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A "chimera" theory on the origin of dicyemid mesozoans: evolution driven by frequent lateral gene transfer from host to parasite

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Abstract

The phylogenetic status of the enigmatic dicyemid mesozoans is still uncertain. Are they primitive multicellular organisms or degenerate triploblastic animals? Presently, the latter view is accepted. A phylogenetic analysis of 18S rDNA sequences placed dicyemids within the animal clade, and this was supported by the discovery of a Hox-type gene with a lophotrochozoan signature sequence. This molecular information suggests that dicyemid mesozoans evolved from an ancestral animal degenerately. Considering their extreme simplicity, which is probably due to parasitism, they might have come from an early embryo via a radical transformation, i.e. neoteny. Irrespective of this molecular information, dicyemid mesozoans retain many protistan-like or extremely primitive features, such as tubular mitochondrial cristae, endocytic ability from the outer surface, and the absence of collagenous tissue, while they do not share noticeable synapomorphy with animals. In addition, the 5S rRNA phylogeny suggests a somewhat closer kinship with protozoan ciliates than with animals. If we accept this clear contradiction, dicyemids should be regarded as a chimera of animals and protistans. Here, we discuss the traditional theory of extreme degeneration via parasitism, and then propose a new "chimera" theory in which dicyemid mesozoans are exposed to a continual flow of genetic information via eating host tissues from the outer surface by endocytosis. Consequently, many of their intrinsic genes have been replaced by host-derived genes through lateral gene transfer (LGT), implying that LGT is a key driving force in the evolution of dicyemid mesozoans.

Keywords: Dicyemid mesozoans; Protistans; Triploblastic animals; Lateral gene transfer; Parasitism; Chimera

1. Introduction

The dicyemid mesozoans, obligate endosymbionts found in the renal system of benthic cephalopods, are one of the simplest multicellular organisms (reviewed by Furuya and Tsuneki, 2003). They consist of one long axial cell surrounded by a single layer of 20–40 multiciliated somatic cells. The axial cell contains a large polyploid nucleus and intracellular stem cells, called axoblasts (Fig. 1). Dicyemids lack distinguishable organs, except for a gonad-like structure that appears during one stage of their life cycle. According to Nouvel (1948), in the late 18th century, Filippo Calvolini of Italy found small worm-like organisms—dicyemid mesozoans—in octopuses. In 1849, Kölliker named them dicyemids, because they produce two types of embryos in their life cycle. In 1876, Van Beneden called them Mesozoa, to express his belief that the group occupied an evolutionarily intermediate position between the Protozoa and the

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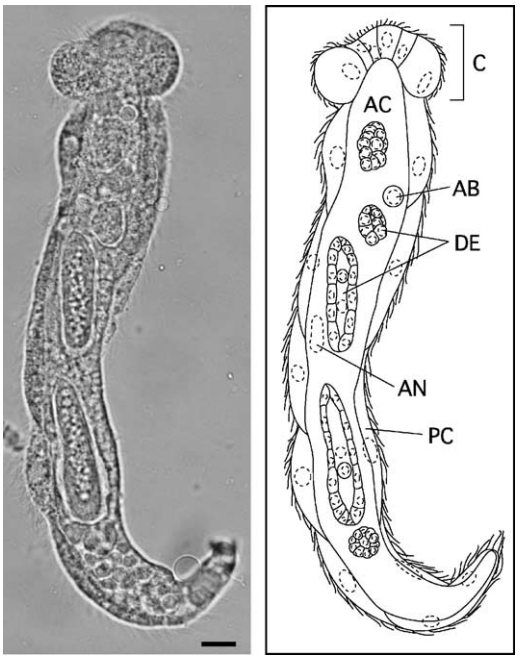

Fig. 1. Dicyemid mesozoans. Light micrograph of nematogen adult (left) and the diagram of the identical adult (right). AB, axoblast (agamete); AC, axial cell; AN, axial cell nucleus; C, calotte; DE, developing embryo; PC, peripheral cell. Bar represents 10 μm.

Metazoa. Some investigators have maintained his position (Beneden, 1882; Hartmann, 1925; Hyman, 1940; Dodson, 1956; Lapan and Morowitz, 1974). Conversely, some have proposed that mesozoans have undergone secondary simplification from a worm-like animal as a result of extreme parasitism (Nouvel, 1947; McConnaughey, 1951; Stunkard, 1954). Consequently, it has long been controversial whether the dicyemids are truly primitive multicellular organisms or secondarily degenerated metazoans. Recent molecular evidence has added to the debate between these two views, and the second view tends to be favored (Katayama et al., 1995; Kobayashi et al., 1999; Pawlowski et al., 1996). In contrast, information on biological traits shows a drastically different aspect of dicyemids. There are no definitive characters supporting a close kinship of dicyemids with animals, while many show an affiliation with protistans. This situation renders the phylogenetic position of dicyemids enigmatic. This paper highlights the contradiction between molecular information and biological traits in the phylogenetic position of dicyemid mesozoans. We propose a new theory that resolves this contradiction rationally, leading to the conclusion that dicyemid mesozoans are a chimera organism of animals and protistans.

2. Background

Molecular sequence data are increasingly used to analyze phylogenetic relationships among eukaryotes. A phylogenetic analysis of 5S rRNA data suggested that dicyemids are more closely related to protozoan ciliates than to multicellular animals (Ohama et al., 1984). Halanych (1991) argued that the 5S rRNA molecule is too small to contain phylogenetic information sufficient for appropriate reconstruction of evolutionary relationships, although his tree also indicated a close relationship between mesozoans and protozoan ciliates. By contrast, 18S rDNA analyses placed the mesozoans as triploblastic animals (Katayama et al., 1995; Pawlowski et al., 1996). However, 18S rDNA phylogenies are sometimes misleading (Loomis and Smith, 1990) and may be inadequate to elucidate relationships among groups more than 500 million years old (Rodorigo et al., 1994). In practice, rRNA- and protein-coding gene-based phylogenies can contradict each other drastically, as in the *Trypanosoma* (Alvarez et al., 1996; Germot and Philippe, 1991) and amitochondrial protozoa like microsporidia (Keeling et al., 2000) and *Entamoeba* (Hasegawa et al., 1993). The validity of molecular sequence data for deducing phylogenetic relationships depends on selecting macromolecules that are ubiquitous, have a highly conserved primary structure, and are functionally conserved during evolution (Müller, 1995).

Since the sequences used to construct phylogenies of the dicyemid mesozoans so far are RNA-coding genes, a phylogeny based on representative protein-coding genes is needed to provide more robust data. Microtubules are structures that are characteristic of eukaryotic cells; they are associated with cell movement via major cytoskeleton components, axonemes, and the $9 + 0$ basal body/centriole, suggesting that their evolution may have paralleled that of eukaryotes (Edlind et al., 1996). β-Tubulin sequences from a wide variety of eukaryotic species have been reported (Burns, 1991) and used for phylogenetic analyses (Edlind et al., 1996). In order to elucidate

the phylogenetic relationship between the dicyemids and other eukaryotes, we cloned and sequenced four β-tubulin genes from two dicyemid species as a representative protein-coding gene. In our β-tubulin phylogeny, dicyemid mesozoans were again placed within higher invertebrates, rather than near lower ones, such as platyhelminths, different from the 18S rDNA analysis (Appendix A). In both trees from 18S rDNA and β-tubulin genes, however, the exact position of the dicyemid mesozoans within animals was not supported by reliable bootstrap values because of the poor resolutions. These analyses only indicate that the dicyemid mesozoans are involved in the triploblastic animals, but not in fungi and protists. Most of the molecular information, including the presence of a Hox-type gene discussed below, strongly suggests that dicyemid mesozoans are triploblastic animals. Is this conclusion fully convincing? We suspect that there is still something wrong with it.

3. Theoretical consideration of the status of dicyemid mesozoans

3.1. Are dicyemids triploblastic animals?

Since Whitman (1883) regarded the simplicity of the mesozoans as not at all primitive, but the result of extreme parasitic degeneration, several investigators, such as Stunkard (1954), have strongly maintained this viewpoint. In the last decade, two lines of evidence suggesting that dicyemids evolved degenerately from animals have accumulated. The 18S rDNA phylogeny suggested that dicyemids were triploblastic animals (Katayama et al., 1995; Pawlowski et al., 1996). Recently, the presence of a Hox-type gene, *DoxC*, was reported in the dicyemid mesozoan *Dicyema orientale* (Kobayashi et al., 1999). The analysis of the homeodomain sequence indicated that it has the highest homology with a member of the ‘middle’ group of Hox genes, supporting the 18S rDNA phylogeny. In addition, the so-called ‘spiralian peptide’ motif was confirmed, so the authors advocated the affinity of dicyemids with lophotrochozoans, which consist of brachiopods, annelids, nemertines, platyhelminths, and mollusks including cephalopods, which are the hosts of the dicyemid mesozoans (Aguinaldo et al., 1997; Adoutte et al., 1999). The β-tubulin gene phylogeny presented here leads to a similar conclusion, although the resolution of animals was low (Appendix A). Based on this molecular information, there are grounds for classifying dicyemids as triploblastic animals. It is possible that extreme degeneration occurred to an unimaginable extent via parasitism. This interpretation requires an explanation of such extreme simplicity; dicyemid adults consist of some 30 cells, which are derived from an axoblast or fertilized egg involving at most 5–9 cell divisions. These cells never divide again during the organism’s life (Furuya et al., 1992, 1994). Considering this, one is compelled to postulate that dicyemids evolved by neoteny from an early embryo at the level of a morula. Recent studies in developmental biology have accumulated much knowledge on the body plan and many genes involved in morphogenesis have been identified (e.g. reviewed by Prince, 2002). The loss of some such genes might have been responsible for the extreme degeneration. This approach might elucidate whether the simplicity of dicyemids is really derived from an ancestral animal by parasitic degeneration. Simultaneously, it might be possible to clarify experimentally how the primitive or protistan-like traits were generated or reverted, accompanying the simplification in body construction.

3.2. Why are there so many primitive or protistan-like features?

The extremely simple dicyemid mesozoans lack a nervous system and gut. For this reason, Cavalier-Smith (1993) once placed the phylum Mesozoa in the kingdom Protozoa; this recommendation must show foresight. Now he still gives the Mesozoa the rank of a distinct subkingdom (Cavalier-Smith, 1998). We agree with his proposal, since dicyemids maintain many protistan-like features, and have radical simplification. No articles comprehensively describe the primitive or protistan-like features. Therefore, we summarize these features and discuss them in some detail.

Noting that dicyemid mesozoans had protozoan features, Hartmann (1907) coined the term Moruloidea for them. Since then, the following evidence of their primitiveness has been noted. They have (1) a double-stranded ciliary necklace, (2) tubular cristae in their mitochondria, (3) endocytic ability from the outer surface, (4) an absence of collagen in the extra-

cellular matrix (ECM), (5) cell-to-cell junctions, and (6) distinct phases of asexual (nematogen) and sexual (rhombogen) reproduction.

Freeze-fracture analysis identifies the ciliary necklace, a structural array of integral membrane proteins that has been valuable as a genetically fixed membrane character for addressing phylogenetic questions (Bardele, 1981). The pattern of protein arrangement in protists is remarkably varied, whereas invertebrates, including the Porifera and Cnidaria, have a consistent pattern. Animals are characterized by a triple-stranded necklace, while dicyemid mesozoans share a double-stranded necklace structure with protistan ciliates and opalinids (Bardele et al., 1986).

Generally, animals lack the ability to take in food or particulate materials via their outer surface. In contrast, the dicyemids can take in particulate material, such as ferritin (Ridley, 1968) or host spermatozoa (Nouvel, 1933), from the surface of their peripheral cells by phagocytosis. This characteristic is strikingly different from that of animals. If degeneration in fact occurred, the degenerated ancestor would have had to regain the ability to endocytose material from the outer cell surface, concomitant with the loss of the digestive tract. However, no embryos in animals retain the endocytic ability even in the stage of gastrula.

The shape of mitochondrial cristae is a diagnostic character for taxa, although it is not necessarily crucial; there are a few instances in which the shape of the cristae alternates within the life cycle, as in *Trypanosoma brucei* and certain platyhelminths. Animals, fungi, and plants generally have mitochondria with plate-like cristae, whereas protistans have either tubular or discoidal cristae (Gray et al., 1998). In dicyemids, the cristae are tubular, like those of most protistans, throughout their life cycle, unlike most animals (Ridley, 1968, 1969).

The synapomorphy that is considered crucial to the affiliation of mesozoans to animals is the presence of collagenous connective tissue, but not multicellularity (Willmer, 1990; Cavalier-Smith, 1993, 1998). So far, electron microscopic observation has yet to identify an extracellular matrix (ECM), such as collagen-like structures, in dicyemids (Furuya et al., 1997). Recently, the dicyemid mesozoan *Kantharella antarctica* was observed by electron microscopy using fibronectin, laminin, and type IV collagen antibodies to investigate the ECM (Czaker, 2000). All three ECM components were located intracellularly, but not intercellularly, unlike the typical ECM. Indeed, fibronectin- and laminin-like molecules have also been confirmed in protistans such as kinetoplastid *Leishmania* (Del Cacho et al., 1996) and apicomplexan *Eimeria* (Lopez-Bernad et al., 1996). These observations strongly suggest that this intracellular distribution of ECM components is primitive. The absence of ECM in dicyemids might be responsible for body organization, which does not reach the tissue level typical of animals (Furuya et al., 1997). The only similar case in animals is the turbellarian group Acoela, which lacks an intercellular matrix (Rieger, 1985). Consequently, a relationship between dicyemids and acoelomates must be considered.

With reference to this problem, cell junctions such as gap junctions (cytoplasmic connections) and adherens-like junctions have been confirmed in dicyemids, but typical septate junctions are absent (Furuya et al., 1997). The gap junction is thought to function in cell-to-cell communication and the exchange of molecules between neighboring cells. Although lower animals, such as placozoans and sponges, lack gap junctions, a similar channel system is believed to develop. Even in protistans, such junctions are observed when cell-to-cell union occurs. For example, in order to synchronize the conjugation process and ciliary movement between pairing partners, a cytoplasmic connection is formed during conjugation in ciliates in which a multicellular state is transiently established. The adherens junction has also been discovered in the multicellular structure of non-metazoan cellular slime molds, coupled with a β-catenin homologue (Grimson et al., 2000). Furthermore, it is well known that multicellularity occurred independently many times in the course of evolution, even in protistans (Willmer, 1990; Bonner, 1997). These discoveries outside the animal kingdom show that the potential for cell junction formation had already developed in protistans. Accordingly, intercellular junctions are not necessarily crucial to solve phylogenetic relationships.

Finally, dicyemids have distinct phases of asexual and sexual reproduction. In the nematogen phase, larvae develop asexually from a diploid axoblast, whereas in the rhombogen phase, larvae are produced from a fertilized egg. The former appears protistan-like, although regenerative reproduction is

observed in some animals. This feature is too different from that in triploblastic animals to imagine how dicyemids acquired the alteration of asexual and sexual reproduction.

4. The chimera theory can solve the discrepancy

As mentioned above, dicyemids maintain many protistan-like or extremely primitive features and lack noticeable morphological characters that are shared with animals; however, most of the molecular information obtained so far strongly suggests that dicyemids are true animals. How should this discrepancy be interpreted? Here, we present a new theory to resolve this discrepancy. It may be reasonable to regard dicyemids as a chimera of protistans and animals, in which dicyemids acquired many genes from their host via lateral gene transfer (LGT). Several lines of evidence have recently shown that LGT via phagocytosis occurs with higher than expected frequency (Doolittle, 1998; Schubbert et al., 1997, 1998; Bushman, 2002). For example, the *Tetrahymena* genome project (http://www.tigr.org/tdb/tgi/ttgi/) has determined that approximately 80 genes out of 3500 sequences determined so far came from bacteria, in spite of their free-living mode. Dicyemids are restricted to a renal appendage in cephalopods, where they absolutely depend on their host for all nutrients. They have endocytic ability mentioned above and the uptake of host spermatozoa has been observed repeatedly. Furthermore, the calotte (the most anterior cells) cilia are stiffer, shorter, thicker and more closely set than those of other peripheral cells, and occasionally penetrate the epithelial cells of the renal appendages, resulting in erosion of the tissue (Ridley, 1968). Therefore, they are exposed to a continual flow of genetic information from the host via their food (fragments of the host tissue and spermatozoa). This situation increased the chance of the dicyemid germline genome taking in host DNA. The observation that two β-tubulin genes from *Dicyemodeca* contained a short intron at precisely the same site as in the host gene may reflect such gene flow from the host (Appendix A). This assumption reasonably interprets the inconsistent facts; the presence of lophotrochozoan-like genes, such as Hox-type genes, and many protistan-like features. A certain laterally transferred gene from the host may have driven multicellularization of an ancestral unicellular dicyemid to some extent, and this would have led to multiciliation and polyploidization of somatic nuclei accompanied by DNA rearrangement (Noto et al., 2003), as seen in ciliates. Nevertheless, the intrinsic nature of the putative protistan ancestor might have remained unchanged, resulting in the creation of a ‘chimera.’ In this sense, dicyemids are truly the ‘Mesozoa,’ making this term even more appropriate. Frequent LGT might be an important driving force in the evolution of dicyemids in particular and in host–parasite relationships in general. This viewpoint will be indispensable for clarifying the origin of dicyemid mesozoans.

5. Perspective

To date, only a few genes in dicyemids have been analyzed. If a genome project in dicyemid were carried out, or many more genes were analyzed, we expect there to be two major classes of genes identified: animal-like genes and protistan-like genes. At present, the 5S rRNA gene is the only gene that does not show the affiliation of dicyemids to animals. If our theory is true, it may be a vestige derived from its ancestor. The discovery of additional genes of this type would lend support to our theory. Now we must await the accumulation of such information on the genes of dicyemids.

With reference to this theory, quite recently, frequent LGTs were systematically analyzed in protistan diplomonads (Andersson et al., 2003). The authors suggest that LGT is a likely source accounting for anomalous phylogeny patterns which are observed in different genes. If LGT events are assumed to be frequent in a certain species, an estimation of molecular phylogeny should be cautiously made.

Finally, the collection of completely sequenced mitochondrial genomes has been expanding rapidly (Gray et al., 1998). Generally, mitochondrial DNA (mtDNA) in animals is roughly equal in size, gene content, and genome organization; it ranges from 14 to 20 kb in size and is circular. In contrast, protistan mtDNA is very different from animal mtDNA in that it is extraordinarily diverse in size, form, and gene

content. No knowledge of dicyemid mtDNA is yet available, except for the presence of minicircle DNAs encoding cytochrome oxidase I, II, and III (Watanabe et al., 1999). These minicircles all have relatively long non-coding regions. Extrapolating the size of the entire genome based on the ratio of the known coding and non-coding sequences, dicyemids appear to have mtDNA larger than that of animals. This is inconsistent with the general tendency for parasites to downsize their mt genome as an adaptation to parasitism (Gray et al., 1999; Saccone et al., 2000). Indeed, dicyemids seem to maintain a small number of high-molecular weight mitochondrial genes in germ cells, separately from the minicircles (H. Awata, personal communication). The entire mitochondrial genome of dicyemids must be analyzed in detail to determine their phylogenetic relationship.

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Appendix A. Phylogenetic analysis of dicyemids from β-tubulin gene sequences

A.1. Characterization of β-tubulin genes from two dicyemid species

Four different β-tubulin sequences were characterized. Two β-tubulin sequences were obtained from *Dicyema* sp. (BTdv1 and ψBTdv2) and the other two sequences from *Dicyemodeca antinocephalum* (BTda1 and BTda2). Additionally, a β-tubulin gene was cloned from the host *Octopus vulgaris* (BTov1). No indels were observed except one pseudogene (ψBTdv2) described below. One intron in the two sequences from *Dicyema* sp. and four introns in the two sequences from *D. antinocephalum* were identified (Fig. 2). They are all short in length, ranging from 20 to 35 bp. The site of the first intron was identical among the four sequences. On the other hand, octopus β-tubulin gene carries one long intron the site of which coincides with the third intron of *D. antinocephalum* sequences. One gene from *Dicyema* sp. (ψBTdv2) is thought to be a pseudogene because of three nonsense mutations in the middle regions and two deletions in the 3′ region of the ORF, leading to frameshift mutation. Accordingly, the other three sequences were used for the following phylogenetic analysis. No signature sequence shared between dicyemids and animals was detected.

It was confirmed that these clones were truly derived from dicyemids by Southern blot analysis using

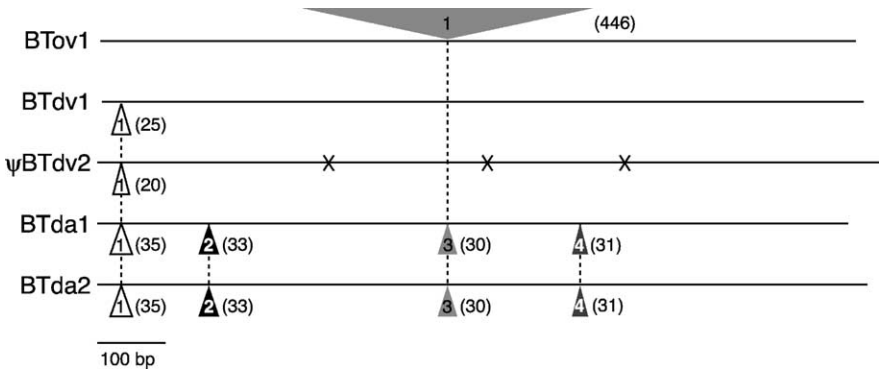

Fig. 2. Map of the β-tubulin genes from dicyemids and the host octopus. BTov1 (1635 bp) was obtained from the host *O. vulgaris*. BTdv1 (1220 bp) and ψBTdv2 (1217 bp) or BTda1 (1310 bp) and BTda2 (1327 bp) were obtained from *Dicyema* sp. or *D. antinocephalum*, respectively. Horizontal lines represent a putative protein-coding region and introns located in the identical sites are shown as triangles in the same colors. Numbers in the triangles and round brackets denote the intron number and their length in base pair, respectively. Crosses in ψBTdv2 represent nonsense mutation.

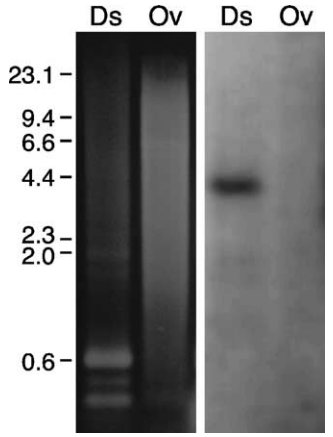

Fig. 3. Southern blot analysis. Each lane contains 1 μg of DNA from *Dicyema* sp. (Ds) or 5 μg of DNA from the host *O. vulgaris* (Ov), which was digested with *Hin*dIII. The filters were hybridized with the radiolabeled BTdv1 probe. A distinct signal is detected only on dicyemid DNA, but not on the host DNA.

one of the cloned homolog as a probe (Fig. 3). In fact, contamination of a small amount of the host tissue is usually unavoidable. However, signals were detected only on the dicyemid DNA by Southern blot analysis, but not on the host DNA. Furthermore, the primers used in this study only amplified a 1.6 kb product from the host DNA, whereas a 1.2 kb product from the dicyemid DNA (data not shown). Both results clearly indicate that the cloned sequence was derived from the dicyemid DNA. On the other hand, a small number of cells of fairly small kinetoplastids, *Bodo* sp. is usually identified in culture medium after maintenance in vitro for 1–2 weeks. The sequence from the *Bodo* sp. was amplified only after the second PCR and was placed in a protistan clade, near to kinetoplastids as expected (Fig. 4). These observations strongly suggest that there is a fairly small amount of contamination in our dicyemid DNA preparation to undetectable extent by Southern blot analysis, if any.

A.2. Phylogenetic analysis of the β-tubulin sequences

The amino acid sequences of β-tubulins from a total of 39 eukaryotes were aligned for phylogenetic analysis. As mentioned above, neither the dicyemid nor the host sequences had deletions or insertions except for one pseudogene, ψBTdv1. Unlike molecules of varying length, such as rDNA, the consistency of these sequences facilitates comparison with others. Based on 378 aligned residues, phylogenies were constructed using the Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods (Fig. 4). Because both trees are almost identical, here MP tree was demonstrated with bootstrap values by MP (left) and NJ (right). When *Trichomonas vaginalis* was used as outgroup, three major branches with high bootstrap values were generated in the MP tree: protistan-plant clade as indicated previously by Edlind et al. (1996), animals, and fungi including microsporidia as shown by Keeling et al. (2000). These large assemblages were monophyletic (bootstrap probability = 73, 97, and 93%, respectively). Fungi formed the sister-group to animals, as in the rRNA phylogeny (Hasegawa et al., 1993). The dicyemid mesozoans were positioned within animals, and outside the protistan-plant clade altogether. Their close affinity with triploblastic animals was supported by high bootstrap values, but the bootstrap confidence level for branchings within animal clade, however, was lower. The exact phylogenic relationship of dicyemids and other animals is still not clear. The phylogeny produced using the ML method was almost identical to the tree presented here although bootstrap value has not been calculated (data not shown). According to our β-tubulin phylogenies, dicyemids group with the triploblastic animals, being consistent with the relationships derived from 18S rDNA data.

Appendix B. Materials and methods

B.1. DNA preparation from dicyemids for PCR

Dicyemids were isolated from two different hosts, *O. vulgaris* and *O. dofleini* collected in Notojima island, Ishikawa prefecture, Japan (Noto et al., 2003). Three different species of dicyemids, *Dicyema japonicum*, *D. misakiense* and *D. acuticephalum*, usually inhabit *O. vulgaris*, whereas *O. dofleini* harbors only one species, *D. antinocephalum* (Furuya, 1999). After isolation and 1 week culture of the dicyemids in Jamarin seawater (JSW; Jamarin Laboratory) containing 10% fetal bovine serum (FBS; Sigma) and 7.625% (w/v) DME/F-12 HAM mixture (Sigma), 10 whole individuals of dicyemids were washed nine times in JSW using a micropipette to remove all octopus host cells, and then digested in 10 mM Tris–HCl, 10 mM

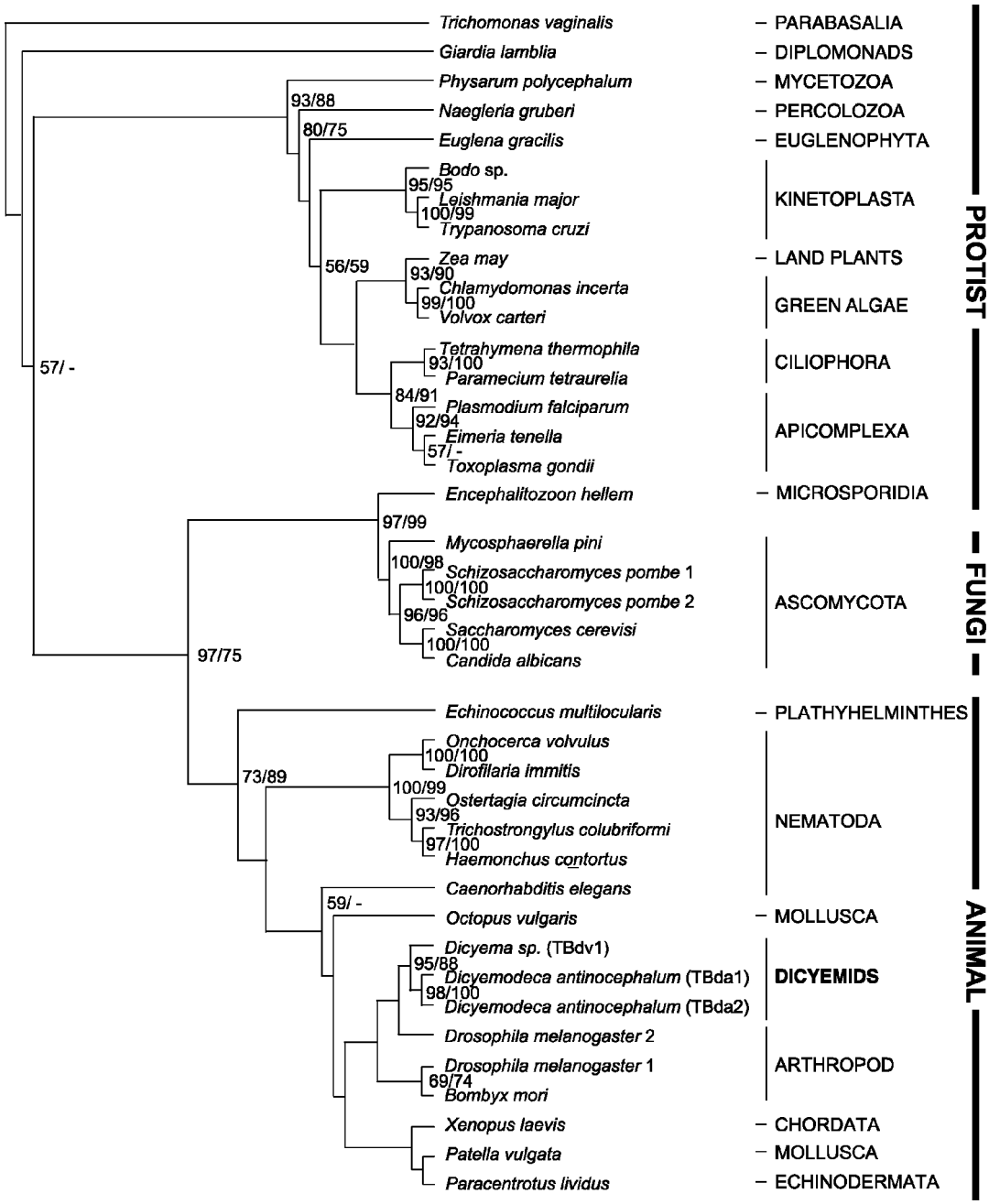

Fig. 4. Molecular phylogenetic consensus tree inferred from β-tubulin amino acid sequences by MP. Parsimony analysis was calculated using the program PROTPARS. Bootstrap resampling was accomplished with the use of the program SEQBOOT (1000 replicates) and CONSENCE. The topology of NJ tree showed almost the same of MP tree. Numbers at each branch indicate bootstrap percentage greater than 50%, which was obtained from MP (left) and NJ (right) analysis. The dicyemid mesozoans were placed within the triploblastic animals supported by high bootstrap values. Accession numbers: AB099885, AB099886, AB099887, AB099888 in dicyemids. AB099884 in the host *O. vulgaris*. AB099889 in the kinetoplastid *Bodo* sp.

EDTA, 150 mM NaCl, and 0.1% SDS at pH 8.0, containing 100 μg/ml proteinase K, at 55 °C for 2 h. Total DNA was extracted with phenol/chloroform, precipitated in ethanol with 2 μl of Pellet Paint Co-Precipitant (Novagen), and dissolved in 10 μl of sterilized water. The DNA was divided in two aliquots, one of which was used for every PCR as a template.

B.2. Amplification, cloning, and sequencing of β-tubulin genes from dicyemids

β-Tubulin genes were isolated by PCR using B-TU1F (5′-CARTGYGGYAACCARATYGG-3′) and B-TU2R (5′-TCCATYTCGTCCATRCCYTC-3′) primers designed to amplify approximately ∼1.2 kbp fragment which account for more than 80% of the dicyemid β-tubulin gene. A mixture of 25 μl of 1× *Taq* DNA polymerase buffer, 0.2 mM dNTPs, 1 μM of each primer, 5 μl of template (sample) DNA solution, and 0.5 U of *Taq* DNA polymerase (Sawady) was put in a thermal cycler for 35 cycles: each cycle consisted of 60 s at 94 °C, 60 s at 50 °C, and 60 s at 72 °C. Cloning and sequencing strategies were described previously (Noto et al., 2003).

B.3. Southern blot analysis

For confirmation of the source of β-tubulin gene, Southern blot analysis using one of clones (BTdv1) as a probe was carried out as described previously (Noto et al., 2003).

B.4. Sequence alignment and phylogenetic analysis

Amino acid sequences were inferred from the PCR product β-tubulin gene sequences, and aligned with homologs from representative species obtained from GenBank. Alignments (378 residues) of the β-tubulin amino acid sequences from *Dicyema* sp., *D. antinocephalum*, *O. vulgaris*, and other taxa were produced using CLUSTAL X (Thompson et al., 1997). These were then modified by eye to optimize them. Phylogenetic trees were constructed using the NJ and MP methods in the programs CLUSTAL X (Thompson et al., 1997) and PHYLIP version 3.6 (Felsenstein, 2002), respectively. All bootstrap values (Felsenstein, 1985) were based on 1000 replicates.

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