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Intraspecific Genetic Variation in the Japanese Loach (*Misgurnus anguillicaudatus*) Revealed by Random Amplified Polymorphic DNA (RAPD) Analysis

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Abstract: To assess the extent of genetic diversity within and between four populations of the Japanese loach, *Misgurnus anguillicaudatus*, an analysis for random amplified polymorphic DNA (RAPD) variations was carried out. Three out of 10 random primers produced consistent and reproducible pattern of RAPD products. Populations showed a nonsignificant difference in their degree of similarity and polymorphism. Difference however, was observed in the primers in producing similarities in the populations. Among the four populations, Futtsu appeared to be an outgroup while the Ueda, Nasu and Nikko populations were genetically closer, as revealed by Nei's unbiased genetic distance values. The RAPD technique was found to be useful in discriminating the populations of Japanese loach.

Key words: Genetic variation, RAPD, PCR, loach

Introduction

The loach, *Misgurnus anguillicaudatus* (Cobitidae: Cypriniformes) is widely distributed in Japan including Hokkaido, Honshu, Shikoku and Kyushu islands as well as in Korea, Taiwan and east coast of Asian continent from Amur river to North Vietnam. Morphological, karyotypic and allozyme studies suggest availability of taxonomically or genetically distinct groups needing clarification of taxonomic relationships in *M. anguillicaudatus* (Oliva and Hensel, 1961; Kimura, 1978). Karyotypic studies revealed at least two different types of loach, tetraploid (in China) and diploid (in Japan) (Li *et al.*, 1983).

The randomly amplified polymorphic DNA (RAPD) technique has been developed simultaneously by Williams *et al.* (1990) and Welsh and McClelland (1990) which involve amplification of genomic DNA by polymerase chain reaction using short random primers.

This method is relatively easier than conventional DNA fingerprinting techniques and allows the examination of genomic variation without prior knowledge of DNA sequences (Williams *et al.*, 1993). The number and size of amplified fragments depend on the length and sequence of the arbitrary primers. Priming sites are randomly distributed throughout the genome and polymorphism in such sites result in differing amplification products detected by the presence and absence of fragments. Such polymorphism are less variable (Hadrys *et al.*, 1992), and inherit in a Mendelian fashion (Stott *et al.*, 1997) and can be used as genetic markers in discriminating the populations. Many authors have pointed out that the application of the RAPD technique may be more successful in differentiating fish between subspecies and strains, or populations of the same species (Gomes *et al.*, 1998; Mamuris *et al.*, 1998; 1999). RAPD analysis was found to be more sensitive than mtDNA analysis in studying intra-population variation in tilapia. Bardakci and Skibinski (1994) and Naish *et al.* (1995) opined that the RAPD method was more suitable than multi locus DNA fingerprinting in studying heterozygosity in aquacultured tilapia. The intraspecific genetic variation in four populations of the Japanese loach have been examined in the present study by RAPD analysis. This study reveals that RAPD technique can be used to discriminate the Japanese loach populations genetically.

Materials and Methods

Fish samples: Japanese loach samples (each of five fish), were collected from four different populations of the species:(1) Ueda (Ueda city, Urano river, Nagano prefecture); (2) Nasu (Nasu town, Naka river, Tochigi prefecture); (3) Nikko (Nikko city, Naka river, Tochigi prefecture); and (4) Futtsu (Futtsu town, Iwase river, Chiba prefecture).

Extraction of genomic DNA: For isolation of genomic DNA, approximately 1 g of fish tissue was homogenized and digested with proteinase-K in extraction buffer (100mM Tris.HCl, pH= 8.0, 10mM EDTA and 50mM NaCl and 1% SDS) overnight at 37°C. The tissue lysate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1 respectively) and once with chloroform:isoamylalcohol (24:1 respectively). DNA was precipitated first using 0.6V of isopropanol, paletted by centrifugation, then resuspended in TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH= 8.0) and then reprecipitated by adding two volumes of ethanol in the presence of 0.3M sodium acetate, and paletted by centrifugation. The pellets were then washed with 70% ethanol, air-dried and resuspended in an appropriate volume of TE buffer. DNA was quantified using a spectrophotometer and stored in freezer.

PCR amplification and gel electrophoresis: Ten 10-mer random primers (Operon Technologies Inc., Table 1) were used for RAPD amplifications. Conditions for RAPD amplification reactions were maintained essentially following Williams *et al.* (1993). In brief, PCR reactions were performed in a 25µL reaction mix containing 100-200µM each of dATP, dCTP, dGTP and dTTP, 2.5µL of 10X PCR buffer (Taq), 2.0 µL of 2 mM $MgCl_2$, 0.1-0.2 µM primer, 0.5 unit of Ampli Taq DNA polymerase (Takara, Japan) and 10-30 (ng) of fish genomic DNA. DNA amplification was performed in an oil-free thermal cycler (Model Gene Amp PCR Systems 9700, PE Applied Biosystems). The reaction mix was preheated at 94°C for 4 minutes followed by 40-45 cycles of amplification (94°C for 1 min, 35°C for 1 min and 72°C for 2 min). The last cycle was followed by an elongation step for 10 min at 72°C. A negative control reaction was prepared for each primer to identify contamination of reaction (if any) with non target DNA. The PCR conditions were optimized by manipulating different parameters before performing final amplification reactions.

An aliquot of 10µL of amplified product from each sample was separated electrophoretically on 1.4% agarose gels containing ethidium bromide in 1 X TAE buffer (40mM Tris-acetate, 1 mM EDTA). DNA bands were observed under UV light on a trans-illuminator and photographed by a Gel Cam polaroid

Table 1: Sequence and operon codes of the random primers used to study genetic variations in four populations of *Misgurnus anguillicaudatus*

Primer codes	Sequence (5′ to 3′)
OPB01	GTTTCGCTCC
OPB02	TGATCCCTGG
OPB03	CATCCCCCTG
OPB04	GGACTGGAGT
OPB05	TGCGCCCTTC
OPC01	TTCGAGCCAG
OPC02	GTGAGGCGTC
OPC04	CCGCATCTAC
OPC05	GATGACCGCC
OPC06	GAACGGACTC

Table 2: Summary of bandsharing-based similarity indices within and between four populations of loach

Similarity (S_i) between individuals of the same population	
Ueda	66%
Nasu	54%
Nikko	59%
Futtsu	51%
Average	58%
Similarity (S_{ij}) between random pairs of individuals of different populations	
Ueda-Nasu	56%
Ueda-Nikko	58%
Ueda-Futtsu	48%
Nasu-Nikko	55%
Nasu-Futtsu	45%
Nikko-Futtsu	47%
Average	51%

camera. A molecular weight marker DNA was run on either side of the gel.

Data analysis: The similarity index values (S_{xy}) between the RAPD profiles of any two individuals on the same gel were calculated from RAPD markers scored manually based on the presence (1) or absence (0) of bands of the same molecular weight according to the following formula:

$$S_{xy} = 2n_{xy}/(n_x + n_y),$$

where n_{xy} is the number of fragments shared by individuals x and y and n_x and n_y are the number of fragments scored for each individual (Lynch, 1990). Within population similarity (S_i) was calculated as the average of S_{xy} across all possible comparisons between individuals within a population. Between population similarity (S_{ij}), was calculated as the average similarity between randomly paired individuals from populations I and j (Lynch, 1991). The bandsharing similarity between population pairs across primers were analyzed by a two-way analysis of variance (ANOVA). The frequencies of polymorphic loci across primers in different populations were also subjected to a two way ANOVA (Zar, 1996).

Distance measured were computed from both the frequency of polymorphic loci and bandsharing data. Nei's (1978) unbiased genetic distance values were computed from frequencies of polymorphic markers using the POPGENE (Version 1.31) computer package (Yeh *et al.*, 1999). Lynch's (1991) analogue of Nei's unbiased genetic distance (D'_{ij}) was computed manually from mean similarity within (S_i) and between-population (S_{ij}) samples using the formula:

$$D'_{ij} = -\ln[S'_{ij}/\sqrt{(S_i S_j)}].$$

Cluster analysis of distance was done via UPGMA (Unweighted Pair- Group Method of Analysis (Sneath and Sokal, 1973) and a dendrogram was drawn using POPGENE (Version 1.31) (Yeh *et al.*, 1999) computer program.

Results

No product was amplified with 7 of the 10 random primers or the amplifications were not repeatable. Three primers produced RAPDs with varied patterns (Fig. 1 A and B) and the results were consistent and reproducible and, therefore, were used in the analysis. The number of fragments generated per primer varied between 2 and 10 with size ranging from 0.2 kb to 3.5 Kb. Among the primers, primer OPC05 gave DNA profiles with more bands than primers OPC04 and OPC06.

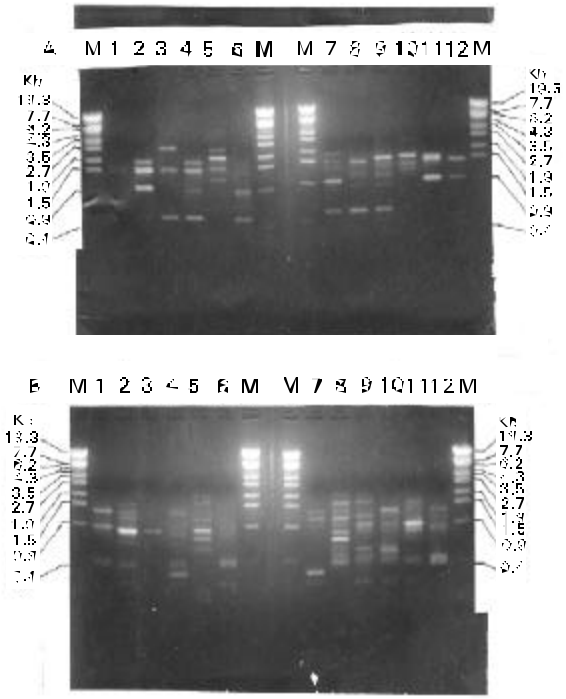

Fig. 1: RAPD profiles of the Japanese loach *M. anguillicaudatus* sampled from four populations. A: Primer OPC04 and B:Primer OPC05. The PCR products were run on agarose gels and stained with ethidium bromide. M: Molecular weight marker (lambda DNA-EcoT14 digest); samples 1, 2 and 3 from Ueda, 4, 5, 6 from Nasu, 7, 8, and 9 from Nikko, and 10, 11, and 12 from Futtsu population.

The values for intra-population similarity indices (S_i) were higher, ranging from 51 to 66 % than inter-population similarity indices (S_{ij}), ranging from 47 to 58 % (Table 2). Within-population similarity indices (S_i) for Ueda population were found to be higher than those for all other populations followed by that of Nikko population. The S_i value for Futtsu was found to be the lowest (Table 2). No significant difference was observed in bandsharing similarities between different population pairs. However, significant difference was

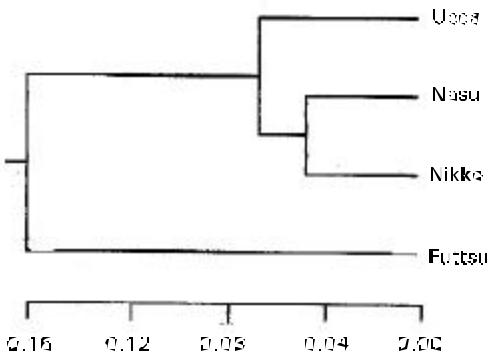

Fig. 2: UPGMA dendrograms for Japanese loach, *M. anguillicaudatus* based on Nei's (1978) unbiased genetic distance computed from RAPD marker frequencies.

Table 3: Percentage of polymorphic loci across primers in the four populations of loach, *M. anguillicaudatus*

Populations	Primers			
	OPC04	OPC05	OPC06	Mean ± SD
Ueda	60	100	50	70.0± 26NS
Nasu	80	100	70	83.3± 15NS
Nikko	60	77	71	69.3± 8.6NS
Futtsu	100	50	71	73.6± 25NS
Mean± SD	75.0± 19*	81.7± 23*	65.5± 10*	

* Significantly different (P< 0.05) NS= Not significant

Table 4: Summary of Nei's (1978) unbiased genetic distance values based on RAPD marker frequencies (upper diagonal) and Lynch's (1991) analogue of Nei's unbiased genetic distance (D'_{ij}) based on bandsharing similarity indices (lower diagonal) among loaches sampled from Ueda, Nasu, Nikko, and Futtsu population.

Populations	Ueda	Nasu	Nikko	Futtsu
Ueda	---	0.058	0.088	0.246
Nasu	0.063	---	0.051	0.140
Nikko	0.073	0.025	---	0.155
Futtsu	0.232	0.153	0.154	---

observed in bandsharing similarities produced by the primers. Primer OPC06 produced higher similarities as compared to those of primer OPC04 and OPC05 (P< 0.05). The percentages of polymorphic loci across primers in the populations of loach are presented in Table 3. No significant difference was observed in polymorphism of the four populations. The overall polymorphism produced by primer OPC06 was the lowest while that produced by primer OPC05 was the highest.

Genetic distance values are presented in Table 4. The genetic distance values for the population pairs of Ueda-Futtsu, Nasu-Futtsu, and Nikko-Futtsu were found to be higher than those for Ueda-Nasu, Ueda-Nikko and Nasu-Nikko. The cluster analysis indicated segregation of the four populations of Japanese loach into two groups: Ueda, Nasu and Nikko in one group and Futtsu in the other group (Fig. 2).

Discussion

The RAPD technique has been found to be suitable in discriminating the four populations of the Japanese loach. DNA amplification from all the primers tested in this study were not consistently reproducible which is a very common feature of the RAPD technique (Hadrys *et al.*, 1992; Williams *et al.*, 1993; Allegrucci *et al.*, 1995). Only three out of ten primers produced reproducible amplification products. Data analysis for genetic polymorphism revealed some degree of divergence between the populations. In addition, there was a consistency in genetic relationships with respect to geographic areas, since affinities of populations corresponding to geographic relationships of the collecting sites. Since there exists physical barrier among the populations (except between Nasu and Nikko population) it is likely that the populations will lack homogeneity due to lack of gene flow unlike marine systems where little intraspecific genetic divergence exist due to lack of physical barrier (Ward *et al.*, 1994).

Populations showing higher intra-population similarity and lower frequency of polymorphic loci likely to have less heterozygosity as compared to those showing less intra-population similarity. In other words, populations having higher similarity are more homogenous groups.

To asses the level of genetic variability within each population, pair-wise comparison of DNA profiles of the individuals of the populations was made. The S_i values for Ueda and Nikko population were higher than those for Nasu and Futtsu population. The higher S_i values reflected lower genetic variability within the populations of Ueda and Nikko. From this study, it is likely that *M. angullicaudatus* of Ueda are more homogeneous while those of Futtsu population are less (Table 2 and 3). The less degree of similarity (S_i= 0.51) between the RAPD profiles of Futtsu population could be indicative of a high genetic variability among individuals of this population.

Between population similarity is also an indicator of genetic distance. Similarities between Ueda and Nasu, Ueda and Nikko, and Nasu and Nikko population were higher (Table 2). So, the genetic distances between these population pairs were lower. On the other hand, the similarities between Ueda and Futtsu, Nasu and Futtsu, and Nikko and Futtsu were found to be lower, as a result the genetic distances between these populations were greater (Table 4).

The Nasu and Nikko populations were from the same river system (Naka river). The higher interpopulation similarity and lower genetic distance indicate the occurrence of gene flow between these two populations through random mating.

Bandsharing-based similarity indices are higher for within river system samples (average 58%) than for all comparisons between river systems (average 51%) (Table 2). This implies that individuals within each river system are genetically more similar to each other, as is expected to be, than to individuals from all other river systems.

The size of the minigels used in this study (7X10 cm) limited the accuracy with which closely grouped bands in different lanes could be identified as having different or equal molecular weights, and hence bigger gels are recommended to be used in separating RAPD products. The number of primers and fish samples should also be increased for better discrimination of fish populations genetically using the RAPD technique.

A serious problem arising with the application of the RAPD technique, is the homology between co-migrating bands produced by the same primer in different individuals. Nevertheless, in the present study, homology constitutes a valid assumption, since all individuals belong to the same species (Allegrucci *et al.*, 1995). Despite the fact that no specific markers were found to discriminate Japanese loach populations, the RAPD technique uncovered a good number of polymorphism from three primers selected for population analysis and revealed that nuclear DNA variation in four populations of the Japanese loach is very high.

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