Fish Aqua Sci 12(3), 171-178, 2009

KOSFAS FISHERIES AND AQUATIC SCIENCES
The Korean Society of Fisheries and Aquatic Science

Genetic Phylogeny among Three Species Red Seabream, Black Seabream and Rock Bream Based on Mitochondrial DNA Sequences

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The Perciformes include approximately 40% of all bony fishes and are the largest order of vertebrates. This order includes some of the most economically relevant marine fishes, particularly the red seabream, black seabream and rock bream. A 409 bp fragment of the cytochrome *b* (cyt *b*) gene and 403 bp and 518 bp fragments of ribosomal RNA (12S and 16S rRNA, respectively) were sequenced from five populations of natural and cultured red seabreams, natural black seabream, and natural and cultured rock breams. The mitochondrial DNA sequences were utilized for the genetic identification and population structural analyses of these three species. Phylogenetic relationships of intra- and inter-species were elucidated using three types of molecular genetic markers from three species of the order Perciformes in Korea. We noted no significant differences in the intra-specific variation of the cyt *b* and rRNA genes in each population however, inter-specific divergences were greater than intra-specific variation. Inter-specific variation was induced more by transition than transversion type in the cyt *b* and rRNA genes. The cyt *b* gene and rRNA genes make it possible to determine the inter-species divergence. The rRNA genes have more conserved sequences than the cyt *b* gene. Therefore, these genes are expected to prove useful among species belonging to the different genera or families.

Key words: Red seabream, Black seabream, Rock bream, Mitochondrial DNA, Phylogeny

Introduction

Effective fishery management requires an understanding of the population and stock structure of bioresources. In other to investigate the genetic information of stock structures, we must study the genetic characteristics that demonstrate sufficient variation for the discrimination of intraspecific groups or stocks. Although the identification of the population structures of useful marine bioresources using the genetic characteristics is a crucial pursuit, simple morphological measuring and systematic feature studies have been the most frequently conducted for the several reasons. However, in the past two decades, genetic features as reproductive systems of organisms have been classified at the molecular level by the development of various biochemical analytical methods and the rapid progress of molecular biology.

Mitochondrial DNA (mtDNA) evolves five to ten times more rapidly than chromosomal DNA (Brown et al., 1979); thus very powerful tools must be utilized for species identification, studies of evolutionary populational genetics, and hybridization analyses (Avise et al., 1984; Bartlett and Davidson, 1991; Billington and Hebert, 1988; Saunders et al., 1986). Among the many mtDNA genes, cytochrome *b* (cyt *b*) and ribosomal RNA genes have been successfully utilized to identify genetic variation in many fish species (Carr and Marshall, 1991; Campo et al., 2007; Lee et al., 2000; Li et al., 2008).

The Perciformes include approximately 40% of all bony fishes and are the largest order of vertebrates. They belong to the ray-finned fish and comprise more than 7,000 different species, with varying shapes and sizes, which are distibuted throughout almost all aquatic environments. The red seabream (*Pagrus major*), black seabream (*Acanthopagrus schlegeli*)

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and rock bream (*Oplegnathus fasciatus*), all members of the order Perciformes are economically crucial fisheries resources in Korea. In the case of the order Perciformes, a variety of studies have been conducted, including morphological (Tojima et al., 1995), protein polymorphism (Funkenstein et al., 1990; Reina et al., 1994; Taniguchi and Sugama, 1990) and mtDNA polymorphism (Magoulas et al., 1995) studies; however, no information is currently available for population analysis using molecular genetic markers.

The nucleotide sequence of the mtDNA specific - genes can be routinely and conveniently utilized to determine the species identity of each individual. In this study, we compared segments of the mtDNA cyt *b* and ribosomal RNA genes in the three species of the order Perciformes. We estimated the genetic differences among the five populations on the basis of mtDNA - specific gene sequences and determined the suitable genetic markers for intra- and inter-species analysis.

Materials and Methods

Sampling and mitochondrial DNA extraction

In this study, the mtDNA sequences of red seabream, black seabream and rock bream were analyzed. Five populations were utilized as natural and cultured red sea breams (CN and CC, respectively), natural black seabream (GD), and natural and cultured rock breams (DN and DC, respectively). Ten to 30 individuals per population were collected from Yeochon and northern Jeju in June 1996. The mtDNA was extracted from frozen liver or muscle tissue using a modified alkaline method previously described by Chapman and Power (1984) and stored at -20°C until analysis.

PCR (polymerase chain reaction) amplification and DNA sequencing

PCR amplifications of cyt *b*, 12S and 16S rRNA genes were conducted using an RTC 200 machine (MJ-Research, Watertown, MA, USA) in 50 μL of solution containing 10-50 ng DNA, 1x ExTaq buffer, 0.2 mM dNTPs, 10 μM of each primer, and 0.25 U of Taq DNA polymerase (TAKARA, Osaka, Japan). The primers utilized in this study are the following: for the cyt b gene, the forward Cyt-L (5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT-3') and the reverse Cyt-H (5'-AAA CTG CAG CCC CTG CTC AGA ATG ATA TTT GTC CTC A-3'), for the 12S rRNA gene, the forward 12SA-L1 (5'-TCA AAC TGG GAT TAG ATA CCC CAC TAT-3') and the reverse 12SB-H (5'-TCA CTG CAG AGG CTG ACG GGC GGT GTG T-3') and for the 16S rRNA gene, the forward 16SAR-L (5'-CGC CTG TTT ATC AAA AAC AT-3') and the reverse 16SBR-H (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Zardoya et al., 1995). The amplification conditions used in this study were as follows; initial denaturation for 11 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C with a final 5 min extension step at 72°C.

PCR products were purified using PCR Purification Kits or Gel Extraction Kits (Qiagen, Hilden, Germany) and sequenced on an ABI3100 Prism automatic DNA sequencer with the BigDye 3.1 Termination system (Applied Biosystems, Foster City, CA, USA).

Data analysis

Sequences were edited with the Bioedit Sequence Alignment Editor software (Hall, 1999). The obtained sequence data were aligned with the DNASIS software (version 2.5; Hitachi, Tokyo, Japan) in other to determine the haplotype of the cyt *b*, 12S and 16S rRNA genes. Genetic distances were calculated in accordance with Kimura's formula (1980). A dendrogram was constructed via the unweighted pair group method with the arithmetic mean (UPGMA; Sneath and Sokal 1973) in the PHYLIP software package (version 3.5; Felsenstein, 1993).

Results

Nucleotide sequences of mitochondrial cyt *b* gene

A 409 bp fragment of the cyt *b* gene was determined in the CN, CC, GD, DN and DC populations. Two major haplotypes in each population were represented in Table 1. In the case of intra-species, nucleotide substitutions were detected at three positions (position 132, 180 and 252) between the CN and CC populations, four positions (position 21, 22, 90 and 188) between the DN and DC populations, and one position (position 96) between the GD haplotypes (Table 1). The average nucleotide substitutions among species represented 61 sites between the red seabream (CN and CC) and the black seabream, 77 sites between the red seabream (CN and CC) and the rock bream (DN and DC), and 76 sites between the black seabream and the rock bream (DN and DC) (Table 2). In this study, the majority of nucleotide substitutions in the cyt *b* gene are transition (adenine (A) ↔ guanine (G) or cytosine (C) ↔ thymine (T)) types. The C ↔ T transition type is more abundant than the other type in all populations (Table 1).

Table 1. Aligned mitochondrial cyt *b* gene sequences in five populations, showing variable sites. Dots (·) indicate the same base with those of the haplotype CC01

	Variable nucleotide site
	1111111111111111111111112222222222222222222222222233333333333333333333333333
	1122223333344455566777889999000011222334445667788899011122223334455566677788990011122223334444455667
	4675812480136925814703258140679258914169251476584703829406902581470625814723925470328914780690258914692
CC01	TATTTCCCCAGTCGTAAGTTTCTTCTACCCACTGACCCCTCATCATTTTCTCGCCCTCTCCTTTAATTCCCTTCGACCCTTCTATATTCGCCTCTCAGAGCCTTT
CC02	·····C·····
CN01	·····T·····T·····
CN02	·····T·····
GD01	·····T·TG·A··AG·CT·CCT··ACTT·T··CACTT·T··G·TCC··ATC··T·AAAC··CCC···C·TTCC···ATTCC··CC··A·ATTA·AT····GTCC·
GD02	·····T·TG·A··AG·CT·CCT··ACTTGT··CACTT·T··G·TCC··ATC··T·AAAC··CCC···C·TTCC···ATTCC··CC··A·ATTA·AT····GTCC·
DC01	CCCCA·G··C·CTC·TCTC·G·CC·CCG·TGTCA··TTTA·GC··ACAAACTCTTA·A·AT·CCTCCC···CGTCC··TACTCC·GA·A·T··TAAGAGTA·CCC
DC02	CCCCAG···C·CTC·TCTC·G·CC·CCG·TGTCA··TTTA·GC··ACAAACT·TTA·A·AT·CCTCCC···CGTCC··TACTCC·GA·A·T··TAAGAGTA·CCC
DN01	CCCCA·G··C·CTC·TCTC·G·CC·CCT·TGTCA··TTTA·GC··ACAAACT·TTA·A·AT·CCTCCC···CGTCC··TACTCC·GA·A·T··TAAGAGTA·CCC
DN02	CCCCA·G··C·CTC·TCCC·G·CC·CCT·TGTCA··TTTA·GC··ACAAACT·TTA·A·AT·CCTCCC···CGTCC··TACTCC·GA·A·T··TAAGAGTA·CCC

Table 2. Estimated sequence divergence (%) between each pair (above diagonal) and total numbers of variable sites (below diagonal) based on mitochondrial cyt *b* gene sequences of five populations

	CC01	CC02	CN01	CN02	GD01	GD02	DC01	DC02	DN01	DN02
CC01	-	0.24	0.49	0.24	14.67	14.91	18.83	18.58	18.58	18.58
CC02	1	-	0.73	0.49	14.42	14.67	18.58	18.34	18.34	18.34
CN01	2	3	-	0.24	15.16	15.40	19.32	19.07	19.07	19.07
CN02	1	2	1	-	14.91	15.16	19.07	18.83	18.83	18.83
GD01	60	59	62	61	-	0.24	18.58	18.34	18.34	18.34
GD02	61	60	63	62	1	-	19.07	18.58	18.58	18.58
DC01	77	76	79	78	76	78	-	0.73	0.49	0.49
DC02	76	75	78	77	75	76	3	-	0.49	0.73
DN01	76	75	78	77	75	76	2	2	-	0.24
DN02	76	75	78	77	76	77	2	3	1	-

Table 3. Estimated genetic distances of each pair based on mitochondrial cyt *b* gene sequences of five populations

	CC01	CC02	CN01	CN02	GD01	GD02	DC01	DC02	DN01	DN02
CC01	-									
CC02	0.0025	-								
CN01	0.0049	0.0074	-							
CN02	0.0025	0.0049	0.0025	-						
GD01	0.1655	0.1612	0.1710	0.1698	-					
GD02	0.1694	0.1662	0.1760	0.1727	0.0012	-				
DC01	0.2194	0.2159	0.2264	0.2229	0.2159	0.2226	-			
DC02	0.2162	0.2127	0.2232	0.2197	0.2127	0.2159	0.0037	-		
DN01	0.2165	0.2130	0.2235	0.2400	0.2157	0.2157	0.0025	0.0025	-	
DN02	0.2165	0.2130	0.2235	0.2400	0.2191	0.2191	0.0049	0.0074	0.0025	-

The percentage of sequence divergence within five populations ranged from 0.24 to 0.73%, whereas the inter-species percentage ranged from 14.42 to 15.40% between the red and black seabream, 18.34 to 19.32% between the red seabream and the rock bream, and 18.34 to 19.07% between the black seabream and the rock bream (Table 2).

The genetic distance calculated with the Kimura's two parameters method ranged from 0.0012 to 0.0074 within species, whereas it ranged from 0.1612 to 0.1760 between the red and black seabream, from 0.2127 to 0.2400 between the red seabream and the rock bream and from 0.2127 to 0.2226 between the black seabream and the rock bream (Table 3). The UPGMA dendrogram of five populations constructed on the basis of their population genetic distances was calculated. The UPGMA dendrogram revealed three distinct clusters, as shown in Fig. 1.

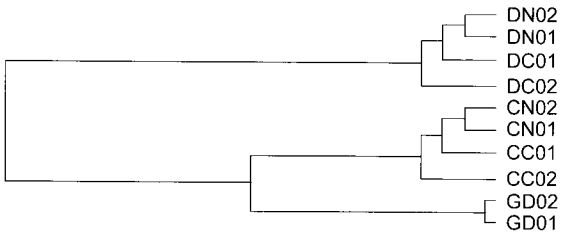

Fig. 1. Genetic relationship constructed by UPGMA in mitochondrial cyt b gene.

Nucleotide sequences of ribosomal RNA

We sequenced 403 bp fragment of the 12S rRNA gene and the 518 bp fragment of the 16S rRNA gene. Two major haplotypes in each population were represented in Table 4. In the 12S rRNA gene, nucleotide substitutions are represented at three positions (position 470, 483 and 489) between the DN and DC populations, one position between the CN and CC populations (position 330) and between the haplotypes of GD (position 479), respectively (Table 4 (a)). In the 16S rRNA gene, nucleotide substitutions were detected only at one site (position 307) between the red seabream populations (Table 4 (b)). Ribosomal genes evidenced fewer base changes as insertions or deletions than those of the cyt *b* gene determined in this study. The average nucleotide substitutions among species represented 41 and 46 sites between the red seabream (CN and CC) and the black seabream, 48 and 45 sites between the red seabream (CN and CC) and the rock bream (DN and DC), and 52 and 54 sites between the black seabream and the rock bream (DN and DC) in the 12S and 16S rRNA genes, respectively (Table 5). These variations in ribosomal genes were caused principally by transitional substitutions (Table 4).

The percentage sequence divergence calculated within species ranged from 0 to 0.25% and from 0 to 0.58% in the 12S and 16S rRNA genes, respectively (Table 5). On the other hand, the percentage sequence divergence of inter-species ranged from 10.17 to 10.42% and from 8.67 to 9.07% between the red and black seabream, from 11.91 to 12.16% and from 8.30 to 8.88% between the red seabream and the rock bream, and 12.90% and from 10.23 to 10.81% between the black seabream and the rock bream in the 12S and 16S rRNA genes, respectively (Table 5).

The genetic distance calculated by Kimura's two parameter method varied from 0 to 0.0025 and from 0 to 0.0058 within species in the 12S and 16S rRNA genes, respectively. In the case of inter-species, it ranged from 0.1106 to 0.1136 and from 0.0929 to 0.0973 between the red and black sea bream, from 0.1309 to 0.1353 and from 0.0882 to 0.0948 between the red seabream and the rock bream, and 0.1436 and from 0.1109 to 0.1176 between the black seabream and the rock bream in the 12S and 16S rRNA genes, respectively (Table 6). The genetic relationships of five populations constructed via UPGMA analysis were divided into three groups (Fig. 2).

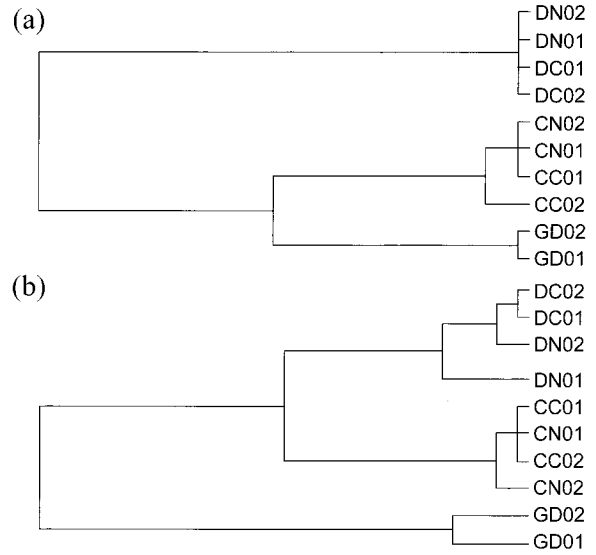

Fig. 2. Genetic relationship constructed by UPGMA in mitochondrial 12S rRNA (a) and 16S rRNA gene (b).

Discussion

The development of techniques for the manipulation of specific DNA fragments has resulted in the identification of a number of genetic markers on the basis of DNA sequence polymorphisms. Fish mtDNA ranges in length from 15.2 to 19.8 kb (Billington and Hebert, 1991) and contains information specifying the two rRNAs (16S and 12S), 22 tRNAs, 13 polypeptides, and a control region (dissociation (D) loop). The proteins for which it encodes include NADH dehydrogenase, cytochrome oxidase, cyt *b*, and ATPases 6 and 8 (Gyllensten and Wilson, 1987). Mitochondrial protein-coding genes were classified as good performers in the resolution of relationships among distant relatives (Zardoya and Meyer, 1996). Among these, cyt *b* gene has several gene sections that are highly conserved among taxa. Also, there are variable parts at the 3' end of the sense strand. The 12S and 16S rRNA genes are subunits of the ribosomal RNA gene in the mtDNA. They are fairly conserved among taxa, but evidence sufficient variations to be useful in population-level research.

The majority of variations in animal mtDNA are

Table 4. Aligned mitochondrial ribosomal gene sequences ((a) 12S rRNA and (b) 16S rRNA genes) in five populations, showing variable sites and deletion events (-). Dots (·) indicate the same base with those of the haplotype CC01

(a)

Variable nucleotide site of 12S rRNA gene

	1111111111112222222222222222222222222222333333333333333333333333444444444444
	3699112333333578111122223334444455555666666700011123333444455577999004555677788
	0017013234568591056901562592345623458024568990191238058903681291901224934930491 39
CC01	TGGTTCACCTCCCCTCTGTGAGCCAGCTTAACAAAA-CAT-GAGAGCTCAAGATACA-TACCTGATAACCCAA-TCGTAGG
CC02	·····
CN01	·····
CN02	·····G·····
GD01	A·A·C·G·TCT··TAAA·C·GA·TTATAAG·T···TAT··GA·CGA·A·TTA··TATTCGT··ACA·G-TT··T-·AA···
GD02	A·A·C·G·TCT··TAAA·C·GA·TTATAAG·T···TAT··GA·CGA·A·TTA··TATTCGT··ACA·G-TT··T-·A····
DC01	·A·C·A·TT·TTA·A··ACAG·ATT··AA·TTGCC·-·G-G·GC··TCA··AGAC·T····TCAT·C·-·TTT·A·A····
DC02	·A·C·A·TT·TTA·A··ACAG·ATT··AA·TTGCC·-·G-G·GC··TCA··AGAC·T····TCAT·C·-·TTT·A·A····
DN01	·A·C·A·TT·TTA·A··ACAG·ATT··AA·TTGCC·-·G-G·GC··TCA··AGAC·T····TCAT·C·-·TTT·A·A·-CA
DN02	·A·C·A·TT·TTA·A··ACAG·ATT··AA·TTGCC·-·G-G·GC··TCA··AGAC·T····TCAT·C·-·TTT·AGA····

(b)

Variable nucleotide site of 16S rRNA gene

	1111111122222222222222222222222233333333333333333333333333333
	1222333334455569355556880000111222336688889999999900011112222233335556667788 88
	8934612467484695606789024234758956723144789012345691470125357890123457028230158
CC01	GCTGATTTTGCTAATTCTCTTGT-CGTAAGATCTGAAAGCTTTTAAACATGTTGAGACCGGCCTCTGCGAG-GCACCTT
CC02	·····A·····
CN01	·····
CN02	·····
GD01	A··TT·····T··G·C·C·C·C·T·····AGCTCAGT·-··G·C---·CC·AC·TAG·AA·TACT·ATATTTAT··T··
GD02	A··TT·····T··G·C·C·C·C·T·····AGCTCAGT·-··G·C---·CC·AC·TAG·AA·TACT·ATATTTAT··T··
DC01	ATATCACACA·AGGC·TCTCCTCT-CCTTA··TG···G·A------··G·CTA-·C··T-·ATG··C··A·A··TGTTCG
DC02	ATATCACACA·AGGC·TCTCCTCT-CCTTA··TG···G·A------··G·CTA-·C··T-·ATG··C··A·A··TGTTCG
DN01	ATATCACACA·AGGC·TCTCCTCT-CCTTA··TG···G·A------··G·CTA-·C··T-·ATG··C··A·A··TGTTCG
DN02	ATATCACACA·AGGC·TCTCCTCT-CCTTA··TG···G·A------··G·CTA-·C··T-·ATG··C··A·A··TGTTCG

attributable to a base substitution, which shows a greater proportion of the transition than the transversion type. A 295 bp fragment of the cyt *b* gene in Atlantic salmon (*Salmo salar*) had mostly harbored transition-type nucleotide differences (McVeigh et al., 1991). Our results demonstrated more frequent transition than transversion type among among the cyt *b* gene and the rRNA genes. The variations between the rock bream and two other species (red seabream and black sesabream) of the Sparidae were more variable among the five populations. Mitochondrial sequence divergence was more variable on the intra-species level than on the inter-species and on the different genus or family levels.

The findings of this study were similar to those of previous studies. Our data demonstrated that the genetic distance within the CC and CN ranged from 0.0025 to 0.0074 in the cyt *b* gene and 0.0025 and 0.0019 in the 12S and 16S rRNA genes, respectively. The genetic distance ranged from 0.00395 to 0.00398 in the red sea bream from the coastal waters of Japan and the East China Sea at 24 isozyme loci (Taniguchi and Sugama, 1990).

The mitochondrial gene sequences in this study, even if they are partial, will serve as basic information regarding genetic variation among the order. Numerous molecular systematic studies have employed variations in this gene to infer patterns of relationship among fish species (Bartlett and Davidson, 1991; Carr and Marshall, 1991; Kocher et al., 1989). The genetic trees among populations or closely related species are reflective of the phylogeny of the organisms (Hillis et al., 1996). Consequently, three mtDNA genes utilized in this study represented favorable genetic markers for systematic genetic

Table 5. Percent sequence divergences (above diagonal) and the number of variation sites (below diagonal) calculated from ribosomal gene sequences of five populations

(a) 12S rRNA gene

	CC01	CC02	CN01	CN02	GD01	GD02	DC01	DC02	DN01	DN02
CC01	-	0.25	0	0	10.17	10.17	11.91	11.91	11.91	11.91
CC02	1	-	0.25	0.25	10.42	10.42	12.16	12.16	12.16	12.16
CN01	0	1	-	0	10.17	10.17	11.91	11.91	11.91	11.91
CN02	0	1	0	-	10.17	10.17	11.91	11.91	11.91	11.91
GD01	41	42	41	41	-	0	12.90	12.90	12.90	12.90
GD02	41	42	41	41	0	-	12.90	12.90	12.90	12.90
DC01	48	49	48	48	52	52	-	0	0	0
DC02	48	49	48	48	52	52	0	-	0	0
DN01	48	49	48	48	52	52	0	0	-	0
DN02	48	49	48	48	52	52	0	0	0	-

(b) 16S rRNA gene

	CC01	CC02	CN01	CN02	GD01	GD02	DC01	DC02	DN01	DN02
CC01	-	0	0	0.19	8.88	8.67	8.49	8.49	8.88	8.67
CC02	0	-	0	0.19	8.88	8.67	8.49	8.49	8.88	8.67
CN01	0	0	-	0.19	8.88	8.67	8.49	8.49	8.88	8.67
CN02	1	1	1	-	9.07	8.88	8.30	8.30	8.67	8.49
GD01	46	46	46	47	-	0.19	10.42	10.42	10.81	10.62
GD02	45	45	45	46	1	-	10.23	10.23	10.62	10.42
DC01	44	44	44	43	54	53	-	0	0.39	0.19
DC02	44	44	44	43	54	53	0	-	0.39	0.19
DN01	46	46	46	45	56	55	2	2	-	0.58
DN02	45	45	45	44	55	54	1	1	3	-

Table 6. Estimated genetic distances of 12S rRNA (below diagonal) and 16S rRNA (above diagonal) genes between each pair based on mitochondrial DNA ribosomal gene sequences of five populations

	CC01	CC02	CN01	CN02	GD01	GD02	DC01	DC02	DN01	DN02
CC01	-	0	0	0.0019	0.0951	0.0929	0.0905	0.0905	0.0948	0.0926
CC02	0.0025	-	0	0.0019	0.0951	0.0929	0.0905	0.0905	0.0948	0.0926
CN01	0	0.0025	-	0.0019	0.0951	0.0929	0.0905	0.0905	0.0948	0.0926
CN02	0	0.0025	0	-	0.0973	0.0952	0.0882	0.0882	0.0926	0.0904
GD01	0.1106	0.1136	0.1106	0.1106	-	0.0039	0.1131	0.1131	0.1176	0.1153
GD02	0.1106	0.1136	0.1106	0.1106	0	-	0.1109	0.1109	0.1154	0.1131
DC01	0.1309	0.1353	0.1309	0.1309	0.1436	0.1436	-	0	0.0039	0.0019
DC02	0.1309	0.1353	0.1309	0.1309	0.1436	0.1436	0	-	0.0039	0.0019
DN01	0.1309	0.1353	0.1309	0.1309	0.1436	0.1436	0	0	-	0.0058
DN02	0.1309	0.1353	0.1309	0.1309	0.1436	0.1436	0	0	0	-

studies among different sparid species. In particular, it is possible to use the cyt *b* gene is possible to determine the intra-species divergence. Ribosomal RNA genes have more conserved sequences than those of the cyt *b* gene. They are expected to prove useful among species belong to different genera or families.

Taniguchi et al. (1995) observed slight genetic changes and improvements in the five red seabream strains for aquaculture. However, we detected no symptoms of inbreeding in the genetic traits. In the case of Atlantic salmon, no evidence was found for the generally reduced variability in the examined farmed lines (Cross and Challanain, 1991; Knox and Verspoor, 1991; Youngson et al., 1991). The farmed and cultured populations evidenced no significant differences, and this was true of both the red seabream and the rock bream. Furthermore, further work will be required to investigate the marked differences in genomic DNA polymorphisms between farmed and cultured populations.

Acknowledgments

This study was supported by the Internship Program of the Biotechnology Research Institute, National Fisheries Research and Development Institute (NFRDI), Republic of Korea (contribution

no. RP-2009-BT-017). Views expressed herein are those of the authors and do not necessarily reflect the views of NFRDI.

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(Received 21 August 2008; Revised 13 October 2008; Accepted 4 September 2009)