Genetic Differences and Geographic Variation in Cuttle Fish (*Sepia esculenta* Hoyle)

Jong-Man Yoon[1†] and Jong-Yeon Kim[2]

[1]*Dept. of Aquatic Life Medicine, College of Ocean Science and Technology, Kunsan National University, Gunsan 573-701, Korea*

[2]*Dept. of Marine Aquaculture and Biotechnology, College of Ocean Science and Technology, Kunsan National University, Gunsan 573-701, Korea*

ABSTRACT : The gDNA isolated from Korean cuttle fish (*Sepia esculenta* Hoyle) from Sockcho (SOCKCHO), Seocheon (SEOCHEON), Incheon (INCHEON) and Vietnamese cuttle fish (VIETNAM), were amplified by PCR. Here, the seven selected primers (BION-A07, BION-A09, BION-A11, BION-A20, BION-B04, BION-B06, and BION-B14) were used to generate the unique shared loci to each population and shared loci by the four cuttle fish populations. In this study, the primer BION-A11 detected 112 shared loci by the four populations, major and/or minor fragments of sizes 300 bp, 400 bp, 700 bp and 1,000 bp, respectively, which were identical in all samples. The dendrogram obtained by the seven primers indicates five genetic clusters: cluster 1 (SOCKCHO 01-SOCKCHO 07), cluster 2 (SEOCHEON 08-SEOCHEON 10), cluster 3 (SEOCHEON 11-SEOCHEON 14), cluster 4 (INCHEON 15-INCHEON 21), and cluster 5 (VIETNAM 22-VIETNAM 28). The shortest genetic distance that displayed significant molecular differences was between individuals 25 and 26 from the Vietnamese cuttle fish (0.025), while the longest genetic distance among the twenty-eight cuttle fishes that displayed significant molecular differences was between individuals SOCKCHO no. 02 and SEOCHEON no. 12 (0.640). Individual of Seocheon and Incheon cuttle populations was somewhat closely related to that of Vietnamese cuttle fish population. Even though it could not be affirmed by a single case, such a result seems to be closely connected that the Korean peninsula is subject to climate changes by global warming. In conclusion, our PCR analyses revealed a significant genetic distance among the four cuttle fish populations.

Key words : Korean cuttle fish, Genetic distance, Genetic cluster, *Sepia esculenta*, Vietnamese cuttle fish.

INTRODUCTION

Cuttle is generally ranked highest among the mollusk species in Korea as a preference food, owing to peculiar taste, flavor and nutritional value. Korean cuttle fish (*Sepia esculenta* Hoyle) is one species of an economically important mollusk species, belonging to the family Sepiidae, and the order Sepioidea. Cuttle fish is widely distributed in Australia, New Zealand, China, and the Far East Asia. In particular, during the summer, this cuttle fish is widely distributed off the coasts of Incheon, Taean, Boryeong, Seocheon, Kunsan, Yeonggwang, and Yeosu, in the Korean peninsula, which are influenced by warm water currents. Cuttle are found from surface waters to the depths of the abyss. The size and type of the mollusk species varies according to habitat including such factors as the temperature and depth of the water, and the availability of nutrients. Basically, the body size of the cuttle fish varies widely according to the environmental conditions. Cuttle fish have a shield-shaped body containing an inner chalky plate, and a small head bearing eight arms and two long tentacles. Generally, the spawning season for this mollusk species starts in March and continues through to June.

As the necessity of cuttle fish increases, the understan-

† Corresponding author: Jong-Man Yoon, Dept. of Aquatic Life Medicine, College of Ocean Science and Technology, Kunsan National University, Gunsan 573-701, Korea. Tel: +82-63-469-1887, Fax: +82-63-469-1887, E-mail: jmyoon@kunsan.ac.kr

ding of the genetics of this mollusk species becomes important. However, little is known about the genetics of cuttle fishes in Korea (Yoon & Kim, 2003). To analyze the genetics of organisms, a number of analytical and molecular techniques have been applied, including morphological standards (Orozco-Castillo et al., 1994), allozyme variation (Smith et al., 1997), and various PCR-based molecular biological techniques. Polymorphisms are determined from specific positions in the banding patterns of the amplified products (Tassanakajon et al., 1998; Yoon & Kim, 2004). Thus, RAPD has been applied to the identification of the genetic characteristics of various species of fish and shellfish (Callejas & Ochando, 1998; Yoon & Kim, 2004; Yoon, 2006). The clustering analysis of the genetic distances between various mollusk species, or populations from different geographic sites, which was performed using RAPD-PCR, is of little quantity (Klinbunga et al., 2000; McCormack et al., 2000; Yoon & Kim, 2003; Yoon, 2006). We carried out clustering analyses to clarify the genetic variations and DNA polymorphisms among four cuttle fish (*Sepia esculenta* Hoyle) geographical populations from Sockcho, Seocheon, Incheon and Vietnam.

MATERIALS AND METHODS

1. Isolation of Genomic DNA, Amplification Condition and Analysis of Genetic Distances

Four geographical populations of cuttle fish (*Sepia esculenta* Hoyle) were obtained from four different regions: Sockcho, Seocheon, Incheon regions of Korea and a site of Vietnam, respectively. Cuttle fish muscle was collected in sterile tubes, instantaneously placed in liquid nitrogen, and stored at −40℃ until the gDNA extraction. The extraction/purification of genomic DNA was performed under the experimental conditions previously described (Yoon & Kim, 2004). PCR analyses were performed on the muscle extract of 28 individuals using seven primers. Seven primers, BION-A07 (5'-GAAACGGGTG-3'), BION-A09 (5'-GGGTAACGCC-3'), BION-A11 (5'-CAATCGCCGT-3'), BION-A20 (5'-GTTGCGATCC-3'), BION-B04 (5'-GGAC TGGAGT-3'), BION-B06 (5'-TGCTCTGCCC-3') and BION-B14 (5'-TCCGCTCTGG-3') were shown to generate the unique shared loci to each population and shared loci by the four cuttle fish populations which could be clearly scored. 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 (Bioneer Corp., Daejeon, Korea) gel containing TBE (90 mM Tris, pH 8.5; 90 mM borate; 2.5 mM EDTA). The concentration of the extracted genomic DNA was measured by optical density at 260 nm by a spectrophotometer (Beckman Coulter, Buckinghamshire, UK). The 100-bp DNA Ladder (Bioneer Corp., Daejeon, Korea) was used as the DNA molecular weight marker. The electrophoresed agarose gels were illuminated by ultraviolet rays, and photographed using a photoman direct copy system (PECA Products, Beloit, WI, USA). A hierarchical clustering tree was constructed using similarity matrices to generate a dendrogram, which was facilitated by the Systat version 10 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

The gDNA isolated from Korean cuttle fish (*Sepiaesculenta* Hoyle) from Sockcho (SOCKCHO), Seocheon (SEOCHEON), Incheon (INCHEON) and Vietnamese cuttle fish (VIETNAM), were amplified several times by PCR reaction. In this study, the seven decamer primers BION-A07, BION-A09, BION-A11, BION-A20, BION-B04, BION-B06, and BION-B14 were used to generate the unique shared loci to each population and shared bands by the four cuttle fish populations. In this study, the decamer primer BION-A07 generated inter-population-specific DNA fragments, approximately 50 bp, 150 bp, 300 bp, 350 bp,

Table 1. Similarity matrix including bandsharing values (BS) and genetic differences calculated using Nei and Li's index of the similarity of Korean cuttle fish and Vietnamese cuttle fish from Korea and Vietnam, respectively

	BS of cuttle fish from Sockcho							BS of cuttle fish from Seocheon							BS of cuttle fish from Incheon							BS of Vietnamese cuttle fish						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	-	0.897	0.810	0.838	0.869	0.80	0.817	0.526	0.504	0.517	0.36	0.414	0.45	0.444	0.533	0.444	0.549	0.523	0.427	0.452	0.520	0.545	0.527	0.554	0.535	0.526	0.524	0.463
2		-	0.790	0.810	0.817	0.804	0.808	0.507	0.531	0.520	0.440	0.446	0.479	0.455	0.551	0.454	0.536	0.492	0.483	0.47	0.538	0.575	0.539	0.554	0.549	0.537	0.524	0.488
3			-	0.841	0.802	0.797	0.812	0.435	0.479	0.475	0.389	0.420	0.454	0.452	0.506	0.435	0.521	0.491	0.48	0.436	0.508	0.562	0.526	0.521	0.549	0.552	0.601	0.498
4				-	0.836	0.888	0.824	0.514	0.518	0.539	0.359	0.391	0.495	0.499	0.499	0.388	0.522	0.488	0.425	0.476	0.513	0.568	0.560	0.607	0.497	0.523	0.576	0.522
5					-	0.800	0.831	0.541	0.539	0.526	0.38	0.393	0.486	0.501	0.503	0.398	0.519	0.553	0.47	0.438	0.496	0.546	0.53	0.559	0.543	0.538	0.538	0.499
6						-	0.849	0.484	0.504	0.477	0.343	0.377	0.513	0.537	0.546	0.425	0.523	0.512	0.445	0.445	0.462	0.645	0.585	0.654	0.560	0.577	0.594	0.545
7							-	0.480	0.483	0.47	0.356	0.468	0.455	0.46	0.531	0.473	0.502	0.477	0.464	0.431	0.490	0.579	0.548	0.606	0.555	0.55	0.559	0.504
8								-	0.760	0.763	0.497	0.495	0.544	0.560	0.49	0.347	0.438	0.443	0.404	0.455	0.401	0.491	0.443	0.471	0.448	0.417	0.438	0.431
9									-	0.856	0.476	0.488	0.553	0.508	0.535	0.402	0.524	0.523	0.443	0.452	0.491	0.46	0.452	0.47	0.449	0.417	0.463	0.412
10										-	0.503	0.467	0.618	0.513	0.496	0.375	0.464	0.443	0.381	0.475	0.438	0.407	0.406	0.418	0.393	0.347	0.363	0.405
11											-	0.765	0.603	0.605	0.609	0.556	0.604	0.561	0.659	0.606	0.544	0.43	0.431	0.403	0.425	0.397	0.384	0.327
12												-	0.639	0.661	0.667	0.598	0.627	0.553	0.648	0.633	0.543	0.484	0.503	0.472	0.497	0.473	0.453	0.215
13													-	0.849	0.699	0.538	0.635	0.588	0.552	0.644	0.572	0.533	0.532	0.45	0.508	0.505	0.489	0.478
14														-	0.682	0.527	0.661	0.635	0.584	0.64	0.595	0.66	0.65	0.63	0.600	0.621	0.600	0.547
15															-	0.700	0.687	0.699	0.644	0.72	0.701	0.673	0.66	0.616	0.603	0.567	0.565	0.539
16																-	0.714	0.645	0.712	0.413	0.699	0.569	0.498	0.462	0.499	0.491	0.495	0.495
17																	-	0.818	0.606	0.745	0.623	0.614	0.623	0.572	0.560	0.554	0.521	0.440
18																		-	0.804	0.717	0.755	0.592	0.578	0.524	0.609	0.608	0.578	0.440
19																			-	0.758	0.743	0.512	0.515	0.475	0.552	0.519	0.506	0.350
20																				-	0.751	0.542	0.543	0.51	0.502	0.495	0.451	0.358
21																					-	0.597	0.543	0.509	0.506	0.500	0.463	0.392
22																						-	0.863	0.833	0.824	0.802	0.798	0.710
23																							-	0.908	0.852	0.827	0.812	0.631
24																								-	0.858	0.834	0.827	0.580
25																									-	0.939	0.917	0.656
26																										-	0.907	0.651
27																											-	0.674
28																												-

and 1,200 bp, respectively, in the Vietnamese populations (Fig. 1A). Especially, the 42 unique shared loci to each population generated by BION-A11 decamer primer were approximately 150 bp, 450 bp, 550 bp, 650 bp, 750 bp and 900 bp, respectively, in Sockcho cuttle fish population, as summarized in Table 2. Interestingly, the decamer primer BION-A20 generated 14 unique shared loci to each population, which were identifying each population, approximately 250 bp, and 350 bp, in Vietnamese cuttle fish populations (Table 2). Especially, the 21 unique shared loci to

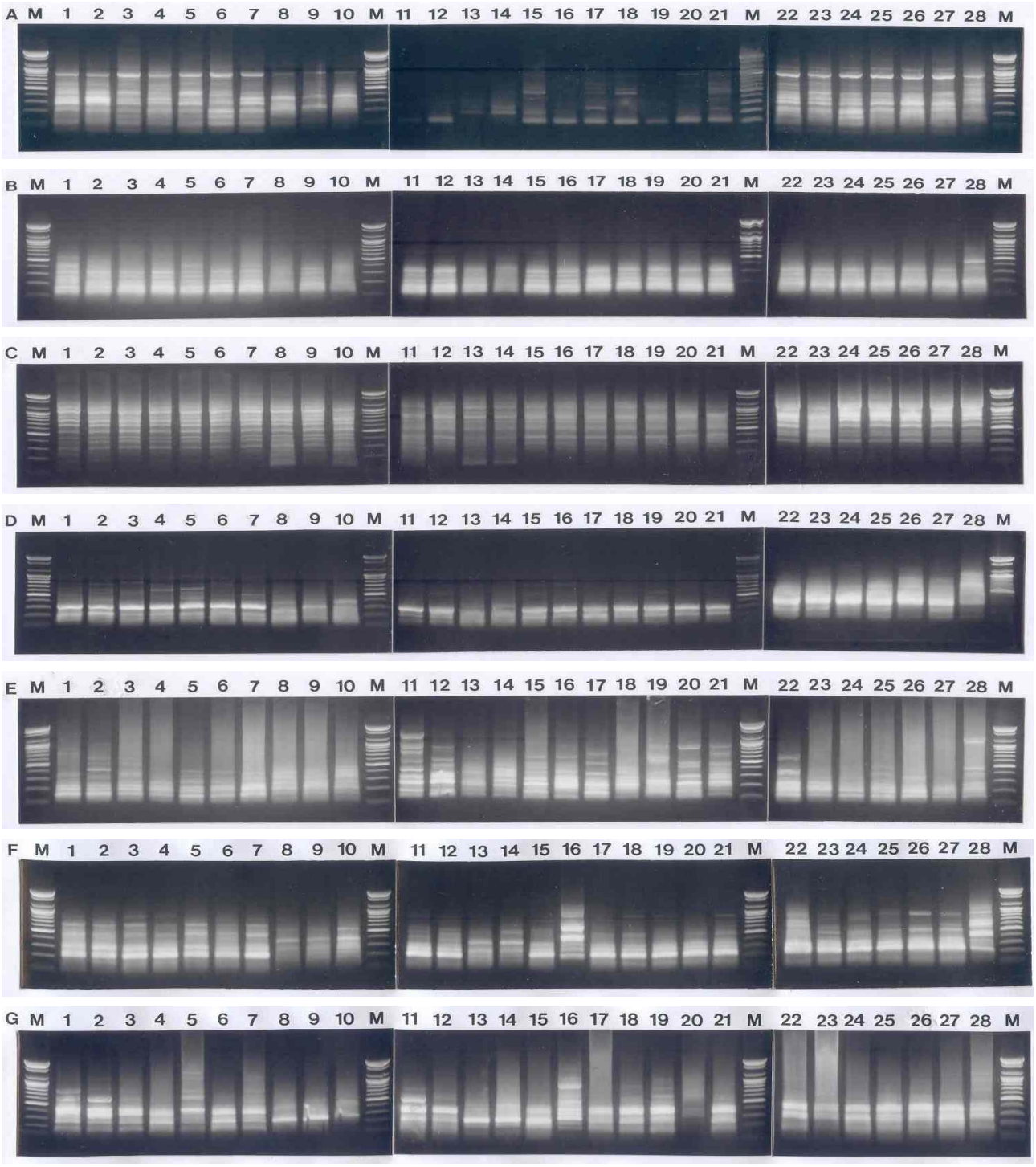

Fig. 1. PCR-based electrophoretic profiles of Korean cuttle fish (*Sepia esculenta*) DNA isolated from Sockcho (lane 01-07), Seocheon (lane 08-14), Incheon (lane 15-21) and Vietnamese cuttle fish population (lane 22-28). Each Individual DNA was amplified by random primer BION-A07 (A), BION-A09 (B), BION-A11 (C), BION-A20 (D), BION-B04 (E), BION-B06 (F) and BION-B14 (G). Amplified products were electrophoresed on a 1.4% agarose gel and detected by staining with ethidium bromide. M, 100 bp Ladder DNA markers.

Table 2. The number of unique shared loci to each population and number of shared loci by the four populations generated by PCR analysis using 7 random primers in Korean cuttle fish and Vietnamese cuttle fish, respectively

Item	No. of unique shared loci to each population				No. of shared loci by the our populations
Primer\population	SOCKCHO	SEOCHEON	INCHEON	VIETNAMESE	Four populations
BION-A07	21	0	0	21	0
BION-A09	0	0	0	0	28
BION-A11	42	0	7	0	112
BION-A20	0	0	7	14	28
BION-B04	0	0	0	0	112
BION-B06	21	0	0	0	28
BION-B14	0	0	0	7	0
Total no.	84	0	14	42	308
Average no. per primer	12	0	2	6	44

each population generated by BION-B06 decamer primer were approximately 100 bp, 450 bp and 650 bp, respectively, in Sockcho cuttle fish population. The decamer primer BION-B14 generated 7 unique shared loci to each population, which were identifying each population, approximately 100 bp in Vietnamese cuttle fish populations. Interestingly, the primer BION-A11 detected 112 shared loci by the four populations, major and/or minor fragments of sizes 300 bp, 400 bp, 700 bp and 1,000 bp, respectively, which were identical in all samples, as summarized in Table 2. For black tiger shrimp, 80 bands ranging in size from 200 bp to 2,200 bp were unambiguously scored (Tassanakajon et al., 1998). It has been reported that a single primer generates 9 to 15 distinct bands. The specific primer was found to be useful for the identification of individuals, which were the result of different DNA polymorphisms (Liu et al., 1998; Yoon & Kim, 2003; Park et al., 2005).

As regards average BS value results, cuttle fish population from Sockcho (0.826) exhibited higher bandsharing values than did fish from Seocheon (0.465), as illustrated in Table 3. This average BS value reported by our study is similar to the value reported for Spanish barbel species (0.71-0.81) (Callejas & Ochando, 1998). However, our reported BS values between the two geographical cuttle fish populations are inconsistent with the previously reported results (Yoon & Park, 2002). The average bandsharing value recorded in our study is also higher than the average value between the two oyster populations (0.282± 0.008) (Kim et al., 2004).

In this study, the dendrogram obtained by the seven primers indicates five genetic clusters: cluster 1 (SOCKCHO 01-SOCKCHO 07), cluster 2 (SEOCHEON 08-SEOCHEON 10), cluster 3 (SEOCHEON 11-SEOCHEON 14), cluster 4

Table 3. Multiple comparisons of average bandsharing values among Korean and Vietnamese cuttle fish (*Sepia esculenta* Hoyle) populations from four regions were generated according to the bandsharing values and similarity matrix

Population	SOCK-CHO	SEO-CHEON	IN-CHEON	VIET-NAMESE
SOCKCHO	0.826	0.465	0.485	0.549
SEOCHEON	-	0.606	0.538	0.463
INCHEON	-	-	0.698	0.528
VIETNAMESE	-	-	-	0.795

(INCHEON 15-INCHEON 21), and cluster 5 (VIETNAM 22-VIETNAM 28). Among the seven Vietnamese cuttle fishes, the shortest genetic distance that displayed significant molecular differences was between individuals 25 and 26 from the Vietnamese cuttle fish (genetic distance =0.025), while the longest genetic distance among the twenty-eight cuttle fishes that displayed significant molecular differences was between individuals SOCKCHO no. 02 and SEOCHEON no. 12 (genetic distance=0.640). Relatively, individual of Seocheon and Incheon cuttle populations was closely related to that of Vietnamese cuttle fish population. In the present study, RAPD-PCR analysis revealed a significant genetic distance among four cuttle fish populations. The existence of population differentiation and DNA polymorphisms among four cuttle fish populations were detected by RAPD-PCR. This shows that the method is one of the adequate tools for comparing the DNA of individuals, species and/or populations of prawn. Even if it cannot be affirmed by only a case, such a result seems to be closely connected that the Korean peninsula is subject to warm climatic changes by global warming. In the case of blacklip abalone, cluster analysis of the pairwise population matrix generated from RAPD data showed that geographically close populations tended to cluster together (Huang et al., 2000). A neighbor-joining tree based on genetic distances between populations using the RAPD-PCR method indicated the relationships between three mud crab species (Klinbunga et al., 2000), among which there were large intraspecies and interspecies differences between geographic populations. RAPD data analysis of genetic distance and parsimony methods, family clustering, and analysis of molecular variance were applied to the study of genetic relationships between a few of species within a genus. It was reported that the species relationships revealed by the RAPD-PCR approach should be consistent with previously obtained data using morphological affinities (Nebauer et al., 2000). From what has been said above, a dendrogram revealed close relationships between individual identities within four geographical populations.

As mentioned above, the potential of this analysis to identify diagnostic markers for the identification of four mollusk populations has also been demonstrated (Tassanakajon et al., 1998; McCormack et al., 2000; Yoon & Park, 2002: Yoon & Kim, 2003; Jung et al., 2004). In spite of the problems with variability and reproducibility, a large number of genetic studies have used RAPD-PCR because it is a relatively fast, reliable, and simple method for investigating a variety of genomic DNAs for polymorphisms, and for revealing genetic diversity within a population; additionally, it does not require prior knowledge of the genome (Welsh et al., 1991; Orozco-Castillo et al., 1994; Klinbunga et al., 2000). Thus, RAPD-PCR analysis revealed a significant genetic distance among the four cuttle fish populations.

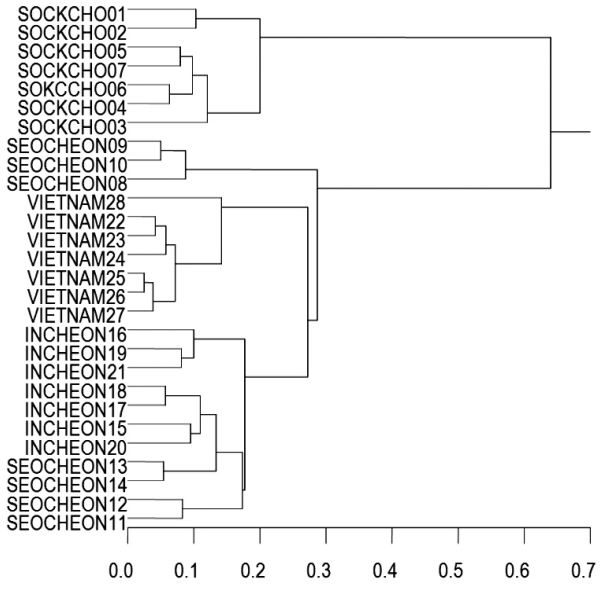

Fig. 2. Hierarchical dendrogram of genetic distances obtained from four geographic cuttle fish populations. The relatedness among different individuals of Korean cuttle fish from Sockcho (SOCKCHO 01-SOCKCHO 07), Seocheon (SEOCHEON 08-SEOCHEON 14), Incheon (INCHEON 15-INCHEON 21) and Vietnamese cuttle fish (VIETNAM 22-VIETNAM 28) generated according to the bandsharing values and similarity matrix as in Table 1.

ACKNOWLEDGEMENTS

We thank our laboratory colleague, Ms. S. A. Kang and other undergraduate students for their assistance with sample collection, PCR techniques, and statistical analyses. Particular thanks go to the anonymous reviewers who assisted us with thorough and far-reaching criticisms. This paper was supported by research funds of Kunsan National University (2010).

REFERENCES

Callejas C, Ochando MD (1998) Identification of Spanish barbel species using the RAPD technique. J Fish Biol 53:208-215.

Huang BX, Peakall R, Hanna PJ (2000) Analysis of genetic structure of blacklip abalone (*Haliotis rubra*) populations using RAPD, minisatellite and microsatellite markers. Mar Biol 136:207-216.

Jung HT, Kim J, Shin JA, Soh HY, Choi SD (2004) Genetic relationship of the five venerid clams (Bivalvia, Veneridae) in Korea. J Aquacult 17:251-257.

Kim JY, Park CY, Yoon JM (2004) Genetic differences and DNA polymorphism in oyster (*Crassostrea* spp.) analysed by RAPD-PCR. Korean J Genet 26:123-134.

Klinbunga S, Ampayup P, Tassanakajon A, Jarayabhand P, Yoosukh W (2000) Development of species-specific markers of the tropical oyster (*Crassostrea belcheri*) in Thailand. Mar Biotechnol 2:476-484.

Koo JG, Jee JH, Kim JM, Kang JC (2004) Effect of water temperature on survival, growth and inter-molt period of tiger crab, *Orithyia sinica* (Linnaeus) larvae. J Fish Pathol 17:139-144.

Liu Z, Li P, Argue BJ, Dunham RA (1998) Inheritance of RAPD markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. Furcatus*) and their F_1, F_2 and backcross hybrids. Anim Genet 29:58-62.

McCormack GC, Powell R, Keegan B (2000) Comparative analysis of two populations of the brittle star *Amphiura filiformis* (Echinodermata: Ophiuroidae) with different life history strategies using RAPD markers. Mar Biotechnol 2:100-106.

Nebauer SG, del Castillo-Agudo L, Segura J (2000) An assessment of genetic relationships within the genus *Digitalis* based on PCR-generated RAPD markers. Theor Appl Genet 100:1209-1216.

Orozco-Castillo C, Chalmers KJ, Waugh R, Powell W (1994) Detection of genetic diversity and selective gene introgression in coffee using RAPD markers. Theor Appl Genet 87:934-940.

Park SY, Park JS, Yoon JM (2005) Genetic differences and variations in slipper lobster (*Ibacus ciliatus*) and deep sea lobster (*Puerulus sewelli*) determined by RAPD analysis. Korean J Genet 25:307-317.

Smith PJ, Benson PG, McVeagh SM (1997) A comparison of three genetic methods used for stock discrimination of orange roughy, *Hoplostethus atlanticus*: allozymes, mitochondrial DNA, and random amplified polymorphic DNA. Fish Bull 95:800-811.

Tassanakajon A, Pongsomboon S, Jarayabhand P, Klinbunga S, Boonsaeng V (1998) Genetic structure in wild populations of black tiger shrimp (*Penaeus monodon*) using randomly amplified polymorphic DNA analysis. J Mar Biotech 6:249-254.

Yoke-Kqueen C, Radu S (2006) Random amplified polymorphic DNA analysis of genetically modified organisms. J Biotechnol 127:161-166.

Yoon JM (2006) Genetic differences and variations in freshwater crab (*Eriocheir sinensis*) and swimming crab (*Portunus trituberculatus*). Dev Reprod 10 (1):19-32.

Yoon JM, Kim GW (2003) Genetic differences between cultured and wild penaeid shrimp (*Penaeus chinensis*) populations analyzed by RAPD-PCR. Korean J Genet 25:21-32.

Yoon JM, Kim JY (2004) Genetic differences within and between populations of Korean catfish (*S. asotus*) and bullhead (*P. fulvidraco*) analyzed by RAPD-PCR. Asian-Aust J Anim Sci 17:1053-1061.

Yoon JM, Park HY (2002) Genetic similarity and variation in the cultured and wild crucian carp (*Carassius carassius*) estimated with random amplified polymorphic DNA. Asian-Aust J Anim Sci 15:470-476.

(received 10 July 2010, received in revised from 8 August 2010, accepted 9 August 2010)