African Journal of Biotechnology Vol. 11(77), pp. 14217-14226, 25 September, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2133
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Molecular characterization of two populations of catfish *Clarias batrachus* L. using random amplified polymorphic DNA (RAPD) markers

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Accepted 27 July, 2012

Studies were undertaken to identify genetic relationship and diversity of *Clarias batrachus* L. populations collected from hatchery and wild stocks through random amplified polymorphic DNA (RAPD). 16 decamer primers were used for screening, out of which five produced amplicons. A total of 1376 RAPD bands ranging from 0.2 to 1.36 kb were amplified using five selected primers. The number of amplification products produced by a primer ranged from as low as three to a maximum of 18, with an average of 16 bands per primer. 8.1 to 13.71 polymorphic bands per primer were amplified. The polymorphic bands in these populations ranged from 56.4 to 59.6%. Polymorphic bands per lane within populations ranged from 4.88 to 5.3%. The similarity within the population from wild varied from 0.40 to 0.83 with a mean ± SE of 0.57 ± 0.08. The Jaccard's similarity coefficient ranged from 0 to 0.27. At 0.06 similarity coefficient, two major clusters were formed, which indicates that the genotypes belonging to same clusters were genetically similar and those belonging to different clusters were dissimilar. Significant (P < 0.05) population differentiation indicated some degree of intra- and inters- population genetic variations in two populations of catfish. This might be due to difference in habitat and breeding strategies between the two populations.

Key words: Random amplified polymorphic DNA (RAPD), similarity coefficient, polymorphism, *Clarias batrachus*, primer.

INTRODUCTION

Clarias batrachus L. (vernacular name Magur) was formally described in 1758 as *Silurus batrachus*. It is also known as walking catfish for its ability to "walk" across dry land to find food or suitable environment. They normally inhabit in swamps and marshy areas (CAFF, 2006). This is primarily found in Southeast Asia. *Claria batrachus* L. is a popular and valuable food fish in India and Bangladesh. In some parts of India, particularly in West Bengal and Tripura, it is considered as a medicinal fish and traditionally remained a strike among the pregnant and lactating mothers, and the elderly and children. Owing to its taste and low fat content, the fish is very popular as a patient's dish and prescribed prophylactically to the anemic and malnourished individuals, as well as for the convalescent of the patients due to the nutritional superiority. Intensive *C. batrachus* culture in several Indian states as in rural Bengal and Tripura have much potential towards livelihood development, employment generation, and ensuring nutritional enrichment in the regular diet among the people. This rough and tough species has been studied extensively by many workers in terms of physiology, biochemistry, toxicology, host parasite interaction, pathology, culture characters, as well as its population genetics (Debnath, 2011).

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Table 1. Primer and their code used for PCR amplification.

Name in literature	Primer sequenced	GC (%)
OPA-01	CAGGCCCTTC	70
OPA-02	TGCCGAGCTG	70
OPA-03	AGTCAGCCAC	60
OPA-04	AATCGGGCTG	60
OPA-05	AGGGGTCTTG	60
OPA-06	GGTCCCTGAC	70
OPA-07	GAAACGGGTG	60
OPA-08	GTGACGTAGG	60
OPA-09	GGGTAACGCC	70
OPA-10	GAGATCGCAG	60
OPA-13	CAGCACCCAC	70
OPA-14	TCTGTGCTGG	60
OPA-16	AGCCAGCGAA	60
OPA-17	GACCGCTTGT	60
OPA-18	AGGTGACCGT	60
MS1613R	CCTCTGGGTTCGGTCAAAAC	55

This species has already been categorized as vulnerable based on the International Union for Conservation of Nature (IUCN) red list in India (Dahanukar et al., 2004). The vulnerability of the species requires conservation, recovery and management strategies, which include investigation on current levels of genetic diversity and differentiation within and between populations as a basis for sustainable management recommendations. Moreover germplasm characterization is an important link between the conservation and efficient utilization of genetic resources (Giri et al., 2012). Genetic variability in fishes has been proven valuable for aquaculture and fisheries management, identification of stocks, selective breeding programmes, restoration of ecology and estimating genetic contributions in stocks. Thorough knowledge of genetic variability within the species is considered prerequisite for efficient utilization of biological resources. Morphological characters have been used extensively to study diversity of different forms in the past. In recent years, attempts to study biodiversity at molecular level have gained importance. Molecular techniques help researchers not only to identify the authentication of the genotypes, but also in assessing and exploiting the genetic variability (Whitkus et al., 1994). Randomly amplified polymorphic DNA (RAPD) has been extensively used to study diversity in different fishes (Liu et al., 1998 & 1999). Williams et al. (1998) observed RAPD analysis to be more sensitive than traditional histochemical agarose gel electrophoresis and considered it as the most frequently used molecular methods for taxonomic and systematic analysis of various organisms by many scientists like (Bartish et al. 2000, Garg et al. 2009a).

The present study reports the insight into the relative genetic diversity within two populations of the catfish *C. batrachus* using RAPD marker, which would be useful for better stock management and conservation of these species.

MATERIALS AND METHODS

Sample collection

Live specimens of *C. batrachus* L. were collected from magur hatchery at College of Fisheries, Pantnagar and collected from the fish market of Dineshpur Uttarakhand (India). Liver muscles were taken from these live specimens of *C. batrachus* L. for RAPD marker analysis. These tissue samples were labeled and immediately transferred into ice bucket containing ice packets, and transported to the laboratory where it was stored in ultra-low freezers at -86°C until further analysis.

Isolation of DNA

DNA was extracted by three methods using N-lauroyl sarcosine sodium salt (Sarkosyl), sodium dodecyl sulphate (SDS) and cetyltrimethyl ammonium bromide (CTAB) as detergents, respectively. Total DNA was isolated from liver tissue of cat fish *C. batrachus* L. Homogenized liver tissues (0.25 g) were incubated in CTAB buffer (0.1 M tris, 1.4 M NaCl, 0.02 M EDTA, 1.5% (w/v) CTAB, 0.2% β marcapatoethanol) with 20 µl of 10 mg proteinase K for 4 to 6 h at 60°C with periodic agitations. Total DNA was then purified and precipitated by methods described by Sambrook et al. (1987). Purified DNA was dissolved in 100 µl TE buffer. The concentration of DNA was measured by recording absorbance at 260 nm by using a UV spectrophotometer.

Random amplified polymorphic DNA polymerase chain reaction (RAPD PCR)

Ten oligonucleotides decamer primers from Genei Pvt. Limited Bangalore, India were used as primers (Table 1). Tests were

Table 2. Number of RAPD loci detected by five primers in catfish, C. *batrachus*.

S/N	Primer code	Total number of RAPD loci	Monomorphic loci	Polymorphic loci		Polymorphic information content (PIC)	Average expected gene diversity
				Number	(%)		
1	OPA-06	22	6	16	72.72	0.96	0.25
2	OPA-09	26	9	17	65.38	0.95	0.25
3	OPA-14	14	5	9	64.28	0.97	0.24
4	OPA-17	35	10	25	71.42	0.95	0.21
5	OPA-03	23	6	17	73.91	0.96	0.24
6	Total	120	36	84	70	0.96	0.24

performed for standardizing PCR conditions and finally PCR amplification was carried out at 94°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing for 1 min at 32°C, extension at 72°C for 1 min and final extension at 72°C for 5 min. For standardizing $MgCl_2$ concentration in PCR reactions for getting consistency in amplification products, a concentration gradient from 1.0 to 3.0 mM was applied, and DNA template concentration was optimized for PCR reaction using initially five concentrations of DNA, 5, 10, 20, 40 and 80 ng per PCR reaction. Reaction mixture (25 µl) contained 10 ng genomic DNA, 1× reaction buffer [100 mM Tris, pH 9.0; 500 mM KCl; 0.1% gelatin, 2.0 mM $MgCl_2$, 200 µM (0.2 mM) of each dNTPs (Bangalore Genei, Bangalore, India), 0.4 µM of each primer, 1 unit of Taq DNA polymerase (Bangalore Genei, Bangalore, India)]. 1 µl of the sample (20 ng/µl) was added to the reaction mixture from different fish samples which would act as template. PCR products were subjected to electrophoresis in 1.5% (w/v) agarose (Genei, Bangalore) gel with 1× TBE buffer at 50 V till the dye part was in wells, after that, 100 V for 7 h was applied. The gel was removed and stained with ethidium bromide (EtBr) solution for 10 min, followed by distaining in distilled water for 5 min. The gel was then placed over the surface of UV transilluminator of the gel documentation system and the image was photographed using Gel Documentation system. The sizes of the amplification product were estimated using Standard marker (ø X 174 DNA marker digested with Hae III). All the amplified bands were scored as present or absent for each DNA sample and further, the RAPD reaction results were analyzed using software Gene Profiler. In order to analyze the relatedness among the species, a dendrogram based on unweighted pair group method with arithmetic average (UPGMA) and NEI and Li genetic distance matrix (Nei and Li, 1979) value was obtained. Data were analyzed to obtain Jaccard's similarity coefficients among the isolates by using NTSYS-pc (version 2.11W; Exeter Biological Software, Setauket, NY) (Rohlf, 1993). The SIMQUAL program was used to calculate the Jaccard's similarity coefficients.

RESULTS AND DISCUSSION

PCR amplification of DNA extracted from 16 genotypes of *Clarias. batrachus* L. was performed using five primers following the same protocol. Genotypes 1 - 8 are from wild stock and genotypes 9 - 16 are from hatchery stock. The PCR products run on agarose gel were scored manually. The total numbers of loci amplified from the five primers were 120. This gave an average of 24 loci per primer. The size of amplified products ranged between 0.2 to 1.36 kb and the number of RAPD loci scored, and polymorphism detected by five primers in 16 catfish samples are presented in Table 2.

For primer OPA-06, a total number of 22 bands of amplicons were visualized. Their size ranged from 240 to 1350 bp (Figure 1). Six of the PCR products of size ranging from 240 to 1100 bp were monomorphic, and the primer produced 72.72% polymorphic loci. For Primer OPA-09, the number of amplified products scored was 26 (Figure 2). These products ranged in size from 240 to 1360 bp. Nine of PCR products that ranged from 240 to 1360 bp were monomorphic and the primer produced 65.38% polymorphic loci. The specific major band of 1360 bp was found to be common in both populations. Primer OPA-14 produced 14 PCR products (Figure 3). Their size ranged between 200 to 1360 bp. Five amplicons of size range 200 to 980 bp were seen to be specific, hence monomorphic and 64.28% of loci which are polymorphic were obtained by this primer. For Primer OPA-17, a total of 35 amplification products were amplified. Size of products obtained ranged from 200 to >1360 bp obtained by this primer (Figure 4); ten of which were monomorphic. The monomorphic amplicons ranged between 200 to >1360 bp and exhibited 71.42% of polymorphic loci. For OPA-03, the number of amplified products scored were 23 (Figure 5), and their size ranged from 240 to 1360 bp, of which six were monomorphic. This primer recorded maximum of 73.91% polymorphic loci out of the total five primers studied.

Out of a total of 668 amplified bands produced in wild population, 298 were polymorphic (44.61%) and average number of polymorphic bands varied from 1.5 to 7.5 (Table 3). The average value 298/5 = 59.6% of total polymorphic bands (298) were approximately 20%. In particular, primer OPA-6 produced highest number of fragments; with an average of 7.5 among the primers used. On the other hand, primer OPA-03 produced the lowest number of fragments with an average of 1.5. The number of bands produced per primer within catfish population from hatchery, with five primers used, varied from 3 to 18, with an average of 11.98. DNA polymorphisms were observed not only from 0.60 kb to larger than 1.35 kb, but also from less than 0.20 to 0.32 kb in

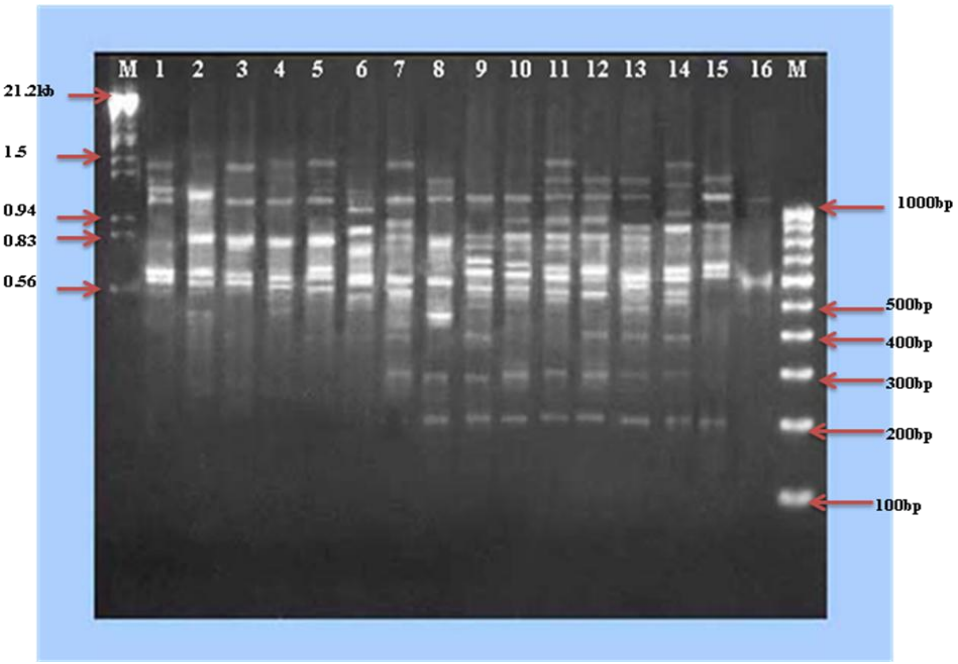

Figure 1. Individual specific RAPD patterns of cat fish amplified by arbitrary OPA-06. Each lane (1 to 16) shows different individual DNA samples from wild stock (lane 1 to 8) and Hatchery stock (lane 9 to 16) [M, Standard marker (ø X 174 DNA marker digested with Hae III)].

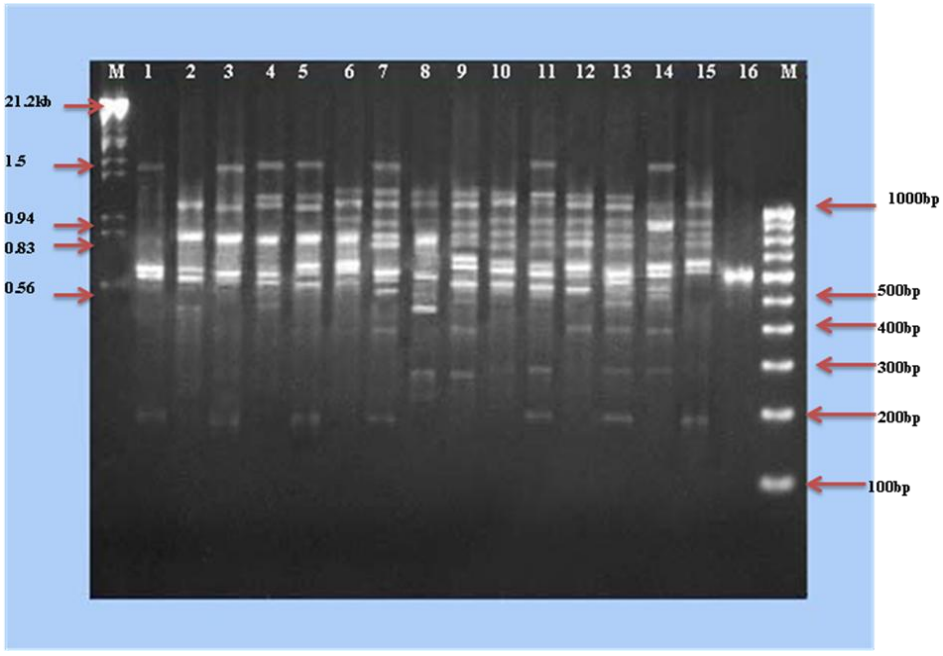

Figure 2. Specific RAPD pattern of cat fish amplified by arbitrary primer OPA-09. Each lane (1 to 16) shows different individual DNA samples (lane 1 to 8 from wild stock and lane 9 to 16 from Hatchery stock). [M, Standard marker (ø X 174 DNA marker digested with Hae III)].

hatchery population (Figure 1). Random primer OPA-9 generated identical band from 0.60 to 1.10 kb and polymorphic bands from 1.09 to 1.35 kb (Figure 2). A total of 708 amplified bands were generated, of which

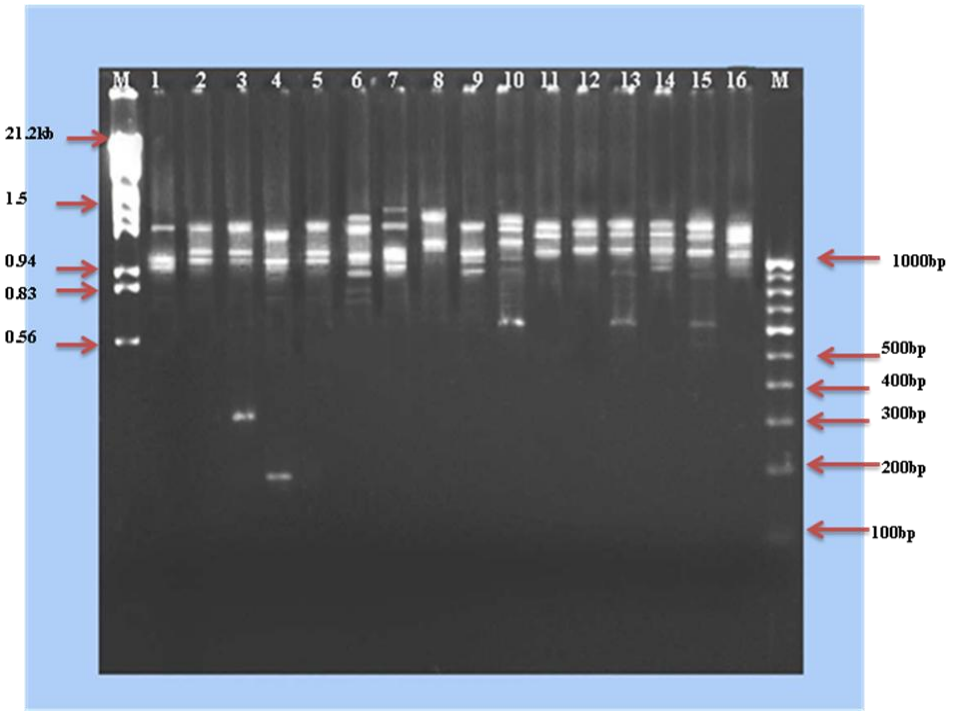

Figure 3. Individual specific RAPD patterns in catfish amplified by arbitrary primer OPA-14. Each lane (1 to 16) shows different individual DNA samples (lane 1 to 8 from wild stock and lane 9 to 16 from Hatchery stock). [M, Standard marker (ø X 174 DNA marker digested with Hae III)].

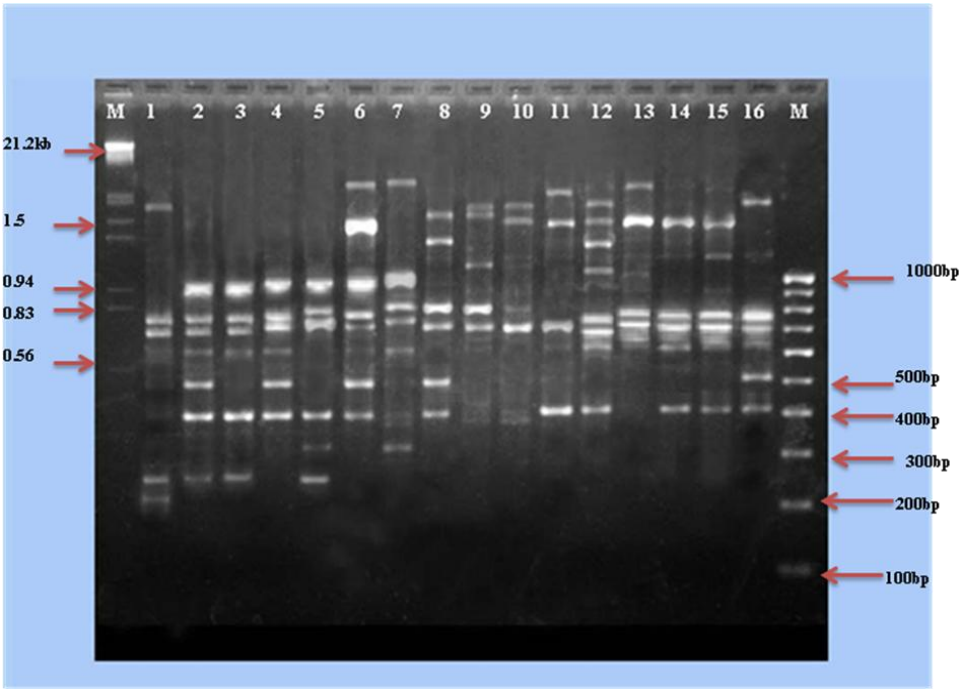

Figure 4. Amplified products were electrophoresed on a 1.4% agarose gel with TBE and detected by staining with ethidium bromide. Individual specific RAPD patterns of cat fish amplified by arbitrary OPA-17. Each lane (1 to 16) shows different individual DNA samples (lane 1 to 8 from wild stock) and Hatchery stock (lane 9 to 16). [M, Standard marker (ø X 174 DNA marker digested with Hae III)].

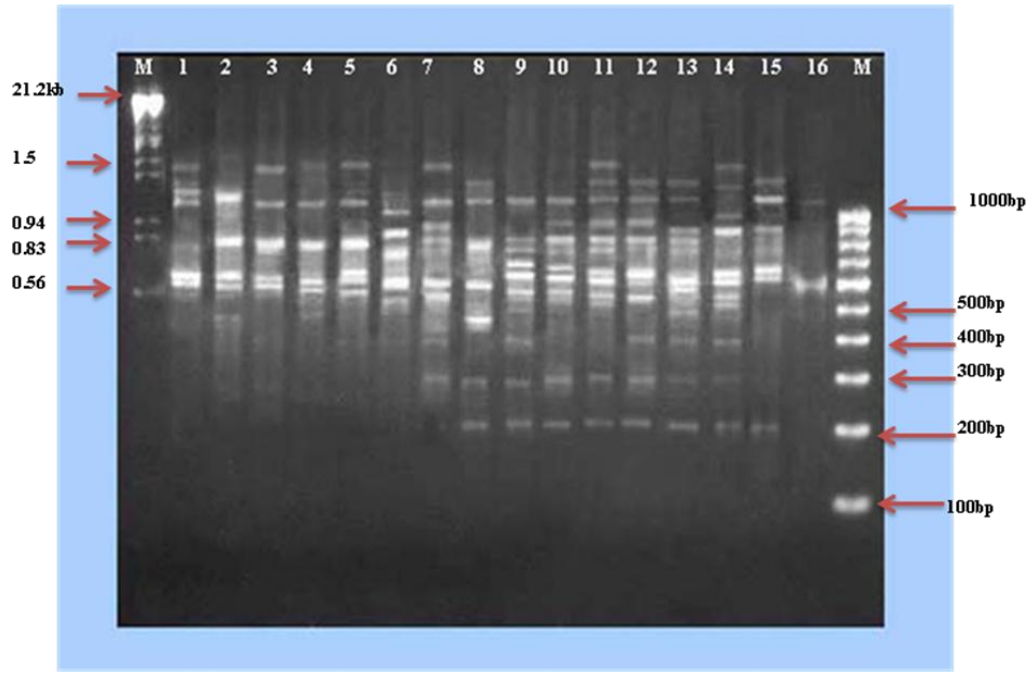

Figure 5. Specific RAPD finger prints generated in catfish by arbitrary primer OPA-03. Each lane (1 to 16) shows different individual DNA samples (lane 1 to 8 from wild stock and lane 9 to 16 from Hatchery stock). [M, Standard marker (ø X 174 DNA marker digested with Hae III)].

Table 3. RAPD profile in wild population of *Clarias batrachus* L.

Primer	Range of band	Total number of band	Number of average band	Total number of polymorphic band	Average number of polymorphic band
OPA-03	3-12	95	8.1	16	1.5
OPA-06	8-17	138	12.4	82	7.5
OPA-09	11-17	157	12.9	57	4.8
OPA-14	6-16	143	11.8	74	6.2
OPA-17	9-18	135	13.0	69	6.3
Total		668	58.2	298	26.3

Table 4. RAPD profile in hatchery population of *Clarias batrachus* L.

Primer	Range of band	Total number of band	Number of average band	Total number of polymorphic band	Average number of polymorphic band
OPA- 03	3-12	101	8.80	30	2.73
OPA- 06	8-17	152	12.52	62	5.24
OPA-09	11-17	150	12.35	80	6.72
OPA-14	6-16	139	12.50	76	6.91
OPA-17	9-18	166	13.71	34	2.82
Total		708	59.91	282	24.42

282 were polymorphic, thus making it 39.83% in this population (Table 4). Averages of 4.88 amplified products were polymorphic in this population. The primer OPA-14 generated the highest number of fragments; with an average of 6.91, amongst five primers used and OPA-03 generated the lowest number of fragments with an

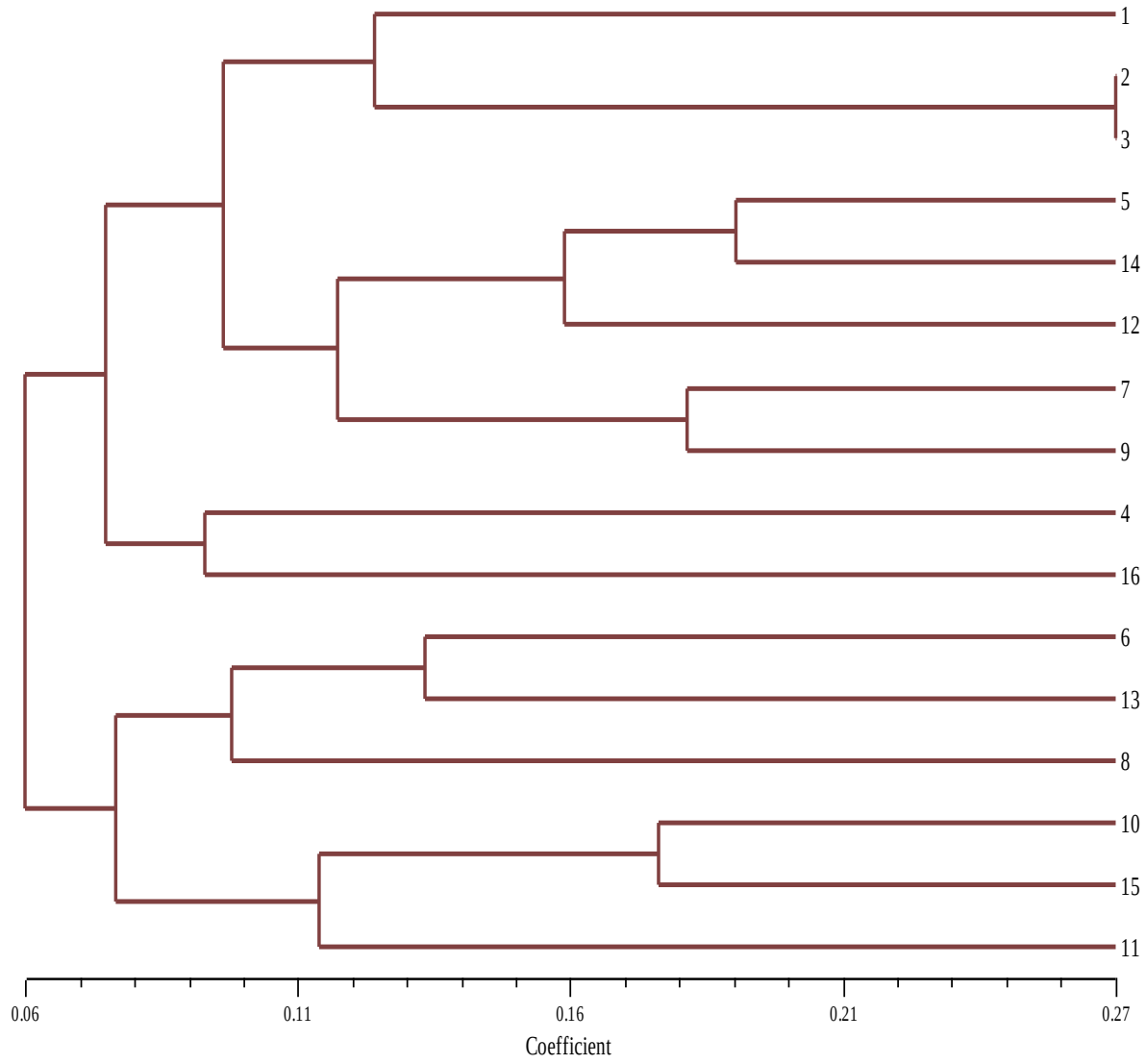

Figure 6. Jacard's coefficient based UPGMA dendogram showing clustering of 16 genotypes of C. *batrachus* based on RAPD primers. The numbers plotted represent individual genotypes.

average of 2.73. Average expected gene diversity was also calculated from the banding pattern of every primer. The average expected gene diversity ranged from 0.25 for primer OPA-06 and OPA-09, followed by 0.24 for primer OPA-14 and OPA-03, to a minimum of 0.21 for primer OPA-17, with an average of 0.24 for all random primers among all the genotypes at all the locus studied.

Associations among the 16 genotypes revealed by UPGMA Cluster analysis are presented in Figure 6. The dendogram separated the 16 genotypes of two populations of *C. batrachus* L. into two major clusters (I and II). These major clusters showed six percent similarity to each other. The major clusters I comprised of six genotypes and could be divided into two sub-groups IA and IB at the demarcation, having approximately 7.5% similarity. Sub group IA was further divided into IA_1 and IA_2, exhibiting approximately 10% similarity. IA_1 comprised of genotypes six and 13 IA_2 comprised of genotype eight only. Sub-group IB of major cluster I was further divided into IB_1 and IB_2 at the demarcation, having approximately 11.5% similarity. IB_1 comprised of genotype ten and 15, showing 18.5% similarity. Sub-group IB_2 comprised of genotype 11 only. The major cluster II comprised of ten genotypes and it was sub- divided into two sub-clusters namely IIA and IIB at the demarcation, having approximately 7% similarity. The sub-cluster IIA was still further divided into two sub-sub-groups IIA_1 and IIA_2 at approximately 9.5% similarity. Sub-group IIA_1 comprised of genotype one only. Sub-sub-group IIA_1** comprised of genotypes two and three, showing 27%

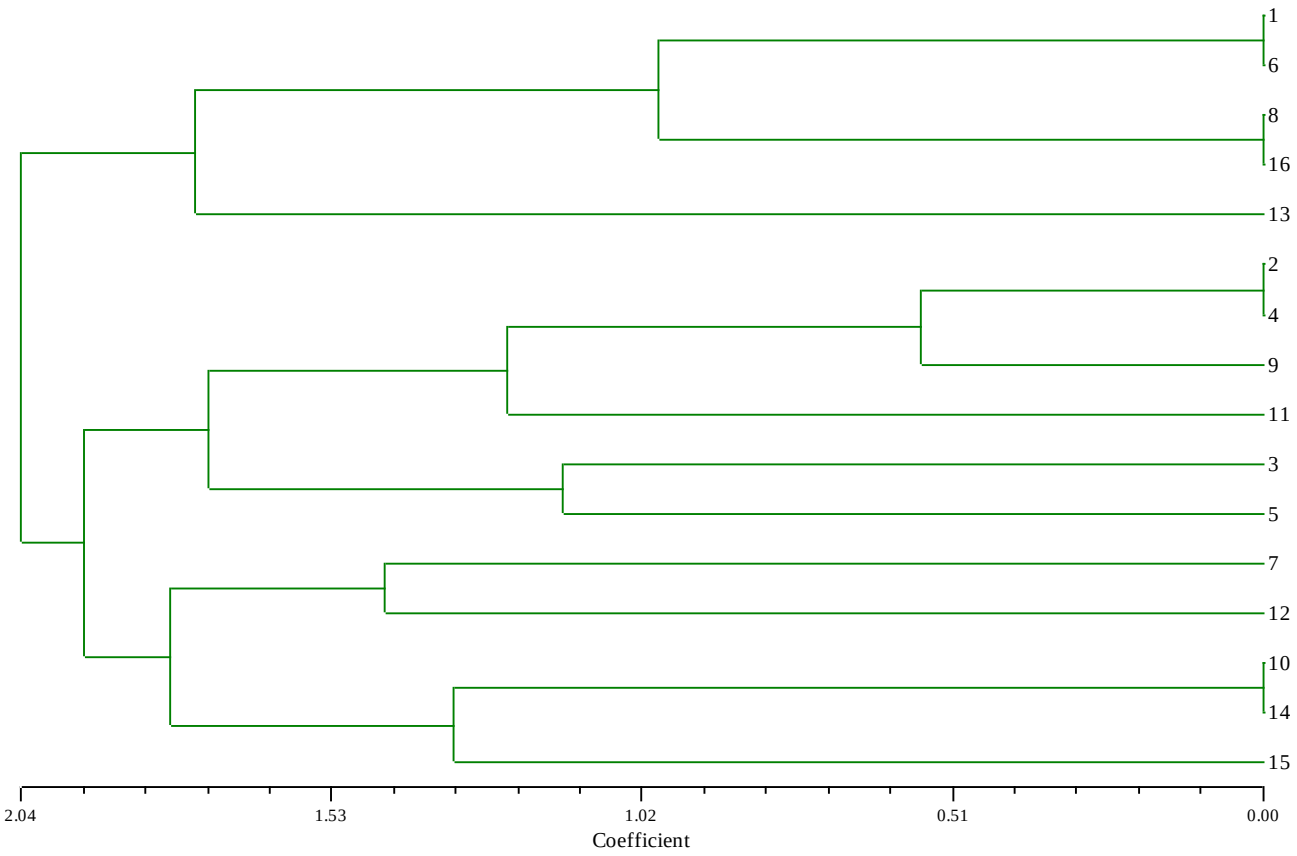

Figure 7. Nei's coefficient based UPGMA dendogram showing clustering of 16 genotypes of C. *batrachus* based on RAPD primers. The numbers plotted represent individual genotypes.

similarity. The sub-group IIA_2 was still further divided into two sub-sub-groups IIA_2* and IIA_2**. IIA_2* comprised of genotype five, 14 and 12, showing 19% similarity between genotype five and 14 and 12. The sub-sub-group IIA_2** comprised of genotype seven and nine, with similarity of approximately 18%. The similarity coefficient values thus indicate the presence of sufficient amount of variability between these genotypes. A maximum of 27% similarity coefficient was observed between genotype three and two, followed by 19% between genotype fourteen and five, and 18% between genotype nine and seven. The maximum similarity coefficient between above genotypes indicates the closeness between most genotypes.

The value of Nei's genetic distance was found to be maximum between genotype ten and seven at 3.05, followed by 2.93 between genotype 12 and four, and 2.89 between genotype six and two. Likewise, the minimum genetic distance value was found to be 0 (minimum) between genotype nine and four, followed by 0.86 between genotype three and two, and by 1.13 between genotype 14 and five. A dendogram reflecting genetic relatedness constructed by unweighed pair group method with arithmetical averages (UPGMA) is shown in Figure 7. It revealed two major clusters I and II at Nei's coefficient value of 2.04. The major cluster I comprised of 11 genotypes and could be divided into two sub-groups IA and IB at the demarcation value of approximately 0.51. Sub-group IA was further divided into IA_1 and $1A_2$. IA_1 comprised of genotypes two, four, nine and 11 while IA_2 comprised of genotype three and five only. Sub-group IB was also further divided into IB_1 and IB_2. IB_1 comprised of genotype seven and 12 at the demarcation value of approximately 0.40, while IB_2 at the demarcation value of approximately 0.30 comprised of genotypes ten, 14 and 15, respectively. The major cluster II comprised of five genotypes and could be divided into two sub-groups IIA and IIB at the demarcation value of approximately 0.25. Sub-group IIA comprised of genotype 13 only. Sub-group IIB was further divided into IIB_1 and IIB_2 at the demarcation value of approximately 0.48 (Figure 7). IIB comprised of genotype one and six and IIB_2 comprised of genotype eight and 16, respectively.

In fisheries, genetic markers have been applied with varying degree of success in stock structure analysis, taxonomy/systematic and selective breeding programmes

(Ward and Grewe, 1994; Carvalho and Hauser, 1994). Commonest use of genetic markers in fisheries is for determining genetic divergence between domesticated and natural fish populations (Waldman and Wirgin, 1993; Ferguson and Dangamann, 1998). The assessment of genetic variation among groups of individuals is prerequisite for useful application of genetic markers at intra-specific level. RAPD profile generated by OPA-03, OPA-04, OPA-05, OPA-02, OPA-11 in tilapia fish (*Oreochromics niloticus*) ranged between 0.3 to 1.8 kb, with six to 17 products (Bardakci and Skibinski, 1994) in comparison to 0.20 to 1.36 kb fragments of *C. batrachus* L. observed in the present investigation. Lower size of PCR products in present study could either be attributed to comparatively higher annealing temperatures of 36°C used for *C. batrachus* L. as compared to tilapia (35°C) (Bardakci and Skibinski, 1994) or difference in the genome of two fish belonging to distinct families. Among the five screened primers, average expected gene diversity varied from 0.21 (OPA-17) to 0.25 (OPA-06, 09) for both the populations of catfish, which indicates that the RAPD markers used in this study was able to reveal high level of diversity within the populations. High levels of genetic diversity found in both the populations may be due to selective advantage for heterozygotes in the population. A second process that would raise the level of genetic polymorphism is the effect exerted by spatial heterogeneity on genetic polymorphism.

Spatial heterogeneity hypothesis states that the spatial differences in the environment results in differential selection for different genotypes in different sub-habitats and thereby results in genetic polymorphism. High levels of genetic variation also reflect broad environmental adaptations in the species that are geographically widely distributed due to the dispersal capacity of long lived larvae in case of shellfishes (Apte et al., 2003). This supports the genetic variation observed within the population of *C. batrachus* L. Significant ($P < 0.05$) population differentiation was observed in each pair of populations, apparently showing the populations to be genetically differentiated to some extent. This could be explained by the difference in habitat and breeding strategies between the populations. Significant population differentiation was also reported in rainbow trout strains collected from broad geographic ranges (Silverstein et al., 2004). A phylogenetic dendogram generated from Nei's (1972) genetic distance suggests separation of the hatchery population from the wild population. Captive management impact over the period in hatchery population might cause a relatively high level of genetic distance and consequently low level of gene flow with the wild population.

Unlike the present study, Leesa-Nga et al. (2000) found the highest genetic distance (0.164) between two wild populations of yellow catfish (*Mystus nemurus*) once they estimated genetic polymorphism in eight-wild and one hatchery stocks from Thailand using horizontal starch gel electrophoresis. A hatchery population may suffer from a bottleneck when only a few marking pairs produce hundreds of offspring that constitute the entire subsequent generation. Moreover, sometimes hatchery owners maintain a polygynous mating system in which a few males monopolize breeding and mate with many females, resulting in a genetic bottleneck in the absence of a demographic bottleneck. A population genetic structure analysis of threatened or ecologically or commercially important fish species is essential for optimizing management strategy or a stock improvement program. Genetic monitoring is necessary for an effective management strategy because a population can suffer severe genetic erosion for example, bottleneck, genetic drift, inbreeding, founder effect etc. without being detected by the traditional demographic monitoring approach.

Our study reveal some degree of intra-and inters-population genetic variations in two populations of *C. batrachus* L. Hatchery managers are required to practice good brood stock management strategy using diverse natural stocks for maintaining the genetic quality of the species in the hatcheries. A management strategy for the population that is on the edge of experiencing a bottleneck should include establishment of fish sanctuaries for *in situ* conservation (conserving species within that habitat), habitat protection from siltation and fragmentation to overcome the vulnerable conditions of this species and helping to maintain their diverse gene pool in nature. The two populations of yellow grouper (*Epinephelus awoara*) showed high level of genetic variation and genetic diversity between populations (Upadhyaya et al., 2006), which showed a high genetic distance of 0.4775. This observation is supportive for a high genetic diversity on the basis of genetic distance (2.04) observed for *C. batrachus* L. in the present study. Similarly, a high level of genetic variation and genetic distance (0.617 to 0.949) was also reported in dinoflagellates (Baillie et al., 2000). The assessment of distinctiveness with range of loci scored and size of distinguish genotypes of *Clarias batrachus* are presented in Table 5. Among the five screened primers, primer OPA-17 distinguished the maximum (ten) number of genotypes as compared to the minimum (five) number of genotypes by primer OPA-14. Genotypes 1, 7, 9, 10, 11, 12, 13, 15 and 16 were distinguished by primer OPA-17, followed by primer OPA-09 which distinguished genotypes 1, 3, 4, 6, 7, 11 and 14. Genotypes 3, 6, 8, 11 and 15 were distinguished by primer OPA-03 and genotypes 3, 8, 11 and 14 were distinguished by primer OPA-06. Primer OPA-14 distinguished the genotypes 3, 4, 6 and 10.

On the basis of information available from the present study, the possible inference can be drawn that good genetic divergence existed in both catfish populations;

although there were some levels of polymorphism among the given population. An elaborated work is needed by using more number of primers and large number of populations from different regions to draw a suitable conclusion. However, some more sensitive techniques like restriction fragment length polymorphism, mini-satellite and micro-satellite, amplified fragment length polymorphism and various sequence techniques, etc may be employed for monitoring of genetic status and breeding programmes of this valuable catfish.

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