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# RFLP Analysis of the mtDNA COI Region in Four Abalone Species

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The cytochrome *c* oxidase subunit I (COI) gene region of mitochondrial DNA (mtDNA) was examined in four abalone species to estimate its utility as a genetic marker using restriction fragment length polymorphism (RFLP) analysis. The utility was evaluated in terms of genetic divergence and relationships among *Haliotis discus hannai*, *H. rufescens*, *H. rubra*, and *H. midae* in both hemispheres of the world. There was clear genetic divergence in the mtDNA COI region between all pairs of the four species. Moreover, relationships among the abalone species were reflected in their geographical distributions and morphological characteristics. Therefore, RFLP analysis of the mtDNA COI region is a suitable genetic marker for the estimation of genetic divergence and relationships among abalone species. However, it is not effective for the evaluation of genetic differences within abalone species.

Key words: *Haliotis* spp., Abalone, Genetic divergence, mtDNA COI, Relationship, RFLP

## Introduction

Abalone species of the genus *Haliotis* are found along the temperate and tropical coasts of most continents and islands. Although abalone is a commercially important shellfish, its taxonomy and systematics remain inconclusive. Moreover, the number of valid species varies extensively depending on the author, e.g., 30 species (Dauphin et al., 1989), 65 species (Lindberg, 1992), 130 species (Cox, 1962), or 150 species (Pickery, 1991). Much of this confusion has resulted from the identification of abalone species mainly by shell morphology. The morphological characteristics of the Haliotidae appear highly conserved, and there is little doubt that they are a monophyletic group (Lindberg, 1992). Therefore, genetic markers are needed to reliably identify abalone species.

Many studies of genetic differences, mainly based on allozyme markers, have been conducted for abalone species (Hara and Fujio, 1992; Brown, 1993; Park and Kijima, 2005). However, this method is problematic because distantly related species cannot be compared, and the phylogenetic tree inferred from isozyme polymorphisms may sometimes differ from those based on nuclear or mitochondrial DNA (Karl and Avise, 1992). Moreover, allozyme markers show lower variability than DNA markers. DNA markers have become available as alternatives to allozyme markers for fast and reliable screening of genetic divergence among and within abalone species and populations (Lee and Vacquier, 1995; Kim et al., 2000, Park and Kijima, 2005). Thus, DNA markers have become essential tools for the genetic identification of abalone species.

Restriction fragment length polymorphism (RFLP) analysis of mtDNA has been used widely to study the genetic divergence and relationships between species and/or populations of fishes (Tabata and Taniguchi, 2000; Mamuris et al., 2001; Ikeda and Taniguchi, 2002) and shellfish (Boudry et al., 1998; Sugaya et al., 2002; Lee and Kim, 2003). The mtDNA is widely used for comparisons between related species, because mutations are accumulated more rapidly there than in most other nuclear regions (Martin and Palumbi, 1993). Intraspecific sequence variation within mtDNA has been used as a powerful tool for the examination of population structure in marine organisms (Carvalho and Pitcher, 1995). With the appropriate choice of gene segments, it is possible to study the DNA sequence variation among individuals local populations, and species (Kocher et al., 1989).

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The cytochrome *c* oxidase subunit I (COI) gene of mtDNA has a moderately conserved sequence variation in various invertebrate taxa (Baldwin et al., 1996; Hoeh et al., 1996). However, an RFLP analysis of the mtDNA COI region has not yet been performed for abalone species. Therefore, the purpose of this study was to estimate the utility of the mtDNA COI region in abalone species as a genetic marker using RFLP analysis. The utility was evaluated using genetic divergence and relationships among and within four abalone species from both hemispheres of the world.

## Materials and Methods

### Sample collection and amplification of mtDNA COI region

Four abalone species were collected from both hemispheres (Table 1). In the Northern Hemisphere, *H. discus hannai* was collected from the northeast coast of Japan and the Yellow Sea coast of China, and *H. rufescens* was collected from the west coast of the USA. In the Southern Hemisphere, *H. rubra* and *H. midae* were collected from the southeast coast of Australia and Cape Town, South Africa, respectively. All samples were collected from natural populations.

Total genomic DNA was extracted from hemolymph using a *SepaGene* kit (Sanko Junyaku, Japan) to avoid harming the abalone. DNA samples were stored at 4°C prior to polymerase chain reaction (PCR) analysis. For amplification of the mtDNA COI region, the following reagents were added to each microtube: 6 μL of template DNA; 5 μL of 10 x buffer (100 mM Tris-HCl [pH 8.3], 30 mM $MgCl_2$, 500 mM KCl); 1.0 μL of each primer (0.1 mM); 5 μL of 2 mM of each deoxyribonucleoside triphosphate (dNTP); and 0.5 units of *Taq* DNA polymerase (Takara, Japan). Deionized water was added to each sample to make up 50 μL. Primers used for the amplification of the mtDNA COI region were: forward primer (5'-TGA TCC GGC TTA GTC GGA ACT GC-3') and reverse primer (5'-GAT GTG TTG AAA TTA CGG TCG GT-3'; Metz et al., 1998). The PCR con-ditions consisted of 35 cycles of denaturation at 94°C for 1 min (1 min for the first denaturation only), annealing at 65°C for 2 min, and extension at 72°C for 2 min (5 min for the last extension only).

### RFLP analysis

The amplified samples were subjected to endonuclease digestion using 20 recognition enzymes: the four-base recognition enzymes *Hae*III, *Hha*I, *Msp*I, *Rsa*I, and the six-base recognition enzymes *Apa*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I (Toyobo, Japan). Digestions were performed directly in the PCR buffer at 37°C for at least 5 h. DNA fragments were separated on 2.0% MetaPhor agarose gel (BMA, Denmark), stained with ethidium bromide, and photographed.

### Nucleotide sequence divergence

The nucleotide sequence divergence calculated from the base substitutions per restriction site (*d*) among the haplotypes was estimated from a restriction site presence/absence matrix using the restriction enzymes and frequencies of each haplotype. The *d* values were calculated from the proportion of restriction fragments shared among haplotypes (*F*) using the fragment method of Nei and Li (1979). Mean nucleotide sequence divergences within (i.e., *dx* and *dy*) and between (i.e., *dxy*) species were calculated following the method of Nei and Tajima (1981). The net nucleotide sequence divergence ($d_A$) was calculated using the following equation: $d_A = dxy - (dx + dy)/2$.

A dendrogram was constructed based on the matrix of $d_A$ using an unweighted pair-group method with arithmetic averages (UPGMA) and the NEIGHBOR program, which accompanies the PHYLIP package (Felsenstein, 1995).

## Results

### Restriction fragment patterns and haplotype frequency

The sizes of the PCR-amplified fragments from the mtDNA COI regions of the four abalone species were approximately 590 bp (Fig. 1a). Polymorphisms of the restriction fragment patterns were observed bet-

Table 1. Collection localities and sample sizes of *Haliotis* spp.

| Hemisphere | Species | Location | Number of individuals |
|---|---|---|---|
| Northern | *H. discus hannai* | Miyagi, Japan | 48 |
| | *H. discus hannai* | Qingdao, China | 30 |
| | *H. rufescens* | California, USA | 5 |
| Southern | *H. rubra* | Tasmania, Australia | 5 |
| | *H. midae* | Cape Town, South Africa | 5 |

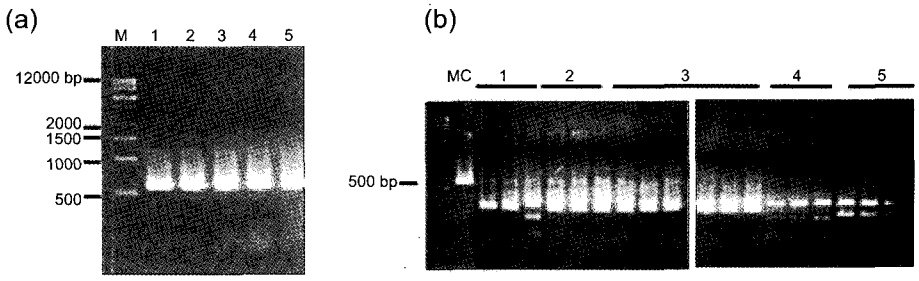


Fig. 1. (a) Amplified fragment (590 bp) of the mitochondrial DNA COI region of four abalone species. (b) Example of restriction fragment length polymorphism produced by digestion of the mitochondrial DNA COI region with *Hha*I for four abalone species. 1, *H. discus hannai* (Japan); 2, *H. discus hannai* (China); 3, *H. rufescens*; 4, *H. rubra*; 5, *H. midae*; M, DNA size marker (500-bp ladder); C, amplified fragment of mitochondrial DNA COI region (590 bp).

Table 2. Fragment patterns produced by the digestion of polymerase chain reaction products with endonucleases

| | Restriction morph and fragment size (bp) | | | |
|---|---|---|---|---|
| *Hha*I | *Hinc*II | *Msp*I | *Rsa*I | *Xba*I |
| A: 310+280 | A: 590 | A: 360+230 | A: 590 | A: 590 |
| B: 310+190+90 | B: 340+200+50 | B: 240+230+120 | B: 500+90 | B: 450+140 |
| C: 340+190+60 | | C: 270+230+90 | | |
| D: 340+250 | | | | |

ween species for five restriction enzymes: *Hha*I, *Hinc*II, *Msp*I, *Rsa*I, and *Xba*I (Fig. 1b). The molecular lengths of fragments were estimated for each restriction enzyme reaction (Table 2).

Digestion of the mtDNA COI PCR fragment with *Hha*I resulted in four patterns, that with *Msp*I resulted in three patterns, and that with *Hinc*II, *Rsa*I, and *Xab*I produced two patterns. For *Hha*I, A type (310 and 280 bp) from *H. discus hannai* and *H. rufescens*, C type (340, 190, and 60 bp) from *H. rubra*, and D type (340 and 250 bp) from *H. midae* were observed. In contrast, B type (310, 190, and 90 bp) was observed from only one sample of *H. discus hannai* from Japan. For *Hinc*II, A type (590 bp) from *H. discus hannai*, *H. rufescens*, and *H. midae*, and B type (340, 200, and 50 bp) from *H. rubra* were observed. For *Msp*I, A type (360 and 230 bp) from *H. discus hannai* and *H. rufescens*, B type (240, 230, and 120 bp) from *H. rubra*, and C type (270, 230, and 90 bp) from *H. midae* were observed. For *Rsa*I, A type (590 bp) from *H. discus hannai* and *H. rufescens*, and B type (500 and 90 bp) from *H. rubra* and *H. midae* were observed. For *Xba*I, A type (450 and 140 bp) from *H. discus hannai*, and B type (590 bp) from *H. rufescens*, *H. rubra*, and *H. midae* were observed. For all five restriction enzymes, the total size of the fragments of each restriction morph was 590 bp.

The composite haplotypes and their frequencies in the four abalone species are shown (Table 3). Haplotypes I and II were found only in *H. discus hannai*, haplotype III was found only in *H. rufescens*, haplotype IV was found only in *H. rubra*, and haplotype V was found only in *H. midae*. No common haplotype was observed among the four species. Therefore, the haplotype frequencies were observed as 1.000 within each species, except for *H. discus hannai* from Japan.

**Nucleotide sequence divergence and species relationships**

The number of shared restriction fragments and the net nucleotide sequence divergence ($d_A$) between species are shown (Table 4). There were one to six common restriction fragments among the species. The highest numbers of common restriction fragments were observed between *H. discus hannai* and *H. rufescens* from the Northern Hemisphere, and between *H. rubra* and *H. midae* from the Southern Hemisphere. The lowest number of common restriction fragments was observed between *H. discus hannai* and *H. rubra*.

The net nucleotide sequence divergence among four abalone species ranged from 0.0160% to 0.1824%. The highest net value was observed between *H. discus hannai* and *H. rubra*, and the lowest between *H. discus hannai* and *H. rufescens*. The mean net value between *H. discus hannai* and *H. rufescens* from the Northern Hemisphere was

Table 3. Haplotype frequency of four abalone species using the mitochondrial DNA COI region

| Haplotype | Composite haplotype[a] | *H. discus hannai* | | *H. rufescens* | *H. rubra* | *H. midae* |
|---|---|---|---|---|---|---|
| | | Japan | China | North America | Australia | Africa |
| I | (AAAAA) | 0.979 | 1.000 | - | - | - |
| II | (BAAAA) | 0.021 | - | - | - | - |
| III | (AAAAB) | - | - | 1.000 | - | - |
| IV | (CBBBB) | - | - | - | 1.000 | - |
| V | (DACBB) | - | - | - | - | 1.000 |

[a]Composite designations are: *Hha*I, *Hin*cII, *Msp*I, *Rsa*I, and *Xba*I.

Table 4. Percent mean nucleotide sequence divergence of four abalone species

| Species | *H. discus hannai* | | *H. rufescens* | *H. rubra* | *H. midae* |
|---|---|---|---|---|---|
| | Japan | China | North America | Australia | Africa |
| *H. discus hannai* | - | 6 | 6 | 1 | 2 |
| *H. discus hannai* | 0.0002 | - | 5 | 2 | 2 |
| *H. rufescens* | 0.0160 | 0.0339 | - | 3 | 4 |
| *H. rubra* | 0.1824 | 0.1284 | 0.0949 | - | 6 |
| *H. midae* | 0.1107 | 0.1155 | 0.0598 | 0.0475 | - |

Upper diagonal = number of shared restriction fragments; lower diagonal = net value between species ($d_A$).

0.0249%, that between *H. rubra* and *H. midae* from the Southern Hemisphere was 0.0475%, and that between both hemispheres was 0.1153%.

An UPGMA dendrogram for the four abalone species was constructed based on the matrix of the net nucleotide sequence divergence ($d_A$; Fig. 2). The degree of genetic divergence between the two species from the Northern Hemisphere was lower than that between the two species from the Southern Hemisphere. Among the four species, the two from the Northern Hemisphere (*H. discus hannai* and *H. rufescens*) were joined at 0.0125, the two from the Southern Hemisphere (*H. rubra* and *H. midae*) were joined at 0.0237, and the two hemisphere clusters were joined at 0.0576.

## Discussion

This is the first molecular survey to evaluate the genetic divergence and relationships among abalone species using RFLP analysis of the mtDNA COI region. The genetic divergence and relationships among the four abalone species examined were very similar to those obtained in previous analyses based on nuclear markers (Brown, 1993; Lee and Vacquier, 1995; Metz et al., 1998; Park and Kijima, 2005).

The isozyme analysis of genetic divergence among the four abalone species resulted in Nei's genetic distances between the two species from the Northern Hemisphere (*H. discus hannai* and *H. rufescens*) of 0.735, between the two species from the Southern Hemisphere (*H. rubra* and *H. midae*) of 1.931, and between the two hemispheres of 3.101. In contrast, the AFLP analysis of genetic divergence resulted in values of dissimilarity between the two species from the Northern Hemisphere of 0.459, between the two species from the Southern Hemisphere of 0.526, and between the two hemispheres of 0.792 (Park and Kijima, 2005). Thus, the dendrogram resulting from each analysis reflected the relationships among the geographical distributions and morphological characteristics of the abalone species.

There was clear genetic divergence between each pair of the four abalone species. Moreover, the topology of the dendrogram constructed from the net nucleotide sequence divergence ($d_A$) corresponded with that constructed from the nuclear markers; that is, the two species from the Northern Hemisphere produced one cluster, the two species from the Southern Hemisphere produced another cluster, and these two clusters were joined (Fig. 2). This indicates that RFLP analysis of the mtDNA COI region is effective for evaluating genetic divergence and relationships among abalone species.

However, RFLP analysis of the mtDNA COI region could not distinguish the genetic differentiation between two local populations of *H. discus hannai*. The frequencies of haplotype I in the two populations were 0.979 and 1.000, i.e., most individuals of the two populations possessed only haplotype I. Therefore, RFLP analysis of the mtDNA COI region is not effective for evaluating genetic differences within *H. discus hannai*. Low levels of genetic divergence within a species may reflect a variety of processes, including gene conversion, strong purifying selection, population bottlenecks,

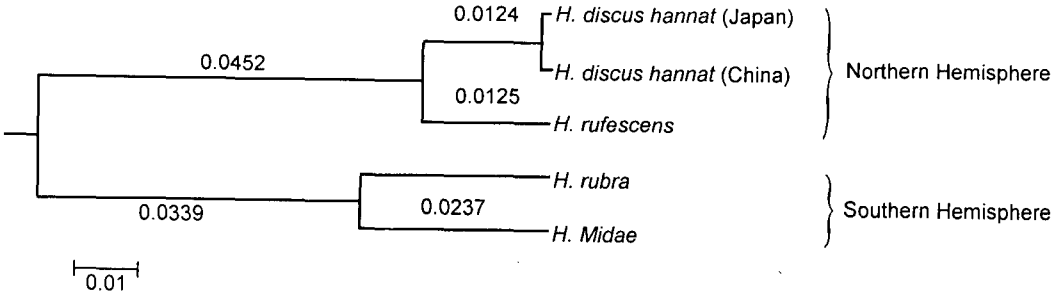


Fig. 2. Dendrogram derived from the net nucleotide sequence divergence ($d_A$) among four abalone species using an unweighted pair-group method and arithmetic averages.

selective sweeps resulting from adaptive evolution, and selection of linked loci in regions of low recombination (Berry et al., 1991).

Although we used only five samples per species, RFLP analysis of the mtDNA COI region was effective in detecting genetic divergence and relationships among the abalone species. This may be because specific genes occur in the mtDNA COI region. In contrast, a low level of divergence within abalone species may have been detected because the mtDNA COI region is extremely conserved within abalone species. To further examine these ideas, detailed surveys with larger numbers of samples and additional species are currently underway.

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