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Genetic Variation in Wild and Cultured Populations of the Sea Squirt *Halocynthia roretzi* Inferred from Microsatellite DNA Analysis

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Abstract

We used nine microsatellite DNA markers to estimate genetic variation among wild and cultured populations of the sea squirt *Halocynthia roretzi*. The loci were polymorphic, with 6-32 alleles, and allelic richness ranged from 6.0 to 26.1 in each population. The wild and the cultured populations had similar mean heterozygosities (H_O and H_E), allele numbers, and allelic richness. One cultured population with softness syndrome had a lower mean in the observed heterozygosity ($H_O = 0.57$) and higher mean inbreeding coefficient ($F_{IS} = 0.261$) than any other populations. This suggests that the loss of genetic variation in the diseased population might be due to increased inbreeding. A neighbor-joining tree and pairwise population estimates of F_{ST} showed moderate genetic differentiation between the wild and the cultured populations. Additionally, the softness syndrome population was genetically divergent from wild populations, but it was genetically close to the cultured populations.

Key words: Sea squirt, *Halocynthia roretzi*, Microsatellite, Population genetics

Introduction

The natural distribution of the solitary ascidian *Halocynthia roretzi* (sea squirt) is restricted essentially to the waters around Korea and Japan, where it inhabits rocky littoral surfaces. *H. roretzi* has received considerable attention due to its high commercial importance in Korea. The Korean aquaculture industry produced 9,300 tonnes of *H. roretzi* in 2007, but this decreased rapidly to less than 1,000 tonnes in 2010 (Ministry of Maritime Affairs and Fisheries, 2010); this decrease has been the result of an unexplained syndrome, which has been termed "softness syndrome". To date, the syndrome has caused the loss of more than $30 million to Korean producers.

The sea squirt are cultivated by the hanging culture method (Rho et al., 1993): seeds from a hatchery are attached to a vertical rope that is hung from a longline, much as oysters are cultivated (Kang et al., 1980). Hatcheries in Korea often use seeds that have been introduced from Iwate in Japan, but the details of the origins and breeding records of the introduced sea squirt are unknown. Also, large numbers of the cultured sea squirt in Korea have died from an unexplained syndrome over the last 20 years (Cho et al., 2008). Each dying Sea Squirt shows thinning of the tunica, and the meat becomes quite soft (thus the name "softness syndrome"). To manage and improve the sustainable production of this sea squirt resource in Korea, it is vital to identify the causative factors responsible for the

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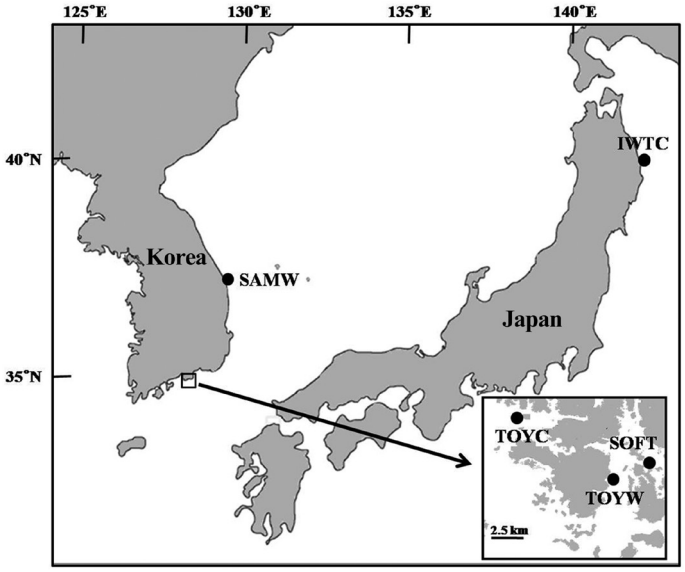

Fig. 1. Geographic locations of sampling sites. SAMW, the wild populations from Samcheok; TOYW, the wild populations from Tongyeong; TOYC, the cultured populations from Tongyeong; IWTC, cultured population from Japan; SOFT, the cultured population from Tongyeong with softness syndrome.

softness syndrome.

A major concern has been focused on genetic changes within cultured populations may threaten the integrity of wild populations (Allendorf and Ryman, 1987). Cultivation to enhance the stock has also been identified as one of the factors responsible for the depletion of wild populations, due to inbreeding, competition, and the spread of disease (Allendorf and Phelps, 1980). Furthermore, genetic variation is beneficial and important for the long-term survival of natural populations, because variation can provide the population with the ability to adapt to changing environmental conditions (Frankel and Soulé, 1981).

Genetic variation within and between populations provides a potential resource for future adaptations (Hurt and Hedrick, 2004). In recent years, molecular markers have been used to observe the genetic variation in sea squirt populations to study phylogeography and population genetics (e.g., Ben-Shlomo et al., 2001, 2006; Tarjuelo et al., 2004; López-Legentil and Turon, 2006). To date, no genetic study in *H. roretzi* by molecular markers has been reported.

In the present study, we investigated genetic variability between the wild and the cultured populations of *H. roretzi* collected in Korea and Japan by analyzing nine microsatellite loci.

Materials and Methods

Samples and DNA extraction

In 2006 and 2007, sea squirt were collected from two wild populations at Samcheok (abbreviated as SAMW) and Tongyeong (TOYW), Korea, respectively, and three cultured populations: a population from Tongyeong (TOYC), a population from Tongyeong with softness syndrome (SOFT), and a population from Iwate, Japan (IWTC) (Table 1, Fig. 1). The

Table 1. Sampling locations, date of collection, the number of samples (N), observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficient (F_{IS}), and mean number of alleles (A_M) used for *Halocynthia roretzi* msDNA analysis

Sampling location	Abbreviation	Date of collection	N	H_O	H_E	F_{IS}	A_M
The Wild							
Samcheok	SAMW	Feb. 2007	96	0.787	0.777	-0.021	12.3
Tongyeong	TOYW	May 2007	90	0.702	0.788	0.105	10.1
The Cultured							
Tongyeong	TOYC	Feb. 2006	96	0.747	0.777	0.043	12.4
Iwate	IWTC	Apr. 2007	96	0.647	0.758	0.139	10.8
Softness Syndrome	SOFT	Apr. 2007	48	0.569	0.779	0.273	10.0

TOYC population, which was introduced in 1989 from Iwate in Japan, originated from third generation spawners (body size > 10 cm) that had been reared continuously. The SOFT and IWTC populations had also been reared continuously, but no information is available about the origin of these populations. Samples of muscle from internal organs were collected from 426 individuals from the five populations. The muscle samples were preserved in 100% ethanol at room temperature until the DNA was extracted. Genomic DNA was extracted from the stored muscle tissues by an automated DNA extraction system (MagExtractor MFX-6100; Toyobo, Japan) with a MagExtractor Genomic DNA Purification Kit (Toyobo). Extracted DNA was dissolved in 80 μL of Tris-EDTA buffer (TE; 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) until genotyping.

Microsatellite DNA analysis

Nine polymorphic microsatellite loci were examined by PCR amplification by primers previously developed for this species (Han et al., 2009). The forward primer of each pair was labeled with one of the following fluorescent dyes: 6FAM, NED or HEX. PCR amplification was performed with a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA, USA) and the following reaction conditions: initial denaturation for 5 min at 95°C, 40 cycles of 30 s at 95°C, 45 s at a primer-specific annealing temperature, 45 s at 72°C, and a final extension step at 72°C for 30 min. The specific annealing temperature of each primer set is given in our previous paper (Han et al., 2009). The PCR products were analysed using an ABI 3130 XL genetic analyzer (Applied Biosystems, Foster City, CA, USA) to screen for polymorphisms.

Analysis of population genetics data

The inbreeding coefficient F_{IS} was calculated from an analysis of variance following Weir and Cockerham (1984) by the GENEPOP program. Observed (H_O) and expected (H_E) heterozygosities per locus were calculated for each population using the programs FSTAT 2.9.3 (Goudet, 2001) and GENEPOP, version 3.4 (Rousset and Raymond, 1995). Pairwise values of F_{ST} were used to estimate genetic differentiation between pairs of populations according to Slatkin (1995) by the program FSTAT 2.9.3 (Goudet, 2001). The significance of F_{ST} values was tested via a permutation method (28,000 permutations).

A matrix of genetic distances, based on the set of allele frequencies in different populations, was constructed according to Nei (1972). A neighbor-joining (NJ) tree was constructed for each replicated matrix by bootstrapping 1,000 replications with NEIGHBOR in PHYLIP version 3.5 (Felsenstein, 1993). The consensus tree was generated following the 50% majority rule by CONSENSUS, also in the PHYLIP package (available at http://www.evolution.genetics.washington.edu/phylip.html).

Results and Discussion

To our knowledge, this is the first reported study on the population genetics of *H. roretzi* in Korea to estimate current levels of genetic variation by nine microsatellite DNA markers. In total, 203 alleles were observed for all nine loci, ranging from 12 alleles at *Haro*26 to 46 at *Haro*15, in all populations. Allele sizes ranged from 125 to 232 bp across all microsatellite loci. As shown in Table 1, the mean number of observed alleles ranged from 10.0 (SOFT) to 12.4 (TOYC), the mean H_O per population ranged from 0.57 (SOFT) to 0.79 (SAMW), and the mean H_E ranged from 0.76 (IWTC) to 0.79 (TOYW). These results indicated that the levels of genetic variation were similar in each of the five populations. No meaningful difference in heterozygosity was found between the wild and the cultured populations, except SOFT, suggesting that the cultured populations of *H. roretzi* showed similar levels of genetic variation to those of the wild populations, at least in terms of the observed levels of heterozygosity. The mean observed heterozygosity of SOFT was the lowest among the different populations. Additionally, SOFT showed a large disparity between H_O and H_E. However, we noted that the mean H_O values were lower than the H_E values for all populations, except SAMW, which might indicate a deficit in heterozygotes. The values of F_{IS} and the evaluations of Hardy-Weinberg equilibrium are shown in Table 1. The SAMW population had the lowest estimate of mean values of inbreeding (mean F_{IS} = -0.021) of the five populations. TOYW, TOYC, and IWTC exhibited moderate mean F_{IS} values, which ranged from 0.043 to 0.139. In contrast, the mean F_{IS} was higher in SOFT than in the other populations.

Most of the sea suirt cultured in Tongyeong area have died during the last 20 years from the “softness syndrome” disease which has had a detrimental effect on the Korean aquaculture industry. Cho et al. (2008) discriminated normal and dying sea squirt by their differential patterns of gene expression. Low genetic variability in the cultured populations, which is caused by increased levels of inbreeding due to the use of a small effective number of parents, may generally lower resistance to various diseases. Although the mean H_O and F_{IS} values for the microsatellite loci did not reveal pronounced differences between the wild and the cultured populations, the results of our study confirmed that the diseased population did show less genetic variation. The population with “softness syndrome” had lower H_O and higher F_{IS} values at each locus compared with the healthy populations. Moreover, the disparity between the H_O and H_E in the diseased population was much higher than that in the healthy populations, suggesting that the diseased population was considerably inbred.

Pairwise population estimates of F_{ST} are presented in Table 2. The F_{ST} values showed significant differences between the wild and the cultured populations, suggesting that genetic differentiation had occurred between these populations. Significant differentiation was inferred for all pairs of the cultured

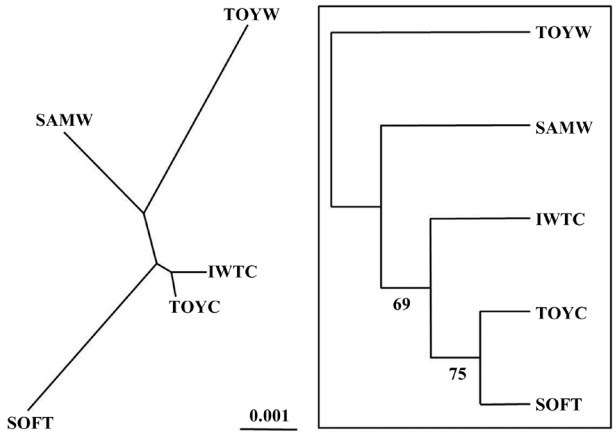

Fig. 2. Neighbor-joining tree showing genetic distance according to Nei (1972) between five sea squirt populations. The inset shows the consensus phenogram with nodal bootstrap values of 1,000 replicates (%). SAMW, the wild populations from Samcheok; TOYW, the wild populations from Tongyeong; TOYC, the cultured populations from Tongyeong; IWTC, the cultured population from Japan; SOFT, the cultured population from Tongyeong with softness syndrome.

populations, except TOYC and IWTC, and for pairs of wild populations. The highest values of F_{ST} were between SOFT and the wild populations. The sampled populations were clustered by the NJ method (Fig. 2). All the wild populations were separated from the cluster of the cultured populations with 100% bootstrap support. These findings indicate that genetic differentiation has occurred between the wild and the cultured populations. The SOFT population was genetically distinct from the other populations, as suggested by the estimates of F_{ST}. Topology of the NJ tree showed that TOYC (Korea) was genetically close to IWTC (Japan), by frequent import of the parent stocks.

Pairwise population estimates of F_{ST} revealed highly significant genetic differentiation between the eastern (SAMW) and southern coastal (TOYW) populations. The NJ tree also indicated that this wild population pair was genetically distinct. These results suggested low levels of gene flow between populations in different regions and long-term isolation between regional populations. Grosberg (1987) and Davis and Butler (1989) found that the larvae of colonial sea squirt were only dispersed over short distances. Thus, our findings suggest that genetic interactions between populations of *H. roretzi* only occur over short distances. This probably reflects the limited duration of the free-swimming stage before it settles into the habitat. Even though our findings indicate strongly restricted gene flow between populations, pairwise tests revealed that the Tongyeong cultured population is very closely related to the Iwate cultured population, possibly due to human-mediated transportation of stocks. As noted in the Introduction of this study, to produce a large number of offspring of *H. roretzi* at the Tongyeong aquaculture sites, many parent and seed have been introduced from Iwate during the last 20 years.

In conclusion, our results demonstrate that genotyping with microsatellite allele frequencies can be applied to the identification of stock and the estimation of genetic variability among and within wild and/or cultured populations. In particular, the results of the analysis suggested that genetic variation had decreased in a cultured population of the sea squirt *H. roretzi* with softness syndrome and that this population had differentiated genetically from the healthy populations. The study of genetic differences among the wild and the cultured populations will help in the development of a sound strategy for the management of the genetic stock programs and the subsequent ecological monitoring of sea squirt communities.

Table 2. F_{ST} values between samples (below diagonal) and probability of differentiation with *P*-value in F_{ST} estimate (above diagonal)

Population	SAMW	TOYW	TOYC	IWTC	SOFT
The Wild					
SAMW		0.001*	0.001*	0.001*	0.001*
TOYW	0.066		0.001*	0.001*	0.001*
The Cultured					
TOYC	0.035	0.045		0.162	0.001*
IWTC	0.042	0.049	0.005		0.001*
SOFT	0.072	0.078	0.032	0.050	

SAMW, wild populations at Samcheok; TOYW, wild populations at Tongyeong; TOYC, cultured populations from Tongyeong; IWTC, cultured population from Japan; SOFT, cultured population from Tongyeong with softness syndrome.
*Significant support for F_{ST} values ($P < 0.05$).

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