Genomic Polymorphisms of Genome DNA by Polymerase Chain Reaction-RAPD Analysis Using Arbitrary Primers in Rainbow Trout

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ABSTRACT

Nuclear DNA was isolated from the sperm cells representing genetic characteristics and genomic polymorphisms of rainbow trout by polymerase chain reaction(PCR) amplification of DNA using arbitrary primers. Genomic DNA fingerprints were generated from rainbow trout sperm DNA by polymerase chain reaction amplification using 20 arbitrary decamers as primers. Out of these primers, 4 generated 17 highly reproducible RAPD markers, producing almost six polymorphic bands per primers. Four of 6 primers tested generated amplified fragments which were polymorphic between different individuals. Polymorphic DNA fragments were reproducibly amplified from independent DNA preparations made from individuals. Rainbow trout was distinctly observed 3 specific DNA markers(2.3, 2.0 and 1.3kb) in bandsharing. Individual fragments generated using the same arbitrary primer, demonstrated that a single primer detected at least three independent genomic polymorphisms in rainbow trout sperm DNA. The RAPD polymorphism generated by this primer may be used as a genetic marker for individual identification. The RAPD-PCR technique has been shown to reveal informative polymorphism in many species of fish. The present results demonstrate that RAPD markers are abundant, reproducible and provide a basis for future gene mapping and MAS in these important aquaculture species using RAPD polymorphic markers. It is concluded that RAPD polymorphisms are useful as genetic markers for fish breed differentiation.

(Key words: Rainbow trout, Polymorphism, Polymerase chain reaction, Random amplified polymorphic DNAs, Bandsharing)

I. INTRODUCTION

Traditionally, genetic progress of fish has been obtained through selecting superior individuals based on their phenotypic records. The selection criteria have been focused mainly on the traits of economic importance. The con-ventional method used for selection was the biometrical approach combined with general genetic principles. The recent advance of genetic techniques with molecular biological methods showed a great potential to accelerate the genetic progress.

Identification of individual or related fish is necessary for efficient selective breeding and

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broodstock management, and for measuring various traits. Fish· genetic selection will benefit from the development of genetic markers for identification and marker-assisted selection. Genetic polymorphisms are playing an increasingly important role as genetic markers in many fields of animal and plant breeding. These polymorphic genetic markers proved useful for individual identification, pedigree analysis, genetic relationship, genetic structure of animal populations and marker assisted selection programs. Especially, DNA markers that are shown to be genetically linked to a trait of interest can be used for gene cloning, pathological diagnostics, and for trait improvement in fish breeding programs. Also, a genetic linkage map in fish is needed to improve efficiency of breeding by marker-assisted selection(MAS) and for identification of economically important genes such as disease resistance genes. The DNA markers most commonly used are restriction fragment length polymorphisms(RFLP)(Smith and Simpson, 1986; Hallerman and Bekmann, 1988; Lloyd et al., 1989; Beckenbach et al., 1990; Geldermann and Ellendorff, 1990; Taggart and Ferguson, 1990; Gold and Richardson, 1991; Ovenden et al., 1993; Clifford et al., 1998). Anonymous low copy number genomic clones are frequently used to visualize polymorphisms. Detection of RFLPs are labrious and incompatible with the high analytical throughout required for many applications. Other polymorphism assays that are based on the polymerase chain reaction (PCR), require target DNA sequence information for the design of amplification primers. The polymerase chain reaction is a rapid, simple, relatively cheap and sensitive procedure for in vitro amplification of specific DNA sequences using appropriate single primers. Genomic fingerprints generated by PCR amplification of DNA using single arbitrary primers have for det-

ecting DNA polymorphisms between organisms (Welsh and McClelland, 1990; Williams et al., 1990; Simpson et al., 1993; Orozco-Castillo et al., 1994; Chung et al., 1995; Kim et al., 1997; Koh et al., 1997). Especially, most fisheries applications of RAPD's have been at the species level(Lilley et al., 1997; Smith et al., 1997; Liu et al., 1998). More recently PCR using arbitrary primers has been applied to the inter- and intraspecies differentiation of trypanosomes(Dias Neto et al., 1993). These primers detect polymorphisms in the absence of specific nucleotide sequence information, and the polymorphisms function as genetic markers. Polymorphisms were scored by the presence or absence of an amplification product at specific positions in the gel (Smith et al., 1997).

In this study, DNA was isolated from rainbow trout sperm and analyzed by 20 randomly amplified polymorphic DNA(RAPD) primers in order to identify genetic characteristics and genomic polymorphisms within the breed and to develop the breed-specific genetic markers by using these RAPD markers. The same arbitrary primer was used in order to examine the relationship of individual amplified sequences to other sequences amplified. Further analysis of the amplified genomic DNA involved direct sequencing of these rainbow trout.

II. MATERIALS AND METHODS

1. Sperm Collection

Rainbow trout DNA samples were obtained from a aquaculture facility at the Department of Aquaculture, Kunsan National University. Preliminary RAPD analysis was performed on genetic DNA samples from a total of rainbow trout as a reference breed using 20 different random primers. The sperm DNA samples collected from mature testis in rainbow trout. Sperm sam-

ples were collected with sterile test tubes from the testes, immediately plunged into liquid nitrogen and stored for further analysis. All glassware, micropipette tips, centrifuge tubes, glass pipettes and solutions were autoclaved to avoid DNA recontamination.

2. Sources of Genomic DNA

Thawed samples of whole sperm were placed into 2ml microcentrifuge tubes, to which an equal volume of Solution I buffer(10 mM Trs (pH 7.6) / 10 mM KCl / 10 mM $MgCl_2$ / Nonidet-P 40) was added, and the mixture was gently by inverting the tube several times. The samples were incubated on ice for 5 min, centrifuged the samples at 2,000 rpm for 30 min. at 4°C to pellet. The supernatant was decented with pellet, resuspended the nuclei in Solution Ⅱ buffer(10 mM Tris(pH 7.6) / 10 mM KCl / 10 mM $MgCl_2$ / 0.5 NaCl /2mM EDTA). Samples were transferred aqueous phase to 1.5ml tube, added 50 μl of 10 % SDS buffer, and added 20μl of 20 mg /ml proteinase K solution. The mixture was gently by inverting the tube and incubated at 37°C for overnight. There was added 400 μl of TE distilled phenol to mixture and centrifuged for 1 min. at 1,000 rpm. 200 μl of phenol and 200 μl of chloroform : isoamylalcohol were added to the mixture. Samples were spun down for 1 min. at 1,000 rpm and added 700 μl of chloroform : isoamylalcohol. The cleard lysates were extracted with 2 volume of ice-cold ethanol, then centrifuged for 5 min. at 4,000 rpm, then precipitated. The DNA pellet was air-dried for 30 min, and then dissolved 200 μl of TE buffer(10 mM Tris-HCl(pH 8.0), 1 mM EDTA). Purity and concnetration of DNA was measured with a spectrophotometer(Beckman DU series 60). Purity was estimated by calculating the ratio of the absorbance measured at 260~280 nm.

3. Primer

Of the 20 arbitrarily selected primers, six random primers were used on the basis of the number and frequency of the polymorphisms producted. The primers, designed for other purpose and chosen arbitrarily for these experiments, were obtained from Genosys, USA. All of these decamer random primers had a G+C content in the range 60~70%. The oligonucleotide primer sequences(5' to 3') were CAGGCCCTTC(P-1), TGCCGAGCTG(P-2), AGGCAGCCAC(P-3), AATCGGGCTG(P-4), GGGTAACGCC(P-9) and GTTGCGATCC(P-20).

4. Amplification Conditions

Amplification reactions were performed in volumes of 25μl contained 20 ng of genomic DNA, 1 X buffer(50mM KCl, 10mM Tris-HCl, 2.0 mM $MgCl_2$, 0.01% gelatin, 0.025% Nonidet P-40, 0. 025% Tween 20), 200μM dNTP(Advanced Biotechnologies, LTD), 0.5 unit of Taq-polymerase (Perkin Elmer Cetus), 0.3 μM primer(Operon Technologies, USA). Amplification was performed in a DNA Thermal Cycler(Perkin Elmer Cetus, USA). This mixture was overlaid with mineral oil, and followed an initial denaturation at 94°C for 3 min. Thermal Cycler programmed for 45 cycles at 94°C for 1 min for denaturation, at 35°C for 1 min for annealing, at 72°C for 2 min for extension, using the fastest available transition between each temperature. Amplification products were analyzed by electrophoresis in 1. 4% agarose gels with TBE(0.09M Tris, pH 8.5 ; 0.09M boric acid; 2.5mM EDTA) and detected by staining with ethidium bromide. The gels were illuminated with UV light and photographed by UV DNA photographic system. Bandsharing was calculated as an expression of similarity of RAPD fingerprints of animals from either the same or different breeds(Jeffreys and Morton, 1987). Bandsharing(BS) of DNA seq-

uences was calculated according to the formula of Jeffreys and Morton(1987): BS=2(Bab) / (Ba+Bb) Where Bab is the number of bands shared by individuals a and b, Ba is the total number of bands for individual a, and Bb is the total number of bands for individual b. Only bands which were readily visible were scored. RAPD profiles were compared only on samples lane in the same gel.

III. RESULTS AND DISCUSSION

When PCR amplification of genomic DNA is performed with short oligonucleotide primers of arbitrary sequence at low annealing temperatures, the amplification products can be generated as DNA fingerprint bands by gel electrophoresis, the patterns being characteristic of both the primers and the template DNA(Welsh and McClelland, 1990; Williams et al., 1990). The RAPD polymorphism generated by random primer P-1 showed fingerprint bands as shown in Fig. 1. The sizes of the bands recorded ranged from 0.56kb to 2.3kb as shown in Fig. 1. The RAPD profiles obtained with pooled DNA of individuals were different(Fig. 2). Nevertheless, 12 of the 27 amplified bands were found to be polymorphic. On average, each random RAPD primer amplified 7.3 bands from rainbow trout. About 26% of total amplified bands from rainbow trout were either specific to rainbow trout. The degree of similarity varied from 0.57 to 0.74 as calculated by bandsharing analysis(Table 2). The average level of bandsharing was 0.695 ± 0.039 within the rainbow trout breed. A specific primer(P-2) was found to be useful in the individual identification, resulting from the different DNA polymorphism among individuals(Koh et al., 1997; Liu et al., 1998). Four primers produced amplified fragments which were consistently polymorphic between the individuals. The
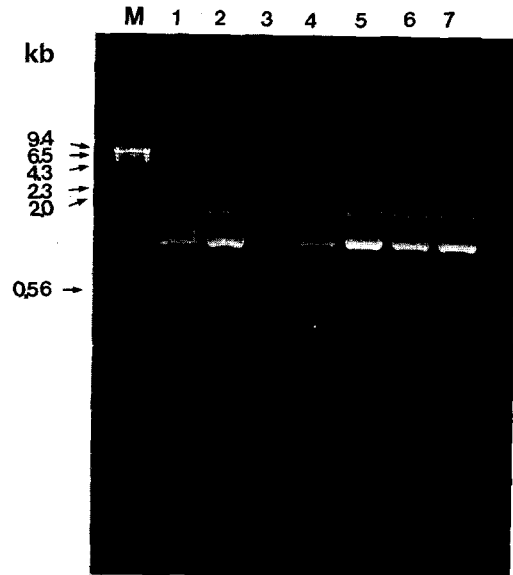
Fig. 1. **Amplification products were electrophoresed on a 1.4% agarose gel with TBE(0.09M Tris, pH 8.5 ; 0.09 M boric acid; 2.5 mM EDTA) and detected by staining with ethidium bromide. Individual specific RAPD patterns of rainbow trout amplified by arbitrary P-2(TGCCGAGCTG). Each lane shows different individual DNA samples. M: Molecular size standard(Lambda DNA marker digested with EcoR I and Hind III).**
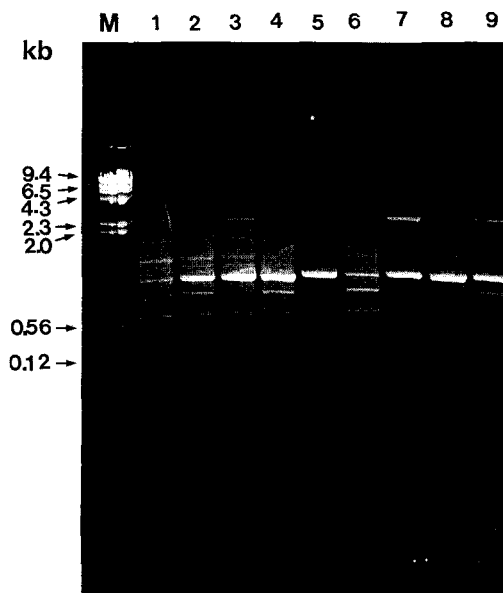
number of intra-strain polymorphisms was greater than that of inter-strain polymorphisms. These workers have found more variation within a strain than between strains considered as a whole. But, Beckenbach et al.(1990) observed that the intraspecific divergence estimates based on sequence were less than the interspecific divergence estimated from restriction fragment analysis.

The other primers P-2 also generated RAPD banding patterns showing DNA polymorphisms (Fig. 1). Another primer, P-4, detected a pair of RAPD major bands, approximately from 0.12 to 2.3kb as shown in Fig. 3. The DNA bands of
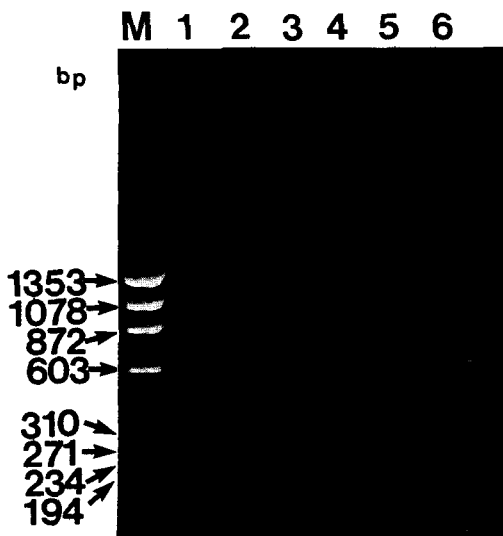
Fig. 2. Specific RAPD patterns of rainbow trout amplified by arbitrary primer P-9(GGGTAACGCC). Each lane(1~6) shows different individual DNA samples. M: 194~1353bp standard ladder(Φ X174 DNA marker digested with EcoR I and Hae III).

Fig. 3. Individual specific RAPD patterns in rainbow trout amplified by arbitrary P-3(AGGCAGCCAC). Each lane shows different individual DNA samples. M: Molecular size marker(Lambda DNA marker digested with EcoR I and Hind III).

from 0.56 to 2.0kb were present in every individuals, but a corresponding band of 2.3kb was absent in 4 of 9 individuals. Therefore, these bands are potentially polymorphic markers. This allele can be used as an potential genetic marker for linkage analysis with economically important traits in fish(Liu et al., 1998). RAPD marker would be particularly useful in marker-assisted selecion(MAS) programs in these important aquaculture species. Liu et al.(1998) reported that some intraspecific RAPD variation was observed for different strains or for individuals. Liu et al.(1998) screened a collection of RAPD markers in catfish and identified 22 primers that revealed 171 strain-specific genetic markers. Also, Johnson et al.(1994) screened a collection of RAPD markers in zebrafish and identified 116

primers that revealed 721 strain-specific genetic markers. Some specific band was independent of sex and individuals, and may be useful as a DNA marker for identifying the breed(Jones, 1983; Lloyd et al., 1989). The number and size of the fragments generated strictly depend on the nucleotide sequence of the primer used and on the source of the template DNA, resulting in a genome-specific fingerprint of random DNA fragment. The potential of RAPDs to identify diagnostic markers for strain identification in mice (Welsh et al., 1991), in parasites(Bishop et al., 1993; Simpson et al., 1993; Dias Neto et al., 1993; Kwon et al., 1997), in livestock(Gwakisa et al., 1994; Koh et al., 1998), in plants(Orozco-Castillo

et al., 1994; Rowland and Levi, 1994) and in fish(Lloyd et al., 1989; Liu et al., 1998) has also been demonstrated. There were species-specific RAPD fragments in rainbow tout and there were differences in frequencies of two primer fragments(P-2, P-3, Table 1), as have been reported in catfish(Liu et al., 1998) and livestock (Koh et al., 1997).

In RAPD profiles of agarose gels using 4 primers, the total number of observed bands produced per primer varied from 4 to 10 with an average of 5.5(Table 1). Especially, primer P-2 generated the highest number of fragments among the primers used with the average of 4. This number of bands was similar to the number reported for Zebu cattle breeds(Gwakisa et al., 1994). The average bandsharing was 0.695±0. 039 within the rainbow tout(Table 2). This value was lower than those of zebu cattle breeds(0. 73~0.79) reported by Gwakisa et al.(1994).This result implies the genetic variation or diversity within the rainbow trout breed as compared
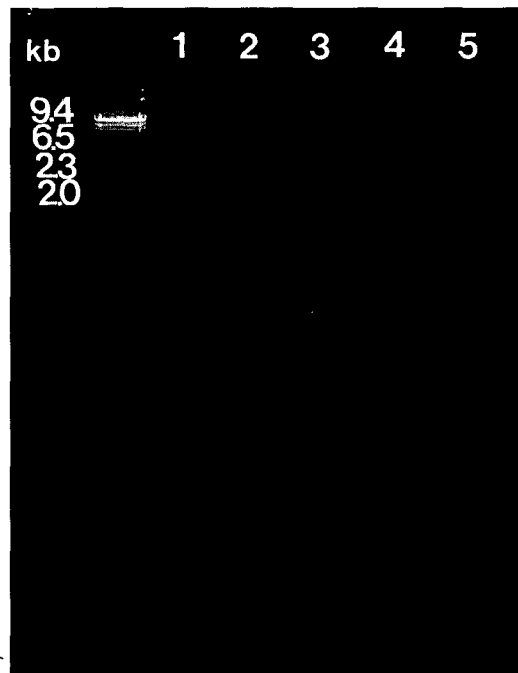
Table 1. Number of RAPD bands for single primer and two primers using agarose gel in rainbow tout

Primers	Number of products	Average
2	7~10	8.5
3	6~9	7.5
4	7~9	8.0
20	4~6	5.0

Table 2. Bandsharing(BS) within the rainbow trout breed(mean or mean±S.E)

Primers	Bandsharing values
2	0.74
3	0.73
4	0.74
20	0.57
Average	0.695±0.039*

*Standard error

Fig. 4. PCR-RAPD products of rainbow trout amplified by arbitrary P-4 (AATCGG-GCTG). Each lane shows different individual DNA samples. M:Molecular size standard(Lambda DNA marker digested with EcoR I and Hind III).

with those from zebu cattle breeds.

Further analysis is required to identify primers that amplify sufficient bands shared by the species to permit a quantitative analysis.

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요 약

PCR-RAPD 기법에 의한 무지개송어 Genome DNA 의 다형현상

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본 연구는 정자세포로부터 분리된 genome내 DNA를 PCR 기법으로 증폭시킨 후 random amplified polymorphic DNA(RAPD) 분석을 통해 무지개송어의 품종내 유전적 특성과 변이성을 해석하고 품종의 특이 유전적 표지를 개발하기 위해서 수행되었다. 20종류의 primer를 사용하여 RAPD 양상을 검색한 후 다형현상의 출현빈도와 band수에 기초하여 이들중 6개의 primer를 선정하여 이용하였다. 그중에 4개의 primer는 17개의 RAPD marker를 나타내었고, 그중 primer당 8개인 48개(28%)의 band가 다형성을 보여주었다. 6개의 primer중 4개는 개체들 사이에 다형성을 나타내는 band를 나타내었다. Bandsharing 의 경우 연어와 비교될 만큼 무지개송어는 3개의 특이적인 DNA marker를 가지고 있었다(2.3, 2.0 및 1.3kb). 같은 무작위 primer를 이용해서 나타난 개별적인 band는 단일 primer가 무지개송어의 정자핵 DNA의 경우 적어도 3개의 독립적인 genome내 다형성을 탐지해 낼 수 있다는 것을 제시하고 있다. 이러한 primer에 의해서 나타난 RAPD 다형성은 개체식별을 위한 유전적 표지인자로서 사용될 수 있는 가능성을 제시하였으며, RAPD-PCR은 많은 어종에서 다형현상을 밝혀내는 기술이라 할 수 있다. 본 연구는 RAPD marker가 풍부하고 재현성이 있으며 RAPD 다형성을 지닌 marker를 사용하여 이러한 중요한 양식대상어종에서 미래의 gene mapping과 MAS를 위한 기초를 제공해 줄 수 있다. RAPD 다형성은 어류의 품종 분화를 위한 유전적 표지로서 유용한 것으로 결론지을 수 있을 것이다.