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[Original report]

Phylogenetic relationships of several pearl oyster species in *Pinctada* (Pteriidae ; Bivalvia) inferred from mitochondrial 12S rDNA and cytochrome *c* oxidase subunit I genes sequences

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Abstract

Molecular phylogenetic analysis was carried out with mitochondrial (Mt) 12S ribosomal RNA (rRNA) and cytochrome *c* oxidase subunit I (COI) genes isolated from five species of the pearl oyster genus *Pinctada*. Nucleotide fragments of approximately 380-440 bp for Mt 12S rRNA and approximately 700 bp for Mt COI genes were amplified by polymerase chain reaction (PCR) using already reported primer pairs for both genes. After sequencing of the amplified PCR products, the phylogenetic relationships among the five species examined could be divided into three groups, namely *P. maxima and P. margaritifera, P. maculata* and *P. albina*, and *P. fucata*. In these species, three specimens of *P. fucata*, one from China and two from Japan (Okinawa and Ishikawa) shared high sequence similarity. These results agree well with the previous data reported from analyses of the nuclear genes of pearl oyster species.

Keywords : mitochondrial genes, 12S rDNA, COI, *Pinctada*, phylogenetic relationship

Introduction

The phylogeny of mollusks, heretofore mostly achieved by employing criteria on a morphological level, have remained controversial, particularly in case of closely related species such as those in the genus *Pinctada*.

The development of polymerase chain reaction (PCR) techniques opened a new age in DNA analysis, and became an alternative and very useful method in establishing phylogeny. Phylogenetic study has been focused on the sequence variations among the species of the genus *Pinctada* referring to the different DNA regions as follows, mitochondrial (Mt) 16S ribosomal RNA (rRNA) (Kobayashi and Masaoka, 2001; Masaoka and Kobayashi, 2005 a), nuclear 18S, 28S ribosomal RNA, internal transcribed spacer (ITS) regions (Masaoka and Kobayashi, 2002 ; 2003 ; 2004 ; 2005a ; 2005b ; He *et al*., 2005 ; Yu and Chu, 2005 ; 2006a ; Yu *et al*., 2005), intergenic spacer (IGS) regions (Masaoka and Kobayashi, 2005c ; 2006a) and other DNA regions (Masaoka and Kobayashi, 2005d ; 2006b ; Yu and Chu, 2006b). However, the confusion regarding the phylogeny of the genus *Pinctada* has yet to be

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completely resolved because of paucity of sequence information. It takes additional investigation with newly detected other DNA region to ensure consistency of phylogeny of them based on the molecular analyses.

The work described herein aimed to offer the representation and assessment of previous studies on the classification and phylogeny of the genus *Pinctada* with two kinds of mitochondrial regions, 12 S rRNA and cytochrome *c* oxidase subunit I (COI) genes. The 12S rDNA sequences have been widely used in phylogenetic studies among vertebrates. In addition, the COI gene region has been reported to be the most conservative protein-coding region in the mitochondrial genomes of animals (Brown, 1985). We believed it would be revealing to analyze this region to infer more clearly the phylogeny of the genus *Pinctada*. The results will provide the common view to elucidate the phylogeny of the genus *Pinctada*.

Materials and Methods

Sample collection and DNA preparation

The species analyzed and their collection sites are listed in Table 1. The specimens were collected in their original habitats, and taken alive to the laboratory. In regard to *P. fucata*, three specimens living in the different sea area were supplied to us for the purpose of solving the confusion on the classification of them. Mitochondrial (Mt) DNA was extracted from adductor muscle tissues of all samples analyzed by the modified TNES-Apatite method (Oohara *et al*., 2000). Small cubes of frozen muscle tissues were ground into pre-warmed extraction buffer (10 mM Tris, 125 mM NaCl, 10 mM EDTA, 4 M Urea, 1% SDS); then proteinase K (20 mg/ml) was added. After incubation at 56℃, 2 g of hydroxylapatite was added to trap DNA. Trapped DNA was then collected with phosphate buffer (1 M sodium phosphate, 0.2 mM EDTA), and Mt DNA was concentrated using QIAGEN-tip 500 (QIAGEN, NRW, Germany).

PCR amplification

An approx. 420 bp fragment of Mt 12S rRNA gene and an approx. 710 bp fragment of COI gene were amplified by PCR. Primers used for these analyses were as follows: F, 5'-GAAACCAGGATTAGATACCC-3'; R, 5'-TTTCCCGCGAGCGACGGGCG-3' (Arnaud-Haond *et al*., 2003) for the 12S rRNA gene, and LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3', HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al*., 1994) for the COI gene. PCR reactions were conducted with 10 ng/μl DNA in 1×PCR buffer containing 1 mM $MgSO_4$, 1.0 U/μl KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan), 200 μM dNTPs, and 0.3 μM of each primer. Thirty-five cycles of 15-sec denaturation at 94℃ (2 min for the first cycle), 30-sec annealing at 55℃ for the 12S rRNA gene and 50℃ for the COI gene, and 1-min extension at 68℃ were performed. Amplified PCR products were isolated on 2% agarose gels, excised under long-wavelength UV light, and extracted using a QIAEX Gel Extraction Kit (QIAGEN, NRW, Germany). The extracted PCR products were cloned into pGEM-T Easy Vectors (Promega, WI, USA). Vectors with insertions were transformed into JM 109 Competent Cells (Promega, WI, USA). After blue/white screening, the inserts of the vectors were sequenced.

Table 1. Localities of the specimens analyzed and GenBank accession numbers of the nucleotide sequences of the 12S rRNA and COI genes isolated from them. Three individuals of each species were analyzed.

Species	Locality	GenBank accession number	
		12S rRNA	COI
P. fucata martensii	Wild populations in Ishikawa, Japan	AB250257	-
P. fucata	Hatcheries in Guangdong, China	AB250258	-
P. fucata	Hatcheries in Aka Island, Okinawa, Japan	AB250259	-
P. maxima	Hatcheries in Aka Island, Okinawa, Japan	AB250255	AB259165
P. margaritifera	Wild populations in Amami Islands, Kagoshima, Japan	AB250256	AB259166
P. albina	Wild populations in Amami Islands, Kagoshima, Japan	AB250260	AB261165
P. maculata	Wild populations in Amami Islands, Kagoshima, Japan	AB250261	AB261166

DNA sequencing

DNA sequencing of the amplified genes was carried out using Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-Deaza-dGTP (Amarsham Biosciences, Tokyo, Japan). Sequencing was performed by the DSQ-1000L DNA Sequencer (Shimadzu Co., Kyoto, Japan). In consideration of individual variability in the level of several nucleotides, experiments were carried out for preparations from three different individuals, and obtained sequences were repeatedly checked for diminishing sequencing errors. Sequence data deposited with DDBJ database Libraries, derived on the basis of different individuals, were adopted in subsequent analyses. Sequence alignments were carried out using ClustalX (Thompson *et al*., 1997), with *Atrina pectinata* and *Crassostrea gigas* as the outgroups, which were selected referring to a report about the evolutionary relationships of bivalvia (Campbell *et al*., 1998).

Phylogenetic analyses

To properly justify our datasets, two kinds of sequence analyses by distance and parsimony were performed using the MEGA 3.1 software packages (Kumer, 2004), respectively. Genetic distances were calculated according to the two-parameter method of Kimura (1980), and the resulting matrix was used to develop a phylogeny of the species examined following the neighbor-joining (N-J) method (Saitou and Nei, 1987). Bootstrap values were obtained after 1000 permutations as mentioned by Felsenstein (1985). We chose the complete mitochondrial genome sequence of *Crassostrea gigas* (Accession No. AF 177226) as an outgroup.

Results

Sequence variations among species

In all samples analyzed, approx. 380-440 bp fragments of the 12S Mt rRNA and approx. 700 bp fragments of the COI genes were amplified by PCR and sequenced. Sequence data obtained were deposited with DDBJ database Libraries (Table 1), and sequence similarities were calculated as shown in Table 2.

According to the aligned sequence of 12S Mt

Table 2. Sequence similarities of the (A) 12S rDNA and (B) COI sequences among *Pinctada* species.

A

Species	Sequence similarity (%)					
	12S					
	A	B	C	D	E	F
A. *P. fucata martensii*						
B. *P. fucata* (China)	98.4					
C. *P. fucata* (Okinawa)	99.2	98.4				
D. *P. albina*	87.9	87.3	87.6			
E. *P. maculata*	87.9	87.6	87.9	99.7		
F. *P. maxima*	74.3	74.4	74.3	71.1	71.3	
G. *P. margaritifera*	79.9	78.9	79.4	77.0	77.3	81.3

B

Species	Sequence similarity (%)*				
	COI				
	A	B	C	D	E
A. *P. fucata*		95.4	94.4	89.4	91.2
B. *P. albina*	78.0		99.1	92.6	94.4
C. *P. maculata*	77.5	99.5		91.7	93.5
D. *P. maxima*	77.0	76.9	75.8		98.2
E. *P. margaritifera*	77.8	77.0	76.6	99.2	

* The values on the upper right are the levels of amino acid sequence similarity, and the values on the lower left are those of nucreotide one.

Fig. 1. Alignment of the nucleotide sequences of the Mt 12S rDNA in comparison with that of *P. fucata martensii* (in GenBank No. AB250257). Dots, nucleotides identical to those of the *P. fucata martensii* ; dashes, deleted nucleotides ; asterisks, nucleotides identical in all specimens. The specific insertion are underlined.

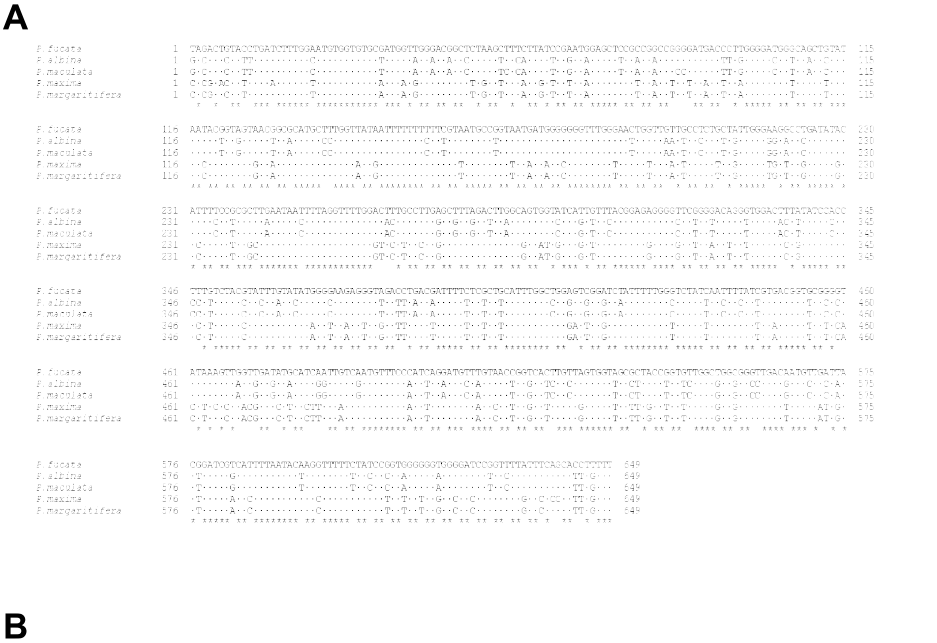

Fig. 2. Alignment of the nucleotide (A) and amino acid (B) sequences of the Mt COI gene in comparison with that of *P. fucata* (in GenBank No. DQ 299941). Dots, nucleotides identical to those of the *P. fucata* ; dashes, deleted nucleotides ; asterisks, nucleotides or residues identical in all specimens.

rDNA (Fig. 1), three specimens of *P. fucata* living in the different sea area were found to have almost identical sequences, with only 3-7 nucleotide variations. In addition, *P. albina* and *P. maculata* shared high homology in their nucleotide sequences. A striking similarity in the sequences between *P. maxima* and *P. margaritifera* was confirmed except for a specific insertion in those of the former species.

Regarding the analysis of *P. fucata*, the complete sequence of the gene deposited in DDBJ was used as a representative of the *P. fucata* species Libraries (Accession No. DQ299941 ; Zhang *et al*., unpublished data, a sample from China without description of the collection site). According to the aligned sequence of COI (Fig. 2), small sequence variations were detected in comparison with that of 12S rDNA. Thus, only 4 nucleotide variations were detected for the COI gene between *P. maxima* and *P. margaritifera*, and *P. albina* and *P. maculata*, respectively.

Phylogenetic analyses based on the mitochondrial gene sequences

To examine the phylogenetic relationships among closely-related species, it is important to define appropriate outgroup species. We focused on the mitochondrial sequences of *Pteria* species that are available on GenBank because it has been classified into Pteridae as well as *Pinctada* species. Unfortunately, GenBank data on the mt DNA of *Pteria* are confined to a certain region of COI gene which is not corresponding to that azalyzed in this study. Therefore, partial sequences of COI region derived from two *Pteria* species, namely *P. hirundo* (Accession No. AF120647) and *P. loveni* (Accession No. AB076925), had to be concatenated for adopting it as an outgroup in this study. However, such a method will not provide reliable and robust phylogenetic information. In fact, the phylogenetic analysis using the concatenated COI sequences of *Pteria* species as an outgroup, resulted in a taxonomically-perplexing phylogenetic tree.

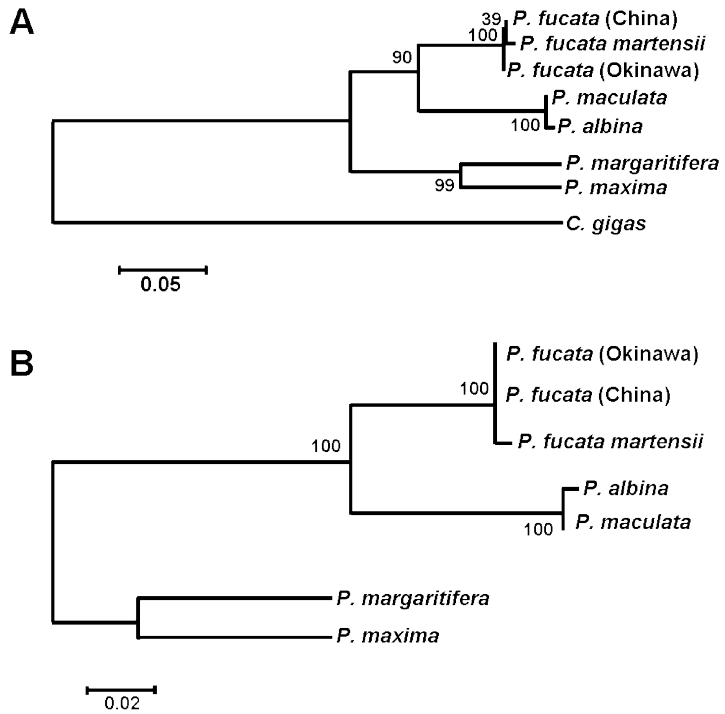

Fig. 3. Phylogenetic relationships of several species of *Pinctada* inferred from 12S rRNA gene sequences analyzed by neighbor-joining method. N-J tree was constructed with pairwise distances calculated following the application of Kimura's two-parameter correction for multiple substitutions. *C. gigas* (A) and *P. maxima* (B) were designated as the outgroups. Numbers represent bootstrap values of 1,000 replications.

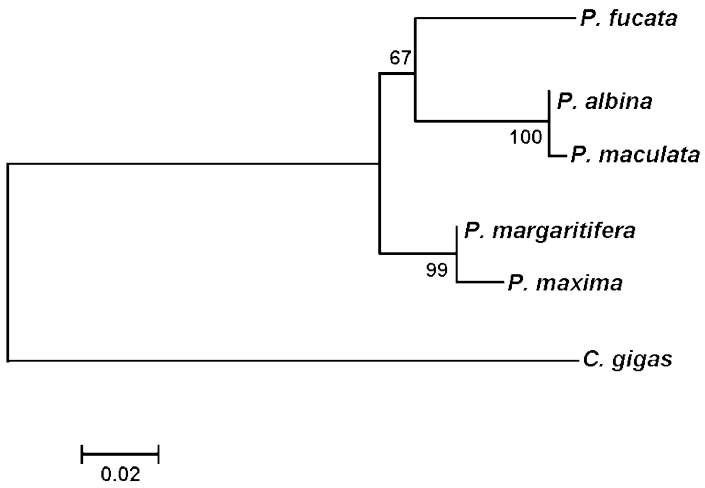

Fig. 4. Phylogenetic relationships of several species of *Pinctada* inferred from COI genes. Neighbor-joining tree was constructed with pairwise distances calculated following the application of Kimura's (1980) two-parameter correction for multiple substitutions. *C. gigas* was designated as the outgroup. Numbers represent bootstrap values of 1,000 replications.

Consequently, we chose the complete mitochondrial genome sequence of *Crassostrea gigas* (Accession No. AF177226) as an outgroup.

Fig. 3A shows the N-J tree obtained by exhaustive analysis of 12S data sets, including inferred gaps, using *C. gigas* as an outgroup. According to the N-J tree, the clade that branched firstly was estimated to be the group of *P. maxima* and *P. margaritifera*. The next branching might have occurred between the group of *P. albina* and *P. maculata*, and *P. fucata* group. The same topology was produced in the most parsimonious tree (data not shown). Another N-J tree based on the nucleotide sequence of 12S rDNA is shown in Fig. 3B with a topological positioning for *P. maxima* as an outgroup instead of *C. gigas*. According to this tree, the examined species were separated into two groups: 1) *P. albina* and *P. maculata* and 2) *P. fucata* group.

Fig. 4 shows the N-J tree estimated on the basis of the exhaustive analysis of the COI data, including inferred gaps, using *C. gigas* as an outgroup. Related to the analysis, only positions of the third codon were compared since they retain higher mutation rates than those of the other two codons in the genes encoding proteins. According to this tree, the species examined were divided into three groups supported by high bootstrap values, showing good coincidence with the results obtained from the ribosomal regions in this study.

Discussion

When we examine genetic differentiations and phylogenetic relationships among species, it is important to take individual variations within the same species into consideration. In the present study, experiments were carried out for preparations from three different individuals, and the obtained sequence were repeatedly checked for diminishing sequencing errors. Consequently, sequence homology of Mt 12S rRNA and COI genes among individuals were over 99% in all samples analyzed, implying that there were few individual variations between individuals for both regions.

Difference in the evolutionary rate among the regions of Mt DNA has not been completely determined in case of the pearl oyster species. The fact that the highest bootstrap values were calculated in the phylogenetic tree inferred from the 12S rDNA sequence enables us to conclude that the 12S rRNA region is suitable for speculations of the phylogenetic relationships of pearl oysters.

Import volume of the so-called Chinese pearl oyster, *P. fucata* of Chinese origin, has recently drastically increased and its importance has been growing for pearl production in Japan because of their high resistance to infection diseases (Wada, 2005). Conspecific position of the populations of *P. fucata* from China and Japan has recently been published in several papers based on the biochemical

or molecular population analyses (Wada, 1982; Kobayashi and Masaoka, 2001; Colgan and Ponder, 2002; Masaoka and Kobayashi, 2002; 2003; 2005a; Atsumi *et al*., 2004; Yu and Chu., 2006a; 2006b). Our phylogenetic placement of *P. fucata martensii* as a geographical niche of *P. fucata* supports these conclusions.

In the recent studies of molecular phylogeny of the pearl oysters, the topological position of *P. maxima* and *P. margaritifera* has been commonly placed at the most primitive position of the clade (Kobayashi and Masaoka, 2001; Masaoka and Kobayashi, 2002; 2003; 2005a; He *et al*., 2005; Yu *et al*., 2005). Masaoka and Kobayashi (2002, 2005a) considered that *P. maxima* might be evolved from the putative ancestral species of the genus *Pinctada*, and that *P. maculata* and *P. fucata* were then evolved from *P. maxima*, on the basis of sequence analysis of 16S rRNA, 18S rRNA and 28S rRNA genes in the samples collected from broad areas in the Atlantic and Pacific. *P. margaritifera* is speculated to be separately derived more recently from *P. maxima*, which is adaptable enough for the colder environment of the subtropical regions. They concluded that genus *Pinctada* might be differentiated from the ancestral species, living in tropical sea area, and after that, their larva widespread to northern area with help of the current. In addition, He *et al*. (2005) also grouped *P. maxima* and *P. margaritifera* in the basal position of the phylogenetic tree with *Crassostrea gigas* as an outgroup, based on their sequence analysis of the ITS-2 regions of rRNA genes of samples collected in southern China. Furthermore, Yu *et al*. (2005) reported similar results on the bases of ITS sequence analysis of the *Pinctada* samples from China, Japan and Australia, with *Pteria penguin* as an outgroup. Our analysis of Mt 12 S rRNA and COI genes in five *Pinctada* species from China and Japan with *C. gigas* as an outgroup clearly fortified the previously-reported phylogenetic relationship. It appears certain that *P. maxima* and *P. margaritifera* constructed one group being separable from the other group of *Pinctada*, even though determination of the ancestral species of *Pinctada* still remain unclear.

More detailed comparison of other regions in the mitochondrial and nuclear DNA among the genus *Pinctada* can inform us the process of evolution and the adaptive divergence of these species more clearly.

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