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Genetic Differences and Variation of Ascidians, *Halocynthia roretzi* von Drasche and *H. hilgendorfi* Oka Identified by PCR Analysis

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ABSTRACT : The seven selected primers OPA-02, OPA-04, OPA-18, OPD-07, OPD-08, OPD-15 and OPD-16 were used to generate unique shared loci to each species and shared loci by the two species. The hierarchical dendrogram indicates three main branches: cluster 1 (RORETZI 01~RORETZI 11) and cluster 2 (HILGENDORF 12~HILGENDORF 22) from two geographic populations of ascidians, *Halocynthia roretzi* and *H. hilgendorfi*. The shortest genetic distance displaying significant molecular difference was between individuals' HILGENDORF no. 14~HILGENDORF no. 19 (genetic distance =0.008). Ultimately, individual no. 02 of the RORETZI ascidian was most distantly related to HILGENDORF no. 21 (genetic distance=0.781). These results demonstrate that the *H. roretzi* population is genetically different from the *H. hilgendorfi* population. From what has been said above, the potential of PCR analysis to identify diagnostic markers for the identification of two ascidian populations has been demonstrated. Generally speaking, using a variety of decamer primers, this PCR method has been applied to identify specific markers particular to line, species and geographical population, as well as genetic diversity/polymorphism in diverse species of organisms.

Key words : Ascidians, Dendrogram, Specific markers, Genetic distance, *Halocynthia roretzi*, *H. hilgendorfi*

INTRODUCTION

The specific markers specific to line, breed, species, genus or geographical population have all also been employed in the identification and discrimination of individuals, species and populations, hybrid parentages and genetic diagnostics (Callejas & Ochando, 1998; Huang et al., 2000; Kim et al., 2000; Yoon & Kim, 2004). The random amplified polymorphic DNA method was used to generate fingerprint patterns for 10 meat species: wild boar, pig, horse, buffalo, beef, venison, dog, cat, rabbit and kangaroo (Koh et al., 1998). Polymorphic bands generated by RAPD-PCR using arbitrary primers have classically been considered to constitute a reliable method for the detection of DNA similarity and/or diversity between organisms (Jeffreys & Morton, 1987; Liu et al., 1998; McCormack et al., 2000; Kim et al., 2004).

Ascidian is one of the most popular marine products in Korea because of their taste and nutritional value, and Koreans consume them in large quantities. Among ascidians, one ascidian (*Halocynthia roretzi* von Drasche) and the other ascidian (*H. hilgendorfi*) are an economically important aquacultural species. In the natural ecosystem, two species of ascidians are widely distributed in the entirety of seawater habitat areas of the East Sea and southern sea in the Korean Peninsula, as well as in several areas in East China. Most representatives of the species live attached by abyss to the underside of rocks, coral heads, and other hard substrates and buried in sandy mud at a variety of depths. Especially, this ascidian is widely cultivated on the coast in Tongyeong, Taean, Boryeong, Seocheon and Shinan in Korean peninsula all the year round. However, in spite

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of their economic and scientific consequences, a little information currently exists regarding the physiological and ecological levels of ascidian species in Korea. In the present study, to elucidate the genetic distances and differences among geographical ascidians populations, we performed a clustering analysis of two ascidian populations collected from two sites of Korea.

MATERIALS AND METHODS

1. Sample Collection and Extraction of Genomic DNA

One species of ascidians from Tongyeong and the other ascidian species from Geojedo were collected in the southern sea of Korea, respectively. These ascidian muscles were collected in sterile tubes, immediately placed on ice, and stored at −40℃ until needed. PCR analysis was performed on the muscle extracts from 22 individuals, using seven arbitrarily selected primers of two decades of different decamer primers. The extraction/purification of genomic DNA was performed under the conditions described previously (Kim et al., 2004; Yoon & Kim, 2004). The purified DNA pellets were then incubation-dried for more than 10 hours, maintained at −40℃ until analysis, then dissolved in the ultra-pure water (JW Pharmaceutical, Seoul, Korea). The concentration of the extracted genomic DNA was measured by its absorbance ratio at 260 nm, with a spectrophotometer (Beckman Coulter, Buckinghamshire, UK).

2. Decamer Primers, Molecular Markers and Amplification Stipulations

The decamer primers were purchased from Operon Technologies, USA. The OPA-02 (5'-TGCCGAGCTG-3'), OPA-04 (5'-AATCGGGCTG-3'), OPA-18 (5'-AGGTGACCGT-3'), OPD-07 (5'-TTGGCACGGG-3'), OPD-08 (5'-GTGTGCCCCA-3'), OPD-15 (5'-CATCCGTGCT-3') and OPD-16 (5'-AGGGCGTAAG-3') were shown to generate unique shared loci to each species and number of shared loci by the two species which could be clearly scored. We used these primers to determine the genetic variations, DNA polymorphisms, genetic diversity, and similarity of the one species of ascidians from Tongyeong and the other ascidian species from Geojedo. PCR was performed using two Programmable DNA Thermal Cyclers (Perkin Elmer Cetus, Norwalk, CT, USA; MJ Research Inc., Waltham, MA, USA). DNA amplification was performed in 25 $\mu\ell$ samples, which contained 10 ng of template DNA, 20 $\mu\ell$ of premix (Bioneer Corp., Daejeon, Korea), and 1 unit of primer. Amplification products were generated via electrophoresis on 1.4% agarose (VentechBio, Korea) gel containing TBE (90 mM Tris, pH 8.5; 90 mM borate; 2.5 mM EDTA). The 100 bp Ladder marker (Bioneer Corp., Daejeon, Korea) was utilized as a DNA molecular weight marker. Bands were detected by ethidium bromide staining. The electrophoresed agarose gels were illuminated by ultraviolet rays, and photographed using a Photoman direct copy system (PECA Products, Beloit, WI, USA).

3. Data Analysis

Bandsharing (BS) values were calculated according to the presence/absence of amplified products at specific positions in the same gel from the PCR outlines. Absence of bands indicates that the priming site is not present, presumably as a result of some alteration in the DNA sequence. The degree of variability was calculated by use of the Dice coefficient (F), which is given by the formula: $F = 2\ n_{ab} / (n_a+n_b)$, where n_{ab} is the number of bands shared between the samples a and b, n_a is the total number of bands for sample a and n_b is the total number of bands for sample b (Jeffreys & Morton, 1987; Yoke-Kqueen & Radu, 2006). The average of within-species similarity was calculated by pairwise comparison between individuals within a species. The relatedness between different individuals in the ascidian species of Korea was generated according to the bandsharing values and similarity matrix. Using similarity matrices to generate a dendrogram facilitated by the pc-package program Systat version 10

(SPSS Inc., Chicago, IL, USA), a hierarchical clustering tree was constructed. Euclidean genetic distances within and between populations were also calculated using the Systat hierarchical dendrogram program version 10. Systat version 10 was also used to obtain other statistical results including means and standard errors.

RESULTS AND DISCUSSION

1. PCR Variations within and between Populations, and Genetic Distances

Here, the seven selected primers OPA-02, OPA-04, OPA-18, OPD-07, OPD-08, OPD-15 and OPD-16 were used to generate unique shared loci to each species and shared loci by the two species. The complexity of the banding patterns varied dramatically between the primers from the two locations. The size of the DNA fragments also varied wildly, from 150 to 2,000 bp (Fig. 1). The bandsharing value between individuals no. 15 and no. 16 was 0.984, which was the highest value identified within the *H. hilgendorfi* species (Table 2). The bandsharing value between individual's no. 08 of the RORETZI ascidian and no. 16 of the HILGENDORF ascidian was 0.124, which was the lowest observed. The 22 unique shared loci to each species generated by OPA-01 decamer primer were in *H. roretzi* ascidian, as summarized in Table 1. Interestingly, the decamer primer OPA-18 generated 33 unique shared loci to each species, which were identifying each species in *H. hilgendorfi* ascidian (Table 1). Especially, the decamer primer OPA-15 generated 11 unique shared loci to each species, which were identifying each species in *H. hilgendorfi* ascidian. Interestingly, the primer OPA-01 detected 11 shared loci by the two species, which were identical in all samples, as summarized in Table 1. Polymorphisms are determined by the banding patterns of primer-amplified products at specific positions (Tassanakajon et al., 1998; Nozaki et al., 2000; Yoon & Kim, 2003). Researchers have studied the sizes of DNA fragments in the PCR profiles of five species of Eastern Pacific abalone (genus *Haliotis*) (Muchmore et al., 1998), black tiger shrimp (*Penaeus monodon*) (Tassanakajon et al., 1998), the brittle star (*Amphiura filiformis*) (McCormack et al., 2000) and cultured and wild shrimp populations (Yoon & Kim, 2003). The specific primer proved useful in the identification of individuals and/or populations, resulting from variations in DNA polymorphisms among individuals/

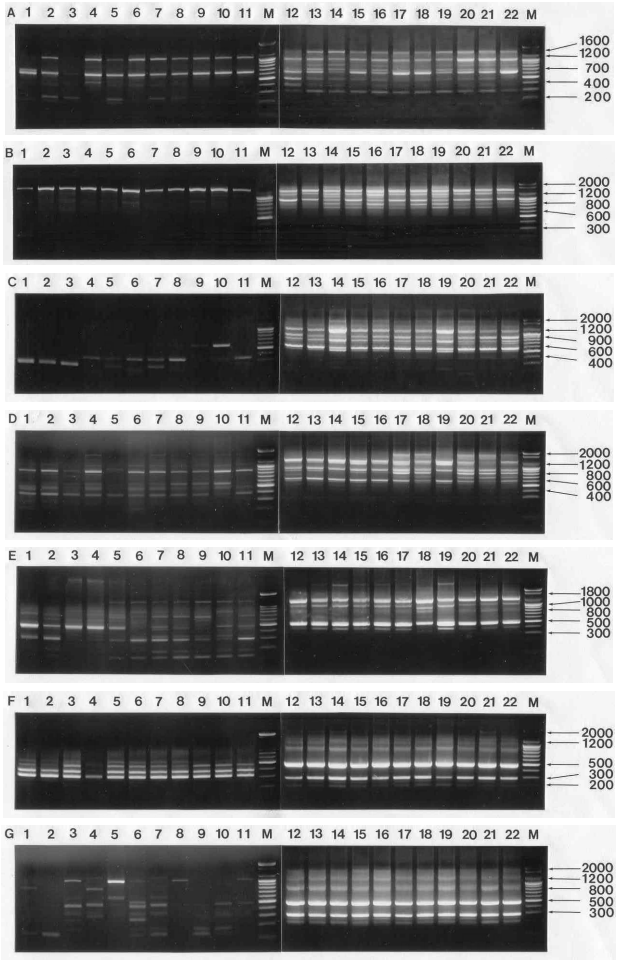

Fig. 1. PCR analysis generated electrophoretic profiles of individual ascidian, *Halocynthia roretzi* and *H. hilgendorfi*. Each lane shows DNA samples extracted from 22 individuals. DNA isolated from one ascidian species (lane 1-11) from Tongyeong in the southern sea and the other ascidian species (lane 12-22) from Geojedo were amplified by oligonucleotide primers OPA-02 (A), OPA-04 (B), OPA-18 (C), OPD-07 (D), OPD-08 (E), OPD-15 (F) and OPD-16 (G). Bands were detected by ethidium bromide staining. The 100 bp Ladder marker was utilized as a DNA molecular weight marker.

Table 1. The number of unique shared loci to each species and number of shared loci by the two species generated by PCR analysis using 7 decamer primers in of ascidians, *Halocynthia roretzi* and *H. hilgendorfi* from Tongyeong and Geojedo, respectively

Item	No. of unique shared loci to each species		No. of shared loci by the two species
Primer\Species	*H. roretzi*	*H. hilgendorfi*	Two species
OPA-02	22	11	11
OPA-04	0	11	0
OPA-18	0	11	0
OPD-07	0	11	0
OPD-08	11	11	0
OPD-15	0	11	0
OPD-16	0	33	0
Total no.	33	99	11
Average no. per primer	4.71	14.14	1.57

Table 2. Similarity matrix, including bandsharing values, calculated using Nei and Li's index, of the similarity of ascidians, *Halocynthia roretzi* and *H. hilgendorfi* from Tongyeong and Geojedo, respectively

	Bandsharing values																					
	Halocynthia roretzi from Tongyeong											*Halocynthia hilgendorfi* from Geojedo										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	-	0.767	0.572	0.544	0.531	0.498	0.623	0.535	0.520	0.505	0.575	0.277	0.250	0.241	0.280	0.254	0.243	0.243	0.239	0.248	0.253	0.219
2		-	0.668	0.568	0.641	0.644	0.723	0.584	0.699	0.643	0.692	0.223	0.227	0.224	0.234	0.234	0.252	0.233	0.223	0.236	0.245	0.253
3			-	0.541	0.633	0.620	0.687	0.512	0.591	0.629	0.598	0.298	0.299	0.306	0.302	0.278	0.332	0.285	0.301	0.292	0.270	0.287
4				-	0.599	0.683	0.733	0.577	0.582	0.642	0.700	0.258	0.255	0.270	0.278	0.278	0.294	0.273	0.265	0.280	0.284	0.297
5					-	0.720	0.752	0.682	0.683	0.718	0.759	0.214	0.218	0.216	0.213	0.213	0.249	0.204	0.215	0.233	0.209	0.270
6						-	0.729	0.688	0.783	0.851	0.685	0.302	0.305	0.283	0.289	0.289	0.329	0.287	0.279	0.312	0.270	0.307
7							-	0.728	0.733	0.784	0.775	0.252	0.250	0.227	0.239	0.239	0.256	0.256	0.226	0.261	0.266	0.237
8								-	0.656	0.728	0.762	0.159	0.137	0.130	0.150	0.124	0.145	0.145	0.130	0.167	0.151	0.145
9									-	0.863	0.673	0.253	0.259	0.247	0.240	0.240	0.285	0.243	0.245	0.247	0.224	0.262
10										-	0.747	0.260	0.263	0.233	0.226	0.226	0.270	0.247	0.231	0.254	0.231	0.248
11											-	0.199	0.199	0.208	0.205	0.205	0.230	0.209	0.207	0.211	0.216	0.231
12												-	0.948	0.913	0.927	0.927	0.936	0.907	0.900	0.950	0.947	0.948
13													-	0.942	0.940	0.940	0.964	0.925	0.951	0.945	0.942	0.933
14														-	0.972	0.972	0.954	0.882	0.974	0.910	0.924	0.924
15															-	0.984	0.965	0.894	0.957	0.921	0.935	0.937
16																-	0.965	0.910	0.957	0.921	0.935	0.953
17																	-	0.913	0.939	0.957	0.954	0.971
18																		-	0.919	0.911	0.925	0.919
19																			-	0.897	0.911	0.888
20																				-	0.984	0.970
21																					-	0.966
22																						-

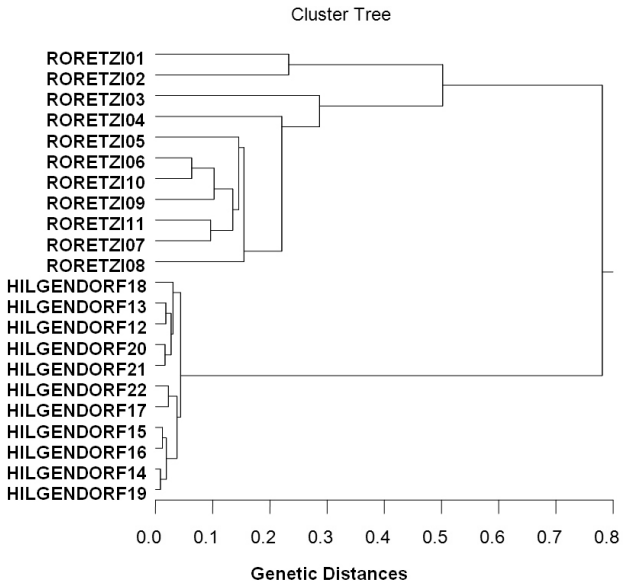

Fig. 2. Hierarchical dendrogram of genetic distances, obtained from two geographic populations of ascidians, *Halocynthia roretzi* (RORETZI 01～RORETZI 11) and *H. hilgendorfi* (HILGENDORF 12～HILGENDORF 22) from Tongyeong and Geojedo, respectively. The relatedness between different individuals in the ascidians was generated according to the bandsharing values and similarity matrix.

populations (Liu et al., 1998; Yoon & Kim, 2003; Kim et al., 2004; Yoon & Kim, 2004). The PCR method, using various primers, was applied to the identification of three endemic Spanish barbel species: *Barbus bocagei*, *B. graellsii* and *B. sclateri* (Callejas & Ochando, 1998). Results indicated that *Barbus bocagei* and *B. graellsii* were more closely related to each other than they were to *B. sclateri*.

In our study, PCR analysis has revealed a significant genetic distance between two population/species pairs. PCR enabled us to detect the existence of population/species discrimination and genetic variation in two geographic populations of ascidians, *H. roretzi* and *H. hilgendorfi*. The hierarchical dendrogram indicates three main branches: cluster 1 (RORETZI 01～RORETZI 11) and cluster 2 (HILGENDORF 12～HILGENDORF 22) from two geographic populations of ascidians, *H. roretzi* and *H. hilgendorfi*. The shortest genetic distance displaying significant molecular difference was between individuals' HILGENDORF no. 14～HILGENDORF no. 19 (genetic distance=0.008). Ultimately, individual no. 02 of the RORETZI ascidian was most distantly related to HILGENDORF no. 21 (genetic distance=0.781). These results demonstrate that the *H. roretzi* population is genetically different from the *H. hilgendorfi* population. Phylogenetic relationships among 5 *Haliotis* species and one hybrid were conducted by calculation of the distance coefficient and construction of a phylogenetic tree based on RAPD data (Kim et al., 2000). These branched off into two clusters: cluster I was formed by *H. discus hannai, H. discus, H. gigantea, H. sieboldii*, and the hybrid, which was subsequently re-divided into two sub-clusters. From what has been said above, the potential of PCR analysis to identify diagnostic markers for the identification of two ascidian populations has been demonstrated. In invertebrates, cluster analysis of the pairwise population matrix, generated from genetic data, showed that geographically close populations tended to cluster together in the blacklip abalone (Huang et al., 2000). This confirms that the method is a suitable tool for DNA comparisons, both within and between individuals, species, and populations. Generally speaking, using a variety of decamer primers, this PCR method has been applied to identify specific markers particular to line, species and geographical population, as well as genetic diversity/polymorphism in diverse species of organisms (McCormack et al., 2000; Yoon & Kim, 2003; Kim et al., 2004).

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