The Phylogenetic Position of *Rhopalura ophiocomae* (Orthonectida) Based on 18S Ribosomal DNA Sequence Analysis

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The Orthonectida is a small, poorly known phylum of parasites of marine invertebrates. Their phylogenetic placement is obscure; they have been considered to be multicellular protozoans, primitive animals at a "mesozoan" grade of organization, or secondarily simplified flatworm-like organisms. The best known species in the phylum, *Rhopalura ophiocomae,* was collected on San Juan Island, Wash. and a complete 18S rDNA sequence was obtained. Using the models of minimum evolution and parsimony, phylogenetic analyses were undertaken and the results lend support to the following hypotheses about orthonectids: (1) orthonectids are more closely aligned with triploblastic metazoan taxa than with the protist or diploblastic metazoan taxa considered in this analysis; (2) orthonectids are not derived members of the phylum Platyhelminthes; and (3) orthonectids and rhombozoans are not each other's closest relatives, thus casting further doubt on the validity of the phylum Mesozoa previously used to encompass both groups.

Introduction

The Orthonectida are rare, multicellular endoparasites found in the internal spaces and tissues of a wide variety of marine invertebrates. There are approximately 23 species in four recognized genera (Kozloff 1992). Orthonectids have neither a gut nor a nervous system. Adult orthonectids are small (<1 mm in length), free-swimming, and ciliated, and in most species have separate sexes. The outer layer of the body consists of a defined number of cells arranged in rings. Some of these cells are ciliated while others may contain few or no cilia, depending on the particular species examined. The inner body consists of a layer of cells containing contractile elements which enclose a mass of developing gametes (Kozloff 1969). Males of at least one species, *Rhopalura ophiocomae* Girard, also contain cells with collagen-like structures that may be paramyosin (Kozloff 1969, 1990, p. 217).

Fertilization and development of the eggs occur within the female's body, and ciliated larvae consisting of two body layers are released and, depending on the orthonectid species, infect turbellarians, nemerteans, gastropods, bivalves, polychaete annelids, brittle stars, or ascidians (Kozloff 1992, pp. 216–220). Once inside a host, the larva releases a mass of internal cells that gives rise to an ameboid, syncytial form called a plasmodium. Germinal cells within the plasmodium give rise to the adults, and in most species a particular plasmodium gives rise to either males or females. The origin of the plasmodium is controversial and most recently Kozloff (1994) considered the matrix of the plasmodium to be the cytoplasm of a host contractile cell.

The phylogenetic position of the Orthonectida remains uncertain. Most authors consider them to be representatives of the Kingdom Animalia (Brusca and Brusca 1990; Kozloff 1990, 1992, pp. 210–220). They traditionally have been considered a class in the phylum Mesozoa (= Middle Animal) along with Class Rhombozoa (equivalent to the class Dicyemida of some authors). Rhombozoans, which are also referred to as dicyemid mesozoans, are ciliated multicellular symbionts living in the nephridia of cephalopod molluscs. Although multicellular, mesozoans are considered to represent a level of body complexity intermediate between "protozoans" (unicellular protists) and "metazoans" (more complex multicellular animals) because of their lack of layered body construction and obvious gastrulation (Brusca and Brusca 1990). The phylum Mesozoa is still recognized in some recent textbooks (e.g., Barnes, Calow, and Olive 1993; Roberts and Janovy 1996), yet several authors have argued that orthonectids and rhombozoans deserve status as separate phyla based on considerations of body structure, particularly of the sexual stages, which are very different in the two groups (Kozloff 1969, 1990; Brusca and Brusca 1990).

In contrast to the widely held view that mesozoans are animals, Cavalier-Smith (1993) argued that they are members of the Kingdom Protozoa, the only protozoans to possess multicellular cell differentiation within their trophic phase. His argument is based on (1) their possession of tubular rather than plate-like mitochondrial cristae, a characteristic they share with other protozoans; (2) the apparent lack of the synapomorphy (collagenous tissue sandwiched between two dissimilar epithelial layers) that in his opinion best defines Animalia; and (3) his comment that mesozoans are no more complex than the multicellular spores produced by members of the phylum Myxozoa, which he also places within the Protozoa.

The status of Myxozoa as protozoans has been questioned by Smothers et al. (1994) who, on the basis of 18S ribosomal data, have concluded that myxozoans share their most recent common ancestor with bilaterally symmetrical animals and are thus metazoans. Based on both ultrastructural and 18S rDNA sequence data, Siddall et al. (1995) have also recently concluded that myxozoans are metazoans, in particular a clade of highly derived parasitic cnidarians. The status of dicyemid

Key words: 18S rRNA gene sequence, Mesozoa, Metazoa, Orthonectida, phylogeny, *Rhopalura ophiocomae.*

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Mol. Biol. Evol. 13(9):1187–1191. 1996

mesozoans (= rhombozoans) as multicellular protozoans has also been challenged recently. Katayama et al. (1995) have reported near complete 18S rDNA sequences for two species of rhombozoans and have concluded that they belong among triploblastic animals. They were unable to establish with certainty their relationship with other bilateral animal phyla.

Of particular interest with respect to the phylogenetic position of orthonectids and rhombozoans is whether or not their characteristic mesozoan grade of organization is primitive or derived. Assuming the former, they might represent a side branch from the lineage leading from protozoan to metazoan grades of complexity. As such they would provide significant information regarding the initial organization of the animal bauplan. For example, their body plans would suggest that early metazoans had solid, not blastula-like bodies, and that an early animal innovation was movement of reproductive cells to the body's interior (Hyman 1940). Stunkard (1954) and others argued that the complex life cycles of these organisms are reminiscent of those of parasitic flatworms and that their simple body plans represent a classic example of secondary loss of body complexity owing to their commitment to a parasitic lifestyle.

The simple body organization of orthonectids provides few meaningful characters, making comparison of morphological data across all animals difficult. The collection of molecular characters such as 18S rDNA sequence data offers a possible way to determine if: (1) orthonectids are more closely aligned with metazoans or protozoans, (2) orthonectids have affinities with parasitic flatworms, and (3) orthonectids and rhombozoans are each other's closest relatives.

Materials and Methods

Specimen Collection

Rhopalura ophiocomae adults were collected from the brittle star *Amphipholis squamata,* collected in Snug Harbor on San Juan Island, Wash. in May 1995. The central disc of brittle stars was removed and the gonadal tissue was teased apart with forceps in filtered seawater. Adults of *R. ophiocomae* fell cleanly away from host tissue and were transferred to clean seawater where they were separated from any small remaining pieces of host tissue. Both sexes were collected in this manner. Individuals derived from a particular brittle star, all of one sex, were placed in 95% ethanol and stored at −70°C.

DNA Extraction

Approximately 70 females of *R. ophiocomae* were rinsed with double-distilled water, and lysed (50 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% SDS, pH 8.0, containing 4 mg/ml proteinase K at 60°C for 1 h; Minchella et al. 1994). Genomic DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated, and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0).

Oligonucleotide Primers

The 18S rDNA "universal primers" described by Medlin et al. (1988), modified only by removal of polylinkers, were selected for the initial polymerase chain reaction (PCR) amplification of the 18S region of *R. ophiocomae.* The sense primer was A (5′-AACCTGGTTGATCCTGCCAGT-3′) and the antisense primer was B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′). The design of additional sequencing primers was based on newly obtained sequence data of the *R. ophiocomae* 18S gene. All primers were produced commercially (Ransom Hill Bioscience Inc., Ramona, Calif.).

DNA Amplification

Polymerase chain reactions were carried out with Multi Core kit reagents and a thermal cycler model 480. A 100-μl reaction contained 10 ng of genomic DNA as template, 10 μl 10 × PCR reaction buffer, dNTPs at 250 μM each, 2.5 units *Taq* polymerase, 2 mM $MgCl_2$, and 0.5 μM of each primer. The reactions were run using the following steps: (1) one cycle of 94°C for 4 min; (2) 30 cycles at 94°C for 1 min, 50°C for 30 s, 72°C for 1 min with a 4 s time increase for each extension time; (3) one 7 min extension cycle at 72°C. PCR products were visualized on an ethidium bromide stained 1.1% agarose gel.

Cloning and Sequencing

The amplified 18S rDNA region was ligated into pCRII (TA cloning kit); this construct was then used to transform *E. coli* (one-shot cells), all according to instructions of the manufacturer (Invitrogen, San Diego, Calif.). Plasmids were isolated by standard alkaline-lysis methods (Promega protocol, Madison, Wis.). The correct size of the insert was confirmed by restriction enzyme digestion and PCR experiments using the universal 18S primers. The insert was sequenced at the University Core DNA Services (373A automated sequencer; University of Calgary) and at the RIMI Multi Users Facility (377 automated sequencer; University of New Mexico) employing cycle sequencing with fluorescent dye terminators (Applied Biosystems, Foster City, Calif.). Sequencing of this fragment was initiated from the Sp6 and T7 sequencing primer sites available on the vector pCRII. The full-length information was resolved by "walking in"; several additional primers were designed from newly obtained sequence data until an overlap between the two strands of the insert was acquired. A consensus sequence of the entire 18S was contig aligned using DNASIS v. 2.1 (Hitachi Software, San Bruno, Calif.).

Phylogenetic Analyses

An alignment of the 18S sequences from *R. ophiocomae* and taxa listed below was first produced using CLUSTAL W (Gibson, Thompson, and Higgins 1994) compiled on an SGI workstation, then modified by eye to optimize the alignment. Areas that could not be satisfactorily aligned (i.e., homology of bases was easily established), were excluded. In addition, there were two large regions in the alignment which were alignable in all taxa except for the myxozoans. Instead of assuming homology between the myxozoan sequences and the others in these regions, we coded them as missing data in the myxozoan sequences, preserving the signal pres-

ent in the data for the remainder of taxa without adding potential "misinformation." The following 18S sequences were used, identified by species name and GenBank accession number: *Amphidinium belauense,* L13719; *Anemonia sulcata,* X53498; *Anthopleura kurogane,* Z21671; *Arabidopsis thaliana,* X16077; *Argopecten irradians,* L11265; *Beroe cucumis,* D15068; *Brachionus plicatilis,* U29235; *Calicophoron calicophorum,* L06566; *Convoluta naikaiensis,* D17558; *Dicyema acuticephalum,* D26530; *Dicyema orientale,* D26529; *Dugesia mediterranea,* M58344; *Glycera americana,* U19519; *Gordius aquaticus,* X87985; *Henneguya* sp., U13826; *Latimeria chalumnae,* L11288; *Lineus* sp., X79878; *Lobatostoma manteri,* L16911; *Meloidogyne arenaria,* U42342; *Moniliformis moniliformis,* Z19562; *Mus musculus,* X00686; *Mytilus trossulus,* L24490; *Myxidium* sp., U13829; *Nephroselmis olivacea,* X74754; *Onchidella celtica,* X70211; *Opisthorchis viverrini,* X55357; *Pinus griffithii,* X75080; *Plumatella repens,* U12649; *Priapulus caudatus,* Z38009; *Sarcocystis gigantea,* L24384; *Schistosoma haematobium,* Z11976; *Strongylocentrotus purpuratus,* L28055; *Strongyloides stercoralis,* M84229; *Sycon calcaravis,* D15066; *Trichoplax adhaerens,* L10828; *Tetrahymena corlissi,* U17356; *Tripedalia cystophora,* L10829; *Volvox carteri,* X53904.

PAUP* 4.0 d38–40 (Swofford 1996) was used to analyze the aligned sequences. Both minimum evolution and maximum parsimony were employed for the analysis. The minimum evolution criterion was used with HKY85 distances (Hasegawa, Kishino, and Yano 1985; Swofford 1996), with and without rate heterogeneity. Rate heterogeneity was modeled using a discrete gamma distribution (Yang 1994) having shape 0.5, with the means of the four rate categories as the relative rates. In addition, log determinant distances (LogDet; Lake 1994; Lockhart et al. 1994; Steel 1994) were used to examine whether base compositional nonstationarity was a factor influencing the topology. Parsimony analyses were done using a 2:1 transition/transversion weighting step matrix, thereby assigning greater cost to transversions than to transitions ("weighted parsimony"). All analyses used heuristic searches with random addition of taxa, and the tree-bisection/reconnection swapping algorithm. All bootstrap values (Felsenstein 1985) are based on 100 replicates, with heuristic searching.

Results and Discussion

Utilizing universal 18S rDNA primers A and B, an 1,853-bp PCR product was amplified from *R. ophiocomae* genomic DNA and cloned. With a total of 10 primers and several replicate sequencing reactions, 98% of the sequence information was obtained in duplicate; some parts were confirmed up to five-fold. Approximately 60% of the length of insert was sequenced from the sense strand, another 50% was obtained from the antisense strand.

BLAST searches (Altschul et al. 1990) disclosed high similarities with ribosomal sequences in GenBank. Highest homologies included small ribosomal subunit-like sequences from higher invertebrates but not from echinoderms, algae, or fungi (results not shown). This indicated that the sequence obtained was indeed 18S rDNA, and that it most likely originated from *R. ophiocomae* DNA and not from potential contaminants such as nucleic acids from echinoderm host (*A. squamata*) tissue, algae, or fungi. The sequence has been deposited in the NCBI GenBank database under accession number U58369. The complete data file in Nexus (D. R. Maddison, personal communication) format is available at ⟨http://biology.unm.edu/~esloker/hanelt96.html⟩.

Nearly identical topologies were obtained using the LogDet and HKY85 models, and with weighted parsimony. Congruent results from different models indicate that the topology is probably the result of phylogenetic signal and not an artifact of the particular methods chosen, since all of these methods make different assumptions concerning the evolutionary process. Figure 1 displays the HKY85 bootstrap consensus tree, which summarizes the results obtained with the three methods. All methods produced topologies that separated protists, plants, and animals, as well as the following well-supported animal clades: diploblastic taxa, triploblastic taxa plus myxozoans, flatworms, and deuterostomes. Within the animalia, the sponges, placozoans, cnidarians, and ctenophores were clearly delineated from triploblastic phyla. The backbone of the triploblastic clade, representing several phyla such as nematomorphs and rotifers, consisted of very short branches and no single resolution was well supported.

Within these trees, the orthonectid *R. ophiocomae* grouped with the triploblastic metazoans, not with the protists, green algae, or diploblastic taxa. This node is supported by bootstrap values of 100%. Furthermore, the dicyemids (rhombozoans) were not the closest neighbor to *R. ophiocomae* (with an average pairwise distance estimate between them of 0.48), thus casting further doubt on the validity of the phylum Mesozoa previously used to encompass both groups.

Rhopalura ophiocomae, dicyemids, and myxozoans formed three distinct lineages within the triploblastic animals; they did not group with the protists. As such, the results of this 18S-rDNA-based analysis did not lend support to the notion that these three phyla are "multicellular protists," a proposal based on the consideration of several intriguing anatomical characteristics (Cavalier-Smith 1993).

With respect to myxozoans, the results of this analysis were similar to those of Smothers et al. (1994), who also placed myxozoans with the metazoans. Our results did not agree with the placement of the myxozoans with the phylum Cnidaria (Siddall et al. 1995), however; the node supporting metazoan affiliation for the myxozoans was supported by a bootstrap value of 100% (see fig.1). Siddall et al. (1995) suggest that inclusion of the putative sister taxon *Polypodium* is essential to the resolution of myxozoans as cnidarians. Initial analyses that included *Polypodium* produced trees with a myxozoan plus *Polypodium* clade, intermediate between the triplobastic and diploblastic taxa, but never within the cnidarians, with a boostrap value of 91%. Additionally, removal of

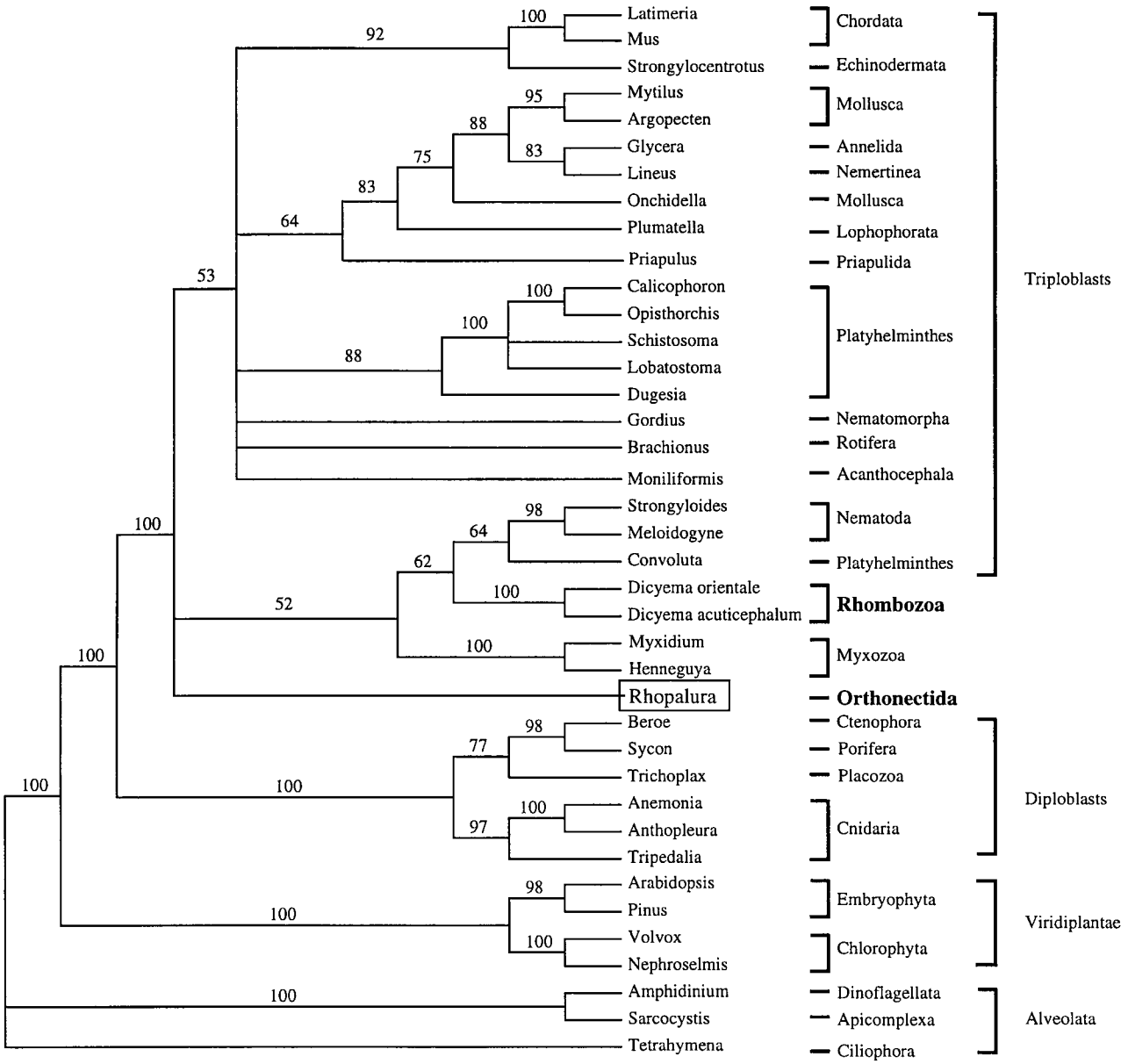

FIG. 1.—Phylogenetic position of the orthonectid *Rhopalura* based on 18S rRNA sequence comparisons with protists, green plants, and 31 animals. The tree shown is the 50% majority-rule bootstrap consensus, and numbers above nodes indicate bootstrap percentages obtained from 100 replicates using HKY85 distances and heuristic searches (minimum-evolution criterion). Branch lengths shown are arbitrary and do not represent evolutionary distance. Brackets indicate traditional classification, and boldface groups are those typically treated as mesozoans. See text for complete species names and GenBank accession numbers.

Polypodium (as in fig. 1) changed the support for a triploblastic clade minus myxozoans from 84% to less than 50%. This dramatic effect suggests long branch attraction involving the *Polypodium* sequence (Felsenstein 1978). In our exploration of the *Polypodium* sequence, it was found to be so different from any existing 18S sequences using BLAST searches that we felt its exclusion was warranted. In addition, we found the nucleotide content of the *Polypodium* sequence (53% G+C) was different from all other taxa, including the other cnidarians (45%). The use of LogDet, as mentioned above, corrects for nonstationarity in base composition. Because the LogDet tree differed only in minor ways from the topology obtained using HKY85 or parsimony, we can conclude that base composition nonstationarity is not influencing the tree-building algorithms.

Affinities between the platyhelminthes clade and *R. ophiocomae* were not supported. Unexpectedly, the acoel flatworm *Convoluta naikaiensis* fell outside of the flatworm clade. The classification of *Convoluta* has been suspect (Katayama et al. 1995), and in our analysis this sequence has the highest pairwise distance estimates of any of the included sequences (0.5–0.6). Katayama et al. (1995) point out the possibility of artifactual grouping of *Convoluta* with other triploblasts that are traditionally considered unrelated, and this may be the case in our study. Nonetheless, based on 18S sequences in hand, it is unlikely that orthonectids represent derived

members of the flatworms as suggested by Stunkard (1954).

Within the triploblasts, affinities among phyla are considerably uncertain and bootstrap values become too low to make reliable assessments about the sister group of the orthonectids. In our experience, this is a general concern that merits consideration in attempts to clarify placement of triploblastic phyla of uncertain phylogenetic affinity. In light of the 18S-based topologies obtained, the anatomical body plan suggests that orthonectids have undergone secondary anatomical simplification, possibly as a consequence of a parasitic lifestyle, a concept forwarded by Stunkard (1954). Moreover, if the patterns revealed by 18S rDNA phylogenies are correct, then at least three exclusively parasitic metazoan phyla (Orthonectida, Rhombozoa, and Myxozoa) may have undergone extensive secondary morphological simplification, including loss of a gut and nervous system.

Further clarification of the issues addressed in this study has to await availability of sequence information from 18S ribosomal RNA genes and perhaps other genes of *R. ophiocomae* and additional orthonectid species. The specimen collection necessary to obtain such data will be hampered by the rare occurrence of orthonectids in general.

Acknowledgments

This study was supported by a grant from the Howard Hughes Medical Institute through the Undergraduate Biological Science Education Program. Paul O. Lewis assisted in aspects of data analysis. He and Lynn A. Hertel critically read this manuscript. David L. Swofford kindly supplied a test version of PAUP*. Stephen A. Stricker had a significant role in location and collection of *R. ophiocomae*. We are grateful to two reviewers, whose comments helped to refine this manuscript.

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MITCHELL SOGIN, reviewing editor

Accepted July 12, 1996