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Phylogenetic Position of a Deep-Sea Ascidian, *Megalodicopia hians*, Inferred from the Molecular Data

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ABSTRACT—Ascidians inhabit both shallow water and the deep sea. The phylogenetic position of deep-sea ascidians has not been sufficiently investigated because of their unusual habitats. The family Octacnemidae is one such enigmatic deep-sea ascidian. In this report, we determined the sequences of the 18SrDNA and a mitochondrial protein gene of *Megalodicopia hians* belonging to the family Octacnemidae, and we analyzed its phylogenetic relationship with other ascidians. A phylogenetic relationship of this family with the families Cionidae and/or Corellidae has been suspected based on a small number of morphological characteristics. However, our results suggested that *M. hians* has a close relationship to the family Corellidae and might originate from them. This is the first report of the molecular phylogenetic analysis of a deep-sea ascidian.

Key words: deep-sea ascidian, molecular phylogeny, Octacnemidae, *Shinkai 2000*

INTRODUCTION

Ascidians (subphylum Urochordata, class Ascidiacea) are sessile marine invertebrates living both in shallow water as well as in the deep sea. The evolutionary relationships of deep-sea ascidians remain controversial, as potentially comparable morphological characteristics between them and other ascidians have been modified as presumable adaptations to their unusual habitats (Berrill, 1950; Kott, 1969). The family Octacnemidae is one such example of deep-sea ascidians. The octacnemids are thought to be a member of the suborder Phlebobranchia (order Enterogona; see Fig. 1) (Tokioka, 1953; Kott, 1985; Monniot *et al*., 1991), although their phylogenetic relationship with the other phlebobranchian families is a matter of controversy. At least two evolutionary hypotheses for the family have been proposed (see below). This issue has been difficult to resolve by considering only the morphological data.

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Molecular analysis based on DNA and amino acid sequences has proven very useful to confirm potential phylogenetic relationships between animals lacking similar morphological characteristics. To date, such analysis has not been undertaken to investigate deep-sea ascidians, since specimens suitable for molecular analysis could not be collected due to their extraordinary habitats and very delicate soft bodies. However, in 2001, fresh samples of an octacnemid ascidian, *Megalodicopia hians*, were collected by means of a submersible, *Shinkai 2000*. These specimens allowed amplification of almost the entire length of the 18SrDNA and a partial portion of a mitochondrial protein gene, and the nucleotide sequences of these portions of the genome were determined. In this report, we provide the results of phylogenetic analyses based on the molecular data and we discuss the systematic position of the family Octacnemidae within the larger ascidian group. This is the first report describing the molecular phylogenetic investigation of deep-sea ascidians.

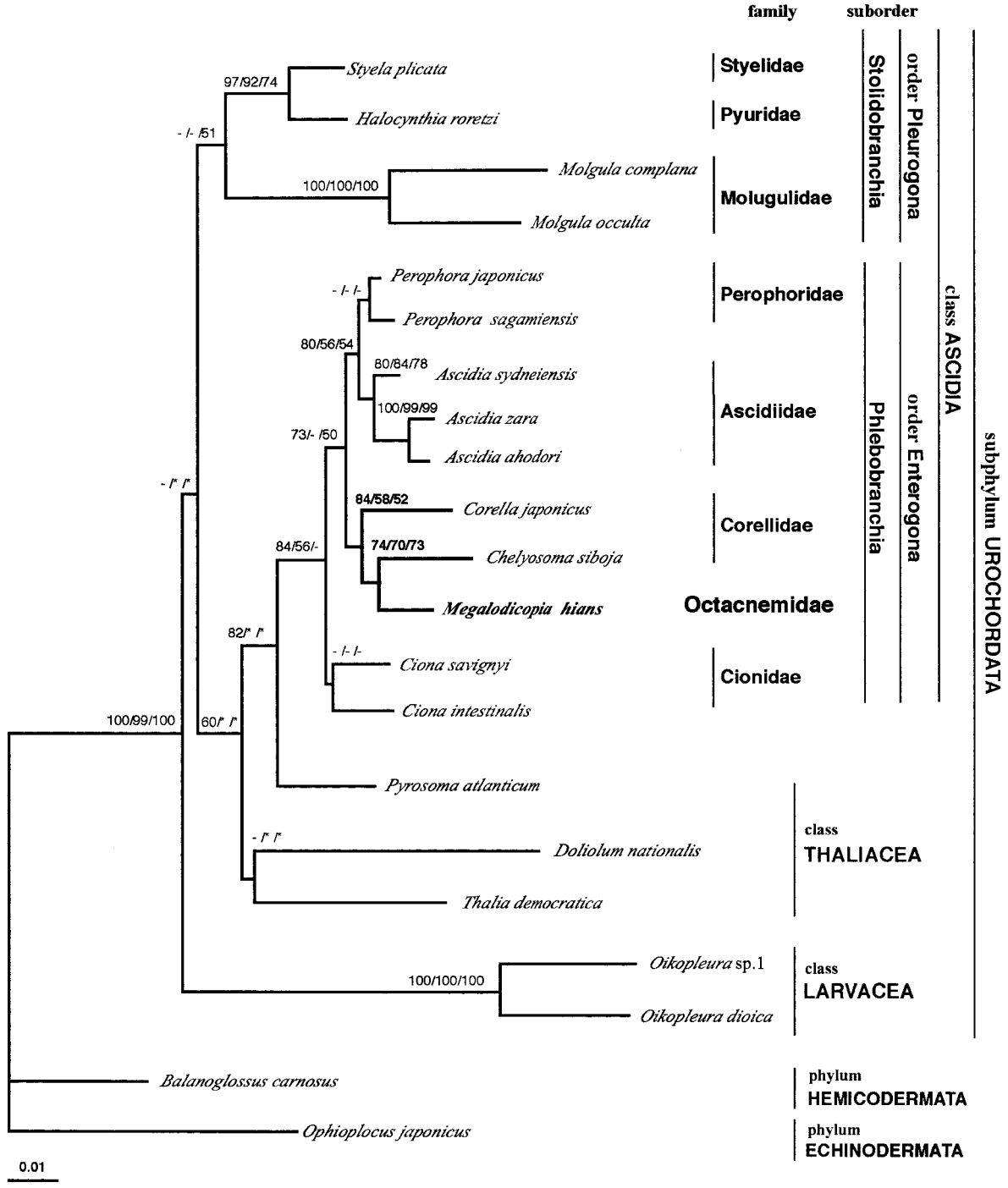

Fig. 1. Ascidian phylogeny estimated from 821 nucleotide sites of 18SrDNA. The NJ tree reconstructed by using the Kimura two-parameter distance is shown here. The numbers on each branch indicate BP values in the NJ/MP/ML trees. The values were calculated based on 1,000 resamplings in the case of NJ and MP and by 100 in the case of ML. Minus marks (–) denote < 50% bp values. Asterisks (*) indicate the nodes that did not appear in each MP and ML tree, or unresolved nodes in the majority consensus tree of 12 MP trees.

MATERIALS AND METHODS

Megalodicopia hians Oka 1918, was collected at Toyama Bay (37°04 N, 137°09 E, Japan Sea), at 841 m and 643 m deep, by means of a submersible, *Shinkai 2000*, of the Japan Marine Science and Technology Center (JAMSTEC). Immediately after collection, the gonad was dissected from a specimen and then fixed by 100% ethanol. From 20 mg of the fixed gonad, the total DNA was extracted with a DNA extraction kit (Qiagen). In order to amplify *M. hians* 18SrDNA, we performed a polymerase chain reaction (PCR) as described in Wada and Satoh (1994). The resulting fragment (approx. 1.8 kbp) of 18SrDNA was directly sequenced using the dideoxy chain termination method employing a cycle sequencing kit

(Pharmacia) and an automated sequencer ABI-377 (Perkin-Elmer). We also PCR-amplified a partial portion (approx. 0.8 kbp) of a mitochondrial protein gene, Cytochrome *c* oxidase subunit I (COI), from *M. hians* with a specific primer set for the ascidian COI gene (S. Yokobori, personal communication). The PCR reaction consisted of 35 cycles of 98°C for 10 sec, 40°C for 30 sec and 72°C for 1 min. The COI fragment was subcloned into pCR 2.1 vector (Invitrogen) and three subclones were sequenced. We collected four popular ascidian species and sequenced their 18SrDNA and COI sequences, as in *M. hians*. The novel sequences determined here were deposited into the DDBJ database (Table 1).

For the phylogenetic analyses, we created three alignment datasets. The first set of data included 821 nucleotide sites of 18SrDNA from 21 deuterostomian taxa; an acorn worm (*B. carnosus*) and a brittle star (*O. japonicus*) were treated as outgroups (see Fig. 1). This alignment was constructed according to Swalla *et al*. (2000) (available from the WWW site: http://chuma.cas.usf.edu/~garey/alignments/swjim2.nex). The second and third alignment datasets consisted of a nearly full portion (1,667 sites) of 18SrDNA and 260 amino acid sites deduced from the COI gene sequence, respectively. The 2nd and 3rd datasets included seven ascidians; five species represented each phlebobranch family, and the remaining two styellid species were used as outgroups (see Fig. 2). The alignments were created using ClustalW (Thompson *et al*., 1994). Gaps and ambiguous sites were excluded from each dataset by visual observation. The alignment data are available

Table 1. Species collected and sequenced genes in this study.

Species	length in base pairs (and Accession No.)	
	18SrDNA	COI
Megalodicopia hians	1,786 (AB075543)	805 (AB104866)
Chelysoma siboja	1,781[p] (AB104872)	805 (AB104867)
Ascidia ahodori	1,798 (AB104871)	802 (AB104870)
Perophora sagamiensis	1,720 (AB104873)	802 (AB104869)
Styela plicata	*PS*	805 (AB104868)

[p], partially sequenced in a previous study (Sawalla *et al*., 2000).
PS, previously sequenced (Hadfield *et al*., 1995).

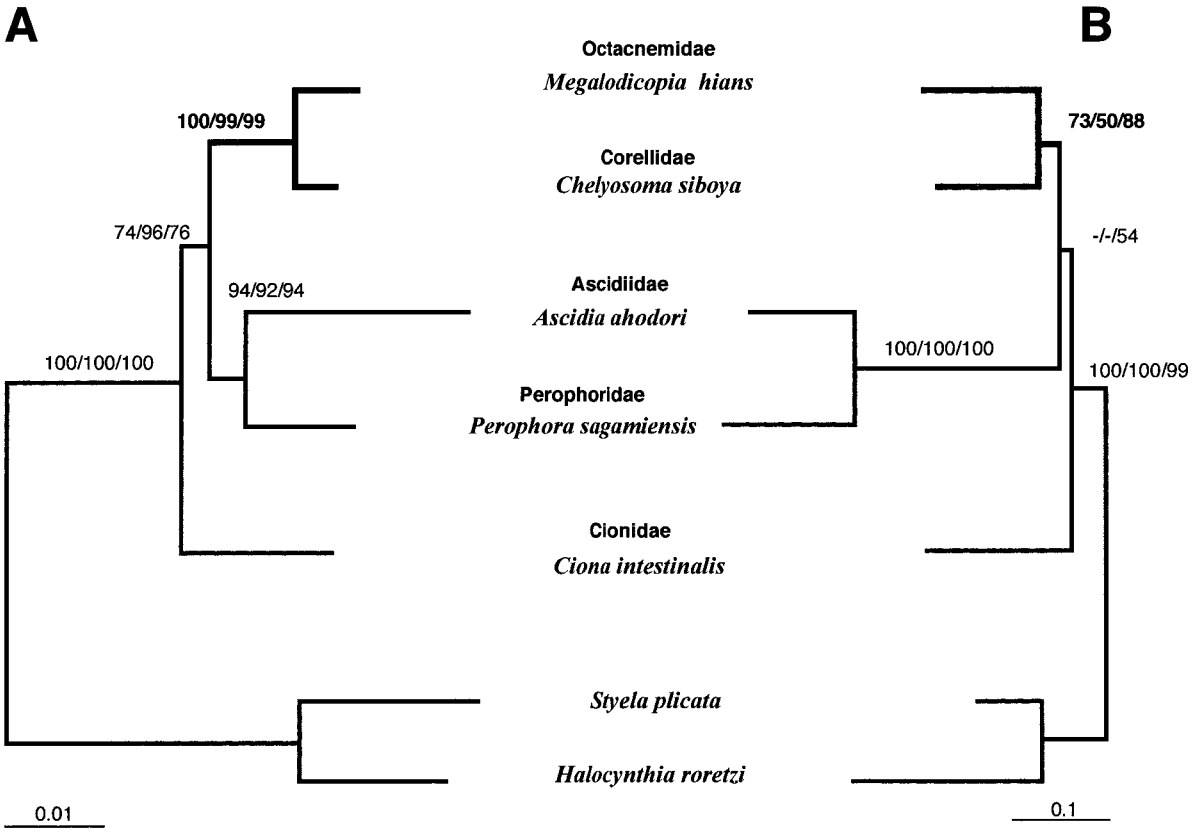

Fig. 2. Phylogeny of phelophobranch families inferred from two molecular datasets. **A.** Phylogenetic tree based on 1,667 nucleotide sites of 18SrDNA. The NJ tree with the Kimura two-parameter distance model is represented. **B.** Phylogenetic tree based on 260 amino acid sites of COI. The NJ tree obtained with the mtREV-F model is indicated. *H. roretzi* COI was reported in Yokobori *et al*. (1999). In both A and B, the bootstrap values are designated in the same manner as that used in Fig. 1.

Table 2. Substitution models tested by ML analyses and BP values supporting monophyly of the *M. hians* and corellids, as obtained by each model.

dataset	substitution models	BP value	
		Cor. Japonica + (*M. hians*+*Che. siboya*)	
821 nucleotide sites of 18SrDNA from 21 deuterostomes	HKY + α = 0.2 ^{ML}	52	73
	HKY + α = 0.5	51	75
	HKY + α = 0.72	51	71
	K2P	52	79
	JC69	61	64
1,667 nucleotide sites of 18SrDNA from 7 ascidians	HKY + α = 0.01 ^{ML}	no specimen	99
	HKY + α = 0.50	no specimen	98
	HKY + α = 0.72	no specimen	98
	K2P	no specimen	99
	JC69	no specimen	98
260 amino acid sites of COI from 7 ascidians	mtREV-F + α = 0.48 ^{ML}	no specimen	88
	mtREV-F	no specimen	100
	Dayfoff	no specimen	95

HKY, Hasegawa-Kishino-Yano; K2P, Kimura's two-parameter; JC69, Jukes-Canter; α, alpha-shaped parameter in gamma distribution; ^{ML}, the model in which the highest -ln L tree occurred and the BP values noted in each Figure. In HKY, TV/TS value, nucleotide frequency, and α were calculated from each dataset. Adding to the calculated value, α = 0.5 (PAUP default) and 0.72 (often used in animal 18S analyses) were also tested. Amino acid frequency and α were also calculated from the dataset.

from the WWW site: http://www.shimoda.tsukuba.ac.jp/~hassei/18SrDNA/align_ds_asc.html.

Based on the alignment datasets, phylogenic trees were constructed by neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods. The tree constructions were performed primarily with PAUP*4.0b10 (Swofford, 1998); however, NJ and ML analysis based on the amino acid data was carried out with Tree-Puzzle 5.0 (Schmidt *et al*., 2002) and Phylip 3.6 (Felsenstein, 2001). In the ML analyses, various substitution and gamma-distribution models were tested. The details of the model settings tested in this study are noted in Table 2.

RESULTS AND DISCUSSION

A nearly full length of 18SrDNA and a partial portion of the COI gene of *M. hians* were PCR-amplified, and their nucleotide sequences were determined. We also sequenced corresponding portions from 4 ascidian species. Information about the specimens and their sequences is noted in Table 1.

In order to clarify the phylogenetic position of the octacnemid among ascidians, we performed phylogenetic analyses. In many ascidians, only the central portion of the 18SrDNA approx. 1 kbp) has been reported (see Swalla *et al.,* 2000). For this reason, we initially performed analyses based on 821 nucleotide sites of 18SrDNA using the NJ, MP, and ML methods (Fig. 1). Fig. 1 shows the phylogenetic tree obtained by the NJ method. The ML tree (obtained with HKY+α=0.2, -ln L=3922.15) was the same as that obtained with the NJ method, except for the position of larvaceans and thaliaceans. In the MP analysis, the twelve parsimonious trees (with a length of 556) were generated, and their majority consensus tree differed slightly from that of the NJ due to the positions of the molgulids and thaliaceans (data not shown). Nonetheless, the trees we obtained were similar to each other and closely matched the results of recent studies of the molecular phylogeny of ascidians (Wada, 1998; Sawalla *et al*., 2000). Without reference to tree construction methods and evolutionary models, our trees resulted that phlebobranch ascidians produced a monophyly clade, in which *M. hians* was included. Of note in this context is that *M. hians* was included in the corellid ascidians. Furthermore, *M. hians* was monophyletic with the corellid species *Chelyosoma siboja*. Relatively high bootstrap confidence supported the corellid clade (84% in NJ) and the clade of *M. hians* plus *Che. siboja* (74, 70, and 73% in NJ, MP and ML; also see Table 2).

To confirm the close relationship between *M. hians* and the family Corellidae, further analyses were performed. We first reconstructed phylogenetic trees based on a long portion (1,667 sites) of 18SrDNA. The resultant NJ tree is shown in Fig. 2A. The topology of the NJ tree is the same as those obtained by ML (-ln L=3851.23) and MP (single parsimonious tree with a length of 273) methods. In these trees, the relationship among phlebobranch families is common to those of the reconstructs from short 18SrDNA data (see Fig. 1) and the monophyly clade of *M. hians* and *Che. siboja* also appeared. The sufficient BP value of the clade in each tree (100, 99, and 99% in NJ, MP, and ML; also see Table 2) indicated that the long 18SrDNA data strongly supports a close relationship of *M. hians* to the family Corellidae. This phylogenetic affinity was supported not only by the 18SrDNA data but also amino acid data from the mitochondrial COI gene. Based on the 260 amino acid sites on

the gene, all of the resultant trees also showed the monophyly clade of *M. hians* and the family Corellidae (NJ represented in Fig. 2B; ML, -ln L=1971.22; MP, a consensus tree of the 4 parsimonious trees with a length of 264). The BP value of this clade was relatively high in the NJ (73%) and ML (88%) trees, and the BP values of ML trees with different substitution models were significantly high (Table 2). The same clade was also constructed by a dataset of 520 nucleotides corresponding to the 1st and 2nd codon sites of the 260 amino acids (data not shown).

Previously, at least two contradicting hypotheses for the phylogenetic position of the family Octacnemidae have been proposed based on morphological studies. Kott (1969) suggested a close relationship between the families Octacnemidae and Corellidae, based on the reasoning that the characteristic gut-loops position, at the right or dorsal edges of the branchial baskets, is common to only these two families. On the other hand, Millar (1956, 1959, 1966) pointed out that the family Octacnemidae most likely evolved from an ancestral form such as *Ciona*, based on an observation of *Octacnermus bythius*; thus, the Octacnemidae might be related to the family Cionidae. Monniot and Monniot (1978) agreed with this view, although they suggested that the octacnemids might not be directly derived from the cionids. To date, the phylogenetic relationship of the octacnemids has remained obscure.

In the present study, we performed molecular phylogenetic analyses containing an octacnemid, *M. hians.* All resultant trees revealed that the species was located at a derived position in the phlebobranch lineage and was monophyletic with corellid(s) (Figs. 1 and 2). In contrast, the position of *M. hians* was far from that of the family Cionidae, as the cionid(s) seems to be the earliest diverged phlebobranch. Thus, as suggested by Kott (1969), our results strongly supported the notion of a close relationship between the family Octacnemidae and Corellidae. In addition to the close affinity, *M. hians* branched away from corellids in the trees, based on a partial 18SrDNA sequence (Fig. 1). This finding suggested that the octacnemids originated from the corellid ascidians, and consequently the family Octacnemidae might be a member of the Corellidae that has adapted to the bathyal fauna. However, at present, usable octacnemid and corellid samples are limited; the unique branch of *M. hians* was shown by a single set of data with a small number of nucleotide sites. Further molecular analyses will provide insight into the evolution of the family Octacnemidae and other obscure deep-sea ascidians.

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