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Phylogenetic Relationships Among Six Vetigastropod Subgroups (Mollusca, Gastropoda) Based on 18S rDNA Sequences

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Complete 18S rDNA sequences were determined for 10 vetigastropods in order to investigate the phylogeny of Vetigastropoda, which is controversial. These sequences were analyzed together with published sequences for nine other vetigastropods and two nerites. With the two nerites as outgroups, the phylogeny was inferred by three analytical methods, neighbor-joining, maximum likelihood, and maximum parsimony. The 18S rDNA sequence data support the monophyly of four vetigastropod superfamilies, the Pleurotomarioidea, the Fissurelloidea, the Haliotoidea, and the Trochoidea. The present results yield the new branching order: (Pleurotomarioidea (Fissurelloidea ((Scissurelloidea, Lepetodriloidea) (Haliotoidea, Trochoidea)))) within the vetigastropod clade.

Keywords: 18S rDNA Sequences; Gastropoda; Mollusca; Phylogenetic Tree Reconstruction; Vetigastropoda.

Introduction

In cases where morphological data are inconclusive, 18S rDNA sequences have been successfully used to infer relationships between molluscan taxa (Bargues and Coma, 1997; Canapa *et al*., 1999; Steiner and Müller, 1996; Winnepenninckx *et al*., 1996; 1998a; 1998b; Yoon and Kim, 2000) since it has several characteristics that make it useful for phylogenetic reconstructions (see Canapa *et al*., 1999; Winnepenninckx *et al*., 1994).

The Order Vetigastropoda, one of the basal groups of the gastropods (Hickman, 1988; Ponder and Lindberg, 1997; Salvini-Plawen and Steiner, 1996), is characterized by several features including ctenidial and epipodial sense organs, and the special structure of the esophagous (Haszprunar, 1988a; 1988b; Ponder and Lindberg, 1996; 1997; Salvini-Plawen and Haszprunar, 1987). The living representatives of this order are usually divided into six subgroups (Haszprunar, 1988a): Pleurotomarioidea and Scissurelloidea (slit-shelled molluscs); Fissurelloidea (keyhole limpets); Haliotoidea (abalones); Trochoidea (trochids and turbans); Lepetodriloidea.

Although numerous morpho-anatomical characters have been used to establish phylogenetic relationships among the vetigastropod subgroups (Haszprunar, 1988a; 1988b; Ponder and Lindberg, 1996; 1997; Salvini-Plawen, 1980; Salvini-Plawen and Haszprunar, 1987; Sasaki, 1998), the systematics of the Vetigastropoda are still controversial. This is due to their remarkable phenotypic diversity and relatively few synapomorphic characters. In order to clarify the phylogeny of Vetigastropoda, investigators have obtained partial 18S (Harasewych *et al*., 1997a; 1997b) and 28S (Tillier *et al*., 1994) rDNA sequences. Harasewych *et al*. (1997b) divided the Vetigastropoda into two subclades, pleurotomariids (Pleurotomarioidea) and non-pleurotomariids (Fissurelloidea + Haliotoidea + Trochoidea) on the basis of partial 18S rDNA sequences. However, the relationships between the three subgroups of non-pleurotomariids were not resolved. Tillier *et al*. (1994) suggested that the Fissurelloidea were a sister group of the Haliotoidea + Trochoidea, on the basis of partial 28S rDNA data from only three vetigastropod subgroups. Therefore more representatives, and more extensive sequence information, are needed to assess accurately Vetigastropod phylogeny.

To clarify the phylogeny of the vetigastropoda, we newly determined the complete 18S rDNA sequences of 10 vetigastropods and analyzed them together with published sequences for nine other vetigastropods and two nerites. The aims of the study were to test the monophyly of each of the vetigastropod subgroups, Pleurotomarioidea, Fissurelloidea, Haliotoidea, and Trochoidea, and to assess

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the phylogenetic relationships between the six vetigastropod superfamilies.

Materials and Methods

Specimens of 10 vetigastropods were collected from natural populations living in the seas around Korea: *M. dilatatum*, *S. diversicolor supertexta*, *O. pfeifferi carpenteri*, *C. hirasei*, *P. japonicus*, and *A. haematragum* from Jejudo; *T. gigas* from Jumunjin; *C. lischkei* from Yangyang; *U.* (*S.*) *thomasi* from Daecheon harbor; *L. coronata coreensis* from Seonyudo. The complete sequences of their 18S rDNA were determined in this study. The sequences of two vetigastropods (*N. discus* and *B. cornutus*) were reported in our previous study (Yoon and Kim, 2000) and the sequences of the remaining seven vetigastropods and two nerites (outgroup species) were obtained from GenBank. The taxa utilized in the present study are listed in Table 1 with their GenBank accession numbers. Voucher specimens are deposited in the School of Biological Sciences, Seoul National University.

Snail feet dipped in 100% ethanol and maintained at −72°C for several weeks were used to isolate genomic DNA by the phenol-chloroform procedure (Sambrook *et al*., 1989). The 18S rDNAs of 10 vetigastropods were selectively amplified by the polymerase chain reaction (PCR) using two oligonucleotide primers, 5′-CCTGGTTGATCCTGCCAG-3′ and 5′-TAATGATCCTTCCGCAGGTTA-3′ (Medlin *et al*., 1988). Thirty cycles of PCR were carried out using a Thermo Cycler (Perkin Elmer Co.) and the following conditions: denaturation at 94°C for 1 min, annealing at 52°C for 2 min, and elongation at 72°C for 3 min. The ends of the amplified PCR fragments were then modified for blunt-ended ligation using T4 kinase and T4 polymerase, ligated into the pGEM-3zf(−) cloning vector and transformed into *E. coli* DH5-α. The recombinant plasmid DNA was purified using a plasmid purification kit (QIAGEN Co.) and used in the sequencing reactions. Both forward and reverse strands were sequenced, on an ABI 310 automated sequencer (Perkin Elmer Co.), using primer walking method and a big-dye terminator sequencing kit (Perkin Elmer Co.). The sequencing primers used in the present study are given in a previous paper (Moon *et al*., 1996).

Sequences were initially aligned with CLUSTAL W 1.6 (Thompson *et al*., 1994) and the alignment was corrected by visual observation. Regions of uncertain alignment were eliminated from the final analyses and alignment gaps were treated as missing data. Accordingly, analyses were limited to reliably aligned regions comprising a total of 1,729 nucleotide positions. These alignments can be obtained from the authors. Phylogenies were inferred by three analytical methods: neighbor-joining (NJ), maximum-likelihood (ML), and maximum parsimony (MP). The program PHYLIP version 3.572c (Felsenstein, 1995) was used to construct NJ (Saitou and Nei, 1987) trees based on the formulas of Kimura (1980) and Jukes and Cantor (1969). ML analyses (Felsenstein, 1981) were performed using the program PUZZLE 4.0.2 (Strimer and von Haeseler, 1996) with the HKY (Hasegawa *et al*., 1985) model based on the previous freshwater gastropod phylogenetic study (Hausdorf *et al*., 2003). For the quartet puzzling method (1,000 puzzling steps), nucleotide frequencies and transition/transversion ratios were estimated from the data set. MP analyses were done with PAUP version 4.0b10 (Swofford, 2002) and performed as heuristic searches using equal character weighting and closest stepwise addition. Bootstrap values (Felsenstein, 1985), representing the robustness of each node in the NJ and MP trees, refer to one thousand replications. Quartet puzzling provided the reliability values for maxi-

Table 1. Species used in the present study, with GenBank accession numbers for sequences. Sequences reported in this paper are marked with an asterisk (*).

NERITOPSINA	
NERITOIDEA	
Neritidae	
1. X91971 *Nerita albicilla*	
2. AF120515 *Theodoxus flaviatilis*	
VETIGASTROPODA	
PLEUROTOMARIOIDEA	
Pleurotomariidae	
3. AF120509 *Entemotrochus adansonianus*	
4. AF120510 *Perotrochus midas*	
FISSURELLOIDEA	
Fissurellidae	
* 5. AF335560 *Macroschisma dilatatum*	
6. AF120513 *Diodora graeca*	
* 7. AF335561 *Tugali gigas*	
SCISSURELLOIDEA	
Scissurellidae	
8. AF120512 *Sinezona confusa*	
LEPETODRILOIDEA	
Lepetodrilidae	
9. AY145381 *Lepetodrilu elevatus*	
HALIOTOIDEA	
Haliotidae	
10. AF082177 *Nordotis discus*	
11. AF120511 *Haliotis tuberculata*	
* 12. AY698072 *Suculus diversicolor supertexta*	
TROCHOIDEA	
Trochidae	
13. X94271 *Monodonta labio*	
* 14. AF335562 *Chlorostoma argyrostoma lischkei*	
* 15. AY698067 *Omphalius pfeifferi carpenteri*	
* 16. AY698073 *Umbonium (Suchium) thomasi*	
* 17. AY698068 *Cantharidus hirasei*	
Turbinidae	
18. AF165311 *Batillus cornutus*	
* 19. AY698070 *Pomaulax japonicus*	
* 20. AY698071 *Lunella coronata coreensis*	
* 21. AY698069 *Astralium haematragum*	

Classification follows Haszprunar's scheme (1988a).

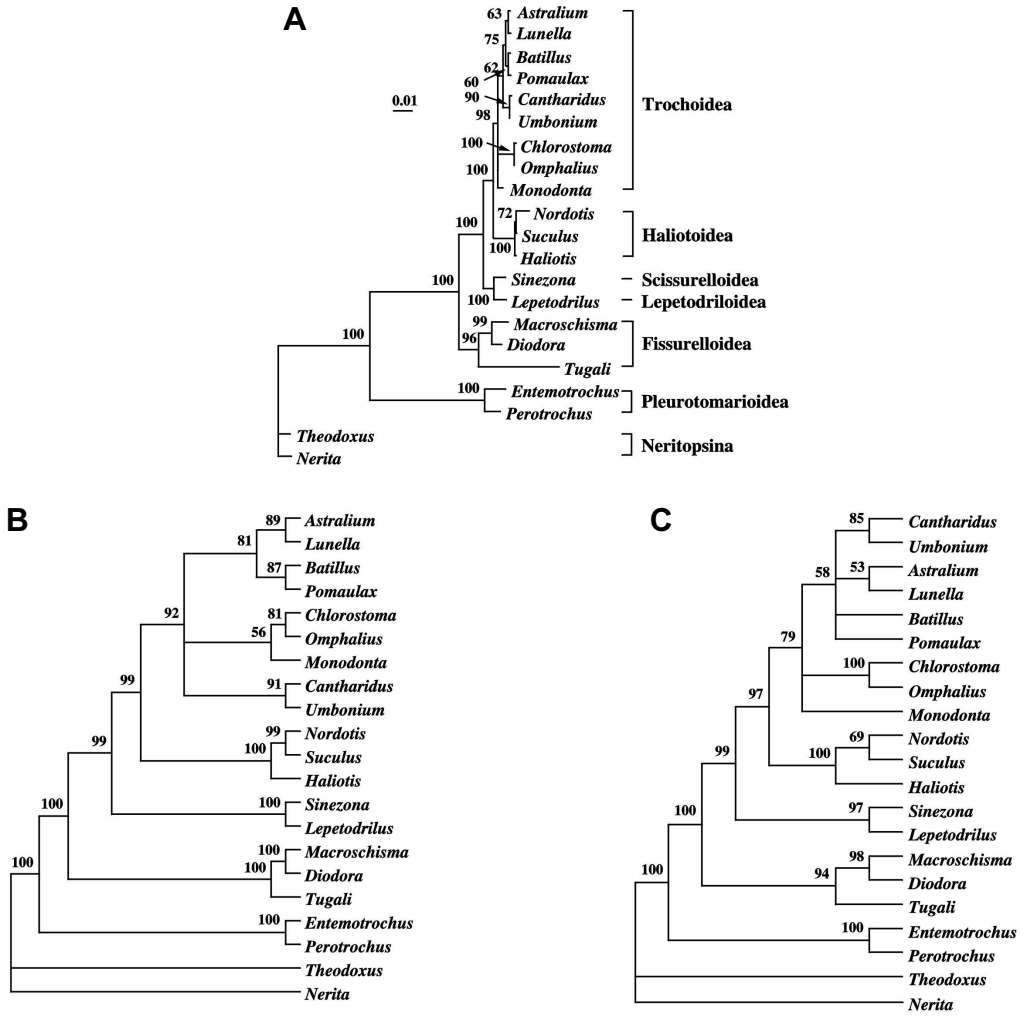

Fig. 1. Phylogenetic trees based on the alignment of complete 18S rDNA sequences for 19 vetigastropods and two nerites. The two nerites, *Nerita albicilla* and *Theodoxus flaviatilis*, were used as outgroups. **A.** Neighbor-joining tree. Bootstrap percentages are shown above branches supported in at least 50% of 1,000 replicates. **B.** Maximum likelihood tree. The numbers of puzzling steps are 1,000 and maximum likelihood quartet puzzling reliability values are indicated above on the nodes. **C.** Majority-rule consensus tree derived from maximum parsimony analysis. The numbers represent the percentage of 1,000 bootstrap replications in which a given node appeared.

mum likelihood analyses (Strimer and von Haeseler, 1996).

Results

Figure 1A shows the neighbor-joining (NJ) tree obtained using the Kimura distances from an alignment of the complete 18S rDNA sequences of 19 vetigastropods and two nerites. Two nerites, *Nerita albicilla* and *Theodoxus flaviatilis*, were used as outgroups because the order Neritopsina formed a stem group in the Hexoglossate Prosobranchia in investigations using morphological (Haszprunar, 1987; 1988a; 1988b) and molecular (Winnepenninckx *et al*., 1998a; Yoon and Kim, 2000) data. The identical topology was obtained using Jukes and Cantor distances. In the vetigastropoda, the Pleurotomarioidea branched off first, and the Fissurelloidea diverged next as an independent clade, before the remaining vetigastropod clade comprising Lepetodriloidea - Scissurelloidea and Haliotoidea - Trochoidea. The Haliotoidea and the Trochoidea formed a sister group with very high bootstrap support (100%). The Lepetodriloidea and the Scissurelloidea also emerged as a reliable sister group taxa, (bootstrap value 100%). In addition, the NJ tree clearly depicted the monophyly of the vetigastropod subclades, Pleurotomarioidea, Fissurelloidea, Haliotoidea, and Trochoidea with high boostrap support (100%; 96%; 100%; 98% respectively).

The maximum-likelihood (ML) tree (Fig. 1B) of the aligned sequences confirmed all major aspects of the NJ tree (Fig. 1A) with minor differences in topology among groups within the trochoidean clades. The ML tree shows

some topological shifts within the trochoidean clade, e.g., the alternative branching order, *Umbonium* - *Cantharidus*, *Monodonta* - *Omphalius* + *Chlorostoma*, and Turbinidae, instead of the order shown in the NJ tree (Fig. 1A), *Monodonta*, *Omphalius* - *Chlorostoma*, *Umbonium* - *Cantharidus* + Turbinidae. However, the bootstrap values that support these nodes are not significant.

Maximum parsimony (MP) analyses of the same data set yielded a single tree of minimum length 613 steps. The tree has a consistency index (CI) of 0.84, and a retention index (RI) of 0.88. A majority-rule consensus tree with bootstrap values for nodes that supported ≥ 50% of 1,000 bootstrap replicates is shown in Fig. 1C. The topology of this MP tree is the same as that of the tree obtained by NJ (Fig. 1A) analysis of the phylogeny of the Vetigastropoda. There are breakdowns of sister group relationship among the turbinid species, *Pomaulax*, *Batillus*, and *Lunella* - *Astralium.* These are incongruent with the results from the NJ analysis and none of them are supported with high bootstrap values.

Discussion

Our 18S rDNA data confirm the monophyly of each of four vetigastropod subgroups, Pleurotomarioidea, Fissurelloidea, Haliotoidea, and Trochoidea. Haszprunar (1988a) considered that the monophyly of the Pleurotomarioidea was due to the commonly derived characters, the reduction of epipodium and the hystrichoglossate radula. Harasewych *et al*. (1997b) also mentioned that pleurotomarioidians are identified on the basis of having dextral, conispiral shells with narrow, generally deep emarginations or "slits" along their outer lips that give rise to a spiral band or selenozone along or near the periphery of the shell. This viewpoint is in agreement with our resolution as well as the results of partial 18S rDNA sequencing (Harasewych *et al*., 1997b). Our trees also support the monophyly of Fissurelloidea. This configuration is certainly consistent with synapomorphic characters such as secondary symmetry and limpet shape, the loss of operculum and pedal gland, the reduction of the left excretory organ, the lack of a coiled caecum in the stomach, the purely extracellular digestion (Salvini-Plawen and Haszprunar, 1987), the reduction of epipodial tentacles, the closed eye-vesicles, the enlarged salivary glands, and the anal gland (Haszprunar, 1988a). The monophyly of Haliotoidea is also supported in the present study and this group has been defined by synapomorphies including the shell with a series of holes, the loss of operculum and pedal gland, the hypertrophied epipodium, and the hypertrophied right shell muscle (Haszprunar, 1988a; Salvini-Plawen and Haszprunar, 1987). Monophyly of the Trochoidea (Trochidae plus Turbinidae) was also confirmed. The Trochoidea are synapomorphically united by their loss of the right ctenidium in relation to the loss of the shell slit (Haszprunar, 1988a; 1988b) and this point is in concordance with the studies based on 18S (Yoon and Kim, 2000) and 28S (Tillier *et al*., 1994) rDNA sequences.

Our results reveal two clearly defined clades among the vetigastropod major subgroups, the pleurotomarioidians and the non-pleurotomarioidians formed by Fissurelloidea, Lepetodriloidea, Scissurelloidea, Haliotoidea, and Trochoidea. This indication supports the result of Harasewych *et al*. (1997b). However opinions among morphologists have differed on this matter. Several workers, such as Haszprunar (1988a; 1988b), Salvini-Plawen and Steiner (1996), Ponder and Lindberg (1997), and Sasaki (1998), unite the Pleurotomarioidea, the Scissurelloidea, the Fissurelloidea, and the Haliotoidea as the Zeugobranchia, but separate independently the Lepetodriloidea and the Trochoidea from them. Therefore, according to the present study, the common morphological features found in these four vetigastropod subgroups, excluding the Lepetodriloidea and the Trochoidea, may be derived convergently rather than comprising synapomorphic characters. According to our findings, Pleurotomarioidians could be regarded as the most primitive vetigastropods, in line with the view of Harasewych *et al*. (1997b) based on partial 18S rDNA data. Many authors have suggested that the Pleurotomarioidea are the most ancestral living gastropods (Boss, 1982; Golikov and Starobogatov, 1975; Taylor and Sohl, 1962; Thiele, 1929; Vaught, 1989). Hickman (1984), in particular, claimed that the radula of Pleurotomarioidea had little in common with that of any living gastropod of more recent geological origin. The combination in pleurotomarioideans of an asymmetrically coiled shell and symmetrically paired pallial organs including gills, auricles, kidneys, and hypobranchial glands has been considered as transitional between extinct, planispirally coiled, bilaterally symmetrical ancestors, generally believed to be bellerophonts, and asymmetrical modern gastropods (Harasewych *et al*., 1997b).

Within the non-pleurotomarioidian vetigastropod clade, the 18S rDNA sequences indicate that the Fissurelloidea diverged first and subsequently the Lepetodriloidea + Scissurelloidea emerged before the remaining two vetigastropod subgroups, the Haliotoidea and Trochoidea. Our results confirm that Haliotoidea and Trochoidea are sister group taxa, which is consistent with the results from 28S rDNA sequences (Tillier *et al*., 1994). Haszprunar (1985) concluded that Haliotidae and Trochoidea are additionally linked by shared aberrant chemosensory structures. However, some investigators of morphology (Hickman, 1984; Hyman, 1967; Knight *et al*., 1960; Vaught, 1989) have suggested that the Haliotoidea resemble more the Pleurotomarioidea and Scissurelloidea than other vetigastropod subgroups. On the other hand, Salvini-Plawen (1980) mentioned that the Haliotoidea are closely related to the Fissurelloidea and the Scissurelloidea, on grounds of shell structure, paired pallial organs, and

dorsoventral retractor bundles. McLean (1984) suggested that the Trochoidea are closely related to the Pleurotomarioidea and this viewpoint accords with the opinion of Haszprunar (1988a) based on derived characters, a shared type of left kidney (papillary sac) and a specialized osphradial cell-type (ciliabottles).

The present study also indicates that the Lepetodriloidea and the Scissurelloidea have a sister group relationship. This result, however, contradict the views of Haszprunar (1988b) and Sasaki (1998) who, on the basis of morphological characters, allocated the Lepetodriloidea to the most basal position, and the Scissurelloidea in the Zeugobranchia within the vetigastropod clade.

In conclusion, NJ, ML, and MP analyses using complete 18S rDNA sequences strongly support the monophyly of the four vetigastropod subgroups, Pleurotomarioidea, Fissurelloidea, Haliotoidea, and Trochoidea. The Vetigastropoda are generally classified into two subclades, the Pleurotomarioidea appearing as the earliest vetigastropod offshoot and the non-pleurotomarioidians comprising the remaining vetigastropods. Therefore, the grouping of Pleurotomarioidea, Fissurelloidea, Scissurelloidea, and Haliotoidea, which is generally accepted, is unreasonable. Within the vetigastropod clade, the branching pattern of (Pleurotomarioidea (Fissurelloidea ((Lepetodriloidea, Scissurelloidea) (Haliotoidea, Trochoidea)))) was strongly supported.

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