Thesis for the Degree of Master of Engineering

Comparison of genetic variability in wild and cultivated olive flounder *Paralichthys olivaceus* in Korea using microsatellite DNA marker

by

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Department of Biotechnology Engineering

The Graduate School

Pukyong National University

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유전자표지를 이용한 한국의 자연산, 양식산 넙치의 유전학적 다양성의 비교에 관한연구

정 종 근

부 경 대 학 교 대 학 원 생 물 공 학 과

넙치(*Paralichthy olivaceus*) 는 아시아지역의 어업과 수산양식에서 중요한 어류이다. 하지만 여러 가지의 원인에 의해서 시간이 지남에 따라 유전적 다양성이 감소하고 있으며 이로 인하여 집단의 생존 및 성장의 부분에 있어서 악영향을 미치고 있다. 이와 같은 수산생물 집단의 구조를 밝혀내기 위해 높은 다형성을 가지는 많은 종류의 유전자 표지가 사용되고 있으며, microsatellite 표지는 집단의 구조분석과 유전적 연관성을 밝혀내는데 있어서 중요한 도구로 사용되고 있다. 본 연구에서는 새로운 34개의 microsatelltes 표지를 넙치의 expressed sequence tags 자료로부터 분리 하였으며, 분리된 표지를 이용 한국의 자연산과 양식산 넙치에 대한 유전적 다양성을 비교하였다. 다양성의 비교를 위해 사용된 표지의 대립유전자의 범위는 2-37 개 (평균 14.09), 관찰치 이형접합체율의 범위는 0.032-0.925 로 나타났으며, 기대치 이형접합체율은 0.031-0.969 의 범위를 나타내었다. 8개의 유전자좌에서 다형성의 결핍에 의한 Hardy-Weinberg 평형 ($P<0.001$)의 이탈이 관찰되었다. 유전적 다양성 비교를 위한 6개의 높은 유전자좌를 확인할 수 있었으며 확인된 유전자 표지를 이용하여 넙치의 집단연구에 이용하였다. 각 집단의 유전적 다양성의 비교에서 기대치 이형접합체율의 범위는 자연산 집단이 0.926 (*KOF002*) - 0.962 (*KOF101*) 로 나타났으며, 양식산 집단은 0.759 (*KOF033*) - 0.976 (*KOF044*) 로 나타났다. 대립유전자의 비교에서는 9.5 (CA) - 22.13 (WG) 의 범위를 나타내었다. 총 대립유전자의 평균개수는 29.75 개 이며, 자연산 집단은 28.17개, 양식산 집단 15.50 개로 나타났다. 각 집단간의 유전적 거리를 비교해보면 CB - CC 간이 가장 가까운 값을 나타내었고, WD - CA 간이 가장 먼 것으로 나타났다. 유전적 거리에 기초한 각 집단이 두개의 군집인 CA, CC, CD, CB, WT 와 WD, WG, WB 로 나누어짐을 확인할 수 있었다. 본 연구에서 이용된 유전자 표지를 이용하여 어류 각 개체의 집단간의 유전학적 다양성을 확인할 수 있으며, 유전자 표지를 이용하여 유전자 연관지도 개발 및 분석과 양적경제형질을 지니는 개체의 선발에 유용하게 이용할 수 있을 것이다.

유전자표지를 이용한 한국의 자연산, 양식산 넙치의 유전학적 다양성의 비교에 관한연구

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Contents

List of figures

List of tables

Ⅰ. Introduction

Oliver flounder (*Paralichthys olivaceus*) is an important fish species in Asia for both fisheries and aquaculture. Market size of this species 42,000 ton last year (2007, Korea national statistical offices). The management of fish stocks would be more effective if it were actually based on true biological stocks rather than arbitrary defined stocks (Carvalho and Hauser, 1994), and there could be serve effects of jumping together populations that have separate population genetics and population dynamics when managing species (Ryman *et al.*, 1995; Bailey, 1997; Frank and Brickman, 2001; Laikre *et al.*, 2005). Large number of cultured olive flounder escape released from aquaculture facilities, they could alter the genetic composition of wild populations by either displacing or interbreeding with them. This is because most hatchery stocks typically show a reduced genetic variability, which may possibly result in the reduction of the population's capability to adapt to new environments (Allendorf and Phelps, 1980). During the last decades, the availability of highly variable genetic markers has facilitated the gathering of large quantities of data on the scale and magnitude of population structuring in marine fishes (H. H. Jakob *et al.*, 2007). But significant and usually temporally stable, levels of genetic divergence has been demonstrated for many species of marine fishes (Ruzzante *et al.*, 1999; Nielsen *et al.*, 2003; Bekkevold *et al.*, 2005; Jørgensen *et al.*, 2005; Ruzzante *et al.*, 2006). A number of mechanisms have been

suggested to explain how population structure can evolve in an environment without any obvious physical boundaries to gene flow. Population genetic structures of a fish species can change over time due to a number of reasons, including overexploitation, introductions of alien species and contamination of native gene pools through introgression, inbreeding, bottleneck effect, environmental pollution, habitat degradation, hydrological manipulations, and global effects such as climatic changes and mutation at various levels (Mohammad. N. I. *et al.*, 2007).

Loss of genetic variation leads to potential harmful effects upon various performance traits such as survival and growth (Allendorf and Utter, 1979; Allendorf and Phelps, 1980), and intensive stocking practices with inappropriate hatchery procedures may result in detrimental genetic impacts on the wild stock accompanied by risk to the continued exploitation of the resource. The rate of loss of genetic variability in a population is based on the effective population size (Primack, 1998), and the magnitude of effective population size is affected by several factors such as a small number of founders, unequal sex ratio in the breeding population, and family size variations (Gall, 1987; Hedrick, 2000).

Historically, satellite DNA was identified as a DNA fraction that sedimented as a strong and localized band, above or below the main band in cesium chloride density gradients, hence its name (Walker *et al.*, 1971). Satellite DNA is found in heterochromatin regions, such as mammalian centromeres, the *D. melanogaster* Y chromosome, and plant subtelomeres and centromeres but may also be found as intercalary DNA. Mini- and microsatellites are tandem repeats composed of short repeat units. The repeat unit size is used as the main

feature to classify a short tandem repeat as a mini- or microsatellite. However, there is at the present time no consensus about the precise definition of both kinds of repeats (B. Charlesworth *et al.*, 1994). Microsatellites are regions of the genome that contain simple tandem repeats with variation occurring in the number of repeats within the region. Microsatellite loci consist of simple tandemly repeated sequences of 1 to 6 base pair in lengths (Tautz, 1989; Weber and May, 1989). Owing to the variation in the number of repeats units, microsatellites may exhibit a high degree of length polymorphism. They also appear to be abundant throughout the genome of eukaryotes. Among the molecular markers available in population genetics, microsatellites emerged as those with finest resolution for labeling of populations and individuals, due to their high variability, abundance, neutrality and codominance (Weber and May, 1989; Tauts, 1989). Microsatellite genotype array has proved to be a powerful tools for accurate genetic assessment of population and pedigree tracing of hatchery populations from various fishery animals (Neff, 2001; Norris *et al.*, 2000; Yu and Guo, 2005; Sugayat *et al.*, 2002; Li *et al.*, 2003). Microsatellites markers have been successfully developed in several fish species including Atlantic eel (Wielogoss S. *et al.*, 2008), catfish (Islam MN. *et al.*, 2007), Atlantic salmon (Dillane E. *et al.*, 2008), sea bream (Lanfant P., 2003) and turbot (Florin A. *et al.*, 2008).

Expressed sequence tags (ESTs) are fragments of mRNA sequences derived through single sequencing reactions performed on randomly selected clones from cDNA libraries. To date, over 45 million ESTs have been generated from over 1400 different species of eukaryotes (Parkinson J *et al*., 2009). EST analysis is not only the most efficient approach for gene discovery. But also

an effective approach for the identification of polymorphic DNA markers such as microsatellites and single nucleotide polymorphism that are highly useful for genetic mapping and comparative gene analysis (He C. *et al.*, 2003; Serapion J. *et al.*, 2004). These ESTs represent a potentially valuable source of gene-based SSR (simple sequence repeat) markers for population genetic analyses (Fig 1). While EST-SSRs are not without their drawbacks, they offer a number of clear benefits, including rapid and inexpensive development and high levels of cross-taxon portability. The automated sizing of flourescently labeled SSR allele is a system in which one of the polymerase chain reaction (PCR) primer to a locus is labeled with one of three colored flourescent labels are separated on sequencer. A series of size standard labeled with a fourth fluorescent tag are employed to allow exact sizing of labeled SSR alleles (Diwan *et al.*, 1997). Reputed advantages of the automated system as compared to sizing of PCR products on standard sequence are; (1) the ability to visualize and analyze several product's in the same size range, but with different fluorescent labels; (2) single base resolution over a wide size range from 75-350 bases; (3) automated sizing; (4) automated data out put; (5) elimination of radioactivity. These advantages increase the efficiency and accuracy of SSR allele size determination (Ziegle *et al.*, 1992; Freqeau *et al.*, 1993; Holgrsson *et al.*, 1994; Scharf JM. *et al.*, 1996).

In this study, several microsatellite loci from olive flounder ESTs were isolation and characterization. Compared genetic variability including allele per locus, allele frequency of genotype and heterozygosity in microsatellite DNA analysis of wild and cultivated population in Korea.

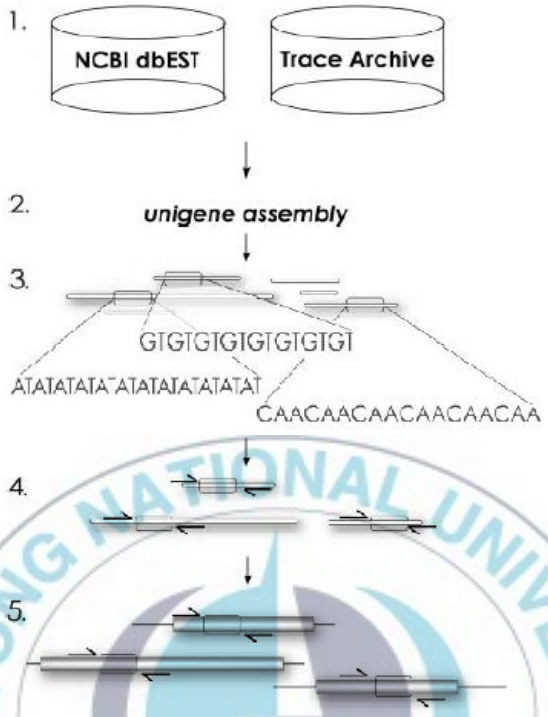


Fig. 1. Simple sequence repeat developed from ESTs (Bouck A. *et al.*, 2007) Deposited into public databases (1). clustered into unigenes used for applications (2). EST-SSRs. Software is used to scan batches of unigenes for SSRs (3). Primers (horizontal arrows) are designed from unigene sequences flanking SSRs (4), which are then used for genotyping (5).

Ⅱ. Materials and methods

1. Data mining for microsatellite markers

The olive flounder EST sequences were downloaded from GenBank (The National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/sites/entrez). The EST data set was scanned and assembled using polEST sequence database (NFRDI, Genetics and Breeding research center. Korea) and generated potential unigenes that contain contigs and singletons from all EST sequences.

2. Primer design and PCR conditions

Primers were designed using the Annhyb program (www.bioinformatics.org). PCR amplification was carried out in a 10㎕ reaction mixture, which include 10pmol of each primer, 100μM of dNTPs, 100μM 5×B.D, 0.5U f-*Taq* (Solgent, Korea) and approximately 10ng of template DNA using MJ Research PTC 200 DNA Engine thermal cycler (MJ Research, Waltham, MA). PCR cycles were as follows: 5 minutes at 95℃, 38 cycles of 30 seconds at 95℃, 30 seconds at 56℃, 30 seconds at 72℃, and final elongation for 10 minutes at 72℃. PCR products were visualized on agarose gels after staining with ethidium bromide.

3. Genomic DNA extraction

DNA was extracted from pectoral fin tissue. Tissues were digested in 100mM NaCl, 20mM Tris-HCl (pH 8.0), 100mM EDTA, with a final concentration of 0.05% SDS and 100㎍/㎖ of Protease K. After protein digestion, the aqueous phase was extracted twice with phenol, twice with phenol/ chloroform/ isoamylalcohol, and once chloroform. DNA was precipitated using ethanol; pellets were washed in 70% ethanol, dried and resuspended in TE (0.01M Tris-HCl pH 7.4, 2.5mM EDTA, pH 8.0).

4. PCR amplification and genotype analysis

PCR amplification was carried out in a 10㎕ reaction mixture, which include 10pmol of each primer, 100μM of dNTPs, 100μM 5×B.D, 0.5U f-*Taq* (Solgent, Korea) and approximately 10ng of template DNA using MJ Research PTC 200 DNA Engine thermal cycler (MJ Research, Waltham, MA). One primer pair was 5' end labeled with fluorescent FAM or NED (Applied Biosystems, Foster City, CA, USA). PCR cycles were as follows: 5 minutes at 95℃, 38 cycles of 30 seconds at 95℃, 30 seconds at 58℃, 30 seconds at 72℃, and final elongation for 10 minutes at 72℃. Fragment size were analysed an ABI Prism 3130*xl* according to the manufacturers protocol. A mix of samples was made by taking 1㎕ of each PCR reaction and diluting with 74 ㎕ ddH_2O. This mix (1㎕) was added to 9㎕ of HiDi formamide and 0.15㎕ of GeneScan 500 Liz standard for genotyping for electrophoresis on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Genescan output files were analyzed using Genotyper 3.7 software (Applied

Biosystems, Foster City, CA, USA).

5. Sample collection

196 adult flounder specimens, based on a total of each 8 sample sets from wild and cultivated were collected and genetically screened (Fig. 2). Individuals averaged 30㎝ in size and were collected in 2005. The localities where samples were collected, population abbreviations, date of sampling, and numbers of individuals sampled are shown in Table 1.

Table 1. The localities where samples were collected date of sampling and number of individuals

	Sampling locality (abbreviation)	Date sampled	No. of individuals
Wild	Taean (WT)	Nov. 2005	32
	Buan (WB)	Nov. 2005	16
	Donghae (WD)	Dec. 2005	16
	Geoje (WG)	Nov. 2005	32
Cultivated	Wando (CA)	Nov. 2005	24
	Jeju-1 (CB)	Nov. 2005	24
	Jeju-2 (CC)	Nov. 2005	24
	Jeju-3 (CD)	Nov. 2005	24

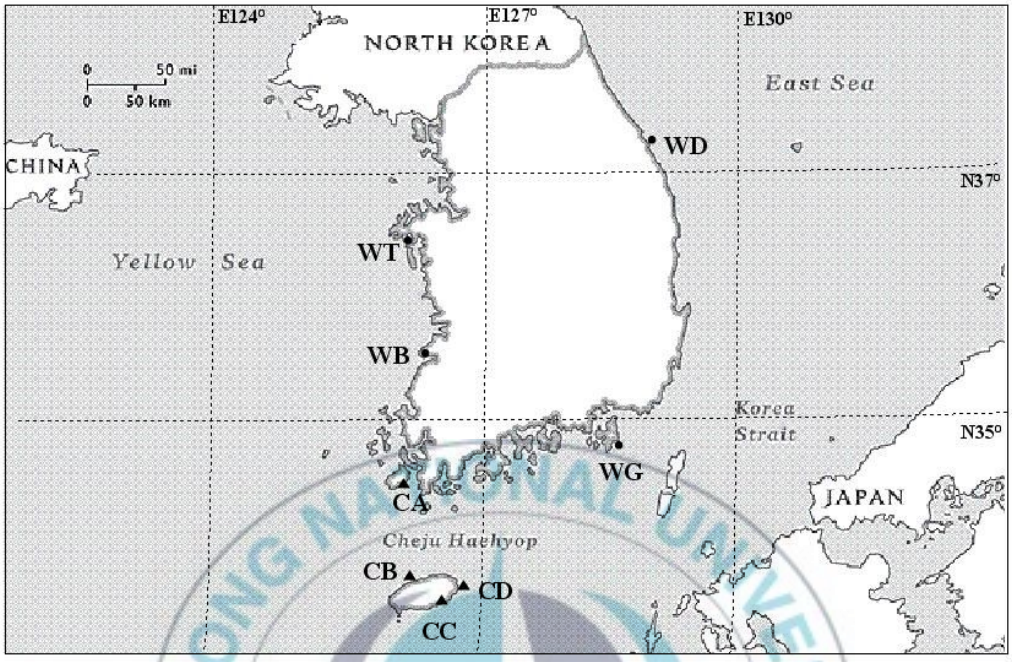


Fig. 2. Map showing location and abbreviated names for four hatcheries (▲) and four wilds (●) sample in Korea

6. Statistical analysis

Allele frequency, number of alleles per locus, expected and observed heterozygosities were calculated using Cervers (Field genetics, ver3.0.3). Hardy-Weinberg equilibrium (HWE), inbreeding coefficient (F_{is}; Weir and Cockerham, 1984), F_{st} values (Weir and Cockerham, 1984), R_{st} values (Slatkin, 1995) were calculated using GENEPOP 3.4 software (Raymond and Rousset, 1995). Values of F_{st} and R_{st} were tested for significant departure from zero using random permutation procedures at 1000 permutations. Genetic differences and relationships among populations were assessed by calculating the Nei's (Nei *et al.*, 1983) chord distance D_a. It has been shown that D_a is one of the most efficient distance measures to obtain tree topology. These procedures were performed on the MSA (Microsatellite analyser, Dieringer and Schlotter, 2003) program. Based on the unweighted of pair-group method with arithmetic means (UPGMA) pair-wise cluster analysis for all 192 individuals was carried out neighbor program in PHYLIP ver 3.68 (Felsenstein, 2008). The tree was visualized using TreeExporer program (K. Tamura, ver 2.12).

Ⅲ. Result

1. Isolation of microsatellites DNA

More than 9,525 olive flounder EST sequences obtained from GenBank. After clustering and assembling of ESTs, 1,069 unigenes were identified, including 4,080 singletons and 1,029 contigs (Table 2). The criteria used in program to identify microsatellites were as follows: 5 repeats for di-, tri-, and tetranucleotide repeats and primer were designed for 25-30 mer, 55-60℃ annealing temperature, total amplified size of 70-200 base pair and 45-60% GC content using programs. Obtained optimal PCR conditions were amplified at high temperatures and were successfully genotype array and finally chose 52 primer.

Length variation of 52 microsatellites was examined first on a panel of 16 unrelated individuals. 34 microsatellites showed specific amplification and polymorphism (Fig. 3, Fig. 4), while the remaining 18 primers exhibited either non specific products or lack of amplification. Dinucleotide repeats were the most abundant, according for 24 loci. Trinucleotide and tetra nucleotide repeats were found at lower within 7 loci and 3 loci.

Table 2. Summary of microsatellite loci from the EST database

Parameter	Value
Total number of ESTs searched	9,525
Total umber of unigene	1,069
Total number of contig	1,029
Total number of singletons	4,080
Total number of unique microsatellite loci	34
Repeat motif	
Dinucleotide	24
Trinucleotide	7
Tetranucleotide	3

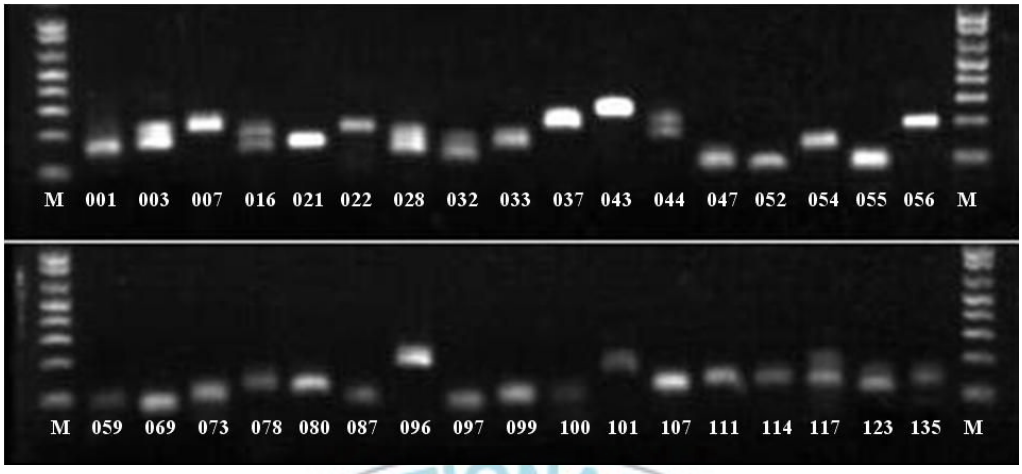


Fig. 3. Amplified PCR product by 34 microsatellite loci

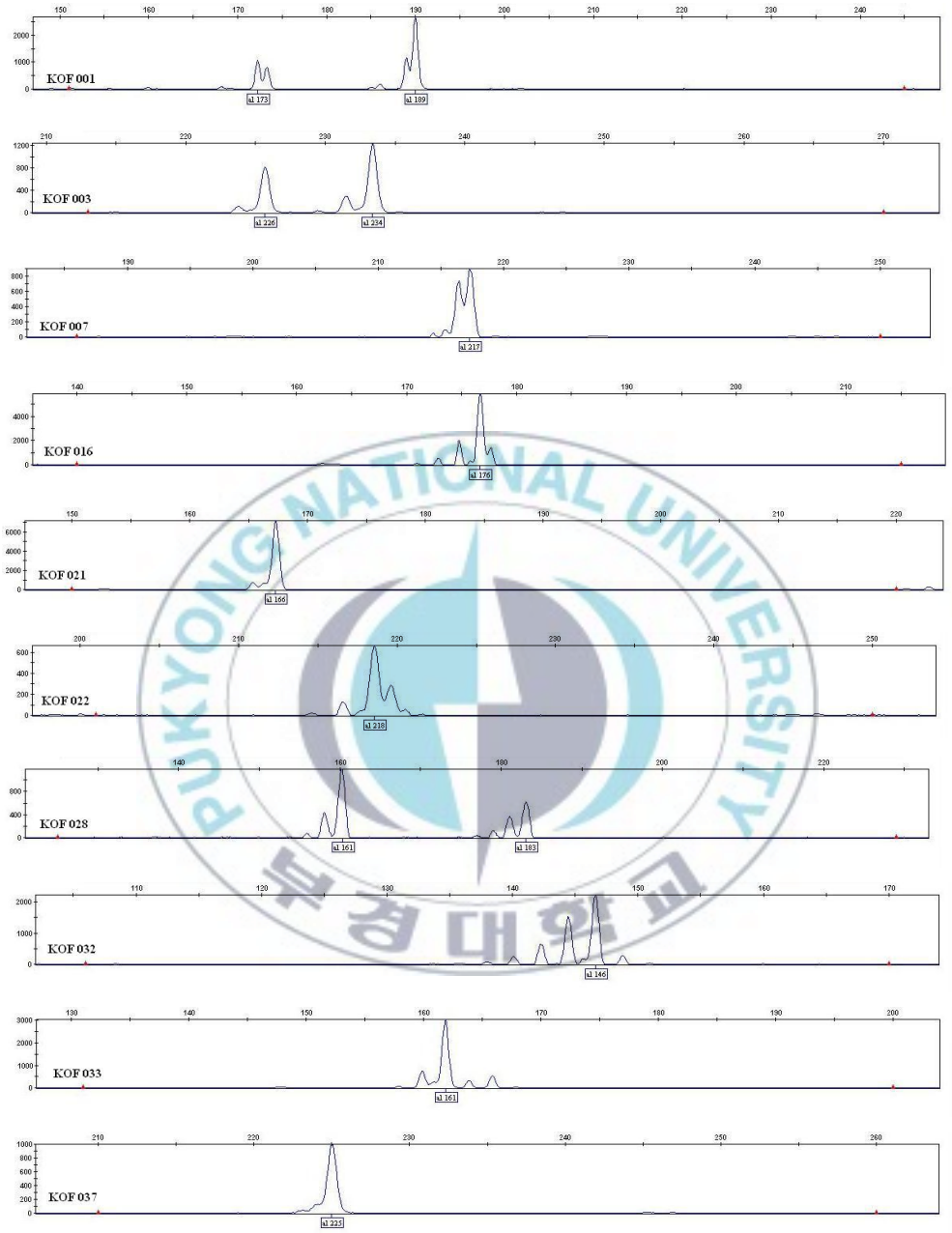


Fig. 4. Electropherogram form of alleles amplified PCR products

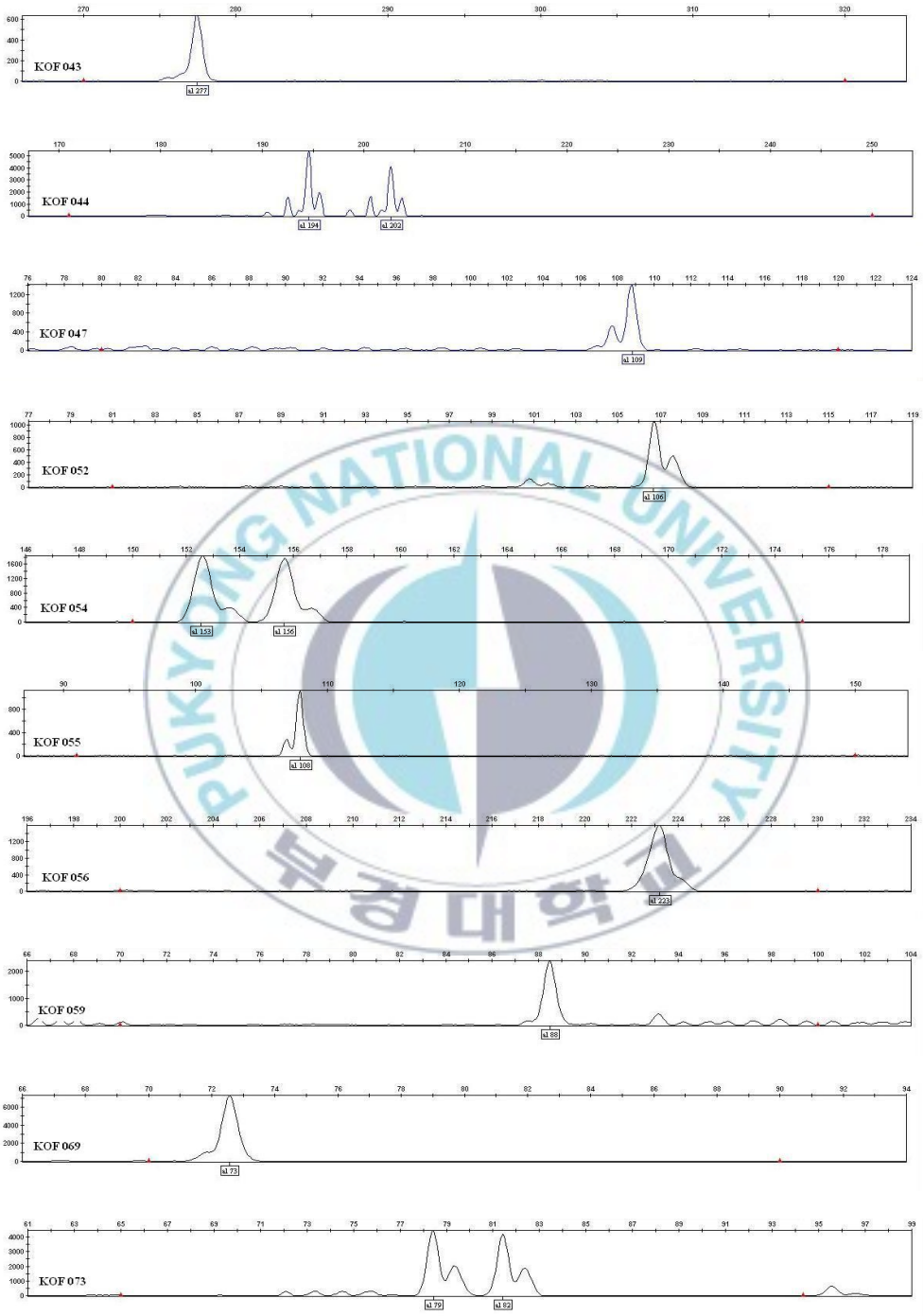


Fig. 3. continued

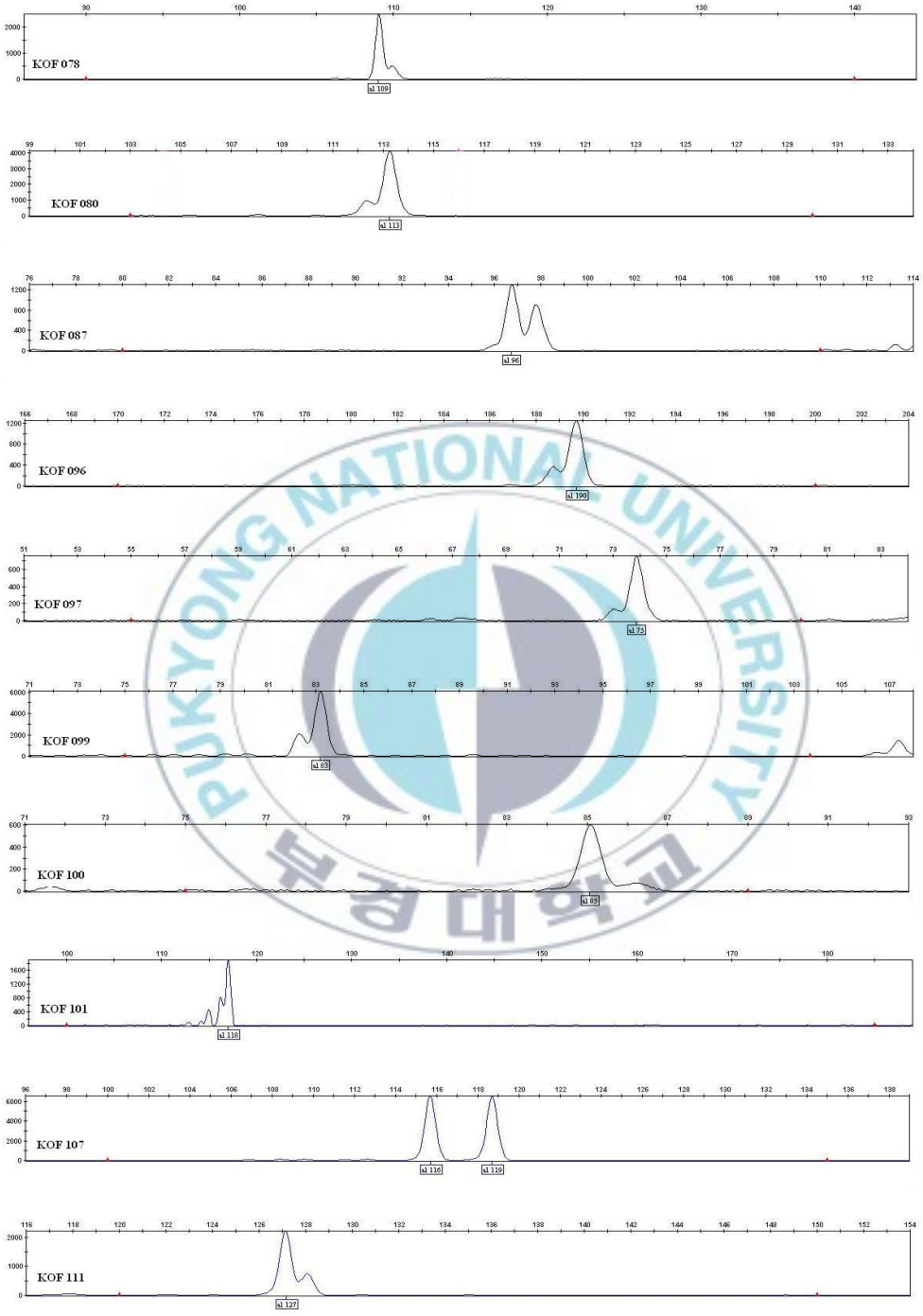


Fig. 3. continued

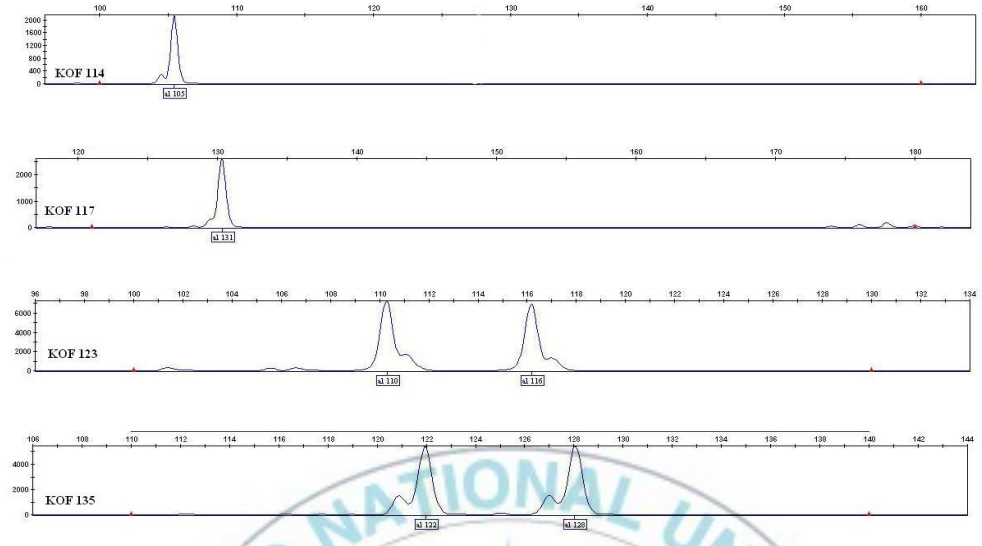


Fig. 3. continued

2. Characterization of microsatellites loci

Table 3 shows optimal annealing temperature, number of alleles, observed heterozyosity (H_o), expected heterozyosity (H_e), *P*-value in characterization of microsatellite loci from oliver flounder. The concept of heterozygosity is commonly extended to refer to the population as a whole. The fraction of individuals in a population that are heterozygous for a particular locus (S. Wright, 1920). It can also refer to the fraction of loci within an individual that are heterozygous. Typically, the H_o and H_e heterozygosities are compared, defined as follows for diploid individuals in a population: H_o where n is the number of individuals in the population, and a_{i1}, a_{i2} are the alleles of individual i at the target locus.

$$H_o = \frac{\sum_{i=1}^{n} (1 \text{ if } a_{i1} \neq a_{i2})}{n}$$

H_e where m is the number of alleles at the target locus, and f_i is the allele frequency of the i^{th} allele at the target locus.

$$H_e = 1 - \sum_{i=1}^{m} (f_i)^2$$

Hardy−Weinberg principle states that both allele and genotype frequencies in a population remain constant from generation to generation unless specific disturbing influences are introduced (W. Weinberg *et al.*, 1908). Those disturbing influences include non-random mating, mutations, selection, limited population size, random genetic drift and gene flow and this value impossible in nature. In the simplest case of a single locus with two alleles: the dominant allele is denoted A and the recessive a and their frequencies are denoted by p and q; frequency (A) = p; frequency (a) = q; p + q = 1. If the population is

in equilibrium, then we will have frequency (AA) = p^2 for the AA homozygotes in the population, frequency (aa) = q^2 for the aa homozygotes, and frequency (Aa) = 2pq for the heterozygotes.

The average allele number at the polymorphic loci was 14.09, with a range from 2 (*KOF069*, *KOF097*) to 37 (*KOF101*). H_o ranged from 0.032 (*KOF069*) to 0.925 (*KOF101*), and H_e range from 0.031 (*KOF069*) to 0.969 (*KOF028*). The most polymorphic loci was *KOF028* and *KOF101*, displaying 36, 37 alleles with H_e 0.969, 0.962. HWE exact test (*P-value*) range from 0.000 to 1.000. At the 8 microsatellite loci (*KOF001, KOF028, KOF037, KOF043, KOF055, KOF111, KOF114, KOF117*) the observed genotype frequencies showed significant departure from HWE expectations (*P<0.001*). Significant linkage deviations from HWE (*P<0.001*) due to heterozygote deficiency were detected 8 loci, which may be due to the presence of null alleles, homoplasy, frequently found in microsatellite loci. The *KOF007, KOF016, KOF032, KOF033, KOF044* and *KOF101*, revealed a high degree of genetic variability. These microsatellite loci harbored an excess 20 of alleles, the H_e value was more than 0.900 and HWE expectations (*P>0.001*). These markers will be useful for parentage and population studies to identify olive flounder.

Table 3. Characterization of microsatellites in olive flounder, *Paralichthys olivaceus*

Locus	Repeat motif	Primer sequence (5'-3') Forward Reverse	Tm (℃)	A	Size range (bp)	H_o	H_e	*P-value*	GenBank accession number
KOF 001	$(ATCT)_{18}$	CTACCATGTTGCAGCTGCCTAA TTGAAGCTTGTGGATCAACTG	56	22	157-245	0.516	0.934	0.000	gi|84096383|
KOF 003	$(AC)_{12}$	TTCAGCCTCAATCACGATTAC ACTGAGACAAACAGGCCATTTTG	56	18	214-254	0.826	0.872	0.140	gi|84096150|
KOF 007	$(GT)_{14}$	AACTATCTCAGCTCACAGCTCTG CCTGACTGTTTGCAGCCCAGAC	56	28	158-249	0.815	0.944	0.055	gi|84095560|
KOF 016	$(AC)_{11}$	GAAGCTCCAGAAGAAGAACTGAG GGTCAGACCAGTCCTGAGTCTG	56	31	152-214	0.872	0.952	0.178	gi|40725186|
KOF 021	$(AC)_{17}$	AACGGCTGAGACAGTGTCAAG CATGACAATGGCCTCTATTCAG	53	27	158-218	0.884	0.873	0.874	gi|12391195|
KOF 022	$(AT)_{12}$	ATCGACATTTTACCTCGACATG AACTTATGTGCATATGAATCTTC	54	22	200-242	0.856	0.926	0.358	gi|12391100|
KOF 028	$(GT)_{29}$	CCTGAGGAAATTCCCTGAAGTC ACTGACTGCTGCTGACTCAAAG	56	36	151-229	0.850	0.969	0.000	gi|12390848|
KOF 032	$(AG)_{17}$	AAGTTGCACCAGGGTCTCTTC TACAAGAGATCTCAACCGGATG	54	25	112-166	0.822	0.933	0.085	gi|5039584|
KOF 033	$(CA)_{18}$	GTCATGTTTCCTGACGGTGCTC CATGCCTCCATGTTTGCAAAAG	56	23	133-193	0.905	0.900	0.870	gi|5039526|
KOF 037	$(CA)_{13}$	AATCTGCCTCAAACTAGAGAAC TGCACATTAGAAGGAGGCTCAG	57	17	223-259	0.581	0.792	0.000	gi|5039280|
KOF 043	$(CA)_{10}$	TAACCTGGGAAAGCAAAGGAAG CTGAGCTCAGTATTAGGGGTAG	57	16	271-315	0.356	0.729	0.000	gi|5018837|
KOF 044	$(GT)_{20}$	ACTGAGTCCTGAGAAGGTGTAC GGTTTGGCCTTTGTTTCTTGAG	56	25	170-242	0.919	0.945	0.248	gi|5018754|
KOF 047	$(AC)_{10}$	ACCAAATGAATTCTGCTGAACAG AACTTGAGTGTCCTCACTAAG	54	8	100-119	0.427	0.475	0.485	gi|2309172|
KOF 052	$(GAG)_5$	CGTACAGAAATCAAGTTCATG ACTGCAGCAGTTTTAAGAGTC	53	8	85-112	0.154	0.635	0.759	gi|12390799|

Table 3. continued

Locus	Repeat motif	Primer sequence (5'-3') Forward Reverse	Tm (℃)	A	Size range (bp)	H_o	H_e	*P-value*	GenBank accession number
KOF 054	$(ATCT)_{18}$	TTACTCCTTCTTCTCCTGTTAC AACTGAGGTTACAGGTCAAAG	56	4	153-168	0.323	0.311	0.129	gi|12390952|
KOF 055	$(AC)_{12}$	TTGCTGGAAACTGCCTCAG TTACTGGTCAGAAAGTCCTGAAG	56	7	99-117	0.270	0.439	0.000	gi|12390954|
KOF 056	$(GT)_{14}$	TGGTCTGATCATAGCGGAAG CAGTGTGACGGTGTAAAACAC	56	5	208-226	0.226	0.242	0.058	gi|12390960|
KOF 059	$(AC)_{11}$	ACACGCTGCATCAACCTGTTAAC AATTGCTTCACATCAGCTTTTC	55	5	72-96	0.255	0.273	0.054	gi|12390976|
KOF 069	$(AC)_{17}$	TTGACCGACTTCAGCACAT AAATGCTGCCATTGTTGTAG	53	2	73-75	0.032	0.031	1.000	gi|40725302|
KOF 073	$(AT)_{12}$	CCTTTGTCAGGCGTGTTGTTTG TTGGGCAAACAAAAGCACAG	54	8	70-94	0.854	0.815	0.003	gi|40726219|
KOF 078	$(GT)_{29}$	ACTGCCTCAGGAAGGTGTGATC TGCTCAGACTTGCTGTGGTCAG	56	16	85-142	0.742	0.835	0.007	gi|5018738|
KOF 080	$(AG)_{17}$	ACTGGTTTAACAGTGATGTCAAG TTGAAGGTTGTGAAGGCCTGTG	54	5	107-119	0.531	0.552	0.766	gi|5018790|
KOF 087	$(CA)_{18}$	ACTGTACAAGGAGGACAAGAAC GGTACCTCCCTCACTGTGAAC	56	6	87-102	0.564	0.655	0.118	gi|55778448|
KOF 096	$(CA)_{13}$	AACTGATGATGATGACGTCATG CTGGCTGCTGAATGGCGTTC	57	8	178-199	0.744	0.782	0.026	gi|84095633|
KOF 097	$(CA)_{10}$	TGTACGTTCCTCCAAGTCTTG CATGGACAAATCTGCATGATG	57	2	63-75	0.158	0.164	0.545	gi|84095634|
KOF 099	$(GT)_{20}$	GTTGAGCCCGAATTAGAGAAC AATGTCTGGCTGCTCGACTG	56	8	80-101	0.656	0.667	0.682	gi|84096249|
KOF 100	$(AC)_{10}$	GGACTGTTTTGTTTCAGCAGAG TTGAAGGCATGAAACAATC	54	4	76-85	0.226	0.297	0.018	gi|84096304|
KOF 101	$(CAG)_5$aagcaaaaa cagttgt$(AGC)_5$	AATTGGTAAGCTAACAGATTCAC TCAGTGCTGCTTCACGACCTG	56	37	104-190	0.925	0.962	0.110	gi|84096413|

Table 3. continued

Locus	Repeat motif	Primer sequence (5'-3') Forward Reverse	Tm (℃)	A	Size range (bp)	H_o	H_e	*P-value*	GenBank accession number
KOF 107	$(TTC)_6$	ACTTGAAGGAAGGAGGATATG TGTGTCTCACAAAAGAAATCTTC	56	7	104-128	0.523	0.534	0.555	gi|84096435|
KOF 111	$(TAT)_5$	CCGGTTTAGCGCTATGTTCCCTCT TGCAAGCCTCTCAGGACTTC	56	6	124-139	0.432	0.682	0.000	gi|84096479|
KOF 114	$(GGGA)_6$	TTGACGTGAGGAGGAGGGTG AATGGAGTCCGTCCGATTAC	56	4	105-155	0.175	0.580	0.000	gi|84096535|
KOF 117	$(CAG)_5$	AGAAAGTCACCAGTGGTGTTG CCCTGATAGTGCTCAGAAGTCAC	53	27	125-179	0.833	0.944	0.000	gi|84096645|
KOF 123	$(GCT)_6$	GTACTAAGGCTGCACCAGTCCA CCTTGTCCTCATCATCTTCATC	54	7	104-122	0.611	0.658	0.037	gi|84096674|
KOF 135	$(ATT)_6$	ACTGGTTGAGGCTGAGACGCA TGACGTCATGTTCAGGGTCATG	54	5	122-134	0.589	0.605	0.263	gi|84096681|

Tm, Optimal annealing temperature; A, number of allele; H_o, observed heterozygosity; H_e, expected heterozygosity

3. Genetic variation in wild and cultivated population

A. Comparison of allele frequency between each population

Recently developed techniques in molecular biology have made it possible to dissect some traits genetically. Genetic linkage maps based on DNA markers are an important tool in these techniques. The use of DNA markers facilitates the eventual positional cloning of the functional genes contributing to the quantitative trait (Collins, 1992). Marker-assisted selection(MAS) and/or marker-assisted gene introgression(MAI) using DNA markers can improve breeding programs for domestic animals and food crops. Also identify the specific allele site was identifying loci influencing quantitative traits in domesticated animals and food crops (Andersson *et al.*, 1994; Georges *et al.*, 1995; Elouafi *et al.*, 2001; Kwon *et al.*, 2001). To identify individual loci controlling traits of economic significance to fisheries, it is presently necessary to construct a genetic linkage map based on molecular markers at a large number of sites in the genome. Allele frequency at all 6 loci differed significant between each four wild and cultivated type to comparison. The *KOF007* had WD, WG the highest number of allele at 21, and 227bp - 239bp allele rage non detected to cultivated type. 241bp allele was specific detected on cultivated type. *KOF016* WD was 24 number of allele and 180bp - 182bp, 186bp - 194bp, 208bp - 214bp raged non detected to cultivated type. *KOF032* had WD the highest number of allele at 20, and 112bp - 116bp allele rage non detected to cultivated type. *KOF033* had WD the highest number of allele at 16, and 133bp - 145bp allele rage non detected to cultivated type. *KOF044*

had WG the highest number of allele at 21, and 236bp - 242bp allele rage non detected to cultivated type. *KOF101* had WG the highest number of allele at 27, and 176bp - 190bp allele rage non detected to cultivated type.

Table 4. Allele frequencies of *KOF007* locus

KOF 007	Population							
	WB	WD	WG	WT	CA	CB	CC	CD
185	-	-	0.0313	-	-	-	-	-
187	-	-	-	-	0.1429	0.0417	-	0.0476
189	-	-	-	-	-	-	-	-
191	-	0.0500	0.0781	-	-	-	-	-
193	-	0.0333	-	-	0.0714	-	-	-
195	0.0333	-	0.0313	0.0333	-	0.1458	0.0208	0.0952
197	-	0.0333	0.0469	0.0333	0.0714	0.0208	0.0208	0.0476
199	0.0333	0.0333	0.0156	0.2000	0.0357	0.0417	0.1042	0.0714
201	-	0.0667	0.0625	0.0333	0.0714	0.0417	-	0.0238
203	-	0.0500	-	-	-	-	-	-
205	-	0.0500	0.0938	-	-	-	0.0208	0.0238
207	0.0333	-	0.0313	-	-	-	0.0208	-
209	0.2667	0.0167	0.0938	0.2333	0.3571	0.1875	0.2917	0.3333
211	0.1000	0.1333	0.0625	-	-	0.0208	0.0833	-
213	0.0333	0.1333	0.0313	0.2000	-	0.2083	0.1667	0.0952
215	-	-	0.0156	-	-	-	-	-
217	0.1000	0.0167	0.0313	0.0333	0.0357	0.0208	-	-
219	0.1333	0.0500	0.0313	0.2000	0.1071	0.0625	0.1042	0.2143
221	0.1000	0.1000	0.0781	0.0333	0.0357	0.1250	0.1458	0.0476
223	-	0.0333	-	-	-	-	-	-
225	-	0.0667	0.0781	-	-	0.0417	-	-
227	-	-	-	-	-	-	-	-
229	0.0333	-	0.0469	-	-	-	-	-
231	-	-	0.0469	-	-	-	-	-
233	0.0333	0.0333	0.0313	-	-	-	-	-
235	-	0.0333	-	-	-	-	-	-
237	0.0333	-	-	-	-	-	-	-
239	0.0333	0.0167	-	-	-	-	-	-
241	-	-	-	-	0.0714	0.0417	0.0208	-
243	0.0333	0.0167	0.0313	-	-	-	-	-
245	-	-	-	-	-	-	-	-
247	-	0.0167	0.0313	-	-	-	-	-
249	-	0.0167	-	-	-	-	-	-
n=	14	21	21	9	10	13	11	10

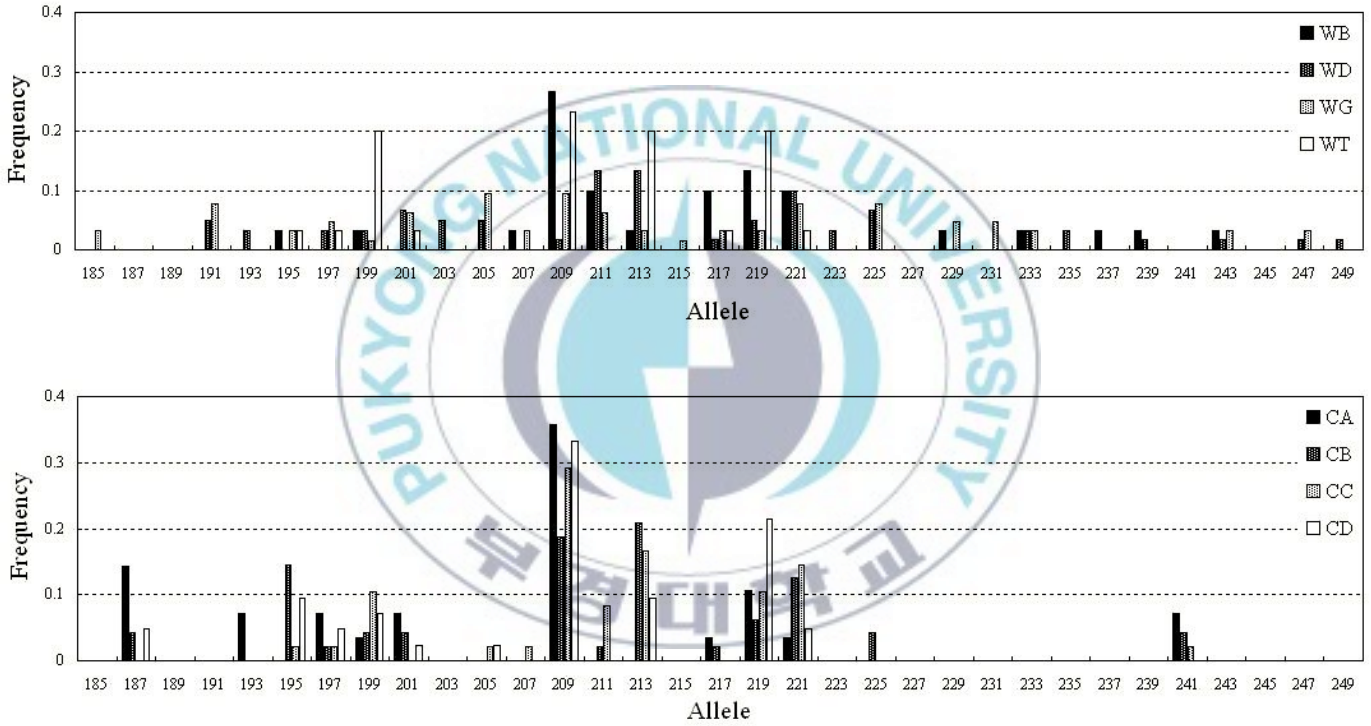


Figure 5. Allele frequency of *KOF007* locus compared to population

Table 5. Allele frequencies of *KOF016* locus

KOF 016	Population							
	WB	WD	WG	WT	CA	CB	CC	CD
152	0.0667	0.0484	0.0192	0.1429	0.1739	0.1190	0.0952	0.0435
154	-	-	0.0192	-	-	-	-	-
156	-	0.0161	0.0385	0.1071	0.1087	0.1905	0.1429	0.1522
158	0.0667	0.0161	0.0385	-	-	-	0.0476	0.1304
160	0.1000	0.1129	0.1154	0.1071	0.1087	0.0714	0.2381	0.0435
162	0.0667	0.0484	0.0192	-	-	-	-	0.0217
164	0.0667	0.0484	0.1538	0.0357	-	-	-	-
166	0.1333	-	0.0577	0.1429	0.1957	0.2381	0.1190	0.2391
168	0.0333	0.0484	-	-	-	-	-	-
170	0.0667	0.0968	0.0962	0.0357	0.1304	0.0238	0.0476	0.1087
172	-	0.0161	0.0385	0.0357	-	-	0.0238	0.0652
174	0.0667	0.0968	0.0769	0.1071	0.1087	0.0952	0.0714	0.1304
176	0.0333	0.0645	0.0385	-	-	-	-	-
178	0.0333	0.0161	0.0385	0.1071	0.0435	0.0952	-	-
180	-	0.0161	0.0385	-	-	-	-	-
182	-	-	0.0192	0.0357	-	-	-	-
184	0.0667	0.0161	0.0192	-	-	0.0238	-	-
186	-	0.0161	0.0192	-	-	-	-	-
188	-	-	0.0385	-	-	-	-	-
190	-	0.0484	-	-	-	-	-	-
192	-	-	-	-	-	-	-	-
194	-	0.0161	0.0577	-	-	-	-	-
196	0.0333	-	-	-	-	0.0238	0.0714	-
198	-	0.0161	-	0.0357	-	-	-	-
200	-	-	0.0385	0.0714	-	-	-	-
202	0.0333	-	-	-	0.0652	0.0952	0.0952	0.0217
204	0.0333	0.0645	-	-	0.0217	-	-	0.0435
206	0.0333	0.0484	-	0.0357	0.0435	0.0238	0.0476	-
208	-	0.0484	-	-	-	-	-	-
210	-	0.0484	-	-	-	-	-	-
212	-	0.0161	0.0192	-	-	-	-	-
214	0.0667	0.0161	-	-	-	-	-	-
n=	17	24	21	13	10	11	11	11

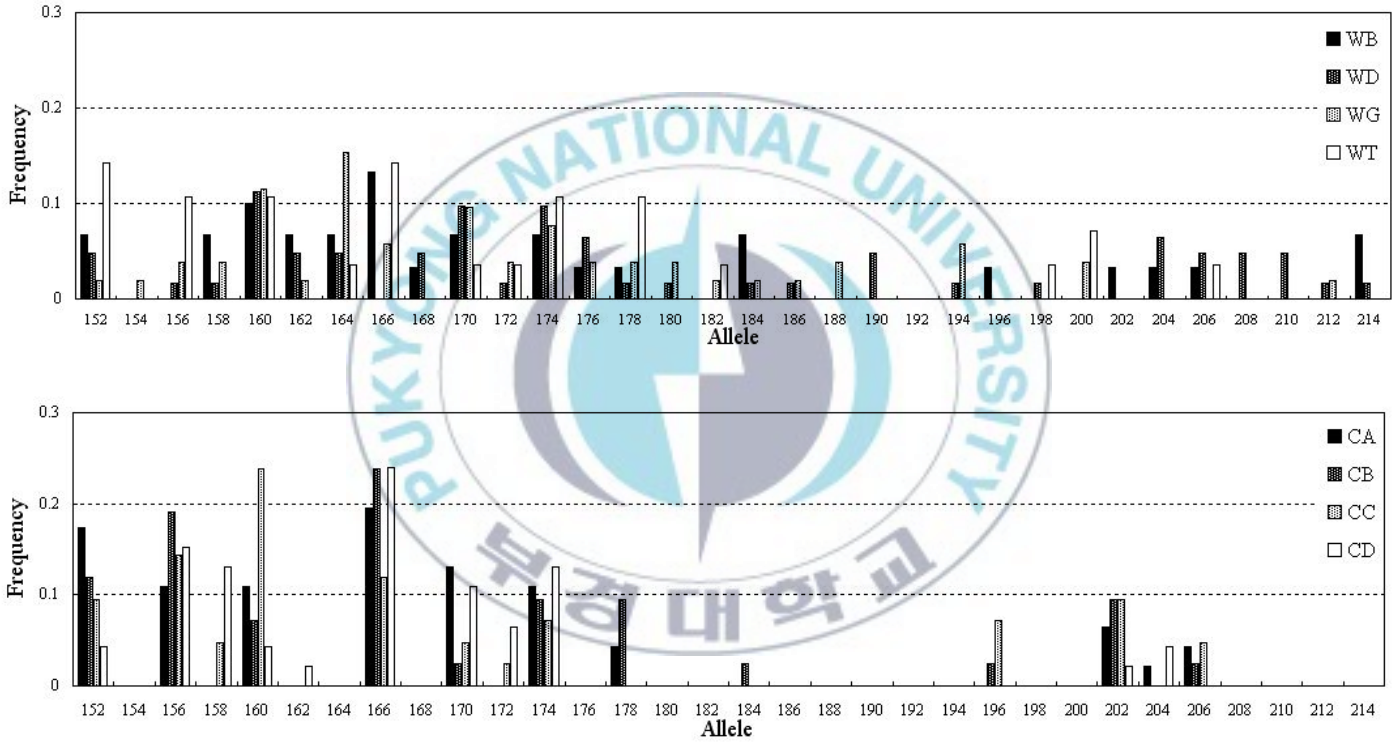


Figure 6. Allele frequency of *KOF016* locus compared to population

Table 6. Allele frequencies of *KOF032* locus

KOF 032	Population							
	WB	WD	WG	WT	CA	CB	CC	CD
112	-	0.0179	-	-	-	-	-	-
114	-	-	-	-	-	-	-	-
116	-	-	-	-	-	-	-	-
118	0.0357	0.0179	0.0208	-	0.0357	0.1316	0.0217	0.0455
120	0.0357	0.0179	0.0208	-	-	-	-	-
122	0.0357	0.0536	-	-	-	0.0789	0.0435	-
124	0.0357	0.0536	0.0417	-	-	-	-	-
126	-	0.0179	0.0417	-	-	-	-	-
128	-	0.0357	0.1458	-	-	0.0789	-	0.0455
130	0.1429	-	0.1042	0.0714	0.3571	0.2895	0.3478	0.2727
132	0.1071	0.0179	0.0625	0.0714	-	0.0263	0.0217	-
134	-	-	-	0.0714	-	-	-	-
136	-	-	-	-	-	-	-	-
138	-	0.0357	0.0208	0.1429	0.0357	-	0.0435	0.0455
140	0.1429	0.1250	0.1250	0.2857	0.0357	0.0789	0.0435	0.0455
142	0.0714	0.1786	0.1042	0.1429	-	0.0263	0.0435	0.0455
144	0.1786	0.0536	0.0625	0.1429	0.1071	0.1053	0.1304	0.2500
146	0.1071	0.0536	0.0417	0.0714	0.0714	0.1053	0.1087	0.0455
148	-	0.0536	0.0625	-	-	-	-	-
150	0.0357	0.0714	0.0833	-	0.1786	-	0.1087	0.0227
152	-	0.0357	-	-	-	-	-	-
154	-	0.0179	-	-	-	-	-	-
156	0.0357	-	0.0208	-	-	-	-	0.0227
158	0.0357	0.0357	-	-	0.0357	-	0.0217	0.0455
160	-	0.0714	-	-	0.1429	0.0789	0.0652	0.1136
162	-	-	0.0208	-	-	-	-	-
164	-	-	0.0208	-	-	-	-	-
166	-	0.0357	-	-	-	-	-	-
n=	13	20	17	8	9	10	12	12

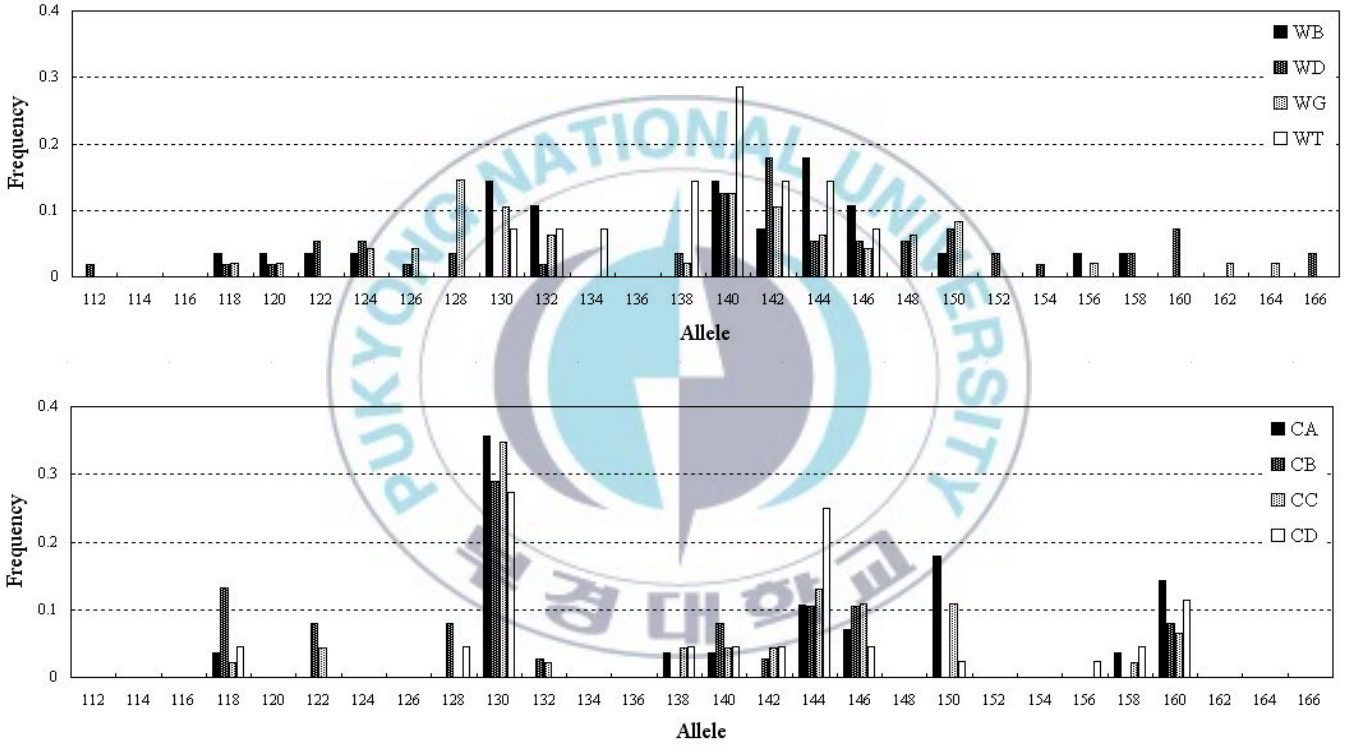


Figure 7. Allele frequency of *KOF032* locus compared to population

Table 7. Allele frequencies of *KOF033* locus

KOF 033	Population							
	WB	WD	WG	WT	CA	CB	CC	CD
133	-	-	-	0.0313	-	-	-	-
135	-	-	-	-	-	-	-	-
137	-	-	-	-	-	-	-	-
139	-	0.0156	0.0161	-	-	-	-	-
141	-	-	-	-	-	-	-	-
143	-	0.0156	-	-	-	-	-	-
145	-	0.0313	-	0.0313	-	-	-	-
147	0.1563	0.1563	0.2258	0.0938	0.0870	0.0435	0.1304	0.0909
149	-	0.0313	0.0323	-	-	-	-	-
151	-	0.0313	0.0323	-	-	-	-	-
153	0.1875	0.0781	0.0806	0.1563	0.1957	0.2609	0.2391	0.2045
155	-	0.0938	0.0484	-	-	-	-	-
157	0.0938	0.0469	0.0484	0.0313	0.0652	0.0870	0.1304	0.1364
159	0.0938	0.1250	0.1935	0.1250	0.0435	0.0870	0.0217	0.1136
161	0.1875	0.1875	0.0968	0.0625	0.1304	0.1304	0.1739	0.1818
163	0.1563	0.1094	0.1290	0.1250	0.1087	0.1957	0.1087	0.1364
165	0.0625	-	0.0161	0.0938	0.1087	0.0435	0.0652	-
167	-	0.0313	-	-	-	-	-	-
169	0.0313	-	-	0.0313	0.0435	0.0435	-	0.0909
171	-	-	0.0161	0.0625	-	-	-	-
173	-	0.0156	0.0323	0.0313	0.0217	-	-	-
175	-	-	-	-	-	-	-	-
177	-	0.0156	-	-	-	-	-	-
179	-	-	-	0.0313	0.0435	0.0435	0.0435	0.0455
181	-	-	-	-	-	-	-	-
183	-	-	-	-	-	-	-	-
185	-	-	-	-	-	-	-	-
187	-	-	-	0.0313	-	-	-	-
189	-	0.0156	0.0323	-	-	-	-	-
191	-	-	-	-	-	-	-	-
193	0.0313	-	-	0.0625	0.1522	0.0652	0.0870	-
n=	9	16	14	15	11	10	9	8

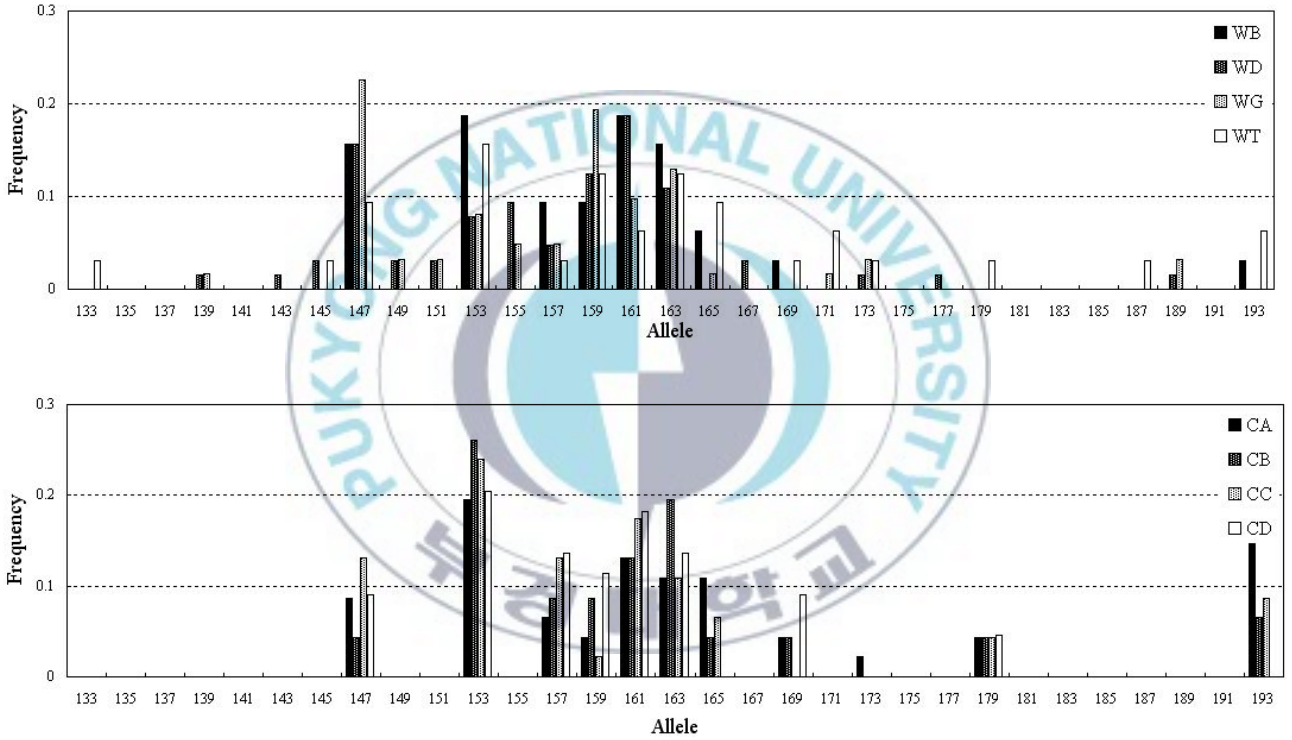


Figure 8. Allele frequency of *KOF033* locus compared to population

Table 8. Allele frequencies of *KOF044* locus

KOF 044	Population							
	WB	WD	WG	WT	CA	CB	CC	CD
170	-	-	-	-	-	-	0.0217	-
172	-	-	-	-	-	-	-	-
174	0.0667	0.0714	0.0417	-	0.1071	0.1429	0.1304	0.1250
176	-	-	-	-	-	-	0.0217	-
178	-	-	-	-	-	-	-	-
180	0.1333	0.0893	0.0417	-	0.0357	-	-	-
182	0.0667	0.1429	0.1250	-	-	0.0952	-	0.0417
184	0.0667	0.0357	0.0417	-	-	-	-	-
186	0.0333	0.0357	0.0208	-	0.1786	0.0476	0.1087	0.0625
188	0.0667	0.0536	0.0625	-	0.1071	-	0.0435	0.0208
190	0.1667	0.1071	0.0417	0.0714	0.2143	0.1190	0.1522	0.1458
192	-	0.0357	0.0625	0.0714	0.0357	-	-	-
194	0.0333	0.0357	0.0208	-	-	-	-	-
196	0.1000	0.0714	0.1042	0.2143	0.1429	0.2619	0.1739	0.2083
198	0.0333	0.0179	0.0625	0.1429	-	0.0476	0.0435	0.1250
200	0.1000	0.0714	0.0833	-	-	0.0952	0.0217	0.0208
202	0.0333	0.0179	0.0625	-	-	-	-	-
204	-	0.0357	0.0417	-	-	-	-	-
206	-	0.0179	0.0208	-	-	-	-	0.0208
208	0.0333	-	-	0.1429	0.1429	0.1190	0.1087	0.1250
210	0.0333	0.0357	0.0208	0.2143	-	-	-	-
212	-	-	0.0417	-	-	-	-	0.0208
214	-	-	0.0208	-	-	-	0.0217	-
216	-	0.0714	-	-	-	0.0476	-	-
218	-	0.0179	0.0417	-	-	-	0.0217	-
220	-	-	-	-	-	-	-	-
222	0.0333	-	-	0.1429	0.0357	-	0.0217	0.0417
224	-	-	0.0208	-	-	-	-	-
226	-	0.0179	0.0208	-	-	-	-	-
228	-	-	-	-	-	-	-	-
230	-	-	-	-	-	-	-	-
232	-	-	-	-	-	-	-	-
234	-	-	-	-	-	0.0238	0.1087	0.0417
236	-	-	-	-	-	-	-	-
238	-	-	-	-	-	-	-	-
240	-	-	-	-	-	-	-	-
242	-	0.0179	-	-	-	-	-	-
n=	15	20	21	7	9	10	14	13

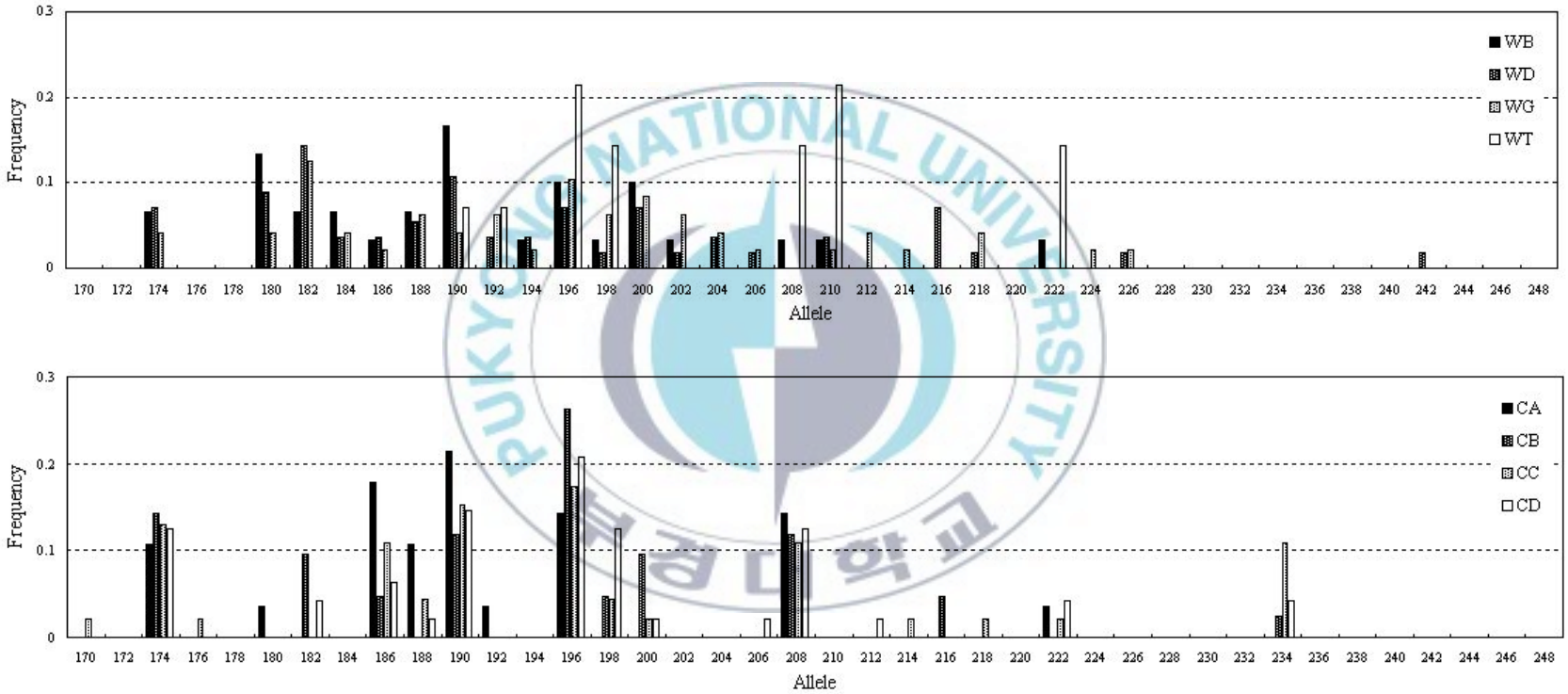


Figure 9. Allele frequency of *KOF044* locus compared to population

Table 9. Allele frequencies of *KOF101* locus

KOF	Population							
044	WB	WD	WG	WT	CA	CB	CC	CD
104	-	0.0167	-	-	-	0.0208	0.0208	-
106	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-
110	0.0333	0.0167	-	0.0938	0.1786	0.1250	0.0208	0.0682
112	-	0.0500	-	0.0313	0.0714	-	-	-
114	-	-	-	-	-	-	-	-
116	-	0.0333	-	-	-	-	-	-
118	-	0.1667	0.0781	0.0625	-	0.1250	0.0625	0.1136
120	-	0.0500	0.0156	-	-	0.0625	-	-
122	0.1333	-	0.0938	0.0625	0.0357	0.0208	0.0417	0.0455
124	-	-	-	-	-	-	0.0417	-
126	-	-	0.0313	-	-	-	-	-
128	-	0.0167	-	-	-	-	-	-
130	-	0.0167	0.0313	-	0.1429	0.1042	0.0625	0.0227
132	-	-	0.0313	0.0938	0.0714	0.0417	0.0208	0.0227
134	0.0333	0.0333	0.0469	-	-	-	-	-
136	-	-	0.0156	0.0625	0.1071	-	0.0833	0.0682
138	-	0.0333	-	-	-	-	0.0208	-
140	0.0333	-	-	-	-	-	-	-
142	0.0667	-	-	0.0313	-	-	-	-
144	-	-	0.0156	-	-	-	-	-
146	-	-	-	0.0313	-	-	-	-
148	0.0333	0.0500	0.0313	-	-	-	-	-
150	-	-	0.0156	-	-	-	-	-
152	0.0333	0.0167	0.0313	0.2188	0.0714	0.1250	0.1667	0.2273
154	0.0333	0.0500	0.0156	0.0313	-	-	-	-
156	0.1000	0.0167	0.0313	0.0625	0.1071	0.0625	0.1250	0.1136
158	0.0333	0.0667	0.0313	-	-	-	-	-
160	0.0333	0.0500	0.0625	-	-	-	-	-
162	0.1333	0.0333	0.0625	0.0313	0.1071	0.1042	0.2292	0.2045
164	0.0667	0.0167	0.0625	-	-	0.0417	0.0208	0.0455
166	0.1333	0.0333	0.0313	0.1250	0.1071	0.1458	0.0625	0.0682
168	-	0.0333	0.0313	-	-	-	-	-
170	0.0333	0.0167	0.0156	-	-	-	-	-
172	0.0333	0.0667	0.0781	-	-	-	-	-
174	-	0.0333	0.0469	0.0313	-	0.0208	0.0208	-
176	0.0333	0.0167	0.0469	-	-	-	-	-
178	-	-	-	-	-	-	-	-
180	-	0.0500	0.0156	0.0313	-	-	-	-
182	-	-	0.0156	-	-	-	-	-
184	-	-	0.0156	-	-	-	-	-
186	-	-	-	-	-	-	-	-
188	-	-	-	-	-	-	-	-
190	-	0.0167	-	-	-	-	-	-
n=	17	26	27	15	10	13	15	11

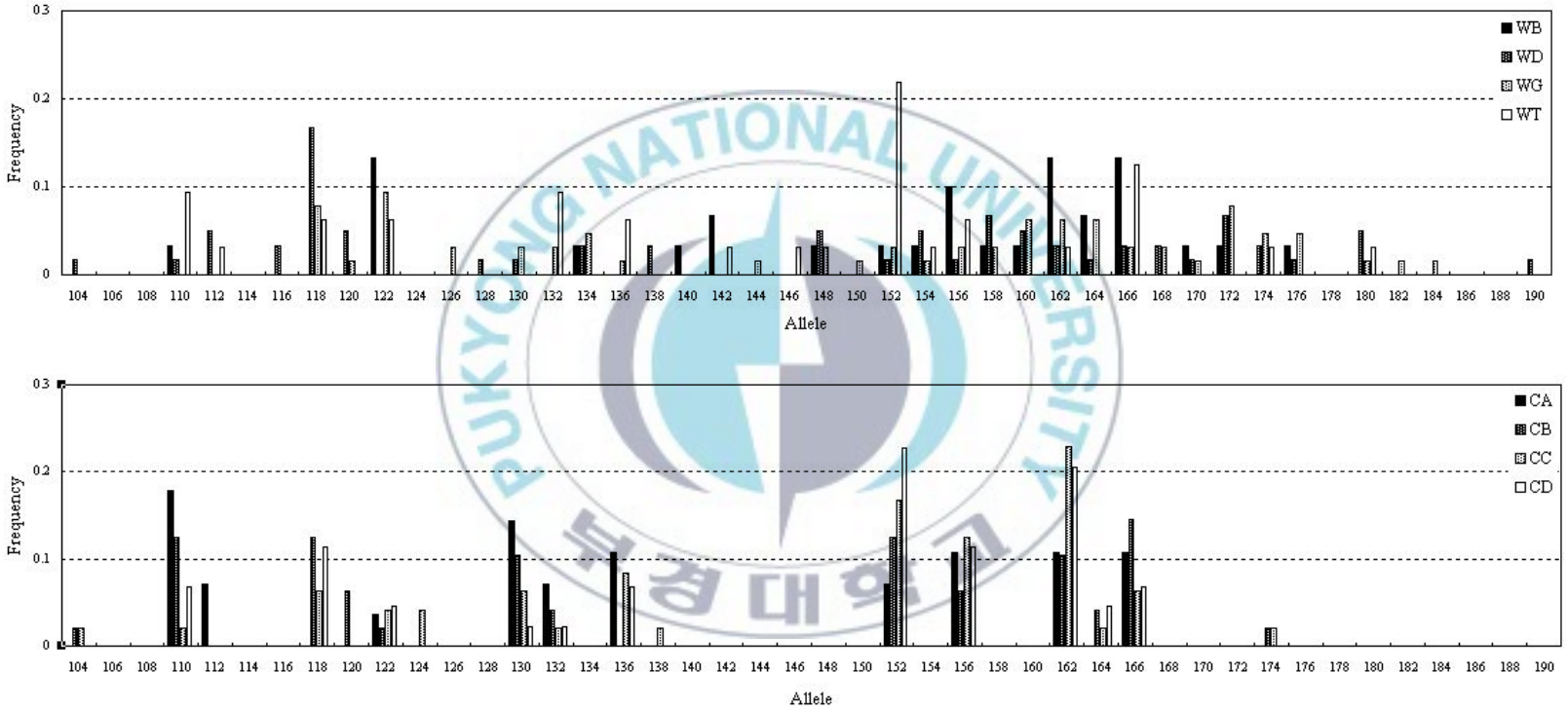


Figure 10. Allele frequency of *KOF101* locus compared to population

B. Genetic variability to each local population

The H_o, H_e, F_{is}, HWE test and number of alleles for the six are summarized in table 10. *F-statistics* describe the level of heterozygosity within a population. More specifically the degree of a reduction in heterozygosity when compared to Hardy-Weinberg expectation (S. Wright, 1920). The measures F_{is}, F_{st}, and F_{it} are related to the amounts of heterozygosity at various levels of population structure. In a simple two-allele system with inbreeding, the genotype frequencies are: p^2+ Fpq for AA; 2pq (1 − F) for Aa; and q^2 + Fpq for aa. The value for F is found by solving the equation for F using heterozygotes in the above inbred population. This becomes one minus the observed number of heterozygotes in a population divided by its expected number of heterozygotes at Hardy−Weinberg equilibrium:

$$F = 1 - \frac{\mathrm{O}(f(\mathbf{Aa}))}{\mathrm{E}(f(\mathbf{Aa}))} = 1 - \frac{\mathrm{ObservedNumber}(\mathbf{Aa})}{n\mathrm{E}(f(\mathbf{Aa}))},$$

where the expected value at Hardy−Weinberg equilibrium is given by

$$\mathrm{E}(f(\mathbf{Aa})) = 2\,p\,q$$

where p and q are the allele frequencies of A and a, respectively. It is also the probability that at any locus, two alleles from a random individual of the population are identical by descent.

The H_o and H_e ranged from 0.571 (*KOF007*, CA) to 1.000 (*KOF044*, CB/CC/CD/WG/WT; *KOF101*, CA/CB) and 0.783 (*KOF016*, CD) to 0.965 (*KOF101*, WG). The number of alleles the *KOF101* had the highest (27) in the WG population. Mean value WD population was 21.17 and CA population 9.83.

Table 10. Summary statistics of variation detected at six microsatellite loci for local population

Locus		Wild				Cultivated			
		WB	WD	WG	WT	CA	CB	CC	CD
KOF007	N	15	30	32	15	14	24	24	21
	H_o	0.733	0.933	0.844	0.600	0.571	0.792	0.917	0.667
	H_e	0.901	0.943	0.955	0.848	0.847	0.889	0.853	0.832
	F_{is}	0.192	0.010	0.118	0.300	0.333	0.112	-0.077	0.202
	P-value	0.292	0.194	0.074	0.039	0.000	0.186	0.788	0.206
	A	14	21	21	9	10	13	11	10
KOF016	N	15	31	26	14	10	13	11	10
	H_o	0.933	0.903	0.846	0.786	0.913	0.857	0.905	0.783
	H_e	0.961	0.954	0.945	0.934	0.913	0.857	0.905	0.783
	F_{is}	0.030	0.054	0.106	0.164	-0.027	0.026	-0.012	0.115
	P-value	0.264	0.201	0.166	0.028	0.370	0.170	0.651	0.570
	A	17	24	21	13	0.376	0.201	0.601	0.562
KOF032	N	14	28	24	7	14	19	23	22
	H_o	0.857	0.857	0.792	0.714	0.857	0.789	0.826	0.864
	H_e	0.923	0.936	0.934	0.901	0.828	0.873	0.843	0.854
	F_{is}	0.074	0.086	0.156	0.221	-0.037	0.098	0.021	-0.011
	P-value	0.632	0.040	0.220	0.230	0.589	0.006	0.383	0.800
	A	13	20	17	8	9	10	12	12
KOF033	N	16	32	31	16	23	23	23	22
	H_o	0.938	0.938	0.839	0.938	0.913	0.739	0.870	0.864
	H_e	0.885	0.905	0.884	0.938	0.900	0.869	0.871	0.876
	F_{is}	-0.061	-0.037	0.052	0.000	-0.015	0.152	0.002	0.015
	P-value	0.988	0.953	0.605	0.588	0.505	0.040	0.212	0.688
	A	9	16	14	15	11	10	9	8
KOF044	N	15	28	24	7	14	21	23	24
	H_o	0.933	0.821	1.000	1.000	0.929	1.000	0.957	1.000
	H_e	0.940	0.944	0.956	0.901	0.886	0.878	0.907	0.896
	F_{is}	0.008	0.132	-0.047	-0.120	-0.050	-0.143	-0.056	-0.119
	P-value	0.380	0.070	0.316	0.728	0.297	0.296	0.808	0.833
	A	15	20	21	7	9	10	14	13
KOF101	N	15	30	32	16	14	24	24	22
	H_o	0.933	0.900	0.938	0.938	1.000	1.000	0.917	0.864
	H_e	0.947	0.954	0.965	0.925	0.918	0.917	0.898	0.882
	F_{is}	0.015	0.057	0.029	-0.014	-0.093	-0.093	-0.021	0.021
	P-value	0.320	0.012	0.181	0.156	0.386	0.538	0.478	0.958
	A	17	26	27	15	10	13	15	11
Mean	N	16	32	32	16	24	24	24	24
	H_o	0.888	0.892	0.877	0.829	0.864	0.863	0.899	0.840
	H_e	0.926	0.939	0.940	0.908	0.882	0.881	0.880	0.854
	F_{is}	0.043	0.050	0.069	0.091	0.018	0.025	-0.023	0.037
	A	14.17	21.17	20.17	11.17	9.83	11.17	12.00	10.83

N, Sample size; A, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity, F_{is}, inbreeding coefficient

C. Genetic variability to wild and cultivated population

Each years, increased fishery industry, as a consequence of the increase of artificial fry production, the potential genetic impact of the release of cultivated-reared fish on the wild fish stocks is a growing concern. This is because most cultivated stocks typically show a reduced genetic variability, which may possibly result in the loss of disease resistance or in the reduction of the population's capability to adapt to new environments (Allendorf and Phelps, 1980). As FAO (1993) has recommended, genetic monitoring for cultivated strains and wild populations is thus required to preserve existing genetic variations in wild populations, and this will in turn contribute to promote exploitable resource conservation and enhancement. Loss of genetic variation in a cultivated strain, as shown by direct DNA examination, is most commonly caused by the limited number of effective parents and/or inbreeding events when the strain was founded (Norris *et al.*, 1999; Iguchi *et al.*, 1999).

The H_o, H_e and F_{is} and number of alleles for the eight loci are summarized in table 11. The wild population of H_o ranged from 0.815 (*KOF007*) to 0.925 (*KOF101*) and cultivated population was 0.759 (*KOF007*) to 0.976 (*KOF044*). The H_e ranged from 0.900 (*KOF033*) to 0.962 (*KOF101*) to wild population and cultivated population was 0.851 (*KOF032*) to 0.906 (*KOF101*). The *KOF033* microsatellite loci had the highest *P-value*, 0.790 in the wild population and cultivated population was 0.437 at the *KOF016* loci. *KOF101* had the highest number of alleles 37 in the wild population and cultivated population was 20 at the *KOF044*.

Table 11. Summary statistics of variation detected at six microsatellite loci for wild and cultivated population

Locus		Population	
		Wild	Cultivated
KOF007	N	92	83
	H_o	0.815	0.759
	H_e	0.944	0.867
	F_{is}	0.137	0.125
	P-value	0.027	0.111
	A	28	16
KOF016	N	86	88
	H_o	0.872	0.864
	H_e	0.952	0.892
	F_{is}	0.084	0.032
	P-value	0.180	0.437
	A	31	15
KOF032	N	73	78
	H_o	0.822	0.833
	H_e	0.933	0.851
	F_{is}	0.120	0.020
	P-value	0.223	0.167
	A	25	14
KOF033	N	95	91
	H_o	0.905	0.846
	H_e	0.900	0.876
	F_{is}	-0.005	0.034
	P-value	0.790	0.164
	A	23	11
KOF044	N	74	82
	H_o	0.919	0.976
	H_e	0.945	0.891
	F_{is}	0.028	-0.096
	P-value	0.093	0.231
	A	25	20
KOF101	N	93	84
	H_o	0.925	0.940
	H_e	0.962	0.906
	F_{is}	0.039	-0.039
	P-value	0.012	0.417
	A	37	17
Mean	N	96	96
	H_o	0.876	0.888
	H_e	0.939	0.880
	F_{is}	0.067	0.012
	A	28.17	15.50

N, Sample size; A, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity, F_{is}, inbreeding coefficient

4. Population differentiation and relationship

In agreement with this high heterogeneity, multilocus F_{st} and R_{st} value were all large and highly significant from zero ($P<0.01$ after Bonferroni correction) (Table 12). Defined F_{st}, as the correlation between two alleles chosen at random within subpopulations relative to alleles sampled at random from the total population (Wright, 1951, 1965). Therefore, F_{st} measures inbreeding due to the correlation among alleles because they are found in the same subpopulation. Hence, F_{st} measures the heterozygote deficit relative to its expectation under Hardy-Weinberg equilibrium (Hartl and Clark, 1997). The Wahlund principle can be stated in terms of variance in allele frequency (Wright, 1943, 1951, 1965; Hartl and Clark, 1997):

$$F_{st} = Vp/[p(1-p)]$$

where p and Vp are the mean and the variance of the allele frequency among subpopulations, considering a two-alleles locus. This positive quantity is the ratio of the observed variance divided by the maximum possible variance.

Slatkin (1995) showed that R_{st} can be defined as follows:

$$R_{st} = (S-S_w)/S$$

where S is the average squared difference in allele size between all pairs of alleles, and S_w, the average sum of squares of the differences in allele size within each subpopulations. These two quantities (S and S_w), and hence R_{st}, can be calculated from the variances of allele sizes, whereas F_{st} will typically be derived from the variances of allele frequencies.

Pairwise multilocus F_{st} and R_{st} estimates are given in Table 12. F_{st} range were 0.006(WD-WG) to 0.047(CD-WD) generally lower than R_{st} value

0.016(CB-CD) to 0.392(CA-WD). The differentiation between some stock was statistically significant (P>0.01). Whereas differentiation between the 3 wild population was lowest (P<0.01). R_{st} values were similar but generally higher than F_{st} values. Genetic distances (D_a) between populations are shown Table. 13. The D_a values ranged from 0.143 to 0.437. The D_a genetic distance was smallest between CB and CC, whereas the largest distance was between WD and CA. The tree generated from the D_a value is shown in Fig. 11. The eight populations fell into 2 clusters: one cluster including the CA, CC, CD, CB and WT population and the other including the WD, WG and WB population.

Table 12. Analysis of genetic differentiation between pairs of samples based on estimates of F_{st} (below diagonal) and R_{st} (above diagonal)

	CA	CB	CC	CD	WB	WD	WG	WT
CA	-	0.031	0.178	0.064	0.212	0.392	0.360	0.096
CB	0.015	-	0.074	0.016	0.128	0.355	0.311	0.066
CC	0.030	0.019	-	0.048	0.027	0.274	0.205	0.135
CD	0.041	0.038	0.034	-	0.064	0.331	0.284	0.105
WB	0.021	0.015	0.017	0.015	-	0.178	0.130	0.135
WD	0.038	0.041	0.042	0.047	0.018	-	0.023	0.288
WG	0.033	0.032	0.032	0.035	0.007	0.006	-	0.266
WT	0.008	0.021	0.028	0.030	0.004	0.020	0.018	-

Wide significance levels were applied using the sequential Bonferrii technique (Rice, 1989; $k=8$)

Table 13. Nei's distance (D_a) between eight populations below the diagonal

	WB	WD	WG	WT	CA	CB	CC	CD
WB	-							
WD	0.287	-						
WG	0.263	0.213	-					
WT	0.322	0.421	0.336	-				
CA	0.303	0.437	0.433	0.255	-			
CB	0.264	0.385	0.328	0.244	0.247	-		
CC	0.253	0.414	0.357	0.289	0.255	0.143	-	
CD	0.260	0.409	0.365	0.299	0.225	0.167	0.145	-

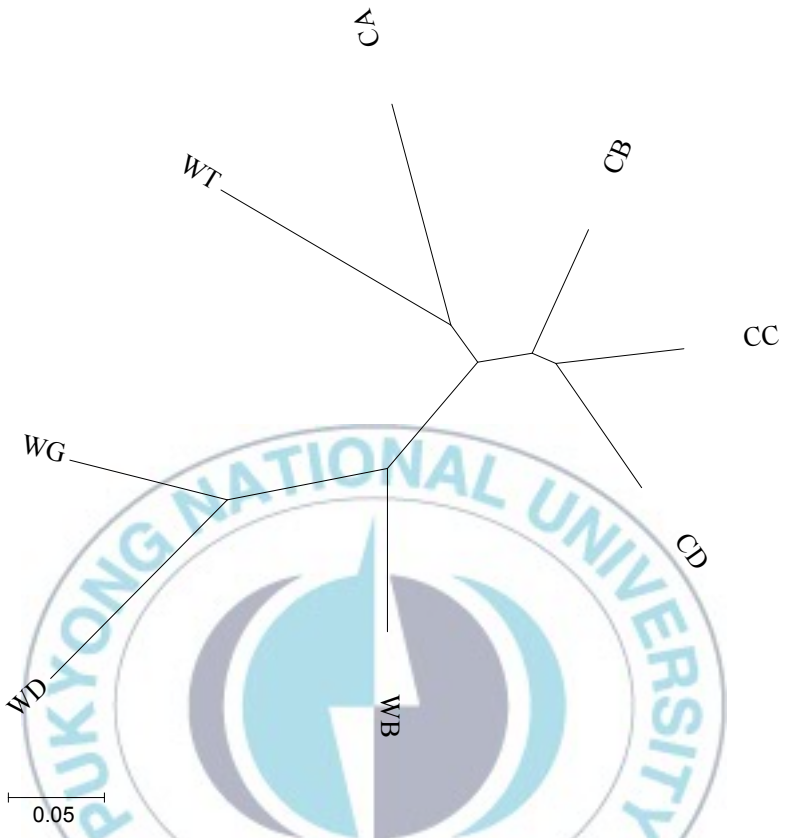


Fig. 11. Tree topology showing the genetic distance among population

Ⅳ. Discussion

Microsatellite profiling technique has been acknowledged as an efficient approach to examine pedigree structure in mixed populations of several aquatic organisms. (Heath DD. *et al.*, 2001; Doyle RW *et al.*, 2001; Norris *et al.*, 2000).

In this study isolated 34 new microsatellite loci olive flounder ESTs to estimated polymorphism. At first, 52 microsatellite loci were isolated, but 18 loci at high annealing temperature, primer target sequence mutation and homozygosity of allele frequency. Characterization of 34 microsatellite loci (A, 2-37; H_o, 0.037-0.925; H_e, 0.031-0.969) were similar to the 52 microsatellite suggested that 30 olive flounder individuals collected from East Sea in Korea (Kim *et al.*, 2009): number of allele range 2 - 30 (mean 15.1), H_o (0.2 - 1.0), H_e (0.20 - 1.0). Considering that significant departure from HWE observed in 8 microsatellite loci was caused by several possible factors such as inbreeding, assortative mating, Wahlund effects, since no other loci showed any significant HWE departures. Alternative, it would be resonable to assume that the HWE departure was caused by random variation.

Result of microsatelite DNA analysis indicated that number of alleles is significantly reduced in the cultivated strain. The average number of alleles per locus in the cultivated and wild population was 15.50 and 28.17. That on average only 55% of the number of alleles were expressed. The farming of

this species has started more than 20 years ago and is still in expansion, but no attempts have been undertaken to assess the genetic status of both wild and cultivated stocks of this species. This work is unusual in that it represents one of a few instances, whereby wild and cultivated sample set of a fish species were studied for microsatellite polymorphism. Generally, farmed and hatchery stocks showed significantly lower allelic diversity than wild stocks (Forst CV., 2006). Small sizes and poor management practices in hatchery are blamed for the loss of genetic variation in the farms stocks. Population genetic theory predicts that a decrease of effect population size increase the rate of both genetic draft and inbreeding. Apart from founder effects, artificial and natural selection in the culture environment might have changed the overall allelic composition of cultivated strain relative to wild population (Mjolnerod *et al.*, 1997).

Microsatellite DNA analysis indicated that the wild and cultivated populations were genetically different. Also WT population was included fewer alleles, most wild alleles than wild population. Significant genetic difference between the wild population and WT population also found and they might have result from released strain population selection procedure. For the same reason, the UPGMA dendrogram showed the WT population was wild population rather than the cultivated population.

The result in study revealed that microsatellite marker is powerful approach to monitor genetic diversity between the wild and cultivated populations of olive flounder investigated. In comparison with the wild and cultivated population significant genetic difference including fewer alleles per locus, allele frequency of genotypes, which indicated a reduction in genetic diversity. It is

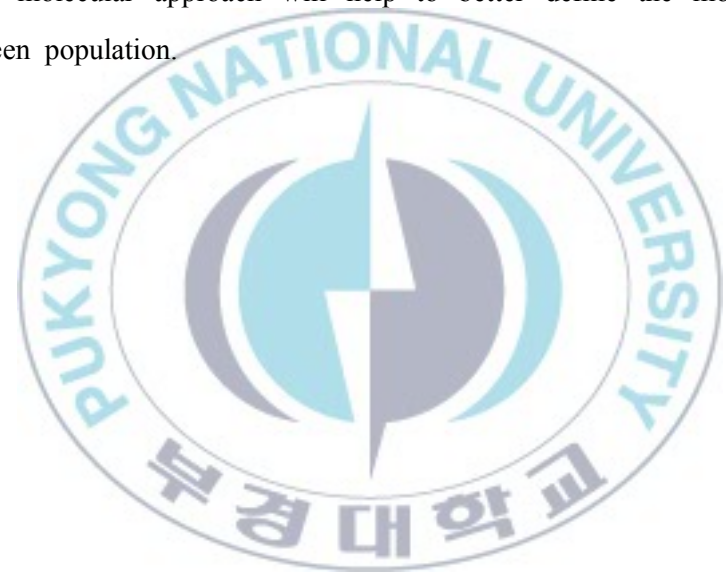
needed to improve the genetic management for all cultivated strains of monitoring the genetic variability. Isolated microsatellite markers will be useful for gene mapping study, marker associated selection and identified economically important quantitative trait loci. These molecular approach will help to better define the mode of gene flow between population.

Ⅴ. Summary

Olive flounder (*Paralichthys olivaceus*) is an important fish species in Asia for fisheries and aquaculture. But change of time due to a number of reasons and loss of genetic variation leads to potential harmful effects upon various performance traits such as survival and growth. The availability of highly variable genetic markers of population structuring in marine fishes and microsatellite markers available in population genetics and powerful tools for accurate genetic assessment of population. This study isolation and characterization 34 new microsatellite from expressed sequence tags database of the olive flounder and comparison of genetic variability in wild and cultivated population. Markers were polymorphic, with 2 to 37 (mean 14.09) number of alleles detected, H_o ranged from 0.032 to 0.925, and H_e range from 0.031 to 0.969. Significant linkage deviations from HWE ($P<0.001$) due to heterozygote deficiency were detected 8 loci. The 6 loci was high degree of genetic variability and these markers will be population studies to identify. Comparison of population each location, the H_e varied from 0.926 (*KOF002*) to 0.962 (*KOF101*). The wild population of H_o ranged from 0.815 (*KOF007*) to 0.925 (*KOF101*) and cultivated population was 0.759 (*KOF007*) to 0.976 (*KOF044*). The H_e ranged from 0.900 (*KOF033*) to 0.962 (*KOF101*) to wild population and cultivated population was 0.851 (*KOF032*) to 0.906 (*KOF101*). The number of alleles ranged was 9.50 (CA) to 22.13 (WD) and total allele

number was 29.75. Wild population to 28.17 was higher than cultivated population to 15.50. Genetic distances (*Dc*) between populations was smallest between CB and CC, whereas the largest distance was between WD and CA. *Dc* value is shown populations fell into 2 clusters: one cluster including the CA, CC, CD, CB and WT and the other including the WD, WG and WB.

Isolated microsatellite markers will be useful for gene mapping study, marker associated selection and identified economically important quantitative trait loci also, these molecular approach will help to better define the mode of gene flow between population.



Ⅵ. Reference

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요 약 문

넙치(*Paralichthys olivaceus*) 는 아시아지역의 어업과 수산양식에서 중요한 어류이다. 하지만 여러 가지의 원인에 의해서 시간이 지남에 따라 유전적 다양성이 감소하고 있으며 이로 인하여 집단의 생존 및 성장의 부분에 있어서 악영향을 미치고 있다. 이와 같은 수산생물 집단의 구조를 밝혀내기 위해 높은 다형성을 가지는 많은 종류의 유전자 표지가 사용되고 있으며, microsatellite 표지는 집단의 구조분석과 유전적 연관성을 밝혀내는데 있어서 중요한 도구로 사용되고 있다. 본 연구에서는 새로운 34개의 microsatelltes 표지를 넙치의 expressed sequence tags 자료로부터 분리하였으며, 분리된 표지를 이용 한국의 자연산과 양식산 넙치에 대한 유전적 다양성을 비교하였다. 다양성의 비교를 위해 사용된 표지의 대립유전자의 범위는 2-37개 (평균 14.09), 관찰치 이형접합체율의 범위는 0.032-0.925로 나타났으며, 기대치 이형접합체율은 0.031-0.969 의 범위를 나타내었다. 8개의 유전자좌에서 다형성의 결핍에 의한 Hardy-Weinberg 평형(*P<0.001*)의 이탈이 관찰되었다. 유전적 다양성 비교를 위한 6개의 높은 유전자좌를 확인할 수 있었으며 확인된 유전자 표지를 이용하여 넙치의 집단연구에 이용하였다. 각 집단의 유전적 다양성의 비교에서 기대치 이형접합체율의 범위는 자연산 집단이 0.926 (*KOF002*) - 0.962 (*KOF101*) 로 나타났으며, 양식산 집단은 0.759 (*KOF033*) - 0.976 (*KOF044*) 로 나타났다. 대립유전자의 비교에서는 9.5 (CA) - 22.13 (WG) 의 범위를 나타내었다. 총 대립유전자의 평균개수는 29.75 개 이며, 자연산 집단은 28.17개, 양식산

집단 15.50개로 나타났다. 각 집단간의 유전적 거리를 비교해보면 CB - CC 간이 가장 가까운 값을 나타내었고, WD - CA 간이 가장 먼 것으로 나타났다. 유전적 거리에 기초한 각 집단이 두개의 군집인 CA, CC, CD, CB, WT 와 WD, WG, WB 로 나누어짐을 확인할 수 있었다. 본 연구에서 이용된 유전자 표지를 이용하여 어류 각 개체의 집단간의 유전학적 다양성을 확인할 수 있으며, 유전자 표지를 이용하여 유전자 연관지도 분석 및 개발과 양적경제형질을 지니는 개체의 선발에 유용하게 이용할 수 있을 것이다.

감사의 글

하나님께 영광 돌리며 도움을 주신 많은 분들의 지도와 가르침이 있었기에 논문이 완성될 수 있었습니다. 먼저 이 논문이 나오기 까지 항상 부족한 저를 배려해 주시고 가르침을 주신 공인수 교수님께 깊은 감사드립니다. 그리고 논문이 완성되기 까지 지도와 격려로 심사해 주신 이형호 교수님과 홍용기 교수님께 감사드립니다. 또한 관심 가져주시고 격려해 주신 김중균 교수님, 박남규 교수님, 김성구 교수님께 감사드리며 유전공학 실험실 연구원님들에게도 고마운 마음 전합니다.

항상 부족하지만 학업과 직장생활을 같이하는데 있어서 많은 격려와 조언을 해주신 명정인 센터장님께 감사드리고, 논문이 완성되기 까지 많은 지도해 주신 이정호 실장님께 감사드립니다. 본 연구의 수행에 진심으로 많은 도움을 주신 노재구 박사님, 김현철 박사님, 박철지 박사님, 민병화 박사님께 감사드리며 육종연구센터 이부연님, 이준님, 김용권님, 이상학님, 이미숙님, 김미숙님, 김경환님, 이장욱님, 하수진님, 최상준님, 김관석박사님, 정선태님, 손세봉님께 머리 숙여 감사드립니다. 많은 실수를 하지만 이해해 주시고 격려해 주시는 본원의 김경길 과장님, 이상준 연구관님, 한현섭 연구관님, 박중연 박사님, 김우진 박사님, 김영옥 박사님, 남보혜 박사님, 공희정 박사님께 감사드리며, 한윤희님, 조현국님, 박은미님, 김현정님, 안상현님께 감사의 말씀을 드립니다.

동생 다음이, 조카 지아 에게도 고마움을 전하며, 부족한 아들이지만 항상 사랑으로 이해해 주시고 염려해 주시는 부모님께 이 논문이 조그만 보답이 되길 바랍니다.

2009년 8월

정 종 근