms and direction of anagenetic evolution in Metazoa, and in the form presented here, it can illustrate the derivation of Cnidaria and Bilateria from a placozoan-like ancestor. A basal position of Placozoa relative to Cnidaria, and diploblasts sister to Bilateria are *cum grano salis* consistent with several recent molecular phylogenetic analyses ([23,27] and this study) encouraging us to reconsider the placula hypothesis in a modern light.

The comparison of Hox/ParaHox-like gene expression patterns in Placozoa and Cnidaria creates a new working hypothesis for the origin of the entoderm, a main body axis, and symmetry. Based on the undisputed evidence that Placozoa are basal relative at least to Cnidaria, the *Trox-2* gene is likely ancestral to Hox/ParaHox-like genes from Cnidaria (as formerly suggested [44,45]). *Trox-2* is expressed at the gastrodermis/epidermis (lower/upper epithelium) boundary in *Trichoplax* [46]. Strikingly, we found similar expression patterns for two putative *Trox-2* descendents in the hydrozoan *Eleutheria dichotoma* (Figure 4). These regulatory gene expression data mirror directly the beginning and ending stage of a modern interpretation of the placula hypothesis. The latter explains the origin of a symmetric bauplan with one or two defined body axes and an internal feeding cavity from a simple placuloid (proto-placozoan–like) bauplan that lacked all of the former characteristics. In the most parsimonious scenario, the expression of a single regulatory gene defines polarity in Placozoa, i.e., the differentiation of a lower versus upper epithelium. According to the proposed "new placula hypothesis," the nonsymmetric placozoan bauplan transforms into a symmetric Cnidaria (or also Bilateria) bauplan by the former ring of epithelia boundary separation transforming into the new "oral" region of the derived symmetric bauplan (Figure 4). This transformation is simply the result of a placula lifting up its feeding epithelium in order to form an external feeding cavity, keeping function and morphology of the epithelium unchanged. In the final stage, the "oral" pole develops specialized organs, such as a mouth and tentacles for feeding (cf. [47]). The latter could be driven by duplication of the regulatory gene, which originally defined polarity in the placula (Figure 4; cf. [48] for review). Observations on extant Placozoa and Cnidaria mirror this scenario almost perfectly (Figure 4).

Although prediction and observation match nicely, one has to note, however, that no gene or even gene family, no matter how important, can provide more than just indirect support for a working hypothesis on a hypothetical animal bauplan that can never be observed. It is important to note that multiple topologies can be consistent with the placula hypothesis and that the form of the extant earliest-branching lineage does not necessarily have to represent the form of the ancestor; we consider the latter, however, the more parsimonious alternative. We also point out that the regulatory gene family mentioned here, Hox/ParaHox-like genes, seems to be absent in sponges [49]. A secondary loss of Hox/ParaHox-like genes in sponges seems plausible, and the work by Peterson and Sperling, 2007 [50] provides some evidence for this assumption. Whether a possible loss of a Hox/ParaHox gene might be related to the reduction of epithelial organization in Porifera [3] remains an interesting speculation.

The Hox/ParaHox loss scenario in sponges is just one of several crucial questions raised by the phylogeny in Figure 2. According to this phylogeny, diploblasts and Bilateria both may have started from a placula-like bauplan as suggested in Figure 4 ("new placula hypothesis"). The shown new placula

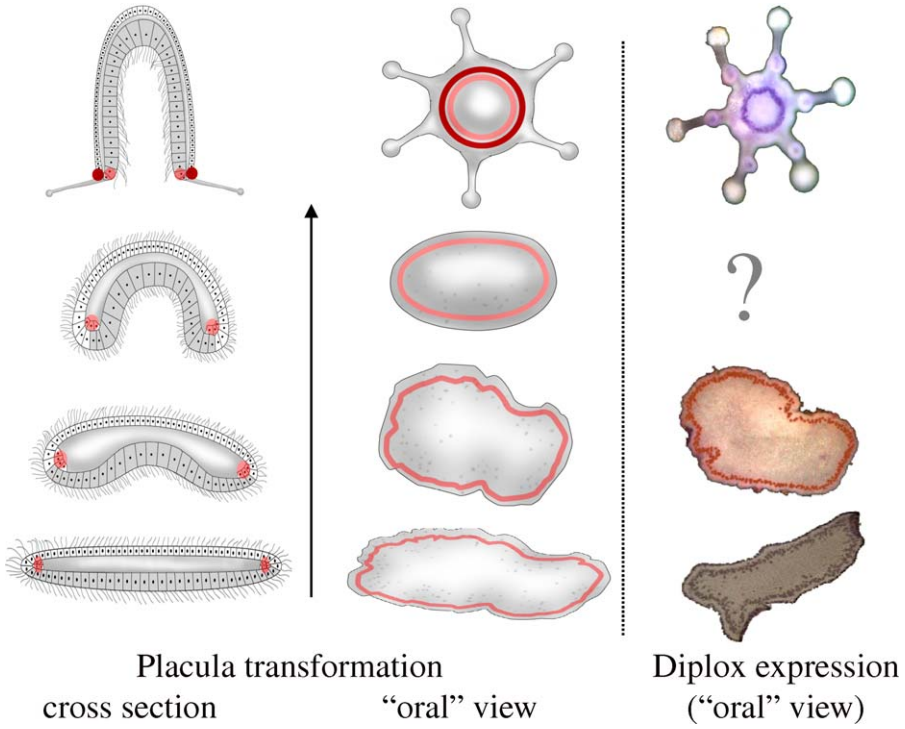

Figure 4. Modern Interpretation and Modification of the Placula Hypothesis of Metazoan Origin

Here, a nonsymmetric and axis-lacking bauplan (placula) transforms into a typical symmetric metazoan bauplan with a defined oral–aboral or anterior–posterior body axis. In the placula transformation, a primitive disk consisting of an upper and a lower epithelium (lower row), which can be derived from a flattened multicellular protist, forms an external feeding cavity between its lower epithelium and the substrate (second row from bottom). The latter is achieved by the placula lifting up the center of its body, as this is naturally seen in feeding *Trichoplax* (i.e., the two *Trichoplax* images derive from a nonfeeding (first row) and feeding (second row) individual. If this process is continued, the external feeding cavity increases (cross section, third row) while at the same time the outer body shape changes from irregular to more circular (see oral views). Eventually, the process results in a bauplan in which the formerly upper epithelium of the placula remains outside (and forms the ectoderm) and the formerly lower epithelium becomes "inside" (and forms the entoderm; upper row). This is the basic bauplan of Cnidaria and Porifera. Three of the four transformation stages have living counterparts in the form of resting *Trichoplax*, feeding *Trichoplax*, and cnidarian polyps and medusae (right column).

The above-outlined transformation of a placula into a cnidarian bauplan involves the development of a main body axis and a head region, which allows the invention of new structures and organs for feeding. From a developmental genetics point of view, a single regulatory gene would be required to control separation between the lower and upper epithelium (three lower rows). If the above scenario were correct, the following empirical data would be congruent with it. In the form of the putative ProtoHox/ParaHox gene, *Trox-2*, in *Trichoplax*, we find a single regulatory gene, marks the differentiation of an as yet undescribed cell type at the lower–upper epithelium boundary in *Trichoplax* [46]. More than one regulatory gene would be required to organize new head structures originating from the ectoderm–entoderm boundary of the oral pole (upper row). Quite noteworthy, two putative descendents of the *Trox-2* gene, *Cnox-1* and *Cnox-3*, show these hypothesized expression patterns (Diplox expression upper row; for simplicity, only the ring for *Cnox-1* expression is shown; see Figure S4 for expression patterns of both genes and Jakob et al. [46,52] for details. *Cnox-1* and *Cnox-3* expression both mark the ectoderm-entoderm boundary at the oral pole in the hydrozoan *Eleutheria dichotoma*. Both genes are expressed in parallel in a ring-shaped manner at the tip of the manubrium, with *Cnox-3* being expressed more ectodermally and *Cnox-1* being expressed more entodermally (unpublished data).

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hypothesis illustrates a potential transition from a non-symmetric, axis-lacking placula into a radial symmetric and head–foot axis organized cnidarian. In a similar way, the placula could also be transformed into a Bilateria bauplan, i.e., a bilaterally symmetric bauplan with an anterior–posterior body axis. One of the easiest models for adopting a bilateral symmetry suggests that the "urbilaterian" kept the benthic lifestyle of the placula but adopted directional movement. The latter almost automatically leads to an anterior–posterior and ventral–dorsal differentiation. The pole moving forward develops a head and becomes anterior, the body side facing the ground carries the mouth and thus by definition becomes ventral. According to the above scenario, the main body axes of diploblastic animals and Bilateria were independent inventions. Whereas an independent evolution of body axes in diploblastic animals and Bilateria seems easily plausible, the independent evolution of other characters (e.g., the nervous system; see below) seems less plausible given our knowledge of the development and morphology of these characters.

We will never observe the hypothetical placula, but we may draw some conclusions from Placozoa, which seem to have retained many of the characteristics of the placula if our interpretation is valid. This scenario draws into question several aspects of animal evolution that will require reinterpretation if this hypothesis is correct. Most notable of these aspects is the evolution of the nervous system, which in the hypothesis in Figure 2, can only be explained by convergent evolution of Cnidaria and Bilateria nervous system organization. According to the placula hypothesis, we suggest that the placula already had the genetic capability and basic building blocks to build a nervous system, and that from here, the final build-up of the nervous system developed via independent, but parallel, pathways in diploblasts and Bilateria. The genome of the placozoan *Trichoplax adhaerens* indeed delivers some notable evidence that the genetic

inventory may precede morphological manifestation of organs [23]. For example, the placozoan genome harbors representatives of all major genes that are involved in neurogenesis in higher animals, whereas placozoans show not the slightest morphological hint of nerve or sensory cells. Quite noteworthy, however, is that placozoans are quite capable of stimuli reception and perception used to coordinate behavioral responses. In this light, the generally accepted unlikely convergent evolution of a nervous system only looks unlikely from a morphological, but not from a genetic and physiological, point of view.

Regardless of the need for reinterpretation of this and other anatomical characters, the findings presented here provide a viable hypothesis for the major cladogenetic events during the metazoan radiation. Given the basal position of Placozoa, we suggest that at least for diploblastic metazoan life, the body plan started with the following: an asymmetric body plan, a most simple morphology (only two steps above basic definition [51]), a single ProtoHox gene, a large mitochondrial (mtDNA) genome, an outer feeding epithelium that gave rise to the entoderm, and the smallest of all known (not secondarily reduced) metazoan genomes. If the placula is also the ancestral state for metazoans (i.e., the common ancestor of Bilateria and diploblasts in Figure 2), then the same could be said for the urmetazoon.

Materials and Methods

Cloning and sequencing of target genes. In order to extend the analyses of Rokas et al. [42] to basal metazoans also, we isolated 13 of the suggested target genes that were missing from the placozoan *Trichoplax adhaerens*. These genes could be amplified by using the primer sets that had worked in the previous study in sponges: TOA04, 05, 06, 09, 10, 11, 13, 15, 16, 17, 21, 25, 33, 48, 53, 56, 57, 59, 62, 65, 67, and 68. In order to obtain sequences of these genes for Placozoa and to characterize variation within Placozoa, we also isolated six of these genes from a second, distantly related placozoan species (Placozoa sp. H2, TunB clone, Tunisia). For cubozoans, we filled gaps in the matrix by isolating three target genes from *Carybdea marsupialis* (Table S5). We amplified target genes from cDNA. For both placozoan species, some 200 healthy growing vegetative animals of each species were used for the isolation of total RNA. Before extraction, animals were washed three times with sterile 3.5% artificial seawater (ASW) and starved overnight to prevent algae contamination. Animals were lysed in 500 µl of fresh homogenization buffer (HOM: 50 mM Tris HCl, 10 mM EDTA, 100 mM NaCl, 2.5 mM DTT, 0.5% SDS, 0.1% DEPC in ultrapure water [Gibco]; pH 8.0). After addition of 25 µg of DEPC-treated Proteinase K, samples were stored for 30 min at 65 °C. The homogenate was squeezed through a needle connected to a 2.5-ml syringe. This protocol significantly increased RNA yield compared to conventional RNA extraction kits. Nucleic acids were isolated by two rounds of phenol/chloroform/isoamyl alcohol (25:24:1) purification. Nucleic acids were dissolved in ultrapure water, and DNA was digested with DNase I (Fermentas). Total RNA was used for cDNA transcription with poly-T primers following the manufacturer's protocol (Invitrogen Superscript II Kit).

Target genes were amplified after initial denaturation (3 min at 94 °C) by 40 rounds of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 75 s, followed by a final elongation step (5 min at 72 °C) using the Bioline Taq system following the manufacturer's recommendations (Bioline). Amplified fragments of the predicted size were purified and cloned into pGEM-T (Promega). Sequencing was performed on a Megabase 500 using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham) or by using the service provided by Macrogen. For further details, see Jakob et al. [46] and Table S5.

For a detailed explanation of the inclusion of sequences in the phylogenetic matrices used in this study, see Table S2, which shows the source of sequences in this study. We constructed two matrices, a small one composed of 24 taxa (see Figure 2) and a large one composed of 73 taxa. For the smaller matrix, we chose nine bilaterian taxa based on the availability of sequence information for a species. We chose three Lophotrochozoa, three Ecdysozoa, and three Deuterostomia as representatives of the Bilateria. Other ingroup taxa include representatives of the four classes of Cnidaria, the three major groups of Porifera (Desmospongiae, Calcarea, and Hexactinellida), Placozoa, and Ctenophora. Since rooting of the tree is critical, we attempted to break up the root by including several outgroup species: two fungal species (*Saccharomyces* and *Cryptococcus*), *Tetrahymena*, *Trypanosoma*, and *Dictyostelium* based on their relevance to the study and the availability of genome-level information. *Trypanosoma* was used as outgroup species in all aspects of the study, but the topology of resultant trees indicates that slime mold or *Tetrahymena* could also be used. To increase the number of placozoan and cubozoan sequences, we PCR amplified several genes as indicated in Table S5. Morphological characters were scored for the taxa in this study as described in Schierwater and DeSalle (2007 [10]; see Table S3). Molecular "morphology" characters were also included for the taxa in this study as scored by Ender and Schierwater, 2003 [8] (see Figure S3). The final partitioned matrices for the smaller (24 taxa) and the larger (73 taxa) can be found in Table S4. In addition to genes already available from whole mitochondrial sequencing (15 genes) and nuclear genes (16 genes), we included 18 genes from the Dunn et al. (2008) study [25]. These genes were chosen on the basis of taxonomic representation being over 50% in the Dunn et al. (2008) study.

For the larger 73-taxon matrix, we included all of the taxa from the Dunn et al. (2008) study (their smaller matrix in their Figure 2; [25]) plus Cubozoa, Scyphozoa, Placozoa, Hexactinellida, Calcarea, *Caenorhabditis*, *Tetrahymena*, *Trypanosoma*, and *Dictyostelium*. For this larger matrix, we filled in character information for these taxa for the 18 Dunn et al. (2008) [25] genes from GenBank as completely as possible. We used Blast scores and existing annotations as criteria for assessing orthology for these added sequences. In this larger matrix, we used only genes from the Dunn et al. (2008) study [25] with greater than 50% taxon representation.

In situ hybridization and immunocytology. RNA in situ hybridization studies were performed as described before [46,52]. For immunocytology studies, polyclonal antibodies were produced to oligopeptides near the C-terminal of the *Trox-2*, *Cnox-1*, and *Cnox-3* proteins. For whole-mount analysis, live animals were fixed for 1 h in 5% formaldehyde in sterile seawater. Immunocytochemistry was performed with anti-*Trox* or anti-*Cnox*, respectively, antisera and goat anti-rabbit-AP (Novagen) or FITC-conjugated goat anti-rabbit antibody (Sigma). Localization of antibody complexes was revealed by staining with NBT and X-phosphate (Roche) or fluorescent microscopy, respectively. Further details will be described elsewhere (S. Sagasser et al. unpublished data).

Alignment. To generate static alignments, we used MAFFT [53], initially with a gap opening penalty of 1.5 and gap extension penalty of 0.123. We also examined the impact of varying gap opening penalties by obtaining alignments using opening penalties of 1.0, 0.5, and 0.1. The alteration of gap penalty only served to alter the number of characters in our matrices and did not severely impact phylogenetic hypotheses.

Phylogenetic analysis. For our 24-taxon matrix, we conducted parsimony, Bayesian, and likelihood analyses as explained below. The 73-taxon matrix was analyzed with Bayesian inference. Phylogenetic trees using static alignment were generated using PAUP v4b10 [54]. Tree searches were accomplished using 1,000 random taxon additions and Tree Bisection Reconnection (TBR). Jackknife measures for node support were obtained using PAUP with 30% character removal and 1,000 repetitions. To examine the effect of character weighting in phylogenetic analysis of this dataset, we implemented character weighting for nucleic acids and amino acid partitions as follows. First, we implemented three schemes for weighting transitions and transversions (100, 10, and 2) for nucleic acids. Second, we used four transformation matrices for amino acid weighting: Gonnet [55], WAG [56], LG [57], and Genetic Identity (GI). Bremer support measures (decay indices) [58], partitioned Bremer and hidden support values [59,60] were generated using TreeRot v3 [61]. The parallel implementation of MrBayes v3.1.2 [62,63] was used for Bayesian inference of phylogeny. Two simultaneous runs with random starting trees were launched for two million generations, each with a 1,000-step thinning, a 10% burn-in, and a temperature parameter of 0.2 so as to lead to better mixing. All three data types (DNA, protein, and morphology) were accommodated in the Bayesian analysis. We employed ML inference in RAxML v7.0.4 [64] using the GTR substitution model for DNA [65,66] along with G-distributed rate heterogeneity [67,68] and the Whelan and Goldman (WAG) amino acid substitution matrix [55] with empirical residue frequencies coupled with G-distributed rate heterogeneity. Node support was evaluated with 1,000 rapid bootstrap replicates [69]. Alternative

phylogenetic hypotheses were compared using the Shimodaira-Hasegawa test [37] and expected likelihood weights [70], as implemented in RAxML.

Supporting Information

Figure S1. Positive or Negative Partitioned Bremer Support for All Nodes under Mitochondrial versus Nuclear Gene Partitions

The shown analysis was done for one of the "plausible" parsimony trees. Other topologies preferred by parsimony analysis gave similar inferences about support. The figure shows whether the partitioned Bremer support values are positive negative or neutral. This figure demonstrates that the nuclear versus mitochondrial partitions all provide similar degrees of support for the various nodes in the tree. Note that over half of the nodes acquire positive support from both partitions (11/21). Most of the negative support in the tree is within the diploblast clade (six out of eight nodes) indicating the instability of the relationships in this clade. Note also that the majority of the negative support comes from mitochondrial partitions further strengthening our contention that the mitochondrial partitions are NOT swamping the nuclear partitions. Nodes at the base of the tree exhibit consistent support from all sources under the shown partitioning scheme. Quite strikingly, nuclear proteins seem to provide the highest positive support of all the characters in the analysis.

Found at doi:10.1371/journal.pbio.1000020.sg001 (70 KB PPT).

Figure S2. Phylogenetic Tree for 73 Taxa Matrix with Bilateria Shown as Major Groups (A) and Including All Taxonomic Names (B)

The 73 taxa are comprised of the 64 taxa from the Dunn et al. (2008) study [25] plus nine taxa added from the present study. Since the topologies within Lophotrochozoa, Ecdysozoa, and Deuterostomia are not discussed in our study, we have represented these as major monophyletic groups in this figure (A).
All included taxa are listed in (B). The blue circles indicate that the support for these nodes are 100% jackknife support for unweighted parsimony analysis and 1.0 posterior Bayesian probability for parsmodel analysis in MrBayes. For four nodes relevant to the present study from this larger analysis, the jackknife values and Bayesian posteriors are listed next to the nodes, respectively.

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Figure S3. 16S rRNA Secondary Structure Prediction

Found at doi:10.1371/journal.pbio.1000020.sg003 (126 KB PPT).

Figure S4. In Situ Expression of Hox-Like Genes *Cnox-1* and *Cnox-3* in the Hydrozoan *Eleutheria dichotoma*

The two Hox-like genes, *Cnox-1* and *Cnox-3*, display differential spatiotemporal expression patterns in the medusa stage. *Cnox-1* (A_1–A_4) is expressed ectodermally in the so-called Nesselring, an area of undifferentiated cells lining the ring canal of medusae (cross section: A_3, A_4). *Cnox-3* expression marks the most ectodermal oral part of the manubrium (B_1, B_2). Staining is with NBT/X-phosphate (A_1, B_1) and fluorescein-labeled probes (A_2, B_2); the scale bar indicates 50 μm. Pictures are reprinted from Jakob and Schierwater (2007) [52].

Found at doi:10.1371/journal.pbio.1000020.sg004 (2.17 MB PPT).

Table S1. Survey of the Literature for Hypotheses Concerning the Major Animal Lineages Discussed in This Paper

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Table S2. GenBank Accession Numbers Used in This Study

Found at doi:10.1371/journal.pbio.1000020.st002 (47 KB XLS).

Table S3. Morphology Data Matrix

Found at doi:10.1371/journal.pbio.1000020.st003 (24 KB DOC).

Table S4. Alignment Matrix for 24 Taxa and 73 Taxa (in Nexus Format)

Found at doi:10.1371/journal.pbio.1000020.st004 (1.70 MB TXT).

Table S5. Disposition of PCR and Sequencing of Placozoan and Cubozoan Genes

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