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Molecular Phylogeny Inferred from Sequences of Small Subunit Ribosomal DNA, Supports the Monophyly of the Metazoa

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ABSTRACT—Recent studies of molecular phylogeny based upon comparisons of the partial nucleotide sequences of 18 and 28S rRNAs have suggested that the metazoa are polyphyletic, i.e., that diploblasts (poriferans, cnidarians, ctenophores, and placozoans) and triploblasts form two separated monophyletic units. In order to examine this hypothesis, we determined almost the complete sequences of small subunit (18S-like) rDNA for two poriferans and a ctenophore. Phylogenetic comparisons of the sequences, together with those of a cnidarian, triploblasts and other eukaryotes, supported the monophyly of the metazoa. Among the diploblasts, the ctenophore showed some similarities to the poriferans.

INTRODUCTION

On the basis of extensive studies of comparative anatomy, embryology, and paleontology, multicellular animals (metazoans) have been divided into three major taxonomic units, namely; animals with three embryonic layers and bilateral symmetry (triploblasts), those with two embryonic layers and radial symmetry (diploblasts) except for bilateral symmetry of anthozoans, and those with extremely loose tissue differentiation (poriferans), the latter sometimes included within diploblasts [cf. 1, 2, 28]. The origin and evolution of these multicellular animals have received considerable attention over the years [1, 2, 8–10, 15, 21, 25, 28]. It is generally accepted that the metazoa arose from protozoa, perhaps 700–1000 million years ago [3]. Models of the metazoan origin suggest that either colonial flagellates [9, 15] or syncytial ciliates [8, 10] became acoel flatworm-like creatures (phylum Platyhelminthes), from which various modern metazoans have diverged. Current debate concerns whether the metazoa are monophyletic or polyphyletic and what the ancestral metazoa were like. Some investigators have insisted that poriferans arose independently from colonial flagellates, whereas other metazoans evolved from syncytial ciliates [8, 25].

Advances in molecular biology have provided some answers to the problems posed by evolutionary biologists. Comparisons based on molecular data, such as the amino acid sequences of certain proteins and the nucleotide sequences of certain RNAs and DNAs, provide powerful tools with which the phylogenetic relationships among animal groups are deducible more objectively than others [13, 20]. In particular, the small ribosomal subunit (16 or 18S) RNA or its gene (16 or 18S rDNA) is ideally suited for phylogenetic studies of distantly related organisms, because it is rich in information and the sequencing methodology permits the rapid accumulation of large databases [5, 19]. A recent study of the molecular phylogeny of

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metazoans based upon comparisons of sequences of 18S rRNAs has suggested that the metazoa are polyphyletic; Cnidarians (diploblasts) arose from a protist ancestry different from the Bilateria (triploblasts) [7]. In addition, analysis of the origin of metazoans, using comparisons of sequences of 28S rRNAs, has demonstrated that triploblasts and diploblasts form two clearly separated units [4]. However, the suggestion of polyphyletic origin of metazoans from the above-mentioned studies [4, 7], was based upon comparisons of partial nucleotide sequences; about 840 bases of 18 and about 290 bases of 28S rRNAs, respectively.

Very recently, Wainright *et al.* [27] reported the evolutionary origin and early branching pattern of the animal kingdom. They inferred the phylogenetic framework by comparing small subunit ribosomal RNA sequences for two poriferans, a cnidarian, a ctenophore, and a placozoan. The authors reported that the animal lineage is monophyletic and that animals and fungi share a common evolutionary history. We also examined the phylogenetic position of diploblastic animals (two poriferans and a ctenophore). The species we examined however differed from those studied by Wainright *et al.* [27].

MATERIALS AND METHODS

Animals

Diploblasts examined in the present study were two poriferans *Sycon calcaravis* Hozawa (class Calcarea, order Sycettida) and *Tetilla japonica* Lampe (class Demospongiae, subclass Tetractinomorpha, order Spirophorida), and a ctenophore *Beroe cucumis* Fabricius (class Atentaculata, order Beroidea). The poriferans were collected in the vicinity of the Tateyama Marine Laboratory, Ochanomizu University, and the ctenophore in Kagoshima Bay. Eggs of *T. japonica* and whole animals of *S. calcaravis* and *B. cucumis* were frozen quickly in liquid nitrogen and kept at −80°C until use.

Isolation of DNA

High-molecular-weight genomic DNA was extracted from the frozen samples by the method described previously [26]. In brief, frozen and powdered samples were lysed in TE buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 8.0) that contained 0.5% sodium dodecyl sulfate. After digesting samples with proteinase K (100 μg/ml) at 50°C for 3 hr, DNA was extracted with phenol, and precipitated in ethanol and an equal volume of 5.0 M ammonium acetate. Samples resuspended in TE buffer were further purified by RNase A digestion (20 μg/ml) at 37°C for 1 hr followed by ethanol precipitation.

Amplification of small-subunit rDNA and sequencing of the amplified DNA

Almost the entire length of the 18S rDNA was amplified by the polymerase chain reaction (PCR) [22] in a thermal cycler (Perkin-Elmer Corp., Norwalk, CT, USA) using primers 0 (5′CTGGTTGATCCTGCCAG3′) and 10 [5′CACCTACGGA(AT)ACCTTG3′]. These primers were designed referring to conserved regions of aligned eukaryote 18S rDNA sequences, with which 18S rDNA of deuterostomes could be amplified. Amplification proceeded in 50 μl of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, with 0.2 mM each dNTP, 50 pmol primers, template DNA (5–50 μg) and 1 U *Taq* DNA polymerase (Toyobo Co. Ltd., Osaka, Japan). One of the primers was kinased prior to PCR at the 5′ terminal phosphate. The temperature regimen for 30 cycles was 1 min at 94°C, 2 min at 42°C, and 3 min at 72°C.

Amplified DNA was purified by electrophoresis in a 0.8% agarose gel and treated with lambda exonuclease (BRL, Bethesda, MD, USA) to obtain single-stranded DNA [11]. With the single-stranded DNA as the template, the nucleotide sequence was directly determined by dideoxy chain-termination [24] using Sequenase ver 2.0 (United States Biochemical Corp., Cleveland, OH, USA) and [^{35}S]-dATP (Amersham Japan, Tokyo, Japan). In addition to primers 0 and 10, primers 8 (5′CCGGAGAGGGAGCCTGA3′), 7 (antisense analog of primer 8), 1 (5′CAGCAGCCGCGGTAATT3′), 9 (antisense analog of primer 1), 3 (5′GCGAAAGCATTTGCCAA3′), 4 (antisense analog of primer 3), 5 [5′GAAACT(TC)AAAGGAAT3′], 6 (antisense analog of primer

5), and 2 [5′ACGGGCGGTGTGT(AG)C3′] were used for sequencing. As shown previously, sequences of 18S rDNA can be determined more accurately than those of 18S rRNA [26].

Comparison of sequences and inferences regarding phylogeny

Sequences were aligned on the basis of maximum nucleotide similarity. Using the aligned sequences, we calculated the evolutionary distance values in a pair-wise manner, as described by Jukes and Cantor [16]. The phylogenetic tree was constructed from an analysis of results by the neighbor-joining method of Saitou and Nei [23]. The degree of support for internal branches of the tree was further assessed by bootstrapping [6].

RESULTS

Almost the entire length of the 18S rDNA of two species of poriferan and a species of ctenophore was amplified by PCR, and the complete nucleotide (more than 1600 nucleotide) sequences of the amplified products of PCR, except for the 5′ and 3′ termini, were determined directly without cloning. The nucleotide sequence data reported in this study will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Database under the following accession number; D15066 for *Sycon calcaravis,* D15067 for *Tetilla japonica,* and D15068 for *Beroe cucumis.*

The nucleotide sequences of the three diploblasts were aligned with those of *Anemonia sulcata, Artemia salina, Homo sapiens* and other eukaryotes [5, 19] on the basis of maximum nucleotide similarity; the alignment of 1269 nucleotides was analysed. Figure 1 shows a phylogenetic tree of a variety of organisms, constructed by neighbor-joining [23]. The tree clearly indicated that the metazoans form an independent, monophyletic unit. In other words, the four species of diploblasts and two species of triploblasts formed a discrete unit of monophyletic origin. This grouping of the metazoa was supported by the high value obtained by bootstrapping (73.8%) [6]. The tree also suggested that animals and fungi share an evolutionary history; the branching of the two groups from the others was supported by the high bootstrapping value (83%).

Because the monophyly of the metazoans was demonstrated in Fig. 1, we further analyzed the phylogenetic relationships between diploblasts and triploblasts by adding data from the flatworm *Schistosoma mansoli* [5] and by taking the yeast *Saccharomyces cerevisiae* as an outgroup. About 60 confidently aligned sites were added in this analysis. These sites were relatively variable and contained information useful to increase the precision of analysis. The structural similarity and evolutionary distance values of these organisms are shown in Table 1. The phylogenetic tree constructed using the distance values, is shown in Fig. 2. The tree suggested that the two poriferan and a ctenophore species form one unit, while the cnidarian species and triploblasts form another. The branching of the former group was supported by the relatively higher value of bootstrapping (62%).

DISCUSSION

The present molecular phylogenetic study based upon comparisons of virtually complete nucleotide sequences of 18S rDNAs showed that the metazoa are of monophyletic origin. Although this study did not include placozoa, the grouping of the metazoans as a discrete taxonomic unit was supported by the high value obtained by bootstrapping. This result corroborates the results of Wainright *et al.* [27] but not the previous suggestion that the metazoa are polyphyletic. A study of the molecular phylogeny of the animal kingdom pioneered by Field *et al.* [7] suggested that cnidarians arise from a protist ancestry different from the bilaterians. However, Lake [18] has studied the animal phylogeny by another systematic method, using the sequence data reported by Field *et al.,* and he concluded that the metazoa are a monophyletic taxon. An analysis by Christen *et al.* [4] suggested that triploblasts and diploblasts form two clearly separated monophyletic units. However, their main conclusion was that the origin of triploblasts is much more ancient, with respect to diploblasts, than was classically assumed.

The evolutionary pathway from unicellular organisms to multicellular animals has been a

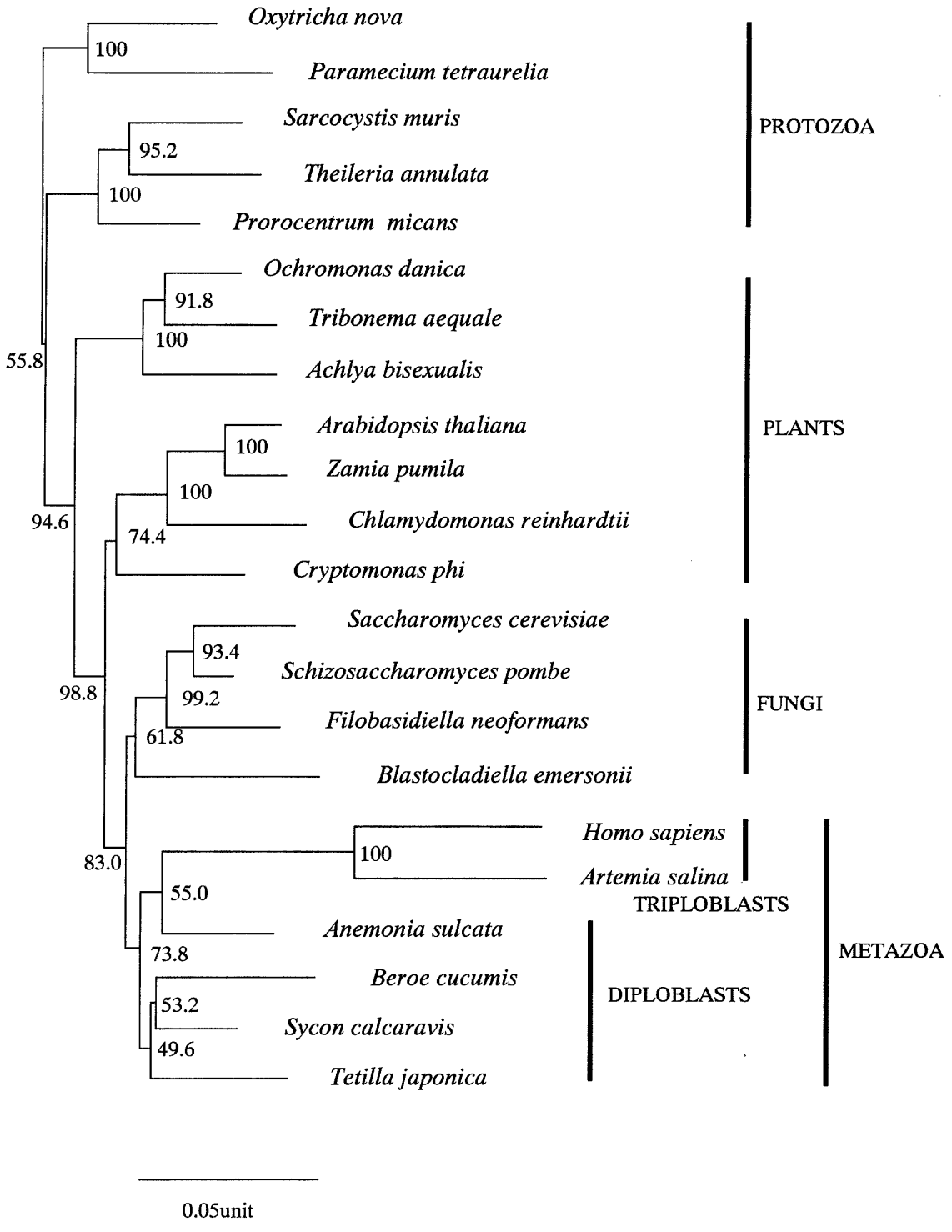

FIG. 1. General phylogenetic tree of eukaryotes, as deduced by neighbor-joining using the fraction of observed substitution differences of confidently aligned sites. The scale bar indicates the evolutionary distance of 0.05 nucleotide substitutions per sequence position. Numbers at each branch indicate the percentage of times a node was supported in 500 bootstrap pseudoreplications by neighbor-joining. The tree demonstrated the monophyletic origin of metazoans, including both diploblasts and triploblasts.

This is an unrooted tree, because we could not include any outgroup organisms. Therefore, we cannot determine where the root of this tree is and what the ancestor of the organisms is.

TABLE 1. Structural similarity and evolutionary distance data for 18S rDNA sequences of metazoans and yeast

Species	*Sy.ca.*	*Te.ja.*	*Be.cu.*	*An.su.*	*Sc.ma.*	*Ar.sa.*	*Ho.sa.*	*Sa.ce.*
Sycon calcaravis		0.0675	0.0749	0.0741	0.1722	0.1527	0.1554	0.0917
Tetilla japonica	86		0.0900	0.0858	0.1828	0.16865	0.1610	0.0951
Beroe cucumis	95	113		0.0875	0.1894	0.1789	0.1875	0.1071
Anemonia sulcata	94	108	110		0.1713	0.1564	0.1564	0.0917
Schistoma mansoni	205	215	223	204		0.1408	0.1345	0.1846
Artemia salina	184	201	212	188	171		0.1203	0.1808
Homo sapiens	187	193	221	188	164	148		0.1837
Saccharomyces cerevisiae	115	119	133	155	218	214	217	

The lower-left half of the table gives the number of substitutions in which gaps are not included. The upper-right half of the table gives the evolutionary distance values (average number of nucleotide substitutions per sequence position) determined by the Jukes and Cantor (1969) [16] formula. The sequence data for *A. sulucata, S. mansoni, A. salina, H. sapiens* and *S. cerevisiae* were obtained from references 5 and 19.

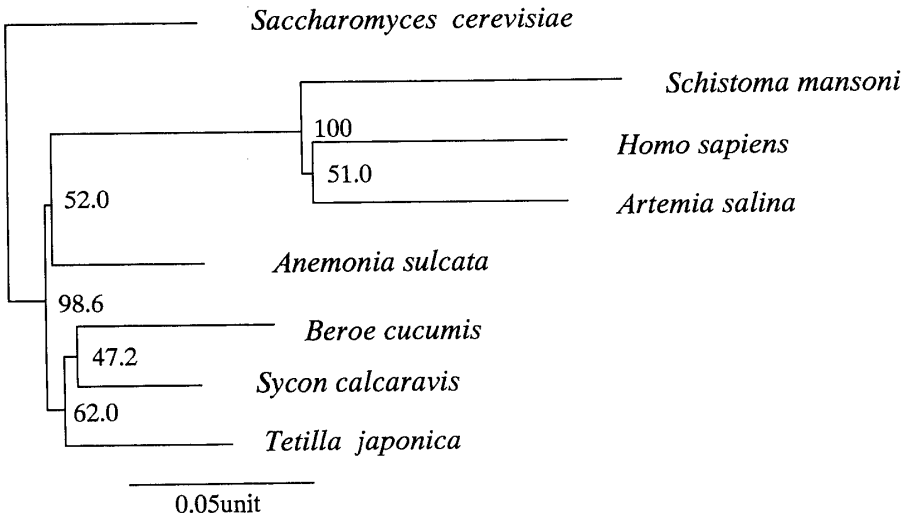

FIG. 2. Phylogenetic relationships of diploblasts with triploblasts (*Schistosoma mansoni, Artemia salina,* and *Homo sapiens*) as deduced by reference to yeast as the out group. The tree was constructed by neighbor-joining. The scale bar indicates an evolutionary distance of 0.05 nucleotide substitutions per sequence position. Numbers at each branch indicate the percentage of times a node was supported in 500 bootstrap pseudoreplications by neighbor-joining. The ctenophore *Beroe* forms a unit with poriferans.

subject of extensive investigation and vigorous discussion for more than a century [1, 2, 8–10, 15, 21, 25, 28]. The emergence of metazoans has been explained by two major theories; the syncytial theory (from a multinucleated ciliate) [8, 10] and the colonial theory (from a colonial flagellate) [9, 15]. Although the present molecular data as well as those shown by Field *et al.* [4] and Christen *et al.* [7] do not allow clarification of this point, the branching among the metazoans shown in Figs. 1 and 2 implies two divergences of the metazoans; one evolved a variety of diploblasts and the other that occurred later, evolved a variety of triploblasts. These two divergences are also suggested from paleontology [3]. The former is the Vendian radiation which gave rise to Ediacaran assemblage, and the latter is the Cambrian explosion. The first Vendian radiation may have occurred soon after multicellular animals derived from other protists. The rapid explosion, together with the relatively

higher rate of substitutions observed in the sequences of 18S rDNA of triploblasts, made it difficult to elucidate the affinity between triploblasts and diploblasts. These may be the reasons why previous studies of partial 18 and 28S rRNA sequences could not show the monophyly of the metazoa.

A phylogenetic tree constructed from the 5S rRNA sequences suggested a slightly earlier emergence of platyhelminthes (*Dugesia japonica*; Tricladida) and nematodes (eg, *Caenorhabditis elegans*) than those of other metazoans, including diploblasts and other bilateralia [14]. However, that view was not supported by a recent comparison of partial 18S rDNA sequences by Katayama *et al.* [17]. Instead, Katayama *et al.* [17] suggested the early emergence of the acoel turbellarians in metazoic evolution; they might be some of the closest multicellular animals to the metazoan ancestors. Although Fig. 2 indicates a grouping of *Schistoma mansoni* (Platyhelminthes, Trematoda) and the two other triploblasts, the phylogenetic relationship between the diploblasts and the acoel turbellarians should be subject of further study based upon the comparison of almost complete sequences of 18S rDNA.

Another suggestion of the present study, was the phylogenetic position of the ctenophore. Classically ctenophores were positioned closely to cnidarians [15]. However, the presence of true mesenchymal muscle cells and gonoducts no longer supports the supposed affinity but suggests its closer relationships to platyhelminths [12]. As evident in Fig. 2, however, the present analysis suggested a close molecular similarity between the poriferans and the ctenophore. This will be examined in further studies.

In conclusion, together with a study of Wainright *et al.* [27], the present study supports the monophyly of the metazoa.

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