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AFLP Fingerprinting Reveals Genetic Variability in Common Carp Stocks from Indonesia

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Abstract

Amplified Fragment Length Polymorphism (AFLP) fingerprinting was used to detect genetic variability in two stocks of common carps (Majalaya carp and Sinyonya carp) and second generation gynogens derived from the Majalaya carp. Primer combinations, E-AAC/M-CAA, E-AGG/M-CAC and E-ACC/M-CTT yielded 299 selectively amplified loci of which 66.2% were polymorphic. Maximum polymorphism was evident in the Majalaya carp and minimum among the gynogens. Four types of AFLP bands which were useful to differentiate the stocks in question were identified. Band Sharing Index (BSI) matrix was generated from the band binary value sets. Maximum average BSI was exhibited by the gynogens and minimum by the Majalaya carp. Inter-stock comparisons showed the gynogens to be closest to the Majalaya and farthest from the Sinyonya stock.

Introduction

Common carp (*Cyprinus carpio* L.) is one of the most important freshwater fishes cultured in Indonesia. Sumantadinata (1995) has described the brief history of common carp culture, morphological characteristics of different stocks and genetic management practices adopted in Indonesia. The

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local names given by fish farmers to various stocks are based on their specific morphological features.

Selective breeding to produce stocks of better growth rate and appearance is practised widely. It is essential to monitor the genetic variations and levels of heterogeneity among the stocks in planning for the appropriate selective breeding strategies. Sumantadinata and Taniguchi (1990) have examined the allele frequencies and genetic variability of six common carps from Indonesia and three from Japan using allozymes. Taniguchi et al. (1992) have made further observations on the genetic variation in six stocks from Indonesia and five from Japan based on morphological and biochemical characters. There has been no report on DNA-level polymorphisms in Indonesian carps. DNA genetic markers have several advantages including requirement of only small amounts of tissue which would permit non-invasive sampling, ethanol-preserved/frozen tissue for DNA extraction and availability of innumerable potential markers (Park and Moran 1994).

The AFLP technique is based on the detection of genomic restriction fragments by PCR amplification. Fingerprints are produced without prior sequence knowledge using a limited set of genetic primers. The number of fragments detected in a single reaction can be "tuned" through the selection of specific primer sets. In short, AFLP technique combines the reliability of restriction fragment length polymorphism (RFLPs) and power of polymerase chain reaction (PCR) (Vos et al. 1995). Several works on the application of AFLP markers in estimating genetic variation and mapping of useful genes in plants have been cited (Cervera et al. 1996; Powell et al. 1996; Sharma et al. 1996; Waugh et al. 1997; Virk et al. 1998). AFLP markers in poultry (Knorr et al. 1999), rat (Otsen et al. 1996) and cattle (Ajmone-Marsan et al. 1997) have also been reported. Application of AFLP fingerprinting in fish is limited (Liu et al. 1998; Takagi et al. 1998; Liu et al. 1999). Our paper describes the generation of AFLP markers and analysis of genetic variation in common carp stocks from Indonesia.

Materials and Methods

Samples

The stocks of common carp (*Cyprinus carpio* L.) were obtained from Freshwater Fish Culture Research Institute, Indonesia. In the present work, we used the following Indonesian stocks for the analysis of AFLP data: 20 individuals of the second generation gynogens originally belonging to the Majalaya stock, 10 individuals of the Majalaya stock and 10 individuals of the Sinyonya stock. The former is referred to as Gynogen (M) in the paper and consisted of second generation individuals resulting from breeding founder (F_0) gynogenetic line. Description of morphological characters of these stocks is given elsewhere (Sumantadinata 1995; Sumantadinata et al. 1994). Sumantadinata et al. (1990) have provided the procedural details of obtaining gynogens of common carp used in the present work.

Genomic deoxyribonucleic acid (DNA) extraction

Genomic DNA was extracted from 10-20 mg of caudal fin snips using the phenol-chloroform-isoamyl alcohol method (Sambrook et al. 1989) with slight modification.

Development of AFLP markers

The AFLP protocol AFLP™ Analysis System I using AFLP Starter Primer Kit (GIBCOBRL/Life Technologies, Cat Nos. 10544-013 and 10483-014) was essentially followed with minor modifications to generate the markers in this work. One hundred nano grams of genomic DNA from each sample were double digested using *Eco*RI and *Mse*I (GIBCOBRL) and ligated with adapter ligation solution and T4 DNA ligase.

The ligation products were diluted ten fold with sterile distilled water and pre-amplified with a mix containing 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 2 mM $MgCl_2$, 0.1 ng/µl BSA, 20µl of pre-amplification mix and 0.5U *Taq* DNA polymerase (GIBCOBRL). Pre-amplification was programmed for 1 cycle of 2 min denaturation (94°C) followed by 20 cycles of 30s denaturation (90°C), 1 min annealing (56°C) and 1 min extension (72°C).

*Eco*RI adapter-ligated primers were 5" end-labelled with [γ-^{33}P] ATP (ICN Biomedicals Inc., CA, USA). Selective amplification reaction was done in a mix containing 50-fold diluted pre-amplification products, 200 mM Tris-HCl (pH 8.4),500 mM KCl, 0.7 mM $MgCl_2$, 0.04 ng/µl BSA, 2.5 ng of labelled *Eco*RI primer,15.08 ng *Mse*I primer and 0.01U *Taq* DNA polymerase enzyme (GIBCOBRL). We have used three primer sets in this study: E-AAC/M-CAA, E-AGG/M-CAC and E-ACC/M-CTT.

The selective amplification profile was as follows: 1 cycle of 30s at 94°C, 30s at 65°C, 1 min at 72°C followed by 12 cycles of 30s at 94°C, 30s at 64°C, 1 min at 72°C, and 10 cycles of 30s at 94°C, 30s at 56°C, and 1 min at 72°C. All PCR amplifications were performed in PE-9600 thermal cycler (Perkin Elmer Corp., Norwalk, CT, USA). DNA size markers were prepared using T7 sequencing kit (Pharmacia Biotech) with minor changes in the protocol.

The AFLP reaction products were loaded on a 6% denaturing polyacrylamide gel (acrylamide and bis in the ratio 19:1; 7.9M Urea; 1x TBE buffer). After electrophoresis, the gels were dried on an ATTO RAPIDRY system for 1h and then exposed to phosphor imaging plates for variable lengths of time depending upon the signal. The imaging plates were scanned using a Bio-image Analyser (BAS 1000, Fuji Photo Films Corp., Ltd, Tokyo, Japan).

Data analysis

For each fish, a binary matrix reflecting AFLP band presence (1) or absence (0) was generated for all the three primer combinations. Estimates of similarity were based on the number of shared amplification products. It was assumed that each band position corresponded to one locus with two

alleles and that if two lines of fish lack a band of certain size, they carry the same allele at that locus. Band Sharing Index (BSI) was calculated by the relationship, $BSI=2N_{ab}/(N_a+N_b)$, where N_a and N_b are the number of bands in individuals a and b, respectively and N_{ab} is the number of bands shared by them (Lansman et al. 1981). We have developed a Microsoft Excel-based BASIC program to rapidly calculate the individual and average BSI values from the binary matrix.

Results

Primer combinations E-AAC/M-CAA, E-AGG/M-CAC and E-ACC/M-CTT revealed a total of 299 AFLPs in 40 fishes (117, 90 and 92, respectively) with a polymorphism of 66.2% (198). The number of bands in individual fish varied from 50 to 61 (E-AAC/M-CAA), 79-93 (E-AGG/M-CAC) and 57 - 67 (E-ACC/M-CTT). Gynogen (M) had 95, 64 and 62, Majalaya 109, 86 and 80, while Sinyonya 107, 83 and 73 AFLP marker loci for the three primer pair combinations, respectively. Average percentage of polymorphic loci was maximum in Majalaya and minimum in Gynogen (M) and E-ACC/M-CTT produced relatively high percentage of polymorphisms (Table 1).

Except for E-AGG/M-CAC, the other two primer combinations yielded as many genotypes as the number of individuals in Gynogen (M), Majalaya carp and Sinyonya carp. E-AGG/M-CAC produced 19 genotypes in Gynogen (M). We considered bands ranging in size from 60 to the largest detected fragment (approximately 400 bp) for analysis. Bands differing even in 1bp could be distinguished by comparing with the DNA size markers. With E-AAC/M-CAA, polymorphisms were generally more abundant in the range of

Table 1. Genetic variability in Indonesian common carps detected by AFLP markers.

Stock	AFLP primer combinations	No. of fish	No. of genotypes*	No. of bands	Loci polymorphism (%)**
Gynogen (M)	E-AAC/M-CAA	20	20	95	34.7(33)
	E-AGG/M-CAC	20	19	64	29.7(19)
	E-ACC/M-CTT	20	20	62	38.7(24)
Majalaya	E-AAC/M-CAA	9	9	109	46.8(51)
	E-AGG/M-CAC	10	10	86	52.3(45)
	E-ACC/M-CTT	10	10	80	58.8(47)
Sinyonya	E-AAC/M-CAA	10	10	107	39.3(42)
	E-AGG/M-CAC	10	10	83	51.8(43)
	E-ACC/M-CTT	10	10	73	45.2(33)

*Individuals with exactly the same band patterns.
**Numbers in parenthesis indicate number of polymorphic bands.

150 - 400 bp, while with E-AGG/M-CAC and E-ACC/M-CTT, polymorphic bands were spread randomly in the gel.

Based on the present data, we have classified the AFLP markers in common carp as follows: (1) MC1-those present in all the individuals of all the stocks; (2) MC2-those present in all the individuals of one stock and absent in all the individuals of others; (3) MC3-those present in at least one individual of one stock and completely absent in others; and (4) MC4-those present in at least one individual of each stock (Fig. 1). All the four types of markers were present in Gynogen (M)-Majalaya and Gynogen (M)-Sinyonya comparisons in all the three primer combinations. Between Majalaya and Sinyonya, MC2 marker type was absent.

In intra-stock comparisons, the average BSI values for the three primer combinations within the respective stocks varied from 0.9065 to 0.9509 in

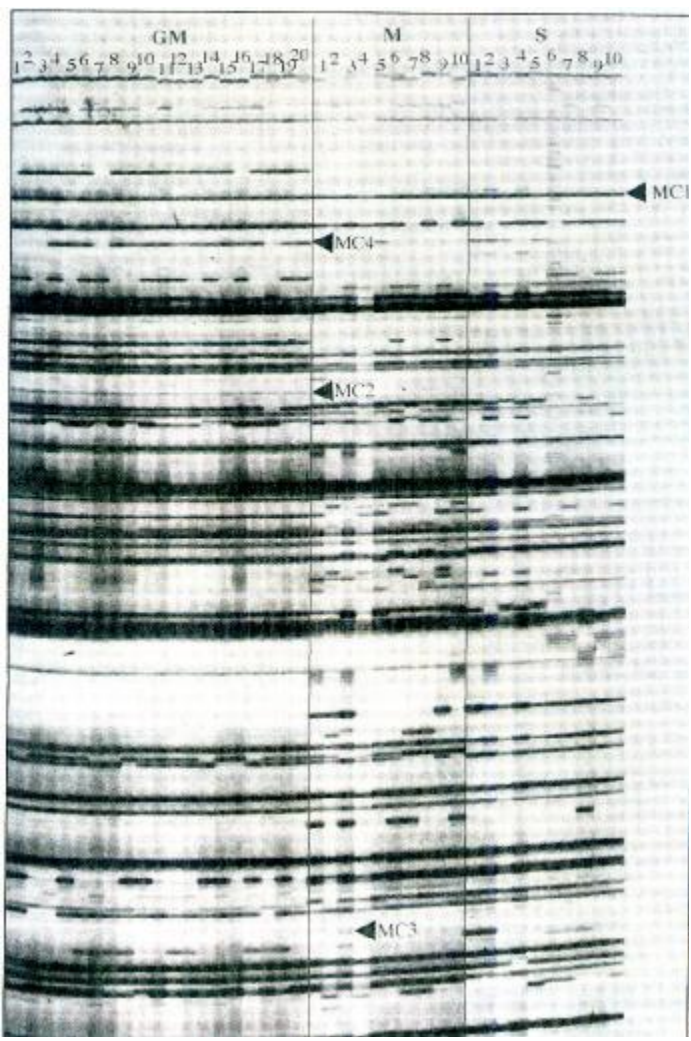

Fig. 1. AFLP fingerprints of genomic DNA of Indonesian common carps generated using primer combination E-ACC/M-CTT. GM, Gynogen (M); M, Majalaya; S, Sinyonya. MC1, MC2, MC3 and MC4 are the four types of AFLP markers respectively.

Gynogen (M), from 0.8298 to 0.8761 in Majalaya and from 0.8548 to 0.8989 in Sinyonya. Gynogen (M) exhibited the highest average BSI value (0.9290) while the Majalaya carp has the lowest (0.8518). This result reflects the high homogeneity of the former and high heterozygosity of the latter. Inter-stock comparisons yielded the following values for BSI: 0.7793-0.8370 between Gynogen (M) and Majalaya, 0.7443 - 0.8301 between Gynogen (M) and Sinyonya and 0.7769 - 0.8336 between Majalaya and Sinyonya. Closest relationship was seen between Gynogen (M) and Majalaya and farthest between Gynogen (M) and Sinyonya (Table 2).

Discussion

Significant advantages of the AFLP technique include its high throughput and high multiplex ratio. Using three primer set combinations we have obtained 299 selectively amplified DNA fragments of which 66.2% were polymorphic. AFLP is comparable with RAPD in not requiring prior sequence knowledge and the loci are dominant. It is comparable with RFLP that genomic DNA is cleaved with restriction endonucleases before detecting selectively amplified fragments. The technique is robust and reliable because stringent reaction conditions are used for primer annealing. Reproducibility is an added advantage, which is at a premium with RAPD markers. The primers used to obtain AFLP markers combine two key characters, (a) they are complimentary to the adapter oligonucleotide, thus allowing a highly specific primer annealing and (b) they are selective, changing the 3" nucleotides allows amplification of a different set of DNA fragments from the same population of pre-amplified fragments (Vos et al. 1995).

Table 2. Average BSI values of AFLPs in three Indonesian common carps (standard deviation given in the parenthesis).

Primers/ Samples	Gynogen (M)	Majalaya	Sinyonya	Gynogen (M) vs Majalaya	Gynogen (M) vs Sinyonya	Majalaya vs Sinyonya
E-AAC/M-CAA	0.9296 (0.019)	0.8761 (0.024)	0.8989 (0.027)	0.8370 (0.015)	0.8301 (0.021)	0.8336 (0.022)
E-AGG/M-CAC	0.9509 (0.020)	0.8496 (0.035)	0.8548 (0.047)	0.8268 (0.022)	0.8214 (0.020)	0.7966 (0.035)
E-ACC/M-CTT	0.9065 (0.025)	0.8298 (0.037)	0.8883 (0.035)	0.7793 (0.035)	0.7443 (0.027)	0.7769 (0.040)
Average	0.9290 (0.021)	0.8518 (0.032)	0.8807 (0.036)	0.8144 (0.024)	0.7986 (0.023)	0.8024 (0.032)

The proportion of polymorphic loci provides a good measure of genetic diversity within a species. As judged from the proportion of polymorphic loci (Table 1), the most diverse group of the stocks examined in our work was the Majalaya carp and the least was Gynogen (M). This observation is further supported by the data on average BSI (Table 2) which shows the maximum value in Gynogen (M) and minimum in Majalaya. AFLP data can also delineate inter-stock relationships. In our study, Gynogen (M) was closest to the Majalaya carp from which it was derived. On the otherhand, average BSI value of Gynogen (M) versus Sinyonya was the lowest. This indicates the greater genetic distance between the Gynogen (M) and Sinyonya stocks.

Liu et al. (1998 and 1999) observed the inheritance and segregation of AFLP markers in channel catfish and blue catfish. They have indicated the potential of these markers in the construction of a genetic linkage and quantitative trait loci (QTL) maps as well as for marker-assisted selection. AFLP fingerprinting provides molecular markers to distinguish different fish populations (Takagi et al. 1998). They observed extremely large genetic divergence between ayu and Ryukyu-ayu populations based on these markers. Our study showed the usefulness of AFLP fingerprinting to test the degree of success of induced gynogenesis in common carp and for differentiating the gynogens from their parental Majalaya stock based on marker type MC2 and MC3. Based on marker type MC3, Majalaya carp can be distinguished from Sinyonya carp.

Selective breeding using small number of females is a common practice among fish farmers in Indonesia. This has resulted in genetic drift and subsequently low average heterozygosity (Sumantadinata and Taniguchi 1990). Our BSI data also indicate relatively high homozygosity among the Gynogen (M) and Sinyonya carp. For the former, it is expected since the sample was the second-generation product of meiotic gynogenesis. Genetic variation is reduced in the second generation of gynogens. Sumantadinata et al. (1994) reported that in Indonesian common carps barring few exceptions, the variance in quantitative characters analysed in the second generation of gynogenetic diploids was always smaller than that of the control-2N. As far as Sinyonya carp is concerned, our results agree with the observations of Sumantadinata and Taniguchi (1990). They have reported about the use of one female with few males for each spawning in Indonesia, which could result in a genetic drift. In West Java, only two fish farmers and two government hatcheries keep the Sinyonya stock, hence probably strong inbreeding has happened in the field. Cluster analysis of AFLP data on more stocks of common carps could make this issue clearer, thereby facilitating reorientation of selective breeding strategies of Indonesian common carps.

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