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Molecular Phylogeny of Poecilostome Copepods Based on the 18S rDNA Sequences

Jihee Kim and Won Kim*

School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

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To elucidate phylogenetic relationships among poecilostome families, 18S rDNA sequence data were generated for seven poecilostome and one cyclopoid copepods by PCR cloning and sequencing techmiques. Phylogenetic trees were constructed by maximum parsimony, neighbor joining, and maximum likelihood methods using cyclopoid sequence as an outgroup. The results from three different analyses showed that the seven poecilostome families were diridel into two groups: Clausidiidae-Myicolidae-Synaptiphillidae-Bomolochidae and Lichomologidae-Chondracanthidae-Ergasilidae. The molecular phylogenies were consistent with those from the morphological characters. Therefore, these analyses provide further evidence for the utility of 18S rDNA sequences in addressing phylogenetic relationships among poecilostome families.

The Poecilostomatoida, represented by about 1,570 species of 47 families and seven genera which lack familial attribution, is one of the most diverse copepod order in terms of gross body morphology (Ho, 1991). Some families of the Poecilostomatoida have been frequently revised because many genera have been recognized as new families (Humes and Boxshall, 1996). In the past, several taxonomists have proposed different schemes to treat families of the Poecilostomatoida (Gooding, 1963; Avdeev, 1978; Gotto, 1979; Ho, 1984; Dojiri and Cressery, 1987). Izawa (1987) suggested that the Poecilostomatoida consisted of two major groups: Antehemicyclops and Antelichomolus groups. Ho (1991) carried out cladistic analysis for 117 morphological characters to elucidate the phylogenetic relationships among poecilostome families. The cladistic analysis supported Izawa's (1987) subdivision that the poecilostomes were divided into two groups (superorder level), and suggested that each of these two lineages gave rise to two subgroups (Fig. 1) (Ho, 1991).

Analyses using morphological characters such as the above contributed significantly to the clarification of poecilostome relationships. However, without accurate identification of homologous characters and determination of character states, it is difficult to identify an reliable evolutionary history of the poecilostome copepods based on morphological characters only. The Poecilostomatoida contains both free living families with typical body forms similar to those of the Cyclopoida and a large number of highly derived families parasiting a coelenterates, fish, molluscs, and the polychaetes (Huys and Boxshall, 1991; Ho, 1991). The identification of homologous characters and determination of character state of the poecilostome families are quite difficult because body forms and appendages are highly modified or reduced depending on their specialized associate types.

Therefore, to complement the study of the phylogenetic relationships among poecilostome families based on the morphological characters, a different approach employing molecular data is necessary. Among the various molecular markers (Friedlander et al., 1992, 1994; Graybeal, 1994), phylogenetic studies based on 18S rDNA sequences have proven to be useful for investigation of evolutionary history of the crustaceans (Kim and Abele, 1990; Abele et al., 1992; Spears et al., 1992, 1994) and other metazoans (Sogin et al., 1986; Moon et al., 1996; Aguinaldo et al., 1997). The versatile systematic utility of 18S rDNA is due to different evolutionary rate among different regions of 18S rDNA (Appels and Honeycutt, 1986; Mindell and Honeycutt, 1990) in conjunction with a large size and a conserved function (Hillis and Dixon, 1991; Olsen and Woese, 1993). Due to such advantages, 18S rDNA sequences are regarded to be useful in assessing the phylogenetic relationships among the poecilostomatoid families which contain highly modified morphological characters.

In this study, we determined the entire sequence of 18S rDNA from seven poecilostomes and one cyclopoid species. Phylogenetic trees were contructed by maximum parsimony, neighbor joining and maximum likelihood methods. The results from the 18S rDNA sequences were compared with those from cladistic analyses of the morphological characters.

*To whom correspondence should be addressed.
Tel: 82-2-880-6695, Fax: 82-2-872-1993
E-mail: wonkim@plaza.snu.ac.kr

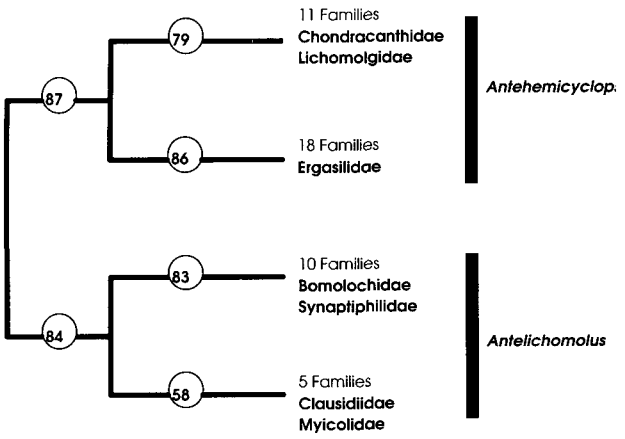

Fig. 1. The phylogenetic scheme represented by Ho (1991). Numbers in the circle of the branch are clade numbers recognized in the cladogram based on the morphological characters.

Materials and Methods

Taxon sampling

Eight copepods were collected to determine the 18S rDNA sequences. Among the seven poecilostome species, three speices, *Acanthochondria shawi* (family Chondracanthidae), *Hemannella longicaudata* (family Lichomolgidae), and *Dermoergasilus amplectens* (family Ergasilidae), were selected from the clade 87 according to Ho's (1991) scheme (Fig. 1). From the clade 84 (Fig. 1), we also chose four species; *Myicola ostreae* (family Myicolidae), *Goidelia japonica* (family Synaptiphilidae), *Conchyliurus quintus* (family Clausidiidae), and *Bomolochus decapteri* (family Bomolochidae). A cyclopoid species (*Doropyrus pinguis*) was used as an outgroup.

Cloning and sequencing

Tissue was homogenized in an extraction buffer (4 M EDTA, 10 mM Tris-HCl, and 1% sodium dodecyl sulfate). The homogenized tissue was incubated at 50℃ for 1 h with proteinase K and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1). After precipitation by addition of two volumes of ethyl alcohol and 1/10 volume of 3 M sodium acetate, the genomic DNA was resuspended in distilled water. Extracted DNA was further purified with GENE CLEAN kit (BIO 101). For the amplification of 18S rDNA, primer 328 (5′-TAC CTG GTT GAT CCT GCC AG-3′) and primer 329 (5′-TAA TGA TCC TTC CGC AGG TTC AC-3′) were used (Medlin et al., 1988). A Perkin Elmer PCR machine was programmed for 1 cycle (94℃, 1 min), 30 cycles (94℃, 1 min; 52℃, 2 min; 72℃, 3 min) and finally extended for 10 min at 72℃. PCR products were prepared for cloning by removing the primers using the GENE CLEAN kit (BIO 101). The ends of the amplified DNA fragments were modified for blunt-ended ligation using T4 DNA Kinase and T4 DNA Polymerase. The fragment was inserted into pGEM-3zf(-) plasmid vector and used to transform JM109 cell line. The 18S rDNA sequences were determined on both strands by the dideoxy chain-termination method (Sanger et al., 1977) using *Taq* Polymerase (Promega) with two plasmid primers and an additional 19 forward and reverse primers (Moon et al., 1994). Sequencing reactions were then run on 6% acrylamide/8M urea denaturing sequencing gels, which were then dried and visualized as described in Sambrook et al. (1989).

Phylogenetic analyses

The eight 18S rDNA sequences were aligned using the Clustal W program (Thompson et al., 1994). The alignment set is available on request from the above e-mail address. All analyses were conducted using PAUP* test version 4d63 (written by D. L. Swofford). Maximum parsimony method was done with coding gaps as missing data and using heuristic searches with tree bisection reconnection (TBR) branch swapping. Neighbor joining method (Saitou and Nei, 1987) was performed using the genetic distances calculated by Kimura 2 parameter model (Kimura, 1980). In maximum likelihood method, the HKY85 substitution model (Hasegawa et al., 1985) was used to reveal the empirical substitution rates. Support for the internodes of the phylogenetic trees was estimated by 100 bootstrap replicates (Felsenstein, 1985).

Results

Alignment set of the eight copepod sequences using the Clustal W program (Thompson et al., 1994) consists of 1,863 sites, 554 of which are variable. Among these variable sites, 360 are phylogenetically informative.

The phylogenetic tree based on neighbor joining analysis is shown in Fig. 2. Poecilostomes are separated into two groups, Clausidiidae-Myicolidae-Synaptiphilidae-Bomolochidae and Lichomologidae-Chondracanthidae-Ergasilidae. Sister group relationships between the Clausidiidae and Myicolidae and between the Lichomologidae and Chondracanthidae were supported with high bootstrap values (100% and 95%, respectively).

An initial search of 360 phylogenetically informative sites using the maximum parsimony method found one minimal length tree requiring 653 steps (CI 0.750, g1 -0.7829) which is shown in Fig. 3. The poecilostomes are grouped into two groups. One clade containing four families, the Clausidiidae, Myicolidae, Synaptiphilidae, and Bomolochidae, was supported with mild strong bootstrap value (73%), while the other clade containing the Lichomologidae, Chondracanthidae, and Ergasilidae did not have a high bootstrap value (58%).

In the phylogenetic tree conducted by the maximum likelihood method, the branching pattern is the same as that from the maximum parsimony analysis (Fig. 3). The result of bootstrap resampling for the maximum

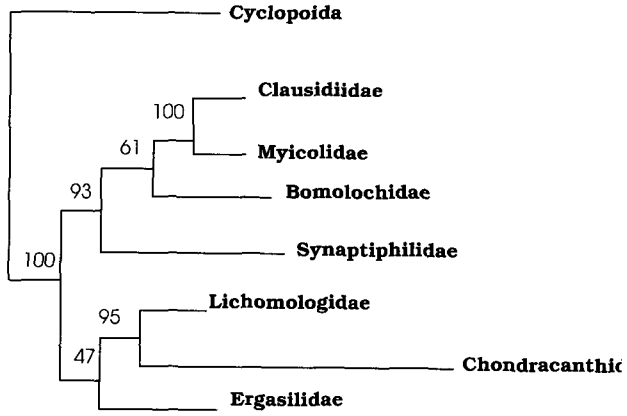

Fig. 2. The neighbor-joining tree of the 18S rDNA sequences. Bootstrap values are shown along branches. The horizontal length of each branch is proportional to the estimated number of nucleotide substitutions.

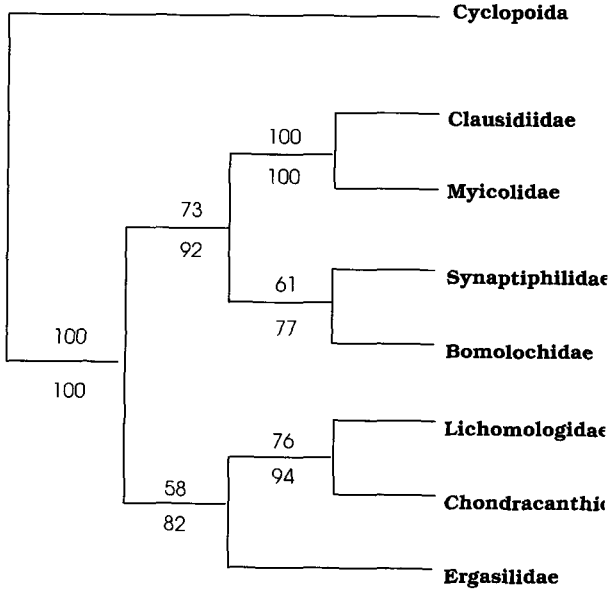

Fig. 3. The phylogenetic tree constructed by maximum parsimony and maximum likelihood methods. The numbers above the branch are from the maximum parsimony analysis with 100 bootstrap replicates and the numbers below the branch are from the maximum likelihood analysis.

likelihood analysis shows stronger support for these two groups, especially for the branch containing the Lichomologidae, Chondracanthidae and Ergasilidae (82% bootstrap value) (Fig. 3, numbers below the branch). Within the two poecilostome subgroups, the sister group relationships of the Clausidiidae and Myicolidae, the Synaptiphilidae and Bomolochidae, and the Lichomologidae and Chondracanthidae are also supported with 100%, 77%, and 94% bootstrap values, respectively.

Discussion

The results constructed by three different methods are generally consistent with one another. The seven poecilostome families are separated into two groups. One group comprises the Clausidiidae, Myicolidae, Synaptiphilidae, and Bomolochidae and the other contains the Lichomologidae, Chondracanthidae, and Ergasilidae (Figs. 2, 3). The bootstrap support for the second group is relatively low in maximum parsimony and neighbor joining analyses, although the monophyly of these three families is supported with 82% bootstrap value in maximum likelihood analysis. The position of the Ergasilidae within the second group was not highly supported while the other two families, the Lichomologidae and Chondracanthidae, were grouped together with high bootstrap values (Figs. 2, 3).

The results from the 18S rDNA sequences are consistent with those from the morphological characters (Ho, 1991). The difference observed is that the sister group relationship between the Bomolochidae and Synaptiphilidae was not shown in neighbor joining tree (Fig. 2). When compared the results with Izawa's proposal (1987), the first group comprising the four families is represented as the Antehemicyclops and the other is represented as the Antelichomolus. Similar to the results from the morphological characters (Ho, 1991), the Myicolidae is placed in the Antehemicyclops group instead of the Antelichomolus in contrast to Izawa's proposal (1987). Although the Ergasilidae was suggested as a new order in the Copepoda because of its unusual naupliar features (Izawa, 1987), based on the 18S rDNA sequences, the Ergasilidae belongs to the Poecilostomatoida and shows a close relationship with the Lichomologidae and Chondracanthidae.

In cladistic framework, congruence of the molecular and morphological data can provide the criterion for an evaluation of homology because taxonomic homology is a statement about characters of common descent in a monophyletic group (Patterson, 1982; Panchen and Smithson, 1987). When the morphological characters employed by Ho (1991) were evaluated, the characteristic that endopod in leg 1 is absent is considered as a homologuous character state for the Chondracanthidae, Lichomolgidae, and Ergasilidae. For the other four families, that leg 5 is composed of two free segments with the distal segment having four elements is regarded as a synapomorphic character state.

The 18S rDNA sequence data strongly indicate that the Clausidiidae is a sister group to the Myicolidae and that the Chondracanthidae and Lichomologidae are in the sister group relationship. Though these four families have the same characteristic that antenulle has seven segments, this character state is regarded as character convergence because these families do not belong to the same clade. The Chondracanthidae and Lichomolgidae are identified by two character states; one segmented endopod with a pointed process in maxilliped and third endopod segmented with five elements in leg 5. The mandible armed with four terminal elements was observed in both the Clausidiidae and Myicolidae. Though the Bomolochidae and Synaptiphil-

idae were shown to be paraphyletic in neighbor joining tree (Fig. 3), these families were grouped together by having six segmented antennule and antenna with claw(s) or spine(s) on penultimate segment.

Generally, the 18S rDNA has been employed to elucidate phylogenetic relationships among remotely related organisms (eg. from phyla to order level) (Field et al., 1988; Spears et al., 1994; Halanych, 1996). Recently, it was shown to be able to resolve patterns of relationships at a family level in several fast evolving taxa (Wen and Zimmer, 1996; Black et al., 1997; Vogler et al., 1997). In this study, the 18S rDNA sequence data were also useful in elucidating the phylogenetic relationships among poecilostome families which show great ecological adaptibility. Therefore, these analyses provide further evidence for the utility of 18S rDNA sequences in addressing phylogenetic relationships among poecilostome families.

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