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Genetic Diversity in Cultured and Wild Populations of the Ascidian *Halocynthia roretzi* Inferred from Mitochondrial DNA Analysis

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Nucleotide sequences of about 500 bp from the 5' end of mitochondrial (mt) DNA Cytochrome Oxidase I (COI) were analyzed to estimate the genetic variation between wild and cultured populations of the ascidian *Halocynthia roretzi* from two sites along the coast of Korea. A total of 25 haplotypes were defined by 21 variable nucleotide sites in the examined COI region. Genetic diversity (haplotype diversity and nucleotide divergence) of wild populations was higher than that of the cultured population. These data suggest that reduced genetic variation in the cultured population may have results from bottleneck effect caused by the use of a limited number of parental stock and pooling of gametes for fertilization. Pairwise population F_{ST} estimates inferred that wild and cultured populations were genetically distinct. The combined results suggest that sequence polymorphism in the COI region would be preferable for estimating the genetic diversity of ascidian populations.

Key words: Ascidian, *Halocynthia roretzi*, Mitochondrial DNA, Cytochrome Oxidase I, Wild and cultured Populations

Introduction

Natural distribution of the ascidian (sea squirt) *Halocynthia roretzi* is essentially restricted to the waters around Korea and Japan, where it inhabits rocky littoral surfaces. This particular species has received considerable attention due to its high commercial value in Korea. The Korean aquaculture industry produced 9,300 tones of *H. roretzi* in 1007, worth approximately US$20 million (Agro-fishery Statistic Annual Report, Korean Ministry for Food, Agriculture, Forestry and Fisheries). Although the annual production of ascidians has been increasing in the past 20 yrs, mortality rates have also remained high due to an unexplained syndrome.

Potential genetic problems affecting culture populations of marine organisms have been previously discussed (Ryman and Laikre, 1991). The main concern is that cultured populations may develop genetic changes that would threaten the integrity of wild populations (Allendorf and Ryman, 1987). Cultivation for stock enhancement has also been identified as one of the factors responsible for the depletion of wild stocks due to interbreeding, competition, and spread of disease (Allendorf and Phelps, 1980). Furthermore, genetic variation is beneficial and important for the long-term survival of natural populations, because it can ensure the maintenance of a high level of fitness by giving the population the ability to adapt to changing environmental conditions (Frankel and Soulé, 1981). Thus, early detection of a loss of genetic variation in ascidian populations, as monitored by appropriate molecular markers, is very important for the prevention of undesirable effects. However, despite its importance to the fishery industry, there is a paucity of data on ascidian genetic information in Korea. Estimation of genetic variation within ascidian populations using molecular markers is vital for differentiation between cultured and/or wild individuals, for the identification of a population's genetic structure, and for developing potential management initiative.

The relatively recent development of new and highly variable genetic markers such as mtDNA has been widely adopted in population genetic studies (Avise, 1998; Zane et al., 2002). Maternally inherited mitochondrial (mt) DNA has higher sequence variability than most single copy nuclear genes, without recombination with nuclear DNA or heterologous

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mtDNA molecules (Brown et al., 1979). Recently, Sekino et al. (2002) detected certain level of variation in mtDNA control region by nucleotide sequence analysis, which was available to detect reductions of genetic variability in all the hatchery strains. Therefore, the analysis of mtDNA has become a method of choice for population genetic analysis of aquatic vertebrates and invertebrates.

The aim of the present study was to estimate genetic variation between wild and cultured populations of ascidian using sequence variation of COI gene in the mtDNA. We hope that our results will assist the design of management or conservation initiatives for breeding programs.

Materials and Methods

DNA extraction

DNAs samples were collected from 67 specimens, including 45 from the wild and 35 cultured individuals. Wild specimens were collected at depth of 10-25 meters using SCUBA (21 from Goseong and 24 from Gangneung), while cultured individuals were collected from farm at Gangneung between February 2008 and April 2008 (Fig. 1). The ascidians are cultured in the hanging culture method, basically same as that of oyster. Muscle tissues were preserved

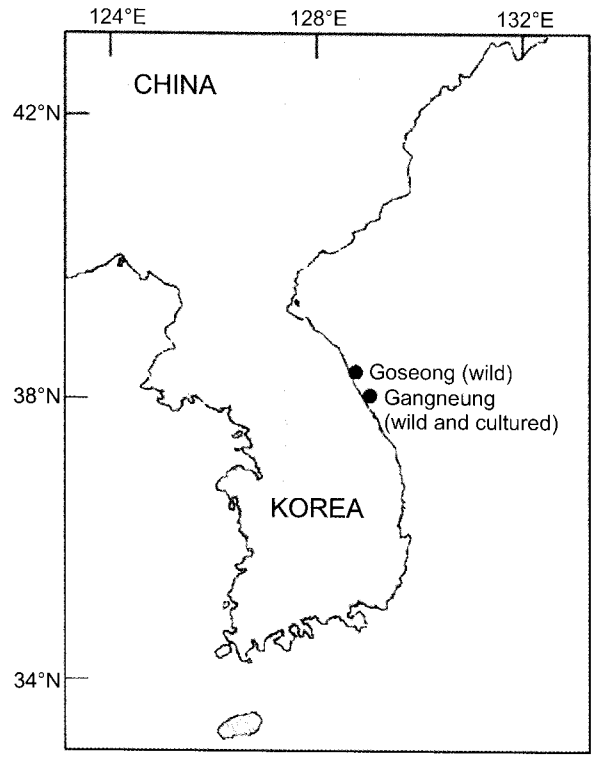

Fig. 1. Geographical locations of sampling sites for ascidian populations.

in 100% ethanol at room temperature until DNA extraction. Total genomic DNA was extracted using a conventional phenol-chloroform method (Sambrook et al., 1989). Extracted DNA was dissolved in 80 μL of Tris-EDTA buffer (TE; 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) until PCR amplification. An ultraviolet spectrophotometer (Shimadzu, Japan) was used to determine the quantity and quality of the isolated DNA. DNA concentration was estimated by measuring absorbance at 260 nm. Protein contamination was estimated by the ratio of absorbance at 260 nm and 280 nm.

PCR amplification and nucleotide sequence analysis

Primers and their sequences for PCR amplification of partial Cytochrome oxidase I (COI) gene and sequence reaction were newly designed based on reported sequences of *Halocynthia roretzi* (Yokobori et al., 1999; GenBank accession number AB024528). They included forward primer, Hr-COI_F, 5'-ATG TTAATGTGATTTTGCGTTGGTTT-3' and reverse primer, Hr-COI_R, 5'-ATCCATCCTTACAGTAAAT ATATGATG-3'. PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) after examining their size and quality with electrophoresis in 1.5% agarose-gel. The direct sequence was performed on PCR products obtained from amplification with the following reaction mixture and cycling condition, a 50 μL of reaction mixture containing 0.5-1 μL of template DNA, 1X PCR buffer, 1 unit of *Taq* DNA polymerase (Qiagen, Germany), 0.2 mM dNTPs and 25 pM each of forward and reverse primers. The cycling condition included pre-cycling denaturation at 94°C for 10 min, following by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, with final-extension at 72°C for 10 min. One μL of PCR products was used for sequencing reaction with a BigDye® Terminator Cycle Sequencing kit version 1.1 (ABI) according to the manufacturer's instruction. The obtained sequence data were aligned by the DNASIS software (Hitachi) to determine the genotypes (haplotypes) of the COI.

Population genetic data analysis

Haplotype and nucleotide diversities within populations, and nucleotide divergence between populations were estimated according to Nei (1987) and Nei and Tajima (1981) based on Kimura's two parameters distance method (Kimura, 1980) using K and DA program in REAP (McElroy et al., 1993). Pairwise F_{ST} values were calculated to estimate genetic distance between populations according to Slatkin and

Hudson (1991) using the Arlequin version 2.000 program package. Significance of F_{ST} values was tested via a permutation method (1023 permutations).

Results and Discussion

Estimated genetic variation and differentiation among specific populations using molecular genetic markers has become important to conservation and management of sustainable yields, and maintenance of genetic diversity (Dunham, 2004). The present study is the first to acquire baseline genetic data using population genetic analysis of ascidians in Korea with which stock production and breeding programs may be enhanced.

As shown in Table 1, direct sequencing of the PCR products revealed 501 bp sequences with 21 variable nucleotide sites defining a total of 25 haplotypes. The nucleotide sequences of the 25 haplotypes were deposited in GenBank under accession numbers FJ590928-FJ590952. The observed 21 variable nucleotide sites of the COI contained 13 transitions and 8 transversions. Substitution at each site was biallelic, suggesting the occurrence of single base substitution between sequences and no saturation of substitutions. Given the observed sequencing variation in the COI region and their highly reproducible sequencing throughput, more variation in this region may be expected as the number of individuals increased, which therefore would be preferable for estimating the genetic diversity of ascidian populations.

The distribution of 25 haplotypes between wild and cultured populations is presented in Table 2. HRO01 and HRO05 haplotypes characterized the Gangneung wild and cultured populations. In addition, HRO02, HRO12, HRO18, HRO24, and HRO25 were specific to cultured population. Hence, HRO03, HRO09, HRO11, and HRO13-HRO17, and HRO19 were specific to the Goseong wild population, and HRO20-HRO23 occurred in only the Gangneung wild population. These results suggest that observed haplotypes were non-randomly distributed, although 7 of the 25 haplotypes were shared between wild and cultured populations.

As shown in Table 3, both haplotype and nucleotide diversity of cultured populations was lower than those of the wild population, suggesting loss of genetic variation in the cultured population. This result might be associated with non-random mating, small effective population sizes and increased bottleneck effect, although no historical data were available for genetic profiles of this cultured species. Several studies using molecular markers have shown that cultured organisms with high fecundity tend to result in a reduction of genetic variability when compared

Table 1. Variable nucleotide positions in the 5' half of mtDNA COI region

Haplotype	24	25	28	33	37	39	46	133	187	197	262	331	334	370	373	382	385	400	409	442	478
HRO01	C	A	G	T	G	C	A	C	T	C	G	T	T	G	A	T	T	T	A	G	T
HRO02	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	C	·	·	·
HRO03	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·
HRO04	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HRO05	·	G	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	C	·	·	·
HRO06	·	G	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·
HRO07	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
HRO08	·	G	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·
HRO09	·	G	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·
HRO10	·	G	T	·	·	·	·	·	C	·	·	·	·	·	·	C	·	·	·	·	·
HRO11	·	G	T	·	·	·	·	·	C	·	·	·	·	·	·	C	·	·	·	·	A
HRO12	·	G	T	·	·	·	·	·	C	·	·	·	·	·	·	C	C	·	·	·	·
HRO13	·	G	T	·	·	·	·	T	C	·	·	·	·	·	·	C	·	·	·	·	·
HRO14	·	G	T	·	·	·	·	·	C	·	·	A	·	·	·	C	·	·	·	·	·
HRO15	·	G	T	·	·	·	·	·	C	·	·	A	·	·	·	C	·	·	G	·	·
HRO16	·	G	T	·	·	·	·	·	C	·	·	·	·	·	C	C	·	·	·	·	·
HRO17	·	G	T	·	·	·	·	·	C	·	·	·	C	·	·	C	·	·	·	·	·
HRO18	·	G	T	·	·	·	G	·	C	·	·	·	·	·	·	C	·	·	·	·	·
HRO19	·	G	·	·	A	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·
HRO20	·	G	·	·	·	T	·	T	·	A	·	·	·	·	·	·	·	C	·	·	·
HRO21	·	G	·	G	·	T	·	T	·	·	·	·	·	·	·	·	·	C	·	·	·
HRO22	·	G	·	G	·	T	·	·	·	A	·	·	·	·	C	·	·	·	·	·	·
HRO23	·	G	·	G	·	T	·	·	C	·	A	·	·	·	·	C	·	·	·	·	·
HRO24	A	G	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	C	·	·	·
HRO25	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

Dots indicate the nucleotide identical to that in the HRO01.

Table 2. Distribution of COI haplotyps in 3 populations. Symbol "-" indicates no detection

Haplotype	Wild		Cultured
	Goseong	Gangneung	Gangneung
HRO01	-	4	11
HRO02	-	-	1
HRO03	1	-	-
HRO04	4	5	3
HRO05	-	4	5
HRO06	1	-	4
HRO07	1	5	5
HRO08	1	-	1
HRO09	1	-	-
HRO10	5	2	1
HRO11	1	-	-
HRO12	-	-	1
HRO13	1	-	-
HRO14	1	-	-
HRO15	1	-	-
HRO16	1	-	-
HRO17	1	-	-
HRO18	-	-	1
HRO19	1	-	-
HRO20	-	1	-
HRO21	-	1	-
HRO22	-	1	-
HRO23	-	1	-
HRO24	-	-	1
HRO25	-	-	1

Table 3. Number of total haplotypes, haplotype diversity (h) and nucleotide diversity ($\pi \pm SD$) in wild and cultured ascidian populations

Population	Total number of haplotypes	h	$\pi \pm SD$
Wild			
Goseong	14	0.9158	0.058 ± 0.0035
Gangneung	9	0.8804	0.058 ± 0.0035
Cultured			
Gangneung	12	0.8588	0.0047 ± 0.0029

with wild populations (Hedgecock and Sly, 1990; Lundrigan et al., 2005). However, wild populations showed high genetic variation with more than 0.85 value of haplotype diversity and weak but moderate value of nucleotide divergence (more than 0.0040). This suggests that examined ascidian populations exhibit an expected signature for a stable and large population, while they also might be observed in an admixed sample of individuals from historically sundered populations (Avise, 2000).

Pairwise F_{ST} estimates revealed significant differrences between wild and cultured populations, and between the two (Goseong and Gangneung) wild populations (Table 4), suggesting that there is pronounced genetic differentiation between wild and cultured populations. These results suggest low gene

Table 4. F_{ST} values between samples (below diagonal) and Symbol "+" indicates significance with $P<0.05$ (above diagonal). Number of permutations was 1023

	Wild		Cultured
	Goseong	Gangneung	Gangneung
Wild			
Goseong		+	+
Gangneung	0.168		
Cultured			
Gangneung	0.220	0.006	+

flow between populations in different regions and long-term isolation between these populations. Also, population-specific haplotypes were observed in each wild population as mention above, although we must point out the small number of wild samples on which analysis was conducted. The larval dispersal of ascidians has been shown to be spatially limited linked to their short free-swimming stage befor settlement (Grosberg, 1987; Davis and Butler, 1989). Such restricted behavior will cause partial genetic isolation in geographically distant regional populations, which ultimately will lead to restricted gene flow (Ben-Shlomo et al., 2006). Our findings suggest genetically distinct traits between wild populations, which maybe indicative of the established short duration of their free-swimming larval stage before settlement. However, further analyses using more samples from different areas would help better elucidate gene flow or genetic drift pattern of ascidians.

In conclusion, our results demonstrated that sequence polymorphism of the mtDNA COI region was a useful marker for analyzing genetic variation in ascidian populations. Genetic profiles of ascidian populations using mtDNA analysis may yield information valuable to various management and conservation initiatives related to breeding programs.

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