MARINE BIOTECHNOLOGY

Phylogenetic Analysis of Six Species of Pacific Abalone (Haliotidae) Based on DNA Sequences of 16s rRNA and Cytochrome *c* Oxidase Subunit I Mitochondrial Genes

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Abstract

Six species of abalones (Haliotidae) are found on the Korean coasts. Identification and characterization of these abalones are usually based on morphologic characters. In this research we compared the partial sequences of the mitochondrial 16S ribosomal RNA and cytochrome *c* oxidase subunit I genes to identify species using molecular data and to determine their phylogenetic relationships. Sequence alignments and phylogenetic analysis revealed that the 6 species fell into 2 distinct groups which were genetically distant from each other and exhibited little internal phylogenetic resolution. One group included *Haliotis discus hannai*, *H. discus discus*, *H. madaka*, and *H. gigantea*, while the other group contained *H. diversicolor supertexta* and *H. diversicolor diversicolor*. The 16S rRNA sequences were relatively more conserved than to the COI sequences, but both gene sequences provided sufficient phylogenetic information to distinguish among the 6 species of Pacific abalone, and thus could be valuable molecular characters for species identification.

Key words: 16S rRNA — cytochrome *c* oxidase submit I (COI) — molecular character — phylogenetic relationship

Introduction

Pacific abalones are economically important marine seafoods in Eastern Asia, with the majority of the annual yield now based on aquaculture production (Zhang et al., 1998). Six species of abalone have been reported to occur along the Korean coasts, *Haliotis discus hannai* Ino, *H. discus discus* Reeve, *H. madaka* Habe, *H. gigantea* Gmelin, *H. diversicolor supertexta* Reeve, and *H. diversicolor diversicolor* Reeve (Kim et al., 1988), but these species are quite similar in external morphology, and natural hybrids are frequently found (Miyaki et al., 1995). Traditional morphologic characters have led to conflicting species identifications, prompting us to examine systematic relationships among the species using molecular data to provide fundamental information for resource management and breeding in artificial crosses.

Traditional studies characterizing these species have relied on morphometric analyses (Ino, 1952). However, it is well known that such characters are environmentally influenced. Genetic studies using isozyme protein electrophoresis have also been employed (Fujino et al., 1979; Sasaki et al., 1980; Arai et al., 1982; Hara, 1992; Hara and Fujino, 1992a, 1992b); however, only a fraction of existing amino acid substitutions for soluble enzymes are resolved, and detecting variation in band patterns of the different developmental stages is difficult because of the amount of tissue required (Lester, 1978; Hedgecock et al., 1982; Lewontin, 1991).

With recent advances in biotechnology, the maximum information available for understanding genetic relationships can be obtained by looking directly at the order of nucleotides in the DNA chain (Kocher et al., 1989; Vacquier et al., 1990; McVeigh and Davidson, 1991; Lee and Vacquier, 1992, 1995's; Ovender et al., 1993; Domanico and Phillips, 1995;

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Table 1. Materials used, Geographical Origin, and Gene Sequences Obtained (GenBank Accession Numbers are Given)

Species	*Origin*	*16S rRNA*	*COI*
H. discus hannai	Pohang, Korea	AY146393	AF060847[a]
H. discus discus	Cheju island, Korea	AY146392	AY146398
H. madaka	Cheju island, Korea	AY146394	AY146399
H. gigantea	Cheju island, Korea	AY146395	AY146400
H. diversicolor supertexta	Cheju island, Korea	AY146396	AY146401
H. diversicolor diversicolor	Cheju island, Korea	AY146397	AY146402

[a]Our sequence is identical to that to already submitted by others.

SY. Lee et al., 2000; YH. Lee, 2000). The unique properties of nucleotide sequence polymorphism of mitochondrial DNA (mtDNA) can provide high-resolution information on the evolutionary relations between taxonomically bound families because mitochondrial genes evolve approximately 10 times faster than single-copy nuclear DNA (Brown et al., 1979); therefore, mtDNA is a useful molecular marker (Brown and Simpson, 1981; Barton and Jones, 1983; Aquadro et al., 1984; Palumbi and Cipriano, 1998).

Mitochondrial 16S rRNA gene and the protein-coding cytochrome *c* oxidase subunit I (COI) gene are reasonably well conserved, and have been sequenced in various invertebrate taxa (Brown, 1985; Bermingham and Lessios, 1993; Brower, 1994). To date COI has proved to be a robust evolutionary marker for determining intraspecific- and interspecific relationships in many marine mollusks (Clary and Wolstenholme, 1985; Baldwin et al., 1996; Hoeh et al., 1996; Boudry et al., 1998). Numerous studies have used 16S DNA sequences to resolve evolutionary relationships at different taxonomic levels (Palumbi and Benzie, 1991; Cunningham et al., 1992; Levinton et al., 1996; Sturmbauer et al., 1996). Thus the COI and 16S rRNA were chosen for this study because of their potential to be phylogenetically informative at lower taxonomic levels.

In the present study, genetic relations among 6 Pacific abalones were investigated using data from DNA sequences. These were obtained for parts of the mitochondrial 16S rRNA and COI genes.

Materials and Methods

Sample Collection. Table 1 lists in sources of materials used. Samples were collected between February and May of 1999 and again in 2000.

DNA Extraction, Amplification, and Sequencing. Total genomic DNA was extracted from mantle musculature of the abalones. The removed musculatures were minced and lysed by TNES-urea buffer (6 or 8 M urea, 10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10 mM EDTA, sodium dodecylsulfate 1% [SDS]) (Asahida et al., 1996). The genomic DNA was precipitated from the lysate with 100% ethanol. Following an ethanol wash the DNA was solubilized in water (or 8 mM NaOH). DNA was diluted with water to a final concentration of 20 ng/μl. The partial regions of the 16S rRNA gene and the COI gene were amplified by polymerase chain reaction (PCR) (Saiki et al., 1988). The primers used for the amplification of the partial 16S rRNA gene were 16SAR (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SBR (5′-CCGGTC TGAACTCAGATCACGT-3′) (Kessing et al., 1989). The primers used for the amplification of partial COI gene were F1 (5′-TGATCCGGCTTAGTCGGAACTGC-3′) and R1 (5′-GATGTGTTGAAATTACG GTCGGT-3′) (Metz et al., 1998). PCR reactions were done in 50-μl volumes. Each reaction contained 5 μl of 10X *EX TaqTM* buffer (Takara), 0.2 mM of each dNTP, 0.4 μM of each primer, 2.5 U of *EX TaqTM* polymerase (Takara), and 5 μl genomic DNA. Amplification was carried out in GeneAmp PCR System (Perkin Elmer). The temperature regimer used for the amplification of partial 16S rRNA gene was 36 repetitions of a 3-step cycle consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute 30 seconds. Denaturation for the first cycle was 4 minutes, and extension for the final cycle was 7 minutes. The temperature regimen of 35 cycles for partial COI gene was 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C with a first denaturation step of 1 minute at 94°C and a final extension step of 7 minutes at 72°C.

Amplification products were checked for size by loading 5 μl on a 2% agarose gel with 0.5 μl/ml ethidium bromide. The remaining product was purified by QIA quick PCR purification kit (Qiagen). These purified products were sequenced directly using the dye-terminator cycle sequencing reaction (PerkinElmer) with ABI DNA sequencer 377. Direct sequencing of the PCR products was performed on all samples. Sequencing reactions were performed according to the manufacturer's instructions. Primers used for the sequencing were the same as those for PCR. All final sequences were obtained from

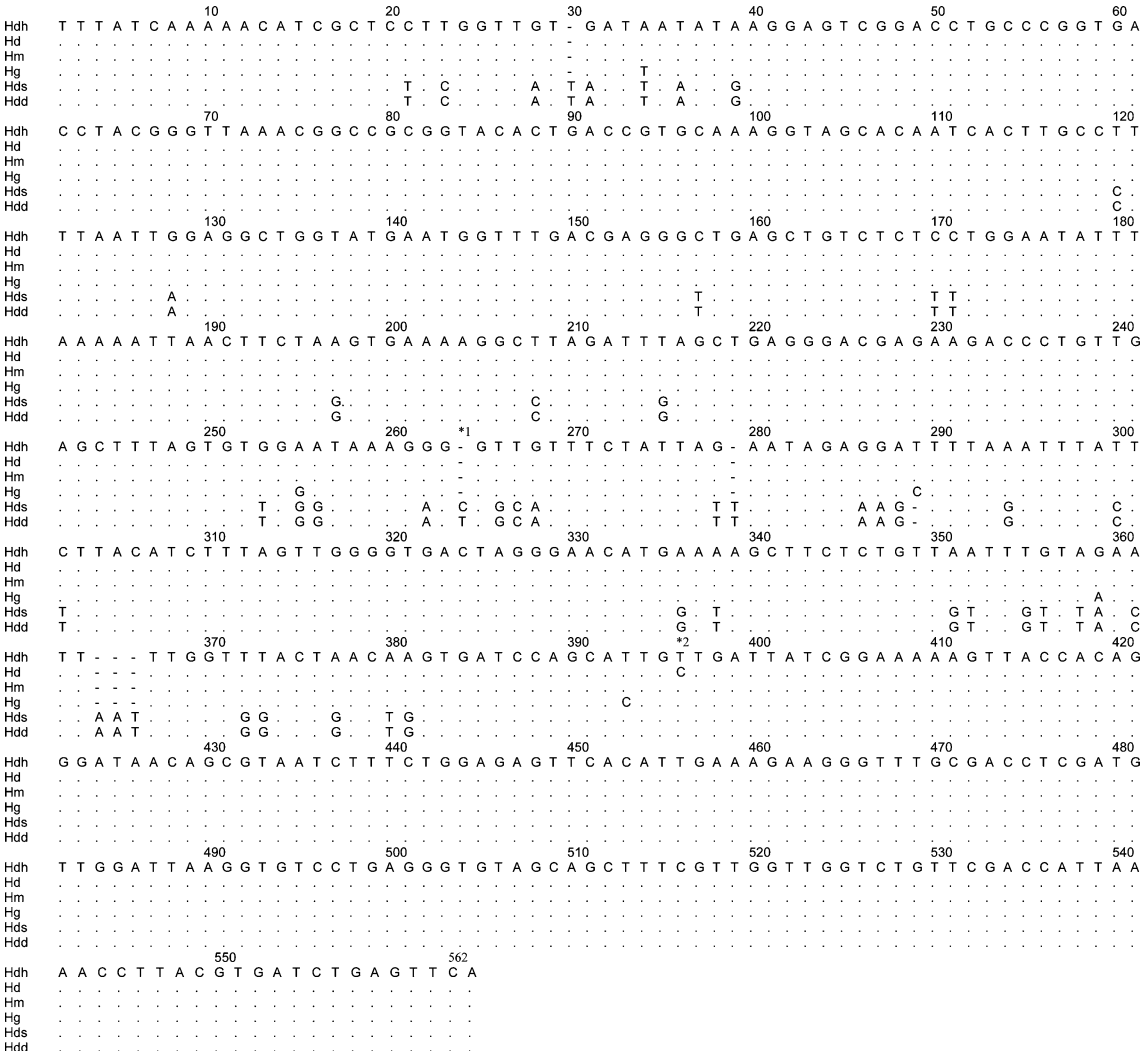

Fig. 1. Alignment of partial DNA sequences of the mitochondrial gene, 16S rRNA of 6 species of Pacific abalone. (Alignment gaps indicated by hyphen. Sites with a nucleotide identical to that on the top line indicated with a dot. Variations between closely related species in each group indicated by*1 and *2.)

both strands for verification. The partial mitochondrial COI and 16S rRNA genes were sequenced from 10 individuals for each of the 6 abalone species.

Sequence Analysis. Sequences were aligned using DNASIS Version 2.5 (DNASIS, 1994–1997) and submitted to GenBank under the accession numbers in Table 1. The extent of sequence difference between individuals was calculated by averaging pairwise comparisons of sequence difference across all individuals. The sequences of the 16S rRNA gene for the 6 species of abalone were aligned to yield a final alignment of 562 bp. The sequences of the COI gene for the 6 species of abalone were aligned to yield a final alignment of 544 bp. Percentages of sequence divergence were determined by dividing the number of variable sites by the total number of sites. Genetic distances were determined by the Kimura 2-parameter method (Kimura, 1980) using the software program MEGA (molecular evolutionary genetics analysis) (Sudhir et al., 2000).

Phylogenetic trees were constructed using the neighbor-joining method in MEGA. The data set was bootstrapped 1000 times to estimate the internal stability of the tree nodes.

Results

Alignment of sequences was simple and unambiguous for both gene regions. For phylogenetic analyses, only one sequence was included for species exhibiting no intraspecific variation. The partial 16S rRNA gene alignment (Figure 1) was 562 bases long including insertions and deletions. The similarity of nucleotide sequences among Pacific abalone species was 91.8% to 100%. Identical 16S rRNA sequences

Table 2. Pairwise Differences Among 16s rRNA Sequences

Species	*1*	*2*	*3*	*4*	*5*	*6*
H. discus hannai (1)	—	1/0	0	3/1	28/15	29/16
H. discus discus (2)		—	1/0	4/1	29/15	30/16
H. madaka (3)			—	3/1	28/15	29/16
H. gigantea (4)				—	27/14	28/15
H. diversicolor supertexta (5)					—	1/0
H. diversicolor diversicolor (6)						—

[a]Values above the diagonal are transitions/transversitions.

were obtained from *H. discus hannai* and *H. madaka*, and these differed from *H. discus discus* sequences at only a single site. *H. diversicolor supertexta* and *H. diversicolor diversicolor* sequences differed from one another at one site. These changes were codon third-position transitional substitutions. But these 2 species differed at many sites from *H. discus hannai*, *H. discus discus*, *H. madaka*, and *H. gigantea* (Table 2). Figure 2 shows relationships among the Pacific abalone species inferred from their partial 16S rRNA sequences.

The partial COI gene alignment (Figure 3) was 544 bases long. In contrast to the 16S rRNA sequence, no 2 species exhibited identical COI nucleotide sequences. There were 2 to 4 nucleotide differences among *H. discus hannai*, *H. discus discus* and *H. madaka*, representing a minimum of 0.37% and a maximum of 0.74% sequence divergence. All changes were codon third-position transitional substitutions. The above species differed from *H. gigantea* at many more sites, with most of the changes being third-position transitions. There were 12 to 14 nucleotide differences, representing 2.20% to 2.57% sequence divergence. *H. diversicolor supertexta* and *H. diversicolor diversicolor* sequences differed from one another at one site, but these two species differed at many sites from the remaining species. There were 107 to 109 nucleotide differences, representing 19.60% to 20.04% sequence divergence (Table 3). The tree in Figure 4 shows relations among the 6 Pacific abalone species based on the partial COI nucleotide sequences. The many nucleotide substitutions seen among the 6 species were all synonymous.

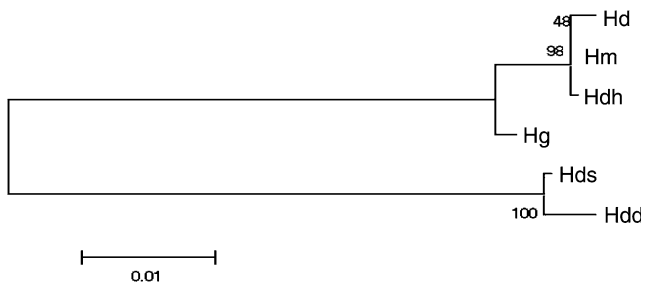

Fig. 2. Tree showing phylogenetic relationships among 6 species of Pacific abalone inferred from DNA sequences of the mitochondrial gene, 16S rRNA. A distance matrix was calculated using the Kimura's 2-parameter model and the tree was constructed using the neighbor-joining approach in MEGA. The data set was bootstrapped 1000 times, and the appropriate bootstrap values were placed on each branch.

Discussion

The 6 species of Pacific abalone were genetically distinct from each other and partitioned into at least 2 groups according to both the 16S rRNA and COI sequences. One group consisted of *H. discus hannai*, *H. discus discus*, *H. madaka*, and *H. gigantea*, and was further subdivided into *H. discus hannai*, *H. discus discus*, and *H. madaka* and *H. gigantea*. The former three species are less clearly defined from both genes data. The second group consisted of *H. diversicolor supertexta* and *H. diversicolor diversicolor* and exhibited relatively little molecular variation between the 2 species. The 2 groups are also distinguished from each other in terms of the major biological features, such as shell shape and the number of chromosomes. Members of the second group are comparatively small in size compared with other species (Yoo, 2000) and have a chromosome number of 2n = 32 compared with 2n = 36 in other species (Arai et al., 1982; Nakamura, 1985; Padermsak et al., 1988; Miyaki et al., 1999).

In general, the genetic divisions identified by our data correspond with the taxonomic subdivisions previously proposed based on morphologic characters. The division pattern based on the 2 mitochondrial gene sequences also agreed with that of the phylogeny constructed for the genus *Haliotis* using DNA sequences of the sperm lysin nuclear gene (Lee and Vacquier, 1995). The DNA sequences of *H. discus hannai*, *H. discus discus*, and *H. madaka*, and those of *H. diversicolor supertexta* and *H. diversicolor diversicolor*, are almost identical to each other based on both mitochondrial genes. Results based on isozyme studies and lysin cDNA

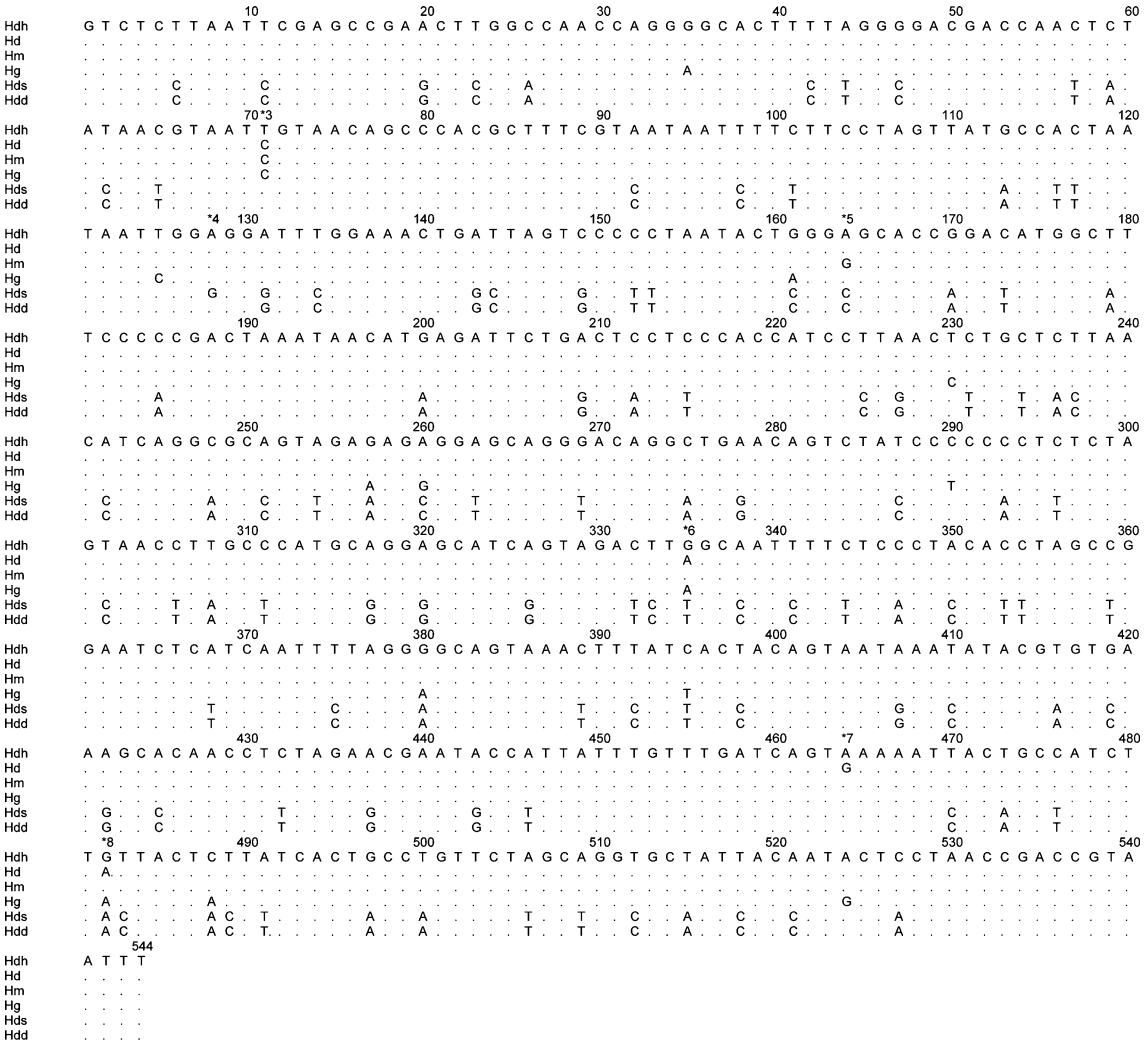

Fig. 3. Alignment of partial DNA sequences of the mitochondrial gene, COI of 6 species of Pacific abalone. (Alignment gaps indicated by a hyphen. Sites with a nucleotide identical to that on the top line indicated with a dot. Variations between closely related species in each group indicated by *3 to *8.)

sequence analysis also indicated the close genetic relationship among these species.

Hara and Fujio (1992a, 1992b) suggested the genetic distance between *H. discus hannai* and *H. discus discus* was at the level of local race based on isozyme studies, and that *H. madaka* might be only one population of *H. discus*. On the basis of morphologic characters, Weber (1928) assigned *H. supertexta* Lischke, 1882 as a synonym of *H. diversicolor* Reeve, 1846.

Lee and Vaquier (1995) were unable to distinguish between *H. diversicolor aquatilis* and *H. diversicolor supertexta* or between *H. madaka* and *H. discus hannai* based on lysin cDNA sequence analysis. They suggested that analysis of other molecular data such as mitochondrial DNA se-

Table 3. Pairwise Differences Among COI Sequences

Species	*1*	*2*	*3*	*4*	*5*	*6*
H. discus hannai (1)	—	4/0	2/0	13/1	66/41	67/41
H. discus discus (2)	0	—	4/0	11/1	67/41	68/41
H. madaka (3)	0	0	—	13/1	67/41	68/41
H. gigantea (4)	0	0	0	—	68/40	69/40
H. diversicolor supertexta (5)	0	0	0	0	—	1/0
H. diversicolor diversicolor (6)	0	0	0	0	0	—

[a]Values above the diagonal are transitions/transversitions. Those below are amino acid differences.

Fig. 4. Tree showing phylogenetic relationships among 6 species of Pacific abalone inferred from DNA sequences of the mitochondrial gene, COI. A distance matrix was calculated using the Kimura's 2-parameter model and the tree was constructed using the neighbor-joining approach in MEGA. The data set bootstrapped 1000 times and the appropriate bootstrap values were placed on each branch.

quences would be necessary to validate the abalone phylogeny based on lysin cDNA sequences.

Recently Sawabe et al. (2002) showed that amplified fragment length polymorphism (AFLP) analysis and repetitive extragenic palindrome (REP)–PCR fingerprinting techniques grouped the 47 *V. halioticoli*–like strains into 3 main clusters which roughly reflect the different species of host abalone from which they were isolated. Cluster 1 included the strains from *H. discus hannai*, cluster 2 from *H. discus discus*, and cluster 3 from *H. diversicolor aquatilis* and H. *diversicolor diversicolor*. This variation in the strength of host species specificity in the abalone gut–microbe relationship suggests that *H. discus hannai* and *H. discus discus* are less closely related than are *H. diversicolor aquatilis* and *H. diversicolor diversicolor*. Overall, it is clear that the genetic relationships among *H. discus hannai*, *H. discus discus*, and *H. madaka*, as well between *H. diversicolor aquatilis* and *H. diversicolor diversicolor* are very close and may require additional analyses based on more polymorphic nuclear loci to determine whether current species designations are accurate.

According to our molecular sequence data, consistent differences shared by 10 individuals per species were found. Position 395 of 16S rRNA sequences and positions 71, 164, 335, 464, and 482 of COI showed differences between *H. discus hannai*, *H. discus discus* and *H. madaka*, and position 263 of 16S rRNA sequences and positions 128 of COI revealed differences between *H. diversicolor aquatilis* and *H. diversicolor diversicolor* (see indels labeled *1 to *8 in Figures 1 and 3). Therefore these differences could be valuable molecular characters for distinguishing among these putative species.

The 16S rRNA and COI sequences of *H. gigantea* are similar to those of *H. discus hannai*, *H. discus discus* and *H. madaka*, but there are 4 to 5 nucleotide differences in the 16S rRNA and 12 to 14 in the COI gene between *H. gigantea* and the other 3 species (Tables 2, 3). These differences are significantly higher than those found among the closely related, *H. discus hannai*, *H. discus discus*, and *H. madaka*, but still represent only a 0.7% to 2.5% sequence divergence. Hara and Fujiro (1992a, 1992b) described the genetic distance between *H. gigantea* and the other 3 species sequences described above at the level of subspecies based on their isozyme studies.

The scientific names for the 6 species of Pacific abalones in the genus *Haliotis* considered here have often been confused. In 1983 Habe reported that the name *H. siboldii* Reeve 1840 was incorrect and that this species represented a variation of *H. gigantea* Gmelin 1791, and registered a new species, previously called *H. gigantea*, as *H. madaka*. In addition, *H. diversicolor aquatilis*, *H. diversicolor supertexta*, and *H. diversicolor diversicolor* are also controversial in their scientific names. K. Stewart proposed that *H. supertexta* Hischke 1882 and *H. aquatilis* Reeve 1846 are subspecies of *H. diversicolor* Reeve 1946 (Lee and Vacquier, 1995). In Japan *H. diversicolor aquatilis* and *H. diversicolor diversicolor* have been mentioned as the most important species with commercial potential (Seibutzudaizukan, 1986), but these same species are refered to as *H. diversicolor supertexta* and *H. diversicolor diversicolor* in Korea. In the illustrated book of the commercial mollusks from the freshwater and continental shelf in Korea, *H. diversicolor supertexta* and *H. diversicolor diversicolor* are reported as representative species, and it is suggested that *H. diversicolor supertexta* has been incorrectly called *H. diversicolor aquatilis*, a species that does not occur in Korea (National Fisheries Research and Development Institute, 1999). For resolving this dispute, morphologic or genetic comparison studies using molecular markers similar to those developed here will be necessary.

In this study the phylogenetic relationship among the 6 species of Pacific abalone inferred from 16S rRNA and COI gene sequences suggests that there are 2 groups of species (*H. discus hannai* to *H. gigantea*, Group A; and *H. diversicolor supertexta* and *H. diversicolor diversicolor*, Group B; Figures 2 and 4). Each group revealed little internal variation, suggesting that the species within each group are closely related. However, we found consistent sequence differences of one to 5 nucleotides among even the most closely related species, indicating the value of the 16S rRNA and COI markers for species identification. Of the 2 markers, the COI gene appeared to have the greatest phylogenetic resolving power.

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