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Practical Manual on Detection of DNA Polymorphism in Fish Population Study

Estu NUGROHO, Motohiro TAKAGI* and Nobuhiko TANIGUCHI

Laboratory of Fish Ecology and Genetics, Department of Aquaculture, Faculty of Agriculture, Kochi University, Monobe, Nankoku, Kochi 783, Japan

Abstract : In order to support an activity of conservation of valuable natural population and biological diversity in aquatic environment, the valuable population genetic data are necessary. In our laboratory, Department of Aquaculture-Kochi University, beside allozymes which have been established for more twenty years ago for revealing those genetic data, some molecular genetic techniques such as Random Amplified Polymorphic DNA (RAPD); Multilocus DNA Fingerprinting; Amplified Restriction Fragment Polymorphism (AFLP); and Variable Number Tandem Repeats (VNTR) of Microsatellite, have also been used.

During study of the DNA polymorphism in several fish species, we conclude that the RAPD and multi-locus DNA fingerprinting (including AFLP) analysis are most useful for pedigree study or species identification, while population genetic parameters will be more accurately estimated by single locus of microsatellite DNA. This paper shows a protocol for detection of fish DNA polymorphism in our laboratory.

Keywords: DNA polymorphism, practical manual, RAPD, Minisatellite, Microsatellite, fish population genetics

INTRODUCTION

Genetic variation knowledge of a species is an important feature of its population both for short term fitness of individuals and the long term conservation of the population through allowing adaptation to changing environmental condition which occurred (Ferguson *et.al.*, 1995). The genetic variation can be observed in the DNA polymorphism (by allelic diversity and heterozigosity). Several approaches to detect the polymorphism of DNA can be implemented, one of them by using molecular genetic techniques (Carvalho and Pitcher, 1995).

Molecular genetics approach was firstly applied in 1950s, starting with experiment of several blood group of salmon and cod (reviewed by de Ligny, 1969), however these serological procedures were not adopted furthermore by fisheries biologists. They were enthusiastically used the electrophoretic procedures revealing genetically determined protein polymorphism. Protein or allozyme electrophoresis provides an indirect assessment of nuclear DNA variability. This technique was found to be quick, reasonably inexpensive, easy and reproducible. However, this analysis is less sensitive and the invasive tissue sampling is generally necessary and more material than most DNA methods. The important point is we should assume that these markers are selectively neutral and that genetic drift is responsible for population differentiation (Ward and Grewe, 1995).

Furthermore, application of procedures for direct assessment of DNA were used. Direct assessment of DNA variability came with the isolation of restriction endonucleases. Initial application of this technology were examined in mitochondrial DNA (mtDNA). The mtDNA analy-

* Present address: Ono Limnological Station, National Fisheries University, Ono, Ube, Yamaguchi 754-13, Japan 109

sis has found favor and is generally assumed to be more powerful than allozyme analysis for revealing population structure because of mtDNA is haploid and maternally inherited. However this analysis is usually treated as a single character, whereas allozyme electrophoresis revealed many independent characters (loci). This technique can be carried out on fresh, frozen or alcohol stored tissue.

Recently, a number of easily assayable and highly variable genetic markers have been developed. Most of them concern to the analysis of repeated sequences (VNTR loci-variable number of tandem repeats). One class of VNTR loci is minisatellite or multilocus DNA fingerprinting. Multi-locus DNA fingerprinting is started in 1985 with the paper 'Hypervariable Minisatellite Regions in Human DNA'. These hypervariable sequences have generally been assayed by using Southern blot analysis with a conserved core repeat probe. This procedure reveals large numbers of loci simultaneously, producing enormous variability in a wide range of organisms (Takagi *et al.*, 1995). However, we can not assign alleles to specific variant fragments because of multiple loci are assayed simultaneously. This will decrease capability to characterize allele frequencies among population or to evaluate fits to Hardy-Weinberg equilibrium (Park and Moran, 1995).

Conjunction with the discovery of a polymerase chain reaction capable of rapid DNA multiplication, a procedure that uses a single short primer of arbitrary sequence to amplify genomic has been used. This procedure which called by Random Amplification Polymorphic DNA (RAPD) has a wide range of potential applications in fisheries (Welsh and McClelland, 1990).

Furthermore, starting in 1980s, Amplified Restriction Fragment Polymorphism (AFLP) was developed (Lin and Kuo, 1996). This technique is based on the combination both of Restriction Fragment Length Polymorphism (RFLP) and Polymerase Chain Reaction (PCR). The AFLP analysis needs smaller genome being analyzed, and has the fewer and simpler fragments amplified than the multilocus DNA fingerprinting. In an effort to simplify the complexity of multilocus DNA fingerprints, isolation of particular VNTR loci has been conducted by many researchers. This class of tandemly-arrayed sequences with repeat unit lengths of only 1 to 4 bp was widely termed as microsatellite (Weber and May, 1989).

Of those several genetics markers, Random Amplified Polymorphic DNA (RAPD), and one class of Variable Number of Tandem Repeat loci, the minisatellites (including microsatellite), have generated a great deal of interest in fisheries research. They are some of the alternatives easily and accurately applied. However, some advantages and disadvantage will be found during application of the techniques. In order to optimize capability of the molecular genetics techniques to detect polymorphism DNA, the purpose of using these techniques should be accurately considered.

Molecular genetic technique application in fisheries are dramatically increasing nowadays. Some experiments have been used the molecular genetic approach, such as utilization of isozyme in the red seabream (Taniguchi and Sugama, 1990), application of RAPD in prawn (Garcia and Benzie, 1995), genus *Anguilla* (Takagi and Taniguchi, 1995), identification of genus Morone (Bosworth *et al.*, 1994) and ayu fish (Takagi *et al.*, 1995) using DNA fingerprinting, mtDNA analysis on study of the amorhead (Martin *et al*, 1992) and development of microsatellite loci from teleostei fish (Brooker *et al.*, 1994), Atlantic salmon (McConnel, *et al.*, 1995), Brook charr (Angers *et al.*, 1995), Rainbow trout (Herbinger *et al*, 1995, Red seabream (Takagi *et al.*).

This article consists of practical protocols to appreciate some molecular techniques as a tool in order to detect the polymorphism DNA in fisheries research.

PREPARING GENOMIC DNA

The primary objective of the isolation process is to recover the maximum yield of high molecular weight DNA avoid of protein and other restriction enzyme inhibitors (Sambrook, 1989). Total genomic DNA can be isolated from fin cutting and blood. Isolation from blood is usually higher quality of DNA resulted than DNA extraction from fin. Preparing DNA samples from fin and blood are as follows:

A. Fin Samples

Extraction

a. Placed fin (5-10 mg in weight) into a 1.5 ml centrifuge tube containing 700.0 μl of the lysis solution (10.0 mM Tris-HCl; pH 7.5; 125.0 mM NaCl; 10 mM EDTA , pH 7.5; 0.5% SDS; 4.0 M Urea).
b. Add 10.0 μg/ml Protein Kinase (PK), vortex, flush centrifuge.
c. Incubate at 37℃ for 12-16 hr.

Purification

a. Add 700 μl of Phenol/Chloroform/Isoamyl Alcohol (25:24:1)
b. Rotamix at 20 rpm. for 10 min., centrifuge at 3,000 rpm. for 10 min.
c. Take a supernatant (aliquots layer) and place into a new 1.5-ml centrifuge tube.
-Repeat step a-c for 3 times.
d. Take a supernatant from c and add a equal volume of Chloroform/Isoamyl alcohol (24:1).
e. Rotamix at 20 rpm. for 10 min., centrifuge at 3,000 rpm. for 10 min.
f. Take a supernatant and place into a new 1.5 ml centrifuge tube.
-Repeat step d-f for 3 times
g. Take a supernatant from f and add a 10% volume of 3M Sodium Acetate and a 2x volume of 95% ethanol.
h. Keep it at -20℃. for 30 min.
i. Centrifuge at 5,000 rpm. for 10 min., throw out the solution carefully.
j. Add 1 ml of 70% alcohol and centrifuge at 5,000 rpm. for 10 min.
k. Throw out the solution and air dried.
l. Resuspended in 100.0 μl TE buffer and kept at 4℃ before used.

B. Blood Samples

Extraction

a. Place a 0.1-0.2 ml blood into a 50 ml tube containing 10.0 ml of physiological solution (0.9-3.0% NaCl; 1.0 mM EDTA, pH 8.0)
b. Centrifuge at 3,000 rpm. for 10 min., throw out the supernatant.
c. Add 10.0 ml of TNE buffer(10.0 mM Tris-HCl, pH 7.5; 0.1 M NaCl; 1mM EDTA), 1 ml of 10% SDS, 10.0 μg/ml Protein Kinase.
Mix gently.
d. Incubate at 58℃ for 4 hr., and at 37℃ for 14 hr.

Purification

a. Add a equal volume of Neutral Phenol (pH 7.0).
b. Rotamix at 20 rpm. for 10 min., centrifuge at 3,000 rpm. for 10 min.
c. Take a supernatant and place into a new tube.
-Repeat step a-c for 2 times.

d. Take a supernatant from c and add a equal volume of Phenol/Chloroform/Isoamyl Alcohol (25:24:1).
e. Rotamix at 20 rpm. for 10 min., centrifuge at 3,000 rpm. for 10 min.
f. Take a supernatant and add a equal volume of Chloroform/Isoamyl Alcohol
g. Rotamix at 20 rpm. for 10 min., centrifuge at 3,000 rpm. for 10 min.
h. Take a supernatant and add a equal volume of Ethyl ether, shake briefly, flush centrifuge.
i. Throw out the supernatant and repeat this step (h-i) for 2 times and dried until all of ether residue was evaporated.
j. Add 10% volume of 3M Sodium Acetate, 2x volume of 95% ethanol, and shacked.
k. Centrifuge at 3,000 rpm. for 10 min., throw out the solution carefully.
l. Add 10.0 ml of 70% alcohol, shacked, centrifuge at 3000 rpm. for 10 min., throw out the solution. Repeat this step one more.
m. Air dried, resuspended in 1.0 ml TE buffer and kept at 4℃.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

According to Park and Moran (1995), this technique is most suitable for pedigree study. The inheritance pattern of the polymorphic bands analysis is based on the presence or absence of main band in each primer (Fig. 1). Another study appreciated by this technique is population genetics, even though not as well as in above study, whereas analysis is based on the comparison main bands of between or within populations.

This technique used the polymerase chain reaction (PCR) to amplify amount of DNA fragment with short single primer sets as chain initiation to reform a nucleotide sequences. In PCR, a minute amount of DNA serves as a template which combined with single primer sequence in the low annealing temperature and four nucleotides (A, C, G, T) in the presence of thermostable polymerase. Firstly, the double strands of the DNA is denatured by heating and become single-strand. Then the primers, which designed

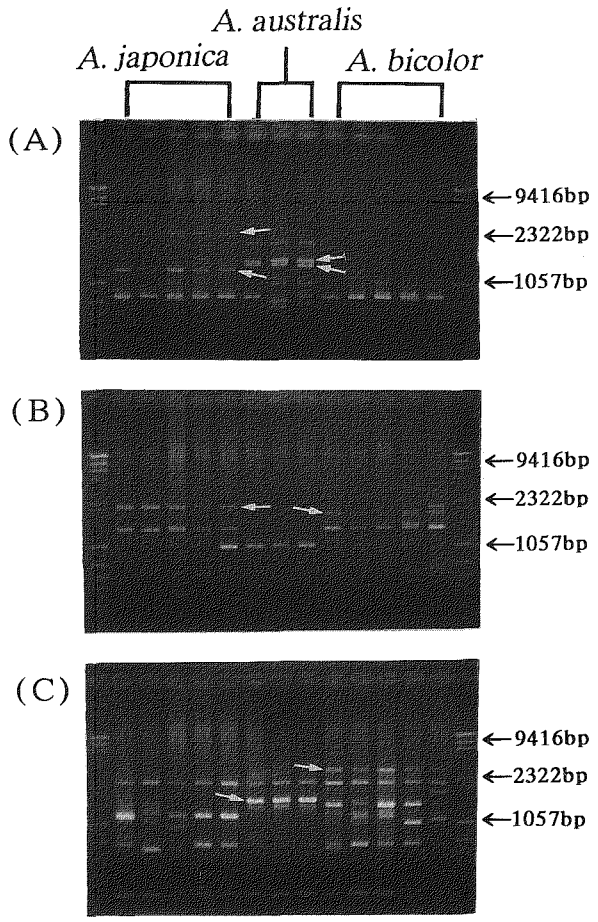

Fig. 1. An example of RAPD-PCR. Presence and absence of main band in primer OPA-11 (A), OPA-12 (B) and OPA-16 (C) as base of the RAPD analysis for identification of *Anguilla sp.* (Figure modified from Takagi and Taniguchi, 1995).

short and randomly to annealing easier (Produced by Operon Technologies, USA), are annealed into the template using cooling. One primer will anneal to one complemental region and the other anneals to the other complement region. If the synthesis occurs across the interest region, the new double stranded DNAs will be performed from the original template. And this process will happen over and over, in the 30 cycles, resulted greater than a millionfold increase in the concentration of the target sequence.

This technique is usually difficult to reproduce the same result in different time of analysis. Dinesh, *et.al.* (1995), however, could improve the reproducibility of RAPD makers using discontinuous polyacrylamide (dPAGE) gel. In briefly, this methodology are as follows (as illustration shown in Fig. 2):

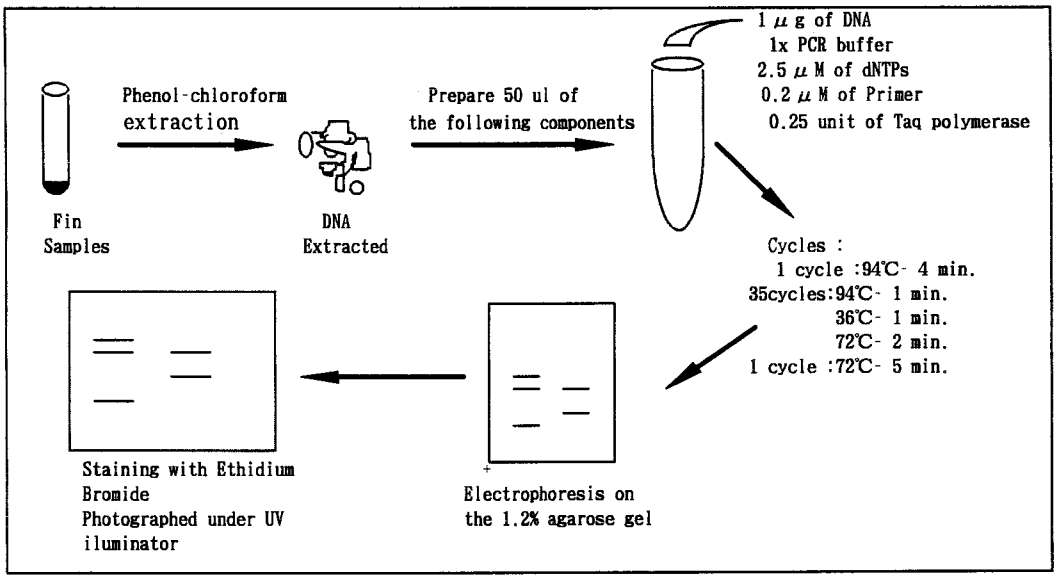

Fig. 2. Schematic illustration of the RAPD.

1. Place the following reaction into a PCR-tube :

DNA template	1.0 μg	Taq Polymerase DNA	0.25 unit
10x PCR-buffer*)	5.0 μl	Distillate water up to	50.0 μl
dNTPs	2.5 mM	*PCR buffer = 10.0 mM Tris-HCl, pH 8.3; 50.0 mM MgC12;0.001% gelatin	
Primer	0.2 μM		

2. Mix gently, centrifuge flush.

3. Place the tube into PCR machine with cycles are as follows:

1 cycle of 94℃ for 4 min.
35 cycles of 94℃ for 1 min.
36℃ for 1 min.
72℃ for 2 min.
1 cycle of 72℃ for 5 min.

4. The PCR products are separated on a 1.2% agarose gel at 15 v/cm for 30 minutes in 1x TBE buffer. The gel is stained with ethidium bromide for 10 min., and photographed on a UV transilluminator.

MULTI-LOCUS DNA FINGERPRINTING (Minisatellite)

This methods is usually used for population genetics study, pedigree analysis and species identification. Analysis is based on the scoring of presence or absence of the numbered bands. For each species, every fragment with a different molecular weight is numbered sequentially, actually each individual is run two-three times on each gel to assure internal consistency.

Many DNA Fingerprinting techniques have been developed in the past years and are generally based on one of two distinct approaches (Gibco BRL, 1995). The first approach is the detection of restriction fragment polymorphism (RFLPs) using Southern hybridization with probes targeted to hypervariable regions of DNA. Numerous probes are available that hybridize to different VNTR loci possessing similar repeat unit sequences. This technique involves the restriction of genomic DNA with endonuclease, followed by electrophoretic, Southern transfer, probe labeling, probe genomic fragment hybridization, and print detection.

The restriction endonucleases cleave DNA molecules at specific recognition base sequences. The recognition sites are palindromic i.e. the order of the bases in a segment of one DNA strand is the reverse of that in the complementary strand. The lengths also vary, with 4-6 bp sequences being relatively commmon. An important point in this step is to choose an enzyme with sites flanking the repeats. Cleavage within a repeat sequence will result in the production of small fragments that may be unresolvable.

The fragments produced by restriction enzyme cleavage is separated into an agarose gel. As notably, if the electroforesis is conducted for longer periods, buffer ion depletion can occur resulting in problems such as DNA band shifting. After that, the separated DNA fragments of different sizes are transferred into nylon membrane support. In this case, we should exercise the caution because of possible variation in performance between different lots.

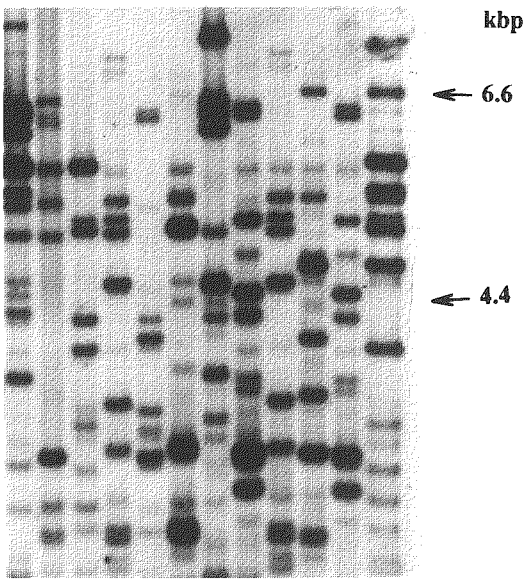

Fig. 3. An example of band patterns of the multilocus DNA fingerprinting for *Carassius langsdorfii*. (Figure modified from Ohara, 1995, unpublished).

Furthermore, the DNA sequences immobilized on membranes are hybridized in order to anneal with a single-stranded labeled probes. There are two types of probe labeling, radioisotope and nonradioisotope such as a horseradish peroxides (HRP). The nonradioistope probe labeling has longer a shelf life than radioisotope probe. Base pair mismatched between membrane-bound specimen DNA and probe can be tolerated with low stringency washes. Finally, autoradiography is used to detect ^{32}P-labeled hybri-

dization probes on the blot membranes using X-ray film, while HRP-labeled blots can be developed after the final wash step using Tetramethyl benzidine (TMB) chromogen.

The second approach is the application of polymerase chain reaction (PCR) to amplify segments of particular DNA sequences using specific or arbitrary primers (labeled during amplification). The PCR products are hybridized to the probes using both dot and reverse dot-blot procedures. The reverse dot-blot technique differs from the standard dot-blot format in that a number of oligonucleotide probes are immobilized on a single membrane strip. Because of the high specific allele copy number nonradioisotop detection procedures are sufficiently sensitive for use with these blot techniques.

In multi-locus DNA fingerprinting, length variation is surveyed at many VNTR loci simultaneously (Fig. 3). Due to the large number of loci examined, each profile of bands is usually highly informative and individual specific. The protocol of DNA fingerprinting with radioisotope probe labeling are as follow (as illustration shown in Figure 4):

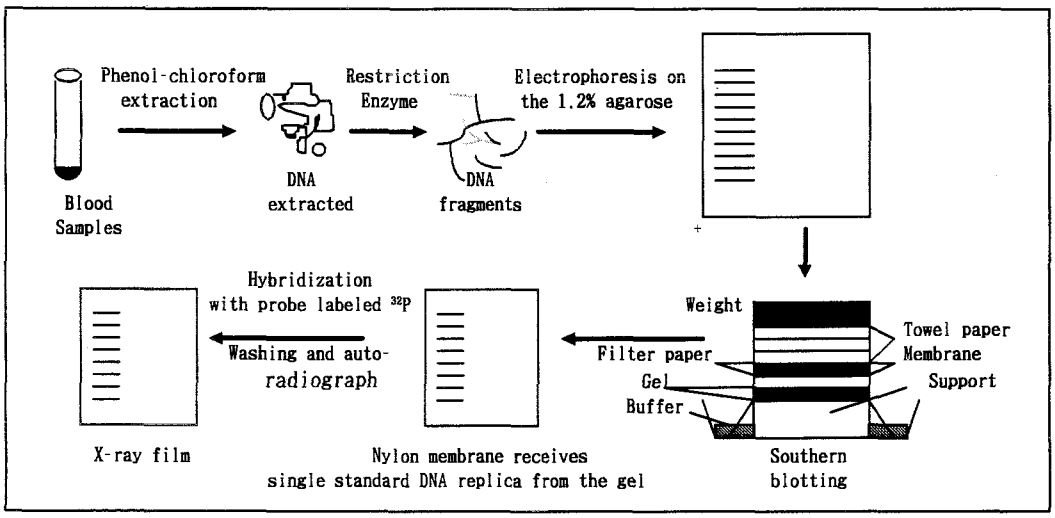

Fig. 4. Schematic illustration of the DNA fingerprinting.

A. Digestion and Gel analysis

1. Take 50.0 μg of total DNA.
2. Add 10% volume of Natrium acetate and 2x volume of 95% alcohol, centrifuge for 5 min. at 5000 rpm.
3. Throw out supernatant, and air dried.
4. Prepare the following reaction into a 1.5 ml tube;
   Genomic DNA ..... 50.0 μg
   10x Buffer ..... 50.0 μl
   Hinf I or *Hae* III ..... 100.0 unit
   Autoclave water up to ..... 500.0 μl
5. Incubate it at 37℃ for 16 hr.
6. Add a equal volume of phenol/chloroform/isoamylalcohol, centrifuge at 10,000 rpm. for 5 min.
7. Take a supernatant, add a equal volume of ethyl ether, centrifuge at 10,000 rpm. for 2 min.

8. Throw out the supernatant, air dried from ethyl ether residual
9. Add 10% volume of 3.0 M Natrium Acetate and 2x volume of 95% ethanol, keep it at -80℃ for 1 hr.
10. Centrifuge at 10,000 rpm. for 5 min., throw out the solution.
11. Rinse it with 1 ml of 70% alcohol, centrifuge at 10,000 rpm. for 5 min., and throw out the solution.
12. Air dried, add 25.0 ul of TE buffer and 1/3 vol. of BPB, keep it at 4℃.
13. Pour 10.0-13.0 μl of the sample onto a 1.2% agarose gel with 1x TAE buffer, at 65v for 60 hr (in a temperature 4℃).
    As a marker is λ DNA-*Hind* III digest.

B. Southern Blotting

1. Take the gel on the area which have only band as well as shown by marker.
2. Soak the gel into the denaturation solution (0.5M NaOH+1.5M NaCl) for 2 hr.
3. Dip the gel into the neutralization solution (1M NH_4Ac+0.03M NaOH) for 1 hr.
4. Set the gel in the blotting equipment such as follows:
   -Place a filter paper on the glass which stand at a support in a container.
   -Place the gel on the filter paper
   -Place a piece of membrane filter, 2 pieces of filter paper on the gel.
   -Then place some towel paper and cover with glass which weighted by stone/iron (1kg).
5. Fill the neutralization solution until glass in the bottom side.
6. Blotting is finished, if the solution go up to the upper glass.
7. Take the membrane and bake at 80℃ for 2 hr.

C. Hybridization

1. Place the paper into a bottle containing 30 ml of the hybridization solution (40% Formamide; 6x SSC; 5 mM EDTA; 0.25 % Skim milk).
2. Incubate and rotate those bottle at 37℃ for 2 hr.
3. Throw out the solution and add 20 ml of hybridization solution and radioisotope labeled probe. Procedure to prepare probe using Bca BEST™ Labeling kit (TAKARA) are as follows :

   YNZ 22 ..... 1.2 μl
   Random primer ..... 2.0 μl
   Water ..... 1.8 μl

   - place it at 95℃ for 3 min., immediately keep on ice for 5 min.
   - add the following components :

   10x buffer ..... 2.5 μl
   dNTPs ..... 2.5 μl
   Water ..... 9.0 μl
   ^{32}P-dCTP ..... 50.0 μCi
   BcaBEST DNA Polymerase 1.0 μl

   - place at 55℃ for 10 min., add 1.5 μl of 0.5M EDTA.
   - keep at 95℃ for 5 min., and immediately place on ice.
4. Incubate at 42℃ for 16h.
5. Wash the paper using twice with 2x SSC for 15 minutes at 55C, and twice with 1x SSC at 55℃ for 15 minutes.
6. The paper was wrapped in plastic wrap and exposed to x-ray film with an intensifying screen at -80℃ until to get a good resolution.

AMPLIFIED RESTRICTION FRAGMENT POLYMORPHISM (AFLP)

The new modification technique of DNA Fingerprinting is now being developed, starting in 1980s (reviewed by Kirby, 1990), is called by Amplified Restriction Fragment Polymorphism (AFLP). This method is suitable for genetic population study, pedigree analysis and species identification. Because of capability of AFLP produced species specific band, this technique also available for species identification. Lin and Kuo (1995) have successfully investigated useful of AFLP technique for genomic DNA of *A. thalianas* and 4 strains of *E. coli.* AFLP technology is a DNA Fingerprinting technique that combines both of Restriction Fragment Length Polymorphism (RFLP) and PCR approaches. It is based on the selective amplification of a subset of genomic restriction fragment using PCR (Gibco BRL, 1995). DNA is digested with 2 restriction endonucleases simultaneously in order to generate small DNA fragment i.e. *EcoR I and Mse* I. In this technique, *EcoR* I and *Mse* I adapters are used to amplify many DNA fragments without having prior sequence knowledge. The amplification consists of two reactions, the first is pre-amplification, genomic DNA are amplified with AFLP primers each having one selective nucleotide. The second is amplification of pre-amplification products with two AFLP primers, each containing three selective nucleotides (The *EcoR* I selective primer is labeling with γ ^{32}P before amplification). Products of the selective amplification are separated on a 6% denaturing polyacrylamide gel (Fig. 5). Briefly, the procedure of AFLP using AFLP$_{TM}$ Analysis System (GiBCO BRL Inc.) are as follow (as illustration shown in Fig. 6):

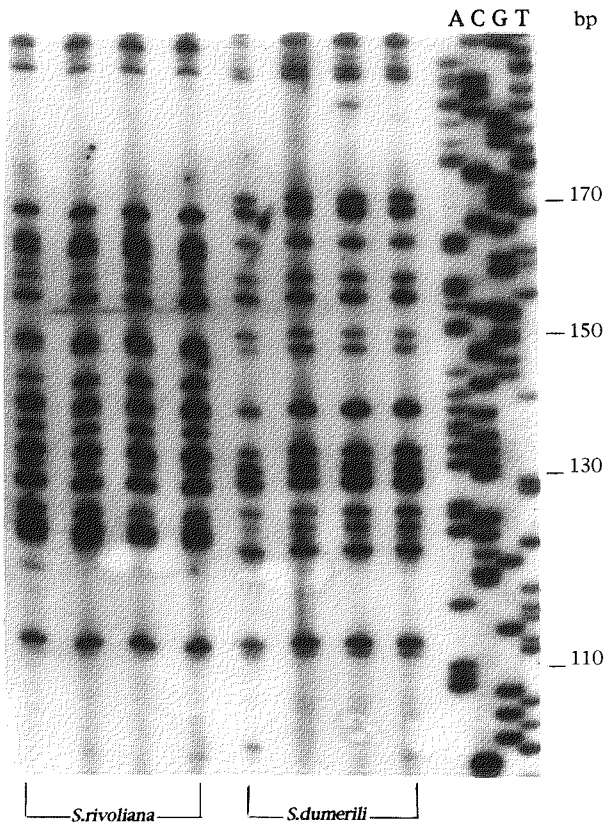

Fig. 5. An example of band patterns of the AFLP analaysis for *Seriola rivoliana and Seriola dumerilli.*

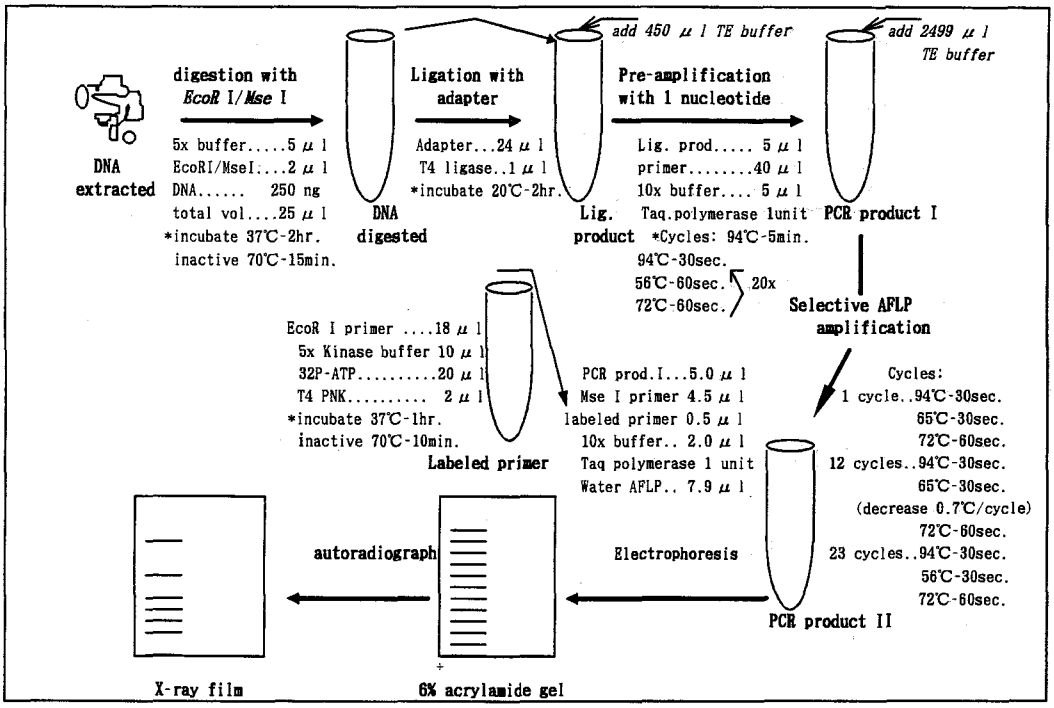

Fig. 6. Schematic illustration of the AFLP method.

A. Digestion and Ligation

1. Prepare the following reaction into a 1.5 ml centrifuge tube :
   5x reaction buffer ..... 5.0 μl
   EcoR I/*Mse* I ..... 2.0 μl
   DNA (250 ng) ..... <18.0 μl
   Water up to volume ..... 25.0 μl
2. Mix gently, centrifuge flush, incubate it at 37℃ for 2 h.
3. Inactive with 70℃ for 15 min. and place it on ice.
4. Add the following reaction : adapter ligation solution ..... 24.0 μl
   T4 DNA ligase ..... 1.0 μl
5. Mix gently, centrifuge flush, incubate it at 20℃ for 2hr.
6. Add 450 μl TE buffer and keep it at -20℃

B. Pre-amplification

1. Place the following reaction into a PCR-tube:
   Ligation product ..... 5.0 μl
   pre-amp primer mix ..... 40.0 μl
   10x PCR buffer for AFLP ..... 5.0 μl
   Taq DNA Polymerase (1 unit/μl ..... 1.0 μl
   Total volume 51.0 μl
2. Mix gently, centrifuge flush and place it at the PCR machine with cycles are as follows:
   1 cycle ..... 94℃ - 5 min.
   20 cycles ..... 94℃ - 30 sec.

56℃ - 60 sec.
72℃ - 60 sec.

Soak temperature is 4℃.

3. Add 2499 μl of TE buffer and keep it at -20 ℃.

C. Labeling *EcoR* I primer

1. Prepare the following reaction into a 1.5 ml centrifuge tube :

EcoR I primer (select one)	18.0 μl
5x kinase buffer	10.0 μl
[γ 32P] ATP (3000 Ci/mmol)	20.0 μl
T4 Polynucleotide kinase	2.0 μl
Total volume	50.0 μl

* Mix gently, centrifuge flush, incubate at 37℃ for 1 hr., inactive at 70℃ for 10 min.

D. Selective AFLP Amplification

1. Place the following reaction into a PCR-tube (for one sample at each primer pair) :

Pre-amplification product	5.0 μl
Mse I primer	4.5 μl
Labeled *EcoR* I primer)	0.5 μl
Water	7.9 μl
10x PCR buffer	2.0 μl
Taq DNA Polymerase (1 unit/ μl)	0.1 μl
Total volume	20.0 μl

2. Place it in the PCR machine with cycles are as follow :

1 cycle	94.0℃ - 30 sec.
	65.0℃ - 30 sec.
	72.0℃ - 60 sec.
12 cycles	94.0℃ - 30 sec.
	64.3℃ - 30 sec.---> the annealing temperature decrease each cycle 0.7℃.
	72.0℃ - 60 sec.
23 cycles	94.0℃ - 30 sec.
	56.0℃ - 30 sec.
	72.0℃ - 60 sec.

3. Add an equal volume of formamide dye (98% formamide; 10mM EDTA; 0.3% bromphenol blue; 0.3% xylene cyanol) to each reaction.
4. Heat the samples at 90℃ for 3 min., immediately place on ice.
5. Loading 2 μl samples onto 6% polyacrylamide gel at 55w until xylene cyanol reach 2/3 part of the gel length.
6. Expose the gel to x-ray film.

MICROSATELLITE

One class of single locus DNA fingerprinting markers that is generally inherited in a Mendelian way is microsatellite (Weber and May, 1989). Recently, this method is being increasingly used to determine polymorphic of the populations because of it was more sensitive and accurate. As notably, development of this technique is still not easy and needs lots of time and cost, especially for primer designing. The uses of radioisotope should also be considered furthermore for application of microsatellite technique.

Microsatellite are short stretches or sequences (their length are usually no larger than 300 base pairs (bp)) of DNA composed of di-, tri- or tetranucleotide repeats arrayed in tandem (Park and Moran, 1994) or different types of repeats, e.g. a GT repeat adjacent to or

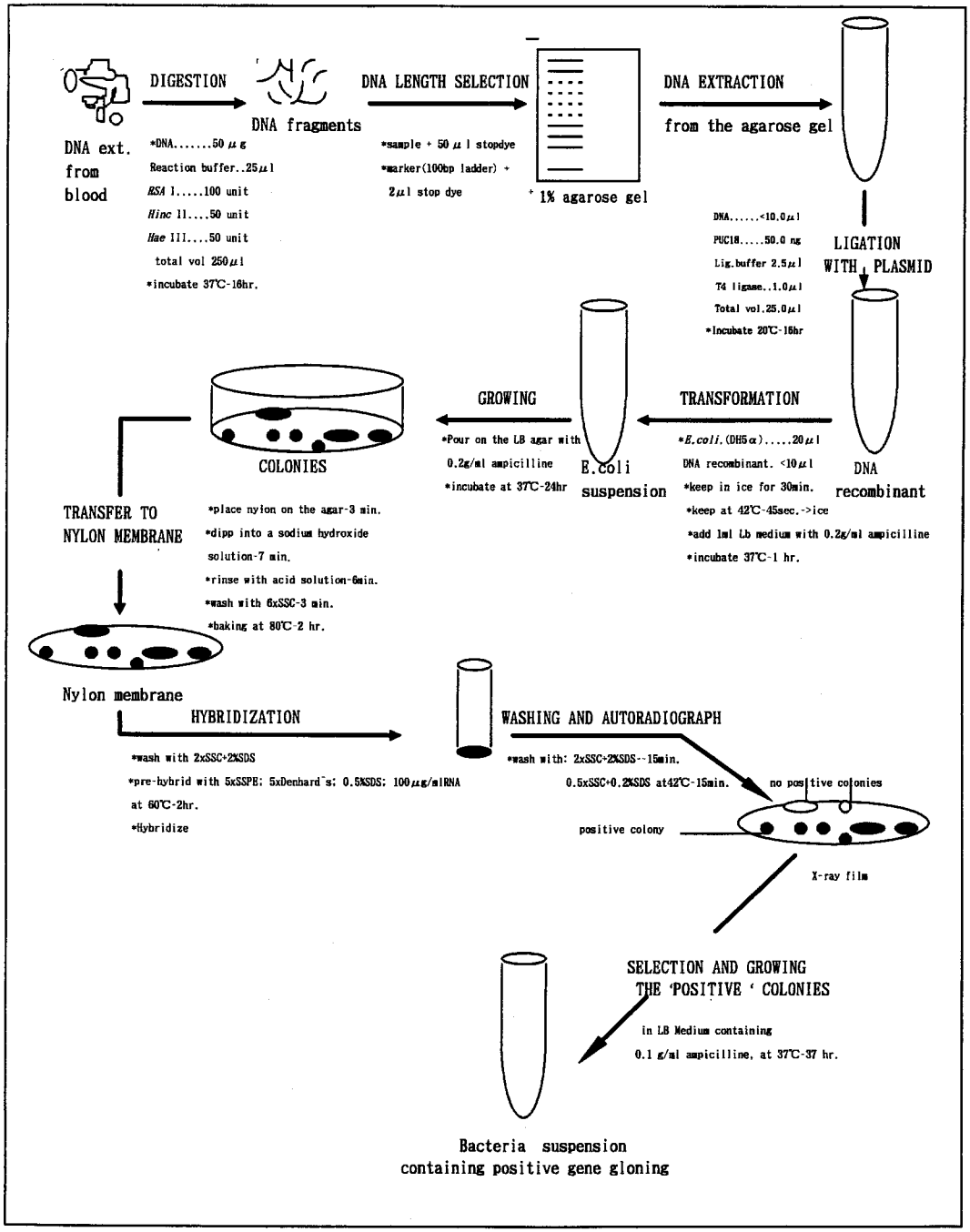

Fig. 7. Schematic illustration of the gene cloning process.

interposed with GA repeats(Wright and Bentzen, 1995). Within vertebrates, the nucleotide repeat (GT)n is believed to be the most common microsatellite (Brenner *et.al.* 1993). They are highly abundant, with frequencies of 10^3 to 10^5 copies, and dispersed at $7\text{-}10^{-100}$ kilobasepair(kbp) intervals in eukaryotic genomes (Wright, 1993).

Each microsatellite locus, tandemly arrayed repeat, is flanked by a unique sequence. If the sequences flanking the microsatellite are known, primers can be synthesized complementary to these flanking sequences. The microsatellite loci can then be isolated with relative ease by PCR amplification from minute quantities of fresh or preserved tissue. Some loci of microsatellite for fish have been developed such as teleost fish (Brooker *et al.*, 1995); Atlantic salmon (McConnel *et.al.*, 1995); brook charr (Bernatchez *et al.*, 1995); Salmonid (McConnell *et.al.*, 1995); bluegill sunfish (Colbourne *et.al.*, 1996); goldfish (Zheng *et.al.*, 1995); red sea bream (Takagi *et.al.*, 1997).

The procedure of primers synthesis and PCR amplification are as follows (as illustration shown in Fig. 7 and 8):

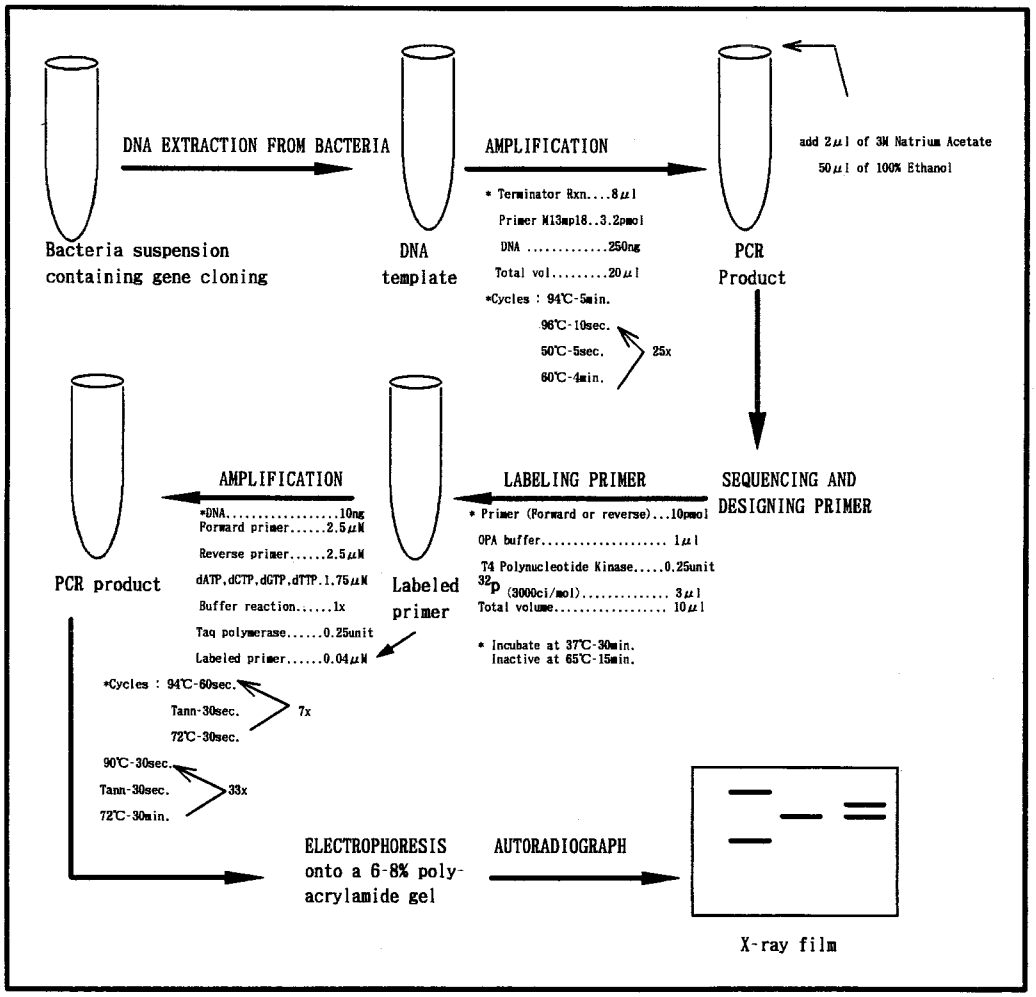

Fig. 8. Schematic illustration of the primer synthesis and amplification of microsatellite.

A. Primers Synthesis

Digestion

1. Place the following reaction into a 1.5 ml tube :
   DNA template ..... 50.0 μg
   Reaction buffer (10x)..... 25.0 μl
   Rsa I (12 units/μl)..... 100 unit
   Hinc II (12 units/μl)..... 50 unit
   Hae III (18 units/μl)..... 50 unit
   Autoclaved Water up to ..... 250.0 μl
2. Mix gently, centrifuge flush, incubate at 37℃ for 14-16 hr.

Fragment length selection

1. Add 50.0 μl of stop dye (0.2% Orange G; 70% glycerol; 10mM Tries); loading onto 1.0% agarose gel. As a marker is 100 bp Ladder which mix with BPB stop dye (0.2% BPB; 0.2% Xylene cyanol; 70% glycerol; 10mM Tris)
2. Cut the gel with DNA length about 300 - 800 bp.
3. Crash the gel on the glass, put into 50.0 ml centrifuge tube.
4. keep it at -80℃ for 60 min.
5. Add 3x volume of neutralized phenol; vortex until the gel become mild.
6. Add 100.0 μl Chloroform isoamyl alcohol, centrifuge at 12,000 rpm. for 15 min.
7. Take a supernatant into the new tube, add 10.0 ml of chloroform / isoamylalcohol.
8. Centrifuge at 12,000 rpm. for 10 min.
9. Take a supernatant, add 20.0 μg/ml glucose and 0.2 M NaCl, keep at -80 ℃ for 1 hr.
10. Take a sub layer solution (0.5-1.0 ml), and place into a 1.5 ml tube.
11. Add 1/1 volume of TE buffer, and add NaCl (final concentration is 0.2 M) and 2x volume of 95% ethanol, keep at -80℃ for 1 hr .
12. Centrifuge 14,000 rpm. for 15 min., throw out the ethanol and rinse with 250.0 μl of 70 % alcohol. The DNA is dried, and resuspended with 20.0 μl of TE buffer.
13. Re-check the DNA using 1.0 % agarose gel.

Ligation

1. Prepare the following reaction into a 0.5ml tube:
   PUC18 (*SmaI*/BAP) (25ng/μl) ..... 2.0 μl
   Ligation buffer (10x) ..... 2.5 μl
   T4 Ligase (5 unit/μl) ..... 1.0 μl
   DNA Template (from above step) ..... <10.0 μl
   Autoclaved water ..... up to volume 25.0 μl
2. Incubate at 23-27 ℃ for 6-16 hr., and keep at 4℃ if it is not used.

Transformation

1. Prepare LB medium and LB agar.
2. Place the following reaction into a 10 ml tube :
   E.coli (DH5α) ..... 40.0 μl
   DNA Recombinant ..... <10.0 μl
3. Keep it at ice for 30 min.
4. Keep it at water bath (42℃) for 45 sec., immediately place it into ice.
5. Add 1.0 ml of LB medium and Incubate at 37℃ for 1 hr using shaker.
6. Pour 100.0 μl -500.0 μl of above solution on the LB agar containing with 0.2g/ml ampicil-

line.
7. Incubate it at 37℃ for overnight.

Hybridization

1. Transfer the colonies on a nylon membrane as follow :
   -place a nylon membrane on the agar for 3 min.
   -transfer the paper to a dilute solution of sodium hydroxide (0.5M NaOH + 1.5 M NaCl) for 7 min.
   -neutralize it twice with 1.5 NaCl + 0.5 M Tris-HCl for 3 min.
   -dip into 6x SSC solution for 3 min., and dried.
   -baking at 80℃ for 2hr.
2. Wash the paper with 2xSSC + 0.2% SDS.
3. Prehybridization with hybridization solution (5x SSPE; 5x Denhard's; 0.5% SDS; 100 μg/ml RNA) at 60℃ for 2hr.
4. Throw out the solution and add 10 ml of hybridization solution and 10 μl radioisotope probe (100ng oligonucleotide $(GT)_{15}$; 6.0 μl of γ^{32}dATP(3000 Ci/nmol); 1x buffer; 5 unit of Polynucleotide Kinase ; at 37℃ for 30 min.) and incubated at 60℃ for 16hr.
5. Wash the paper using twice with 2x SSC + 0.2% SDS for 15 min.; twice with 0.5x SSC + 0.2% SDS at 42℃ for 15 min.
6. The paper was wrapped in plastic wrap and exposed to x-ray film with an intensifying screen at -80℃ for 6hr.

Extraction DNA from Bacteria

1. Pick up and put the positive colonies into 5.0 ml of LB medium containing 0.1g/ml ampicilline.
2. Incubate it at 37℃ for 36 hr.
3. Take a solution into a 1.5 ml centrifuge tube, flush centrifuge, throw out the supernatant. Repeat until all solution is finished.
4. Add 100.0 μl of glucose solution (50.0 mM glucose; 10.0 mM EDTA; 25.0 mM Tris-HCl pH 8.0), keep it at room temperature for 5 min.
5. Add 200.0 μl of Alkali fresh-solution (2N NaOH; 1% SDS), keep it in ice for 5 min.
6. Add 150.0 μl Potassium acetate (3M Potassium acetate; 11.5% Acetic acid), keep it in ice for 5 min.
7. Centrifuge at 12,000 rpm. for 5 min (4℃).
8. Take a supernatant into a 1.5 ml new tube, add a same volume of phenol chloroform.
9. Centrifuge at 12,000 rpm for 5 min., take a supernatant.
10. Add 2x volume of 100% ethanol, centrifuge at 12,000 rpm. for 5 min.
11. Throw out the ethanol and rinse with 70% alcohol and air dried.
12. Resuspend DNA with 50.0 ml of TE buffer.
13. Add 1.0 mg/ml Rnase-A, incubate at 37℃ for 1 hr.
14. Add 30.0 μl of 20% polyethileneglycol and 2.5 M NaCl, keep it at 0℃ for 1 hr.
15. Centrifuge at 12,000 rpm for 10 min., throw out the supernatant.
16. Rinse it with 70% alcohol, air dried.
17. Resuspend with 50.0 μl TE buffer and keep it at 4℃.

Sequencing **(in our lab. using 373A DNA Sequencing System, Applied Biosystem Inc.)**

1. Prepare the following reaction according to kit's manual:
   Terminator Ready React. Mix ..... 8.0 μl

M13mp18 forward or-reverse primer ..... 3.2 pmol
Template double standard ..... 0.5 μg
Distillated Water up to volume ..... 20.0 μl

2. Place it into the PCR machine with cycles are as follows (according to the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit, PERKIN ELMER, Inc.) :
   - 1 cycle ..... 94℃ - 5 min.
   -25 cycles ..... 96℃ - 10 sec.
   50℃ - 5 sec.
   60℃ - 4 min.
3. Put the PCR product and place it into a 1.5-centrifuge tube which containing following solution: 3M Natrium acetate (pH 5.2) ..... 2.0 μl
   95% Ethanol ..... 50.0 μl
4. Mix gently, keep it in ice for 10 min., and centrifuge at 14,000 rpm. for 30 min.
5. Throw out the ethanol and rinse it with 250.0 μl of 70% alcohol and air dried.
6. Keep it at -20℃ before loading onto 6-6.75% acrylamide gel in sequencer according to 373A DNA Sequencing System manual, Applied Biosystem Inc., (loading solution: 5 part of Formamide and 1 part of 25mM EDTA pH 8.0).
7. Designing primer pairs using the DNA-OLIGO (National Bioscience Inc., Version 4.0) software.

B. PCR amplification

1. Prepare labeling forward or reverse primer such as follows :
   Primer (10 pmol/μl) ..... 1.0 μl
   Water ..... 4.5 μl
   Reaction buffer ..... 1.0 μl
   T4 Polynucleotide kinase (5 unit/μl) ..... 0.5 μl
   γ ^{32}P(3000 Ci/pmol) ..... 3.0 μl
   ——> incubate at 37℃ for 30 min., inactive at 65℃ for 15 min.

2. Prepare the following reaction into a PCR tube:
   template DNA ..... 10.0 ng
   forward primer ..... 2.5 μM
   reverse primer ..... 2.5 μM
   dATP, dCTP, dGTP, dTTP ..... 1.75 μM
   reaction buffer ..... 1.0 x
   Taq polymerase ..... 0.25 unit
   Labeled reverse or forward primer ..... 0.25 μM
   Total volume ..... 6.0 μl

3. Place it into the PCR machine with cycles as follows :
   7 cycles ..... 94℃ - 60 sec.
   annealing temperature - 30 sec.
   72℃ - 30 sec.
   33 cycles ... 90℃ - 30 sec.
   annealing temperature - 30 sec.
   72℃ - 30 sec.
4. Add a same volume of deionized formamide stop dye.
5. Heat the reaction at 90℃ for 15 min.

6. Loading 2-5 μl of each sample onto a 6% polyacrylamide urea gel in the 1x TBE buffer, for marker was used M13mp18 sequence (Fig. 9). The gel was exposed overnight at -80 ℃ to X-ray film.

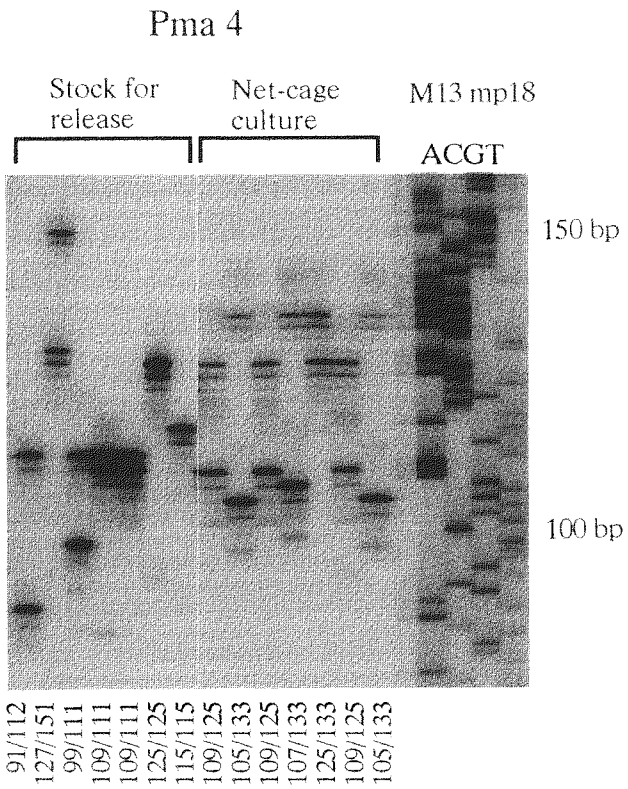

Fig. 9. An example of band patterns of red seabream (*P. major*) in Pma-4 locus microsatellite.

DATA ANALYSIS

In the study of inheritance patterns, generally, the polymorphic bands analysis is based on the presence or absence of the main band. While study on the population genetic structure, the analysis is conducted by calculation of the allele or genotype frequencies using various equations. The following formulas are usually used for both studies;

A. Hardy-Weinberg's equation.

Chi-square analysis is used to test for conformance of genotype frequencies to those expected Mendelian ways using Hardy-Weinberg's equation.

$$X(Chi)^2 = \Sigma \frac{(Ho - He)^2}{He}$$

whereas, Ho = Observed Heterozygosity.
He = Expected Heterozygosity.
The null hypothesis is Mendelian inheritance.

$$He = \text{Sample No x frequency of each allele pairs}$$

For 2 genotype (A,B), allele frequencies of AA:AB:BB = p^2: 2pq : q^2
p,q are allele frequency of A and B respectively.
Briefly, above equation can be summarized as :

$$X\,(Chi)^2 = \frac{(n1 - np^2)^2}{np^2} + \frac{(n2 - 2npq)}{2npq} + \frac{(n3 - nq^2)^2}{nq^2}$$

whereas, n = total sample number.
n1,2,3 = observed number in each genotype.

If alleles variation revealed are too wide, then Hardyweinberg equation calculation is difficult, we can use the following equation to calculate He:

$$He = 1 - (\Sigma(\text{freq. of homozygous allele})^2)$$

and to predict the population observed is Mendelian inheritance or not, it can be calculated as : Ho/He, if Ho is almost same with He, the sample is recognized as Mendelian population.

B. Genetic Identity

$$I = \frac{Jab}{(Ja * Jb)^{0.5}}$$

whereas, Jab : allele frequency in same locus which shared by both populations.
Ja;Jb: allele frequency in population A and B respectively.

C. Nei's Genetic Distance

$$D = -\ln I$$

whereas, D : Genetic distance.
I : Genetic identity.

The following formulas are used for markers which reveal many locus, such as minisatellite and AFLP :

D. Band Sharing Index

$$BSI = \frac{2N_{AB}}{N_A + N_B}$$

whereas, BSI = Band Sharing Index,
N_{AB} = number of shared band by both individuals A and B.
N_A, N_B = Number of shared band by individual A,B respectively.

Other authors have termed as similarity coefficient for the above BSI. The formula of the similarity coefficient and the genetic distance are as follows:

E. Similarity coefficient

$$D = S = \frac{2N_{AB}}{N_A + N_B}$$

whereas, N_A, N_B are the number of fragments in individual A and B.
N_{AB} is number of fragments shared by both individuals.

Its variance can used to test differences between populations by employing the multiple-comparisons among means test (Sokal and Rohfl, 1981). According to (Wetton *et.al.*, 1987) the probability of two randomly chosen individuals would have an identical fragment pattern can be predicted using the following equation :

$$\text{Prob.} = \bar{D}\ \bar{N}$$

$\bar{D}$, $\bar{N}$ are average of similarity index and number of fragments per individual respectively.

This measure assumes the independence of fragments. If the population is consist of only limited number of different homozygous, such as inbreed pure line whereas multilocus genotypes among which the genomes do not mix, that probability can be measured using :
Prob = Σpi
pi = frequency of multilocus genotype i in the population.

According to Davis *et.al.* (1990) the index of population subdivision Fst can be estimated as :

$$Fst = \frac{IDw - IDb}{1 - IDb}$$

IDw = the genetic identities within populations.
IDb = the genetic identities between populations.

The genetic distance between individual pair of populations as determined in ln, the normalized identity between populations (Nei, 1972). According to Lynch (1990) the normalized identity (genetic distance) between populations 1 and 2 can be calculated as:

$$\text{Genetic Distance} = -\ln \frac{IDb(1,2)}{(IDw(1) * IDw(2))^{0.5}}$$

IDb(1,2) = allele frequency in same locus which shared by both populations.
IDw(1);IDw(2) = allele frequency in population 1 and 2 respectively.

While according to Moser (1994) distance between two lines can be calculated as: FD = 1 − BSI

CONCLUSION

Based on the result of various experiments on the markers application which have been conducted in our laboratory, we conclude that those markers were useful to detect DNA polymorphism of fish, eventhought they still have limitations. RAPD was most suitable for pedigree study, while DNA fingerprinting and AFLP were most useful for species identification. The microsatellite was more accurately to study the population genetic by allelic diversity and heterozygosity observed.

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