Close Relationship between *Asterina* and Solasteridae (Asteroidea) Supported by Both Nuclear and Mitochondrial Gene Molecular Phylogenies

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ABSTRACT—Phylogenetic relationships among asteroids remain to be extremely controversial in spite of many morphological and molecular studies have been applied to this issue. In the present study, especially focusing on resolving the relationship of *Asterina* and Solasteridae, we reconstructed the molecular phylogenetic tree of asteroids using nuclear 18S rDNA. A close relationship between *Asterina* and Solasteridae, which has been suggested from analyses of mitochondrial 12S rDNA and 16S rDNA, is supported here by the nuclear 18S rDNA dataset. The support is even stronger when the sequences of mitochondrial rDNAs and nuclear 18S rDNA are combined as a total dataset. The independent support from both nuclear 18S rDNA and mitochondrial rDNAs strongly argues for a close relationship between the *Asterina* and Solasteridae.

Key words: asteroid, molecular phylogeny, rDNA, *Asterina*, Solasteridae

INTRODUCTION

Asteroids (Echinodermata, Asteroidea) are familiar and diverse marine invertebrates. Seven orders of approximately 35 families, 300 genera and 1800 species are recognized (Clark and Downey, 1992; Hendler *et al*., 1995).

Since classification of asteroids have been reformulated in these twenty years (Blake, 1987; Gale, 1987; Clark and Downey, 1992), several molecular phylogenetic studies have been performed to evaluate the phylogenetic relationships (Lafay *et al*., 1995; Wada *et al*., 1996; Knott and Wray, 2000). Although these molecular analyses have not succeeded to provide convincing phylogenetic framework of asteroids so far, the affinity between *Asterina* and *Solaster* is supported with relatively high supporting values (Wada *et al*., 1996).

Traditionally, the Asterinidae and the Solasteridae had been classified into the order Spinulosida, which is characterized by adambulacral mouth frame, reduced marginals,

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lack of true paxillae and arrangement of abactinal ossicles (either reticulated or imbricated) (Spencer and Wright, 1966; Blake, 1981). However, the order Spinulosida had been recognized as a provisional group constituted by assemblage of asteroids which is not included in other orders (Fisher, 1911; Spencer and Wright, 1966), and the two families are classified into different suborders: Solasterids in suborder Eugnathina and *Asterina* in suborder Leptognathina (Spencer and Wright, 1966). Blake (1981) re-examined the classification of the Spinulosida, and transferred Asterinidae from the order Spinulosida to the order Valvatida, mainly based on ossicle morphology of ambulacral column, and he also pointed out that imbricate pattern of abactinal ossicles of *Asterina* has no analogy with the Solasteridae. Since then, nobody has suggested the affinity between Asterinidae and Solasteridae, and Clark and Downey (1992) classified them into different orders, Valvatida and Velatida, respectively.

Therefore the close affinity between *Asterina* and *Solaster* suggested by the molecular phylogenetic analyses using mitochondrial 12S and 16S rDNA (Wada *et al*., 1996) was hard to be accepted from the morphological standpoint. Smith (1997) mentioned that the close relationship between Solasteridae and *Asterina* has never been proposed, and thus even suspected laboratory contaminant in the analyses by Wada *et al.* (1996).

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Table 1. List of specimens analyzed in this study

| Taxon | Sequence accession nos. | | | Rreferences |
	18S rDNA	12S rDNA	16S rDNA	
ASTEROIDEA				
Order Paxillosida				
Family Luidiidae				
Luidia maculata[1]	AB084548*	D63740	D63741	Wada *et al.* (1996)
Luidia quinaria		D63742	D63743	Wada *et al.* (1996)
Luidia foliolata	AF088805			Janies (2001)
Family Astropectinidae				
Astropecten polyacanthus[1]	AB084549*	D63722	D63723	Wada *et al.* (1996)
Astropecten latespinosus[1]	AB084546*	D63720	D63721	Wada *et al.* (1996)
Astropecten irregularis	Z80949			Littlewood *et al.* (1997)
Ctenopleura fisheri		D63732	D63733	Wada *et al.* (1996)
Order Valvatida				
Family Archasteridae				
Archaster typicus[1]	AB084547*	AB084560*	AB084559*	
Archaster angulatus[2]	AB084558*	AB084564*	AB084565*	
Family Ophidiasteridae				
Leiaster leachii[1]	AB084561*	AB084563*	AB084562*	
Certonardoa semiregularis[1]	AB084550*	D63726	D63727	Wada *et al.* (1996)
Family Goniasteridae				
Pseudarchaster parelii	AF088806	D63744	D63745	Janies (2001) Wada *et al.* (1996)
Family Poraniidae				
Porania pulvillus	Z80955			Littlewood *et al.* (1997)
Family Asterinidae				
Asterina pectinifera[1]	AB084551*	D63716	D63717	Wada *et al.* (1996)
Asterina batheri[1]	AB084552*	D63712	D63713	Wada *et al.* (1996)
Asterina coronata japonica[1]	AB084566*	AB084568*	AB084567*	
Asterina minor		D63714	D63715	Wada *et al.* (1996)
Asterina pseudoexigua pacifica[1]	AB084553*	D63718	D63719	Wada *et al.* (1996)
Asterina gibossa	AF088801		Janies (2001)	
Family Acanthasteridae				
Acanthaster planci[1]	AB084554*	AB084569*	AB084570*	
Order Spinulosida				
Family Echinasteridae				
Henricia ohshimai[1]	AB084555*	AB084572*	AB084571*	
Henricia nipponica		D63736	D63737	Wada *et al.* (1996)
Henricia pachyderma		D63738	D63739	Wada *et al.* (1996)
Order Velatida				
Family Solasteridae				
Crossaster papposus[1]	AB084556*	D63730	D63731	Wada *et al.* (1996)
Solaster dawsoni[1]	AB084557*	AB084573*	AB084574*	
Family Pterasteridae				
Pteraster tesselatus[1]	AF088808	AB125598*	AB125599*	Janies (2001)

continued

Order Forcipulatida				
Family Asteriidae				
Asterias amurensis	D14358	D63724	D63725	Wada and Satoh (1994b) Wada et al. (1996)
Coscinasterias acutispina[1]	AB125601*	D63728	D63729	Wada et al. (1996)
Distolasterias nipon		D63734	D63735	Wada et al. (1996)
Aphelasterias japonica[1]	AB125600*	AB084576*	AB084575*	
Family Labidiasteridae				
Rathbunaster californicus	AF088807			Janies (2001)
Family Zoroasteridae				
Heliaster helianthoides	AF088804			Janies (2001)
Order Brisingida				
Family Brisingidae				
Brisingaster robillardi	AF088802			Janies (2001)
OPHIUROIDEA				
Ophioplocus japonicus	D14361			Wada and Satoh (1994b)
Ophiocanops fugiens	Z80954			Littlewood et al. (1997)
ECHINOIDEA				
Strongylocentrotus purpuratus	L28056	X12631	X03553	Jacobs and Grimes (1986) Jacobs et al. (1983)
Paracentrotus lividus		M16500	M16518	Cantatore et al. (1987)
ENTEROPNEUSTA				
Balanoglossus carnosus	D14359	AF051097	AF051097	Wada and Satoh (1994b) Castresana et al. (1998)

* Sequence determined in the present study.
[1] Specimens collected off the cost of Japan, [2] collected off Mauritius

In the present study, especially focusing on the relationship of *Asterina* and Solasteridae suggested by mitochondrial rDNAs (Wada *et al.*, 1996), we re-examined the phylogenetic relationships among asteroid species using nuclear marker: 18S rDNA. In order to disprove the laboratory contaminant suspected by Smith (1997), we re-sampled all the specimens.

MATERIALS AND METHODS

Biological materials and Isolation of genomic DNA
Eighteen species from nine families of asteroids (with asterisk in Table 1) were collected off the coast of Japan and Mauritius (Table 1), and processed to purify genomic DNA. In order to check the laboratory contaminant suspected by Smith (1997), all of these specimens were re-sampled from the field. Genomic DNAs were extracted from gonads or tube feet with a DNeasy Tissue Kit (Qiagen).

Sequencing strategy for 18S rDNAs
Approximately 1.8 kb of 18S rDNA were amplified by the polymerase chain reaction (PCR). The primers used for the amplifications have been described in Wada and Satoh (1994a). Amplification reactions were carried out in 20 µl volumes of a reaction mix with KOD-Plus-DNA polymerase (Toyobo). The temperature regime was 1 min at 94°C, 2 min at 50–60°C, and 5 min at 72°C for 35 cycles.

As the quantity of PCR products of some taxa was insufficient for direct sequencing, the sequences were determined after subcloned into plasmid vector. The amplified DNA fragment was purified by electrophoresis in a 1% agarose gel, and inserted into the vector pBluescript II SK (Stratagene). Multiple copies for the 18S rDNA gene are known to exist in the genome, and most of them maintain identical sequence by means of gene conversion. In some case, however, minor copies (which may be pseudogenes) may be picked up when PCR products are subcloned into plasmid vector (Wada, 1998). In order to avoid using sequences from these minor copies as representative of the species, we partially sequenced three independent clones, and confirmed that they had identical sequences. Since the KOD-Plus-DNA polymerase is a proof-reading DNA polymerase, we ignored errors due to misamplification during PCR. Sequencing was performed on an ABI prism 310 automatic sequencer using an ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems).

Sequencing strategy for mitochondrial rDNAs
Approximately 550 bp for mitochondrial (mt.) 16S rDNA, and 400 bp for 12S rDNA were amplified using primers reported in Wada *et al.* (1996). The temperature regime was 1 min at 94°C, 1 min at 50–60°C, and 1min at 72°C for 35 cycles. Amplified DNA fragments were purified by electrophoresis in a 1% agarose gel, and processed for direct sequencing.

Sequence Alignment and Phylogenetic Analysis
Sequences were aligned using the SeqApp 1.9 manual aligner for Macintosh (Gilbert, 1993). For 18S rDNA analysis, the nucle-
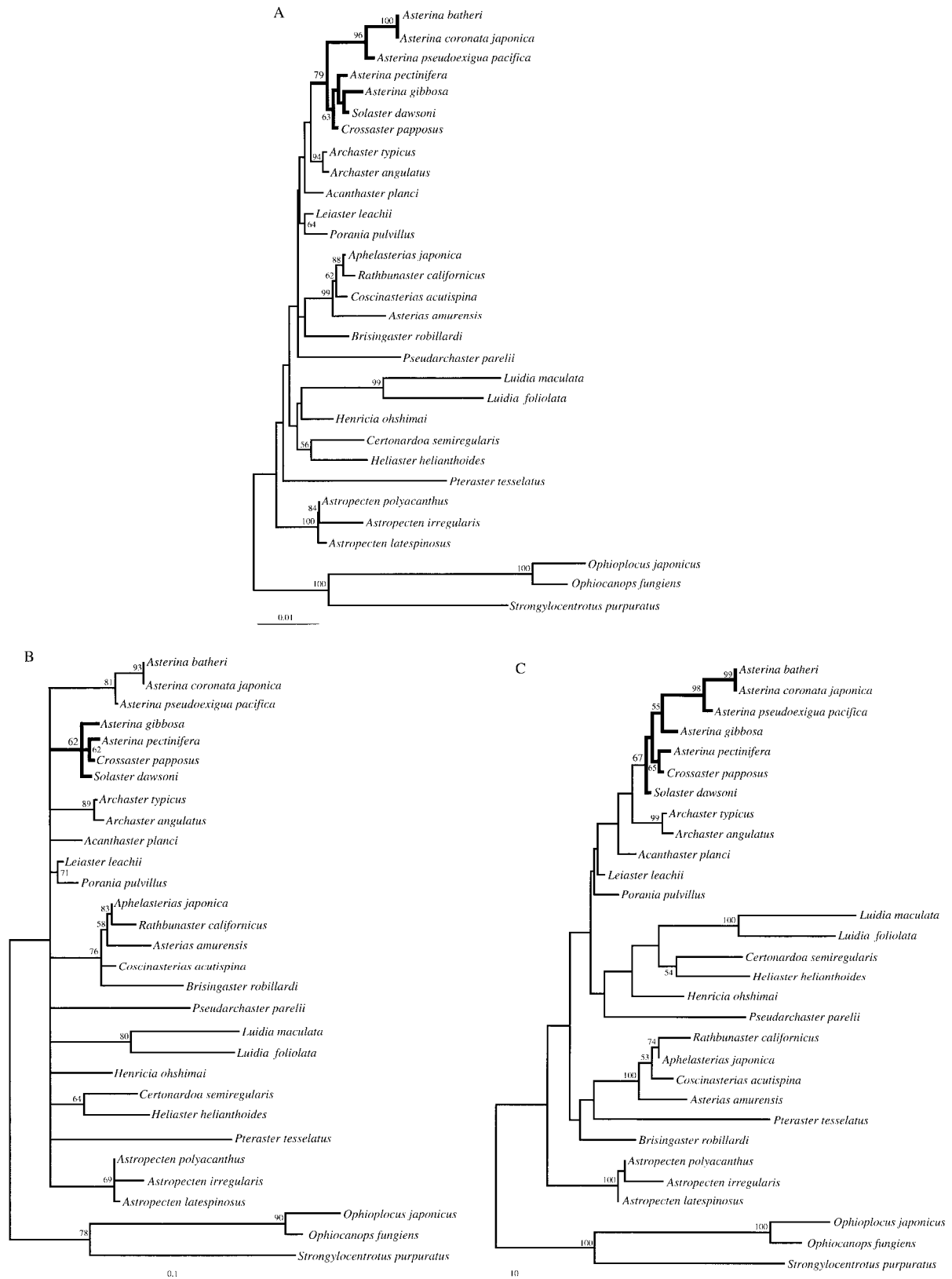
Fig. 1. Phylogenetic hypotheses deduced from analyses of 1669 confidently aligned 18S rDNA sites. (A) A phylogenetic tree obtained by the neighbor-joining method. The numbers (%) at the nodes are bootstrap values from 1000 replicates. The scale bar indicates the branch length. (B) A phylogenetic tree obtained by the maximum likelihood method. The numbers at nodes indicate the support values from quartet puzzling (%). (C) A phylogenetic tree obtained by the maximum parsimony method. The numbers at the nodes indicate bootstrap values from 100 replicates. Nodes with bootstrap of values of less than 50% are not shown.
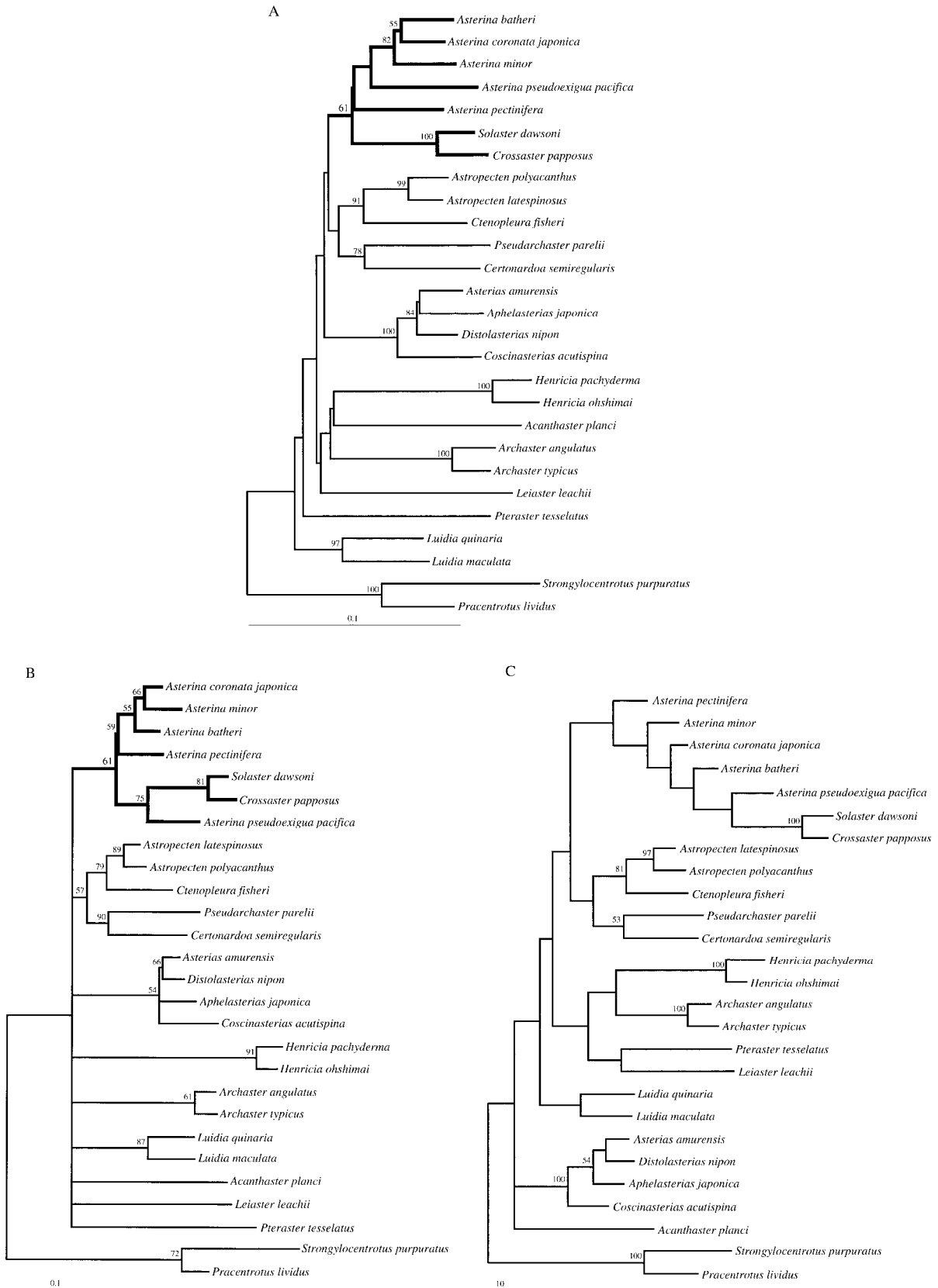
Fig. 2. Phylogenetic hypotheses deduced from analyses of 559 confidently aligned mt. 16S and 12S rDNA sites. Phylogenetic trees obtained by the (A) neighber-joining, (B) maximum likelihood and (C) maximum parsimony methods. The support for the trees is the same as in Fig. 1. In this MP analysis, each transversion is weighted as equal to two transitions.
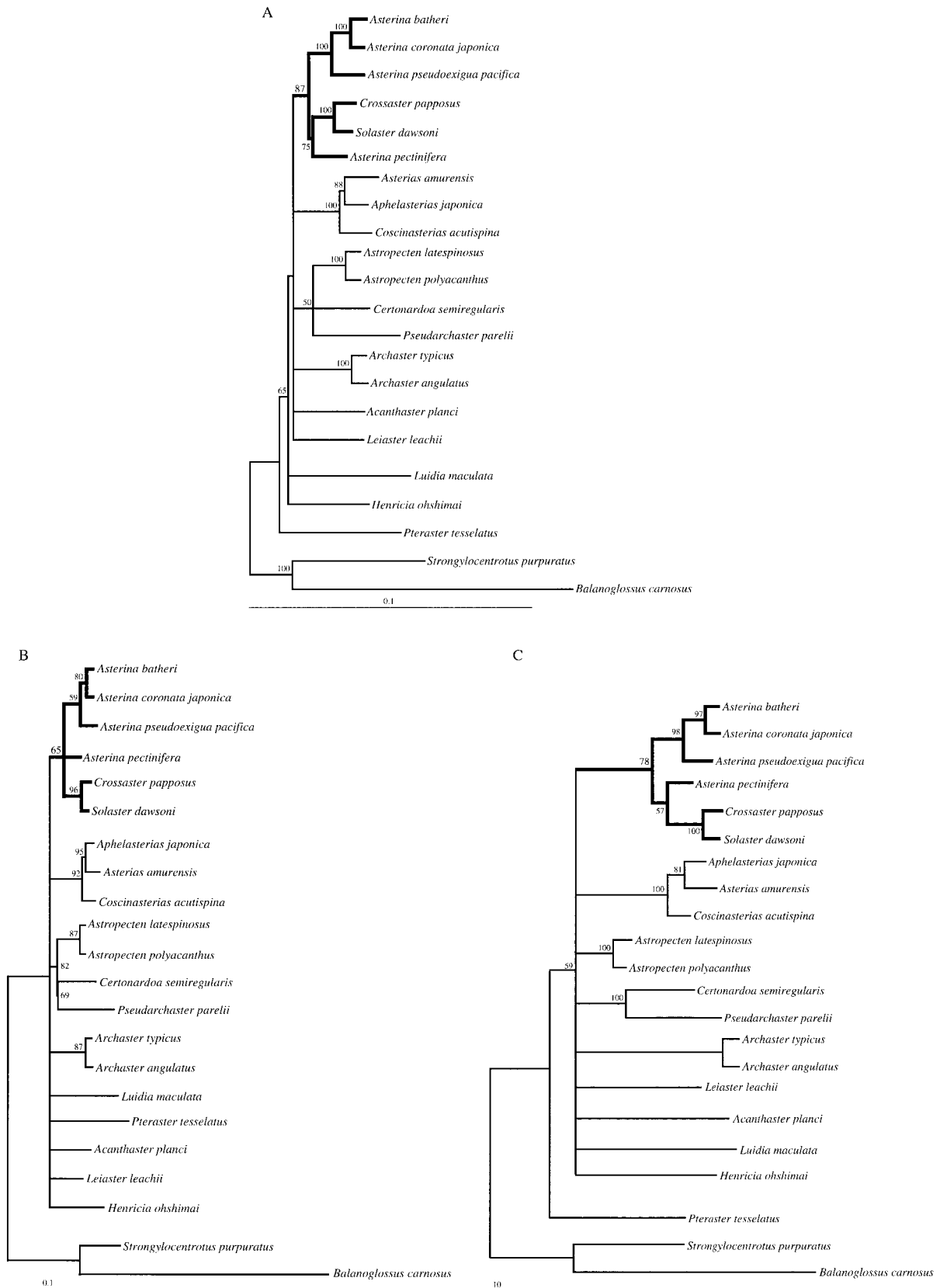
Fig. 3. Phylogenetic hypotheses deduced from analyses of nuclear 18S rDNA, mt. 12S and mt. 16S rDNA with 2327 confidently aligned sites. Phylogenetic trees obtained by the (A) neighbor-joining, (B) maximum likelihood and (C) maximum parsimony methods. The support for the trees is the same as in Fig. 1. In this MP analysis, each transversion is weighted as equal to two transitions.

otide sequences were aligned with outgroup sequences from two brittle stars, *Ophioplocus japonicus* and *Ophiocanops fugiens*, and one sea urchin, *Strongyrocentrotus purpuratus* (Table 1). Confidently aligned 1669 nucleotide sites were used for analyses. In mitochondrial 12S and 16S rDNA analysis, two sea urchin, *Strongylocentrotus purpuratus* and *Paracentrotus lividus* (Table 1) were used as outgroup. Confidently aligned 559 nucleptide sites were processed for phylogenetic analyses. In total dataset analysis combining 18S rDNA and mt. rDNAs, sequences were aligned with one sea urchin species, *Strongylocentrotus purpuratus* and one hemichordate, *Balanoglossus carnosus*.

Phylogenetic trees were constructed using the neighbor-joining (NJ; Saitou and Nei, 1987), maximum likelihood (ML; Felsenstein, 1981), and maximum parsimony (MP) methods. Clustal X (Thompson *et al.*, 1997) was used for the NJ method. Evolutionary distance was calculated according to Kimura's two-paramerter method (Kimura, 1980), and gaps and insertions were excluded from the analyses. ML analyses were performed with Tree-Puzzle 5.0 (Schmidt *et al.*, 2002). Substitution rate heterogeneity was corrected by introducing Gamma-distributed rates for variable sites. We followed the HKY85 model (Hasegawa *et al.*, 1985) for substitution process. PAUP 4.0b10 (Swafford, 2002) was used for the maximum parsimony analyses. MP analyses were constructed with transversion weighted equal to two transitions. The confidence for each node was estimated by bootstrap resampling (Felsenstein, 1985) for NJ and MP, and by quartet-puzzling for ML.

RESULTS

18S rDNA sequences

In this study, we determined the nucleotide sequences of 18S rDNA for 17 species included 9 families which cover five of the seven orders recognized in the classification by Clark and Downey (1992; Table 1).

Although 18S rDNA analyses have made a significant contribution for resolving phylogenetic relations (e.g. Wainright *et al.*, 1993; Aguinaldo *et al.*, 1997), some problems have been suggested to use rDNAs as a molecular marker. First, because rRNA functions by forming the secondary structure, the mutation rate is not uniform among sites (Wheeler and Honeycutt, 1988; Hills and Dixon, 1991). In order to overcome this problem, we performed ML analyses by correcting substitution rate heterogeneity introducing Gamma-distributed rates for variable sites. Second, heterogeneity of GC contents may also lead to a failure in recovering correct tree (Hasegawa and Hashimoto, 1993). We confirmed that the GC contents of the sequence used in the present analyses are between 51% and 56%.

Fig. 1 shows phylogenetic trees constructed by using the NJ, ML and MP methods. In these trees, the close affinities of species belonging to the same family, such as those in Luidiidae, Astropectinidae and Archasteridae, were well supported. In contrast, the confidence values for the branches linking different families were lower. Thus, 18S rDNA barely resolved the relationships among higher taxa.

A striking exception is a monophyly of the group that includes *Asterina* (Asterinidae) and two species of Solasteridae (*Solaster and Crossaster*). The monophyly of these groups is consistently supported by NJ and MP methods with relatively high bootstrap values. Although it is not sup-

ported by ML method, a close relationship between two Asterinidae species and two Solasteridae species is supported.

Mitochondrial rDNA sequences

Since affinity between Asterinidae and Solasteridae is suggested by Wada *et al.* (1996) based on mt. rDNA sequences, we analyzed mt. 12S and 16S rDNA sequence dataset with higher taxonomic density. About 550 bp of mt. 16S rDNA and 400 bp of 12S rDNA were sequenced for nine species in the present study (Table 1). The results are similar to those of 18S rDNA, namely, relationships between higher taxa were not resolved except for close relationship between *Asterina* and Solasteridae (Fig. 2). Although the early divergence of Luidiidae is supported by the previous analyses (Wada *et al.*, 1996), this conclusion is not supported in the present analyses of higher taxonomic density. The close relationship between *Asterina* and Solasteridae is recovered from all methods, although bootstrap support from MP analysys is lower than 50%.

The total dataset of 18S rDNA and mt. 16S and 12S rDNA sequences

Finally, we analyzed twenty species for which both nuclear 18S rDNA and mt. 12S and 16S rDNA sequences were determined (Table 1). Even in these analyses of the total dataset, the phylogenetic relationships among higher taxa were not resolved with satisfying confidence values (Fig. 3). However, the monophyly of the *Asterina* and Solasteridae is supported consistently in all trees by all three methods with higher confidence values than in the analyses described above (Fig. 3).

DISCUSSION

In this study, we tried to resolve the phylogenetic relationship of higher taxa in the Asteroidea by analyzing 18S rDNA sequences. Although even in the analyses where 18S rDNA sequences were combined with those of mt. rDNAs, most of the interfamilial relationships were not resolved, the close relationship between *Asterina* and Solasteridae is consistently supported with relatively higher confidence values. The independent results based on nucleotide sequences of nuclear 18S rDNA and mitochondrial rDNAs support the close relationship between the five *Asterina* species and two species of Solasteridae (one *Solaster* and one *Crossaster*) (Fig. 1, 2). This is even strongly supported when the two datasets were combined (Fig. 3). While nine genera are recognized in the family Asterinidae (Clark and Downey, 1992), we investigated only the genus *Asterina* from Asterinidae. Thus, we cannot be certain whether the family Asterinidae is a monophyletic group. Since, in the analyses by Knott and Wray (2000), monophyly of the Asterinidae is not supported, we should be careful to regard *Asterina* as a representative of the family Asterinidae.

Lafay *et al.* (1995) analyzed the first 400bp of 28S

rDNA in nine taxa. NJ and MP analyses supported the idea that Solasteridae species, represented by *Crossaster*, are more closely related to *Henricia* and *Echinaster* than to *Asterina*. However, the branches leading to *Henricia* and *Echinaster* are quite long, and this may disturb the position of these branches. In the ML tree, which is less sensitive to substitution rate heterogeneity, the sister group of *Asterina* and *Crossaster* was significantly supported. Thus, 28S rDNA analyses and the present results do not contradict each other. In analyses of mt. tRNAs and COI by Knott and Wray (2000), the close relationship between Asterinidae and Solasteridae is not recovered. However, in ML trees of their analysis, a sister group relationship between *Patiriella* (probably synonym of *Asterina*; Hart *et al.*, 1997) and Solasteridae is suggested, although with bootstrap values of less than 50%. In fact, the phylogenetic status of either Asterinidae or Solasteridae is barely resolved in Knott and Wray (2000).

From the aspect of morphology, there is little support for the affinity between Asterinidae and Solasteridae. Regarding the Solasteridae, Blake (1981, 1987) and Gale (1987) agreed on its close relationship with Pterasteridae. However, no affinity is suggested between Solasteridae and Pterasteridae in the present analyses, neither in Knott and Wray (2000). Rather, NJ and ML results of total dataset in the present study suggest that *Pteraster* branched off from the rest of the species in the early stage of the asteroid evolution. Regarding the phylogenetic status of the Asterinidae, Blake (1987) classified it into the superfamily Ganeriacea with the other two families (Ganeriidae and Poraniidae). Although Gareriidae is not included in the present study, we could not detect any signals that suggest the phylogenetic affinity between *Asterina and Porania.*

Blake (1981) re-examined the phylogeny of Spinulosida, and found significant differences of three families including Asterinidae to the rest of the families of the Spinulosida, especially in ossicle morphology of ambulacral column, and thus classified them into different order Valvatida. However, it should be noted that in the same article he also pointed that the presence of metapaxilae on abactical ossicles favors the affinity of Asterinidae to Solasteridae and Echinasteridae (Blake, 1981). Since the phylogenetic status of the Echinasteridae was not resolved in the present study, we hesitate to stress the metapaxillae as a phylogenetically useful character. Careful observation may be necessary to evaluate the present results from a morphological point of view, and on the other hand, more robust phylogenetic framework should be recovered from molecular phylogenetic studies in order to examine phylohgenetic usefulness of each morphological character.

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