Applied Biological Research 16 (2): 134-142; (2014)
DOI: 10.5958/0974-4517.2014.00001.9

GENETIC DIVERSITY IN DIFFERENT STOCKS OF GRASS CARP (*Ctenopharyngodon idellus*) INFERRED BY RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS IN INDIA

T.L.S. Samuel Moses*, Y. Basavaraju[1], K.N. Prabhudeva[2], V. Vinay Kumar[3] and R. Prasad Naik[4]

Department of Aquaculture, College of Fisheries, Karnataka Veterinary Animal Fisheries Sciences University (KVAFSU), Mangalore - 575 002, Karnataka (India)
[1]Director of Extension, KVAFSU, Bidar - 585 403, Karnataka (India)
[2]Fisheries Research and Information Centre (I), KVAFSU, Hebbal, Bangalore - 560 094, Karnataka (India)
[3]Regional Centre of CMFRI, Matsya Bhavan, Bhidiya Plot, P.B. No. 17, Veraval - 362 269, Junagadh, Gujarat (India)
[4]Regional Centre of Marine Product Export Development Authority, Vishakhapattinam - 530 003, Andhra Pradesh (India)
*****e-mail: *samuel.aquaculture@gmail.com*

(Received 12 January, 2014; accepted 26 March, 2014)

ABSTRACT

Four different stocks of grass carp from different geographical locations *viz.*, Kolkata, Kaikaluru, BRP and Thanjavur representing varied environmental conditions were collected from Kolkata (West Bengal), Kaikaluru (Andhra Pradesh), Shimoga (Karnataka) and Thanjavur (Tamilnadu). The stocks were marked using fin cauterization for easy identification during the growth behaviour studies. Growth behavior sudies conducted under same environmental and management conditions. RAPD analysis was performed for different stocks of grass carp. Of the 100 decamer primers screened, seven primers (OPA 1, OPA 18, OPB 11, OPB 19, OPD 11, OPD 18 and OPE 10) gave consistent and at least two polymorphic bands. The RAPD analysis showed that Kolkata stock had 2.74 to 14.72% higher polymorphic bands in comparison with other stocks. The Kolkata stock was found closely related to Kaikaluru stock but distantly related to Thanjavur stock. Growth trials indicated better performance of Kolkata stock in comparison to other stocks. The r^2 value between the % polymorphism and mean body weight attained by different stocks of grass carp showed that these two parameters were positively correlated.

Key words: Genetic diversity, grass carp, polymorphism, random amplified polymorphic DNA (RAPD) markers

INTRODUCTION

India is the 2nd largest aquaculture producer in the world next to China (FAO, 2013). Aquaculture production in India is 4.5 million tonnes (FAO, 2013) and freshwater aquaculture contributes nearly 70% of Indian total inland fish production (ICAR, 2006). Grass carp (*Ctenopharyngodon idellus*, Valenciennes, 1844) worldwide is the 2nd largest extensively farmed fish and its production in 2011 was around 4.5 million metric tonnes (FAO, 2013). Grass carp was first introduced in India at

CIFRI, Cuttack in 1959 (Jhingran, 1991). In India, grass carp is normally farmed along with other Indian major carps as one of the component species in composite fish culture and contributes around 5% of total fish stocked (ICAR, 2006). Apart from contribution to the total aquaculture production, the grass carp, as an efficient biological agent, is used for aquatic weed control in open water bodies.

Random amplified polymorphic DNA (RAPD) is a PCR based technique used to assess genetic variation at DNA level without prior sequence information (Williams *et al.,* 1990). RAPD has been employed to assess genetic variation in various fish species like Discus (Koh *et al.,* 1999), Hilsa shad (Brahmane *et al.,* 2006), black tiger prawn (Tassanakajon *et al.,* 1997), rohu (Islam *et al.,* 2004), flounder (Liu *et al.,* 2007), common carp (Basavaraju *et al*., 2007), kalibaus (Mostafa *et al.,* 2009), catfish (Garg *et al.,* 2009), etc. Brahmane *et al.* (2006) employed RAPD technique to assess genetic variation in Hilsa shad (*Tenualosa ilisha)* in different locations of four Indian rivers namely Ganga, Yamuna, Hooghly and Narmada. They utilized 12 samples from each location and 6 RAPD primers. Five species of Masheer fish from penisular India were analyzed by Mohindra *et al.* (2007) by RAPD analysis and 69 primers screened.

Growth performance is a quantitative genetic parameter controlled by dominant and additive genes. Since addictive genetic effect is inherited so it has great significance in genetic improvement of fish species. In addictive genetic effect, genes in different loci contribute in additive fashion to quantitative genetic parameters including growth. In traditional genetic method for growth improvement the stocks are selected while in RAPD analysis each band is presumed to represent one locus as a gene (Tave, 1993). More number of genes responsible for better growth behaviour in addictive fashion and markers such as RAPD pattern have been correlated and thereby form a base for marker assisted selection (Liu and Cordes, 2004; Liu, 2007). Basavaraju *et al.* (2007) found a positive correlation between growth performance and RAPD polymorphism in different stocks of common carp and opined that RAPD polymorphism can be used in marker assisted selection. Inbreeding is a common problem faced at carp breeding centers in India and is responsible for poor growth performance of grass carps (ICAR, 2011). Genetically, inbreeding increases homozygosity in offspring or, in other words, decreases heterozygosity. Estimating the level of heterozygosity and RAPD polymorphism along with growth behaviour of grass carp may provide key information about the genetic status of grass carps. The assessment of genetic diversity in different stock of grass carp not only may give idea about the present genetic status of grass carp but may also help in updating the information on genetic diversity in different stock. This may, in turn, help in developing appropriate strategies for genetic management and improvement. The present study was conducted with the objectives to assess the growth behaviour and genetic diversity in grass carp to know the level of homogeneity/inbreeding (within population).

MATERIALS AND METHODS

Collection and growth behaviour of different stocks grass carp

Four different stocks of grass carp (*Ctenopharyngodon idellus*) fingerlings which are reproductively isolated and present in different environmental conditions for a minimum period of 15-20 years were collected from four breeding centers in India. The stocks were named after the place of their collection *viz.,* Kolkata stock from Shaw aqua India Kolkata (West Bengal), Kaikaluru stock from A to Z Fish Seed Farms, Kaikaluru (Andhra Pradesh), Bhadra Reservoir Project (BRP) stock from National Fish Seed Farm, Shimoga (Karnataka) and Thanjavur stock from Himalayam Aqua Farm, Thanjavur (Tamilnadu). Grass carp fingerlings were transported by road/rail using oxygenated plastic covers filled with ¼ of water and maintained at Fisheries Research and Information Centre, Bangalore (India). The fish were quarantined and acclimatized by following the standard protocols

(Basavaraju *et al.,* 2003*)*. Marking/tagging is important in genetic studies especially when different groups/stocks are to be grown together. The stocks were marked by cauterization of fins as per Basavaraju *et al.* (1998). The left pelvic fin was cauterized for Kolkata stock, the left pectoral fin was cauterized for Kaikaluru stock, and for the BRP stock right pectoral fin was cauterized. Thanjavur stock was not cauterized.

Growth trials were conducted in triplicates in 150 m^2 ponds for 120 days. Ponds were initially limed @ 250 kg ha^{-1}. The fingerlings of all stocks were stocked in the same ponds. The stocking density followed was 100 fingerlings per 150 m^2 pond, comprising of 25 fingerlings from each stock. Stocked grass carp were fed @ 25% of body weight with aquatic plants *Hydrilla* and *Lemna* in equal proportion. Sampling was done at fortnightly interval to check and maintain water quality and health. The feeding schedule was revised after sampling as per requirement. Various water quality parameters such as temperature, dissolved oxygen, pH, alkalinity and free CO_2 were estimated as per Chattopadhyay (1998) and ammonia concentration assessed by standard method (APHA, 1998). Water quality was maintained in optimum range during the whole growth periods. At the end of 120 days growth trial, the body weight and length of all the surviving fishes were recorded. Harming of fishes was avoided and maximum care taken to ensure their well being during the experiment. There was no conflict of interest in carrying out the whole experiment.

DNA extraction and RAPD-PCR analysis

Caudal fin samples were collected as per standard procedure (Basavaraju *et al.*, 2007). For this, a minimum of 30 samples were collected from each stock and a minimum of 10 samples collected from each ponds for each stock with an aim to select samples having good quality DNA. Fin samples were collected at the end of 90 days growth. DNA extraction was carried out by following salt precipitation method (Nguyen *et al.*, 2006) and DNA samples in sterilized double distilled water were stored at -20^0C. DNA quality was check by using agarose gel electrophoresis and spectrophotometer (Biotek, USA). DNA samples having values close to 1.8 (ratio of A_{260}/A_{280}) were chosen for the present study. DNA was quantified at A_{260} in a spectrophotometer (Biotek, USA). A total of 100 decamer primers namely OPA 1-20, OPB 1-20, OPC 1-20, OPD 1-20, OPE 1-20 (Bioserve, India) were used for screening. The screening was carried out for a minimum of five samples from each stock. Primers were selected based on the total number of bands and total number of consistent polymorphic bands. The primers which gave at least 5 bands and at least 2 consistent polymorphic bands were selected for RAPD analysis. The 7 primers fulfilling aforementioned criteria were selected for further studies. Best 7 primers namely OPA 01, OPA 18, OPB 11, OPB 19, OPD 11, OPD18 and OPE10 with primer sequence as CAGGCCCTTC, AGGT GACCGT, GTAGACCCGT, ACCCCCGAAG, AGCGCCATTG, GAGAGCCAAC and CACCAG GTGA, respectively, were selected. RAPD-PCR was carried on 30 samples from each stock. RAPD-PCR was carried out in Mastercycler (Eppendorf, USA) with 25 ng DNA, 2.5 mM dNTP's, 25 mM $MgCl_2$, 10x assay buffer, 10 µM primer, 1U *Taq* polymerase and remaining amount of sterilized double distilled water to the final volume of 20 µL. PCR conditions were maintained as per Nguyen *et al.* (2006) with initial denaturation at 94°C for 3 min., followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 36°C for 1 min. and extension at 72°C for 2 min. Final extension was carried out at 72°C for 5 min. Negative controls were also maintained for each amplification. PCR products were resolved in 1.4% agarose gel having ethidium bromide (80 µL 100 mL^{-1} agarose gel). PCR products were mixed with 10 µL loading dye (30% glycerol and bromophenol blue solution) and 25 µL of this mixture was loaded in agarose wells. Gel electrophoresis was carried out in gel electrophoresis system contain 1X TBE buffer at 100 V and documented using gel documentation unit (BioRad, USA).

The bands were scored visually for their presence or absence, and data collected was analyzed using population genetic analysis software POPGENE (Yeh, 1997). The percentage of polymorphism was calculated for each stock for each primer as per the formula:

$$\text{Percentage of polymorphism} = \frac{\text{Total number of polymorphic bands} \times 100}{\text{Total number of bands}}$$

Heterozygosity was calculated using the formula: $h_j = 1 - p^2 - q^2$ and $H = \Sigma_j^L hj/L$, where, h_j = heterozygosity per locus, p and q = allele frequencies, H = average heterozygosity for all loci and L = total number of loci. Heterozygosity, unbiased measures of genetic identity and genetic distance were calculated as per Nei (1973) and Nei (1978) using the genetic data analysis software POPGENE (Yeh, 1997). Dendrogram was constructed as per genetic distance data using MEGA 5 software (Tamura *et al.*, 2011**)**. Data on growth of all the stocks were analyzed using one way ANOVA. Percentage polymorphism and growth of different stocks were correlated using r^2 (correlation coefficient of determination) to find out the correlation between the percent polymorphism and the mean average body weight attained by different stocks of grass carp.

RESULTS AND DISCUSSION

The grass carp was introduced from China into different states of India, including Karnataka and Tamilnadu, during 1959-1965 (Chandrasekharaiah, 1989; Sreenivasan, 1989; Natarajan and Menon, 1989). However, there is no published literature available on the genetic status of grass carp introduced in different states of India. In present study fish stocks from diverse geographic locations, reared for more than 15-20 years, were procured. These stocks appeared to be the output of varied genetic management practices and different genetic-environmental interactions prevailing in different breeding centers. The growth performance of grass carp revealed significant difference in body weight in different stocks with highest mean body weight (40.83±13.63 g) and survival rate (94%) in Kolkata stock (Table 1). Kolkata stock, followed by Thanjavur stock, showed better growth performance than other stocks. The performance of BRP stock was poor. Since all the grass carp stocks were grown under similar environment and management conditions, the only source of variation possibly appeared to be the genetic variation existing in the stocks. Basavaraju *et al*. (2003) also found such difference in growth rate in different stocks of common carp from different geographical locations. The significant difference in survival rate in different grass carp stocks possibly appears to be the outcome of genetic variation in different stocks.

In the present study 100 primers were screened and 7 best primers, based on their consistency, total number of bands and number of polymorphic bands, were selected (minimum of 2 polymorphic bands). Thirty samples from each stock were used to assess genetic variation using RAPD technique which is sufficient for conclusive results. Dinesh *et al. (*1996) differentiated 3 species of tilapia with 13 RAPD markers using 14 samples from each species. Tassanakajon *et al.* (1997) also used RAPD technique for determining the genetic variation in wild population of black tiger prawn (*Penaeus monodon*) in Thailand and selected 6 best primers out of 200 ten-base primers

Table 1: Mean body weight (g) attained by different stocks of grass carp at end of growth trials

Stocks/Ponds	Pond 1		Pond 2		Pond 3		Overall average	
	Weight ±SD	SR%*	Weight ±SD	SR%	Weight ±SD	SR%	Weight ±SD	SR%
Kolkata stock	34.94±11.81^{1}	96.0^{2}	43.20±15.65^{3}	92.0^{4}	44.40±12.24^{5}	96.0^{6}	40.83±13.63^{7}	94.0^{8}
Kaikaluru stock	32.50±11.65^{1}	84.0^{2}	28.42±13.70^{3}	88.0^{4}	38.57±16.37^{5}	92.0^{6}	33.06±12.16^{7}	88.3^{8}
BRP stock	24.10±08.82^{1}	92.0^{2}	24.73± 06.77^{3}	86.0^{4}	26.95±07.60^{5}	88.0^{6}	25.25±06.74^{7}	89.6^{8}
Thanjavur stock	33.64±14.55^{1}	88.0^{2}	40.50±14.53^{3}	84.0^{4}	43.80±20.99^{5}	84.0^{6}	39.30±16.93^{7}	85.0^{8}
Mean	31.29± 11.70^{1}	90.0^{2}	34.21±12.66 ^{3}	87.5^{4}	38.43±14.3^{5}	90.0^{6}	34.61±12.36^{7}	89.2^{8}

*SR% = Per cent survival rate; SD = Standard deviation;

The columns superscripted with different number are significantly different at 5% level of significance.

screened for RAPD analysis. Barman *et al.* (2003) employed RAPD technique to study genetic variation in 4 Indian major carps, namely catla, rohu, mrigal and kalbasu, by using 40 arbitrary primers and selected 6 primers using 6 samples from each species. Islam *et al.* (2004) using RAPD technique differentiated 3 different natural populations and one hatchery stock of Indian major carp (*Labeo rohita)* in Bangladesh. Further, they chose 5 primers which gave better polymorphism out of 45 primers for screening. Basavaraju *et al.* (2007) analyzed genetic diversity of different stock of common carp (*Cyprinus carpio)* using RAPD technique. Garg *et al.* (2009) used RAPD technique to study the genetic diversity in two different populations of catfish (*Mystus vittatus)* in Bhadwada reservoir at Bhopal and Mohinisagar reservoir at Gwalior of Madhya Pradesh, India and reported genetic variation within and between the two populations while using 10 RAPD primers and 20 samples from each population.

In present study, among the selected 7 primers, OPD 11 and OPD 18 gave a maximum of 8 bands. Primers OPB 11, OPB 19 and OPE 10 yielded 6 bands and all the primers showed better polymorphism in different grass carp stocks (Table 2; Fig. 1). Kolkata stocks showed maximum polymorphic loci of 74.59% and Kaikaluru stock minimum polymorphism of 59.87% (Table 3). Heterozygosity, assessed on the basis of RAPD profile, revealed highest heterozygosity in Kaikaluru stock. The higher polymorphism for Kolkata stock was accompanied by better growth performance. Genetic diversity in different stocks is primarily related to the number of foundation stock. The use of highest number of brooders for seed production in West Bengal may be the reasons for the observed higher polymorphism and better growth performance in Kolkata stock (Nalini and Krishnan, 2011). The quantitative traits like growth are not governed by a single gene but rather involve a number of multi-genes (Tave, 1993). The additive effect alone does not appear to be responsible for better growth in Kolkata stock. Higher polymorphic bands (loci) [2.74 to 14.72% higher] in Kolkata stock may be responsible of its better growth performance in additive fashion. Thanjavur stock also had high polymorphism than Kaikaluru and BRP stocks. Kaikaluru and BRP stocks showed low polymorphism and poor growth performance. Low polymorphism in Kaikaluru and BRP stocks may be attributed to the inbreeding, probably due to the maintenance of lower number of brood stocks in comparison to other stocks. The growth and polymorphism of different stocks were found positively correlated. The r^2 value of 0.64 indicated positive correlation between per cent polymorphism and growth performance of carp stocks. Basavaraju *et al.* (2007) also found positive correlation between polymorphism and growth performance in different stocks of common carp.

Table 2: Number of polymorphic bands, number of monomorphic bands, percent polymorphic bands for the selected seven primers for four stocks of grass carp

Primers/stocks		Kolkata stock	Kaikaluru stock	BRP stock	Thanjavur stock
OPA-01	P	4	3	3	4
	M	2	3	3	2
	%P	66.0	50.0	50.0	66.0
OPA18	P	3	4	4	2
	M	2	1	1	3
	%P	60.0	80.0	80.0	40.0
OPB 11	P	5	2	3	2
	M	1	1	1	1
	%P	83.0	66.0	75.0	66.0
OPB 19	P	5	3	3	5
	M	1	2	2	1
	%P	83.0	60.0	60.0	83.0
OPD11	P	7	4	4	4
	M	1	2	3	2
	%P	87.5	66.0	57.1	66.0
OPD 18	P	6	5	4	8
	M	2	3	2	0
	%P	75.0	62.5	50.0	100.0
OPE10	P	4	2	4	4
	M	2	4	2	1
	%P	66.0	33.0	66.0	80.0

P = Polymorphic loci; M = Monomorphic loci; %P = Percent polymorphic loci.

Table 3: Heterozygosity, number of polymorphic loci and percentage of polymorphic loci for the four stocks of the grass carp

Stocks	Heterozygosity ±SD	Polymorphic loci (No.)	Percent polymorphism (%)
Kolkata stock	0.25±0.19	34	74.59
Kaikaluru stock	0.32±0.15	23	59.87
BRP stock	0.25±0.19	25	62.68
Thanjavur stock	0.24±0.18	30	71.85

SD = Standard deviation

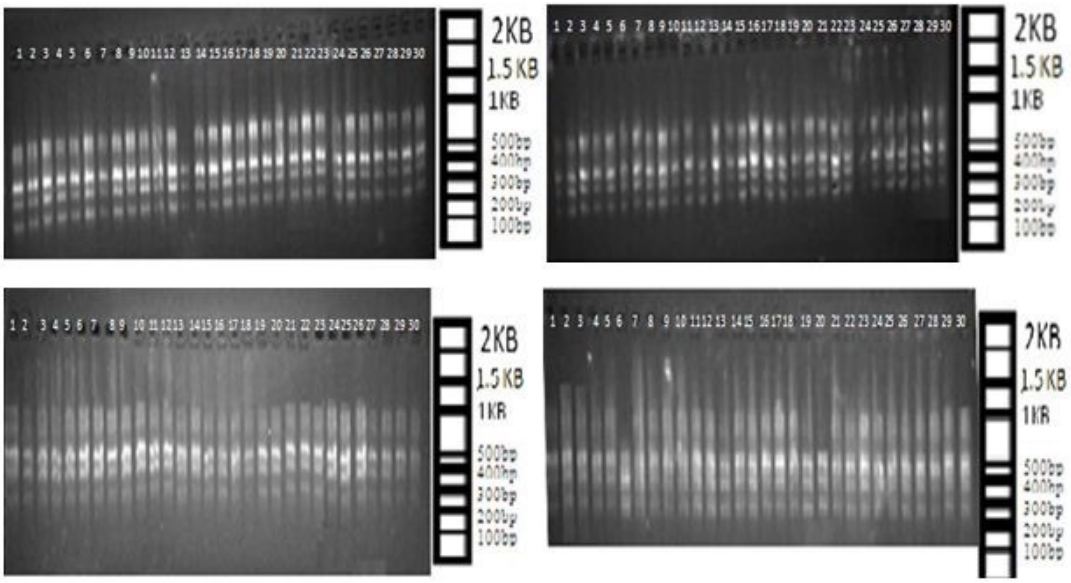

Fig. 1: RAPD profiles of various grass carp stocks using OPA18: Upper left) Kolkata stock, upper right) Kaikaluru stock, lower left) BRP stock and, lower right) Thanjavur stock

BRP and Kaikaluru stocks exhibited maximum genetic distance of 0.2409 and minimum genetic identity of 0.7859 (Table 4). BRP stock originated from Kolkata over several generations so might have gained slightly adapted generations. Further, there was no cross introduction of other stocks in BRP. However, the genetic similarity/divergence may be due to the size and extent of diversity in foundation stock which was higher in Kolkata stock than BRP stock as the seed production was higher in Kolkata than in BRP stock (Nalini and Krishnan, 2011). In randomly collected samples the number of primers used was uniform. Kaikaluru and Kolkata stocks had maximum genetic identity of 0.868 and minimum genetic distance of 0.1416 which may be attributed to cross breeding between Kaikaluru and Kolkata stocks. Thanjavur stock showed higher genetic distance than all other stocks, perhaps due to the non-mixing of other grass carp stocks with Thanjavur stock.

Table 4: Unbiased measures of genetic identity and genetic distance in four different stocks of grass carp

Stocks	Kolkata stock	Kaikaluru stock	BRP stock	Thanjavur stock
Kolkata stock	****	0.868	0.8585	0.8555
Kaikaluru stock	0.1416	****	0.7859	0.8073
BRP stock	0.1525	0.2409	****	0.828
Thanjavur stock	0.1561	0.214	0.1888	****

Genetic identity (above diagonal) and genetic distance (below diagonal).

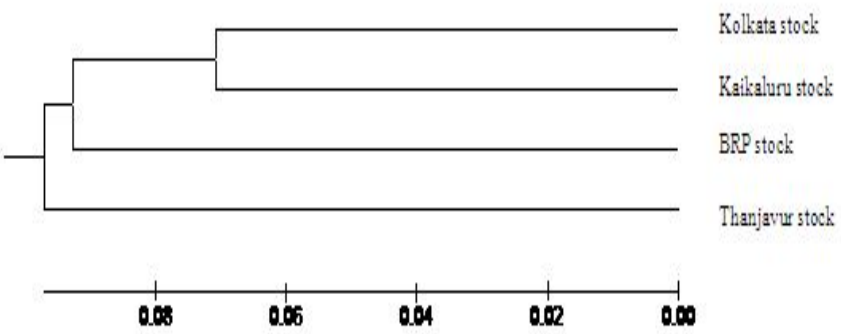

Fig. 2: Dendogram based on unbiased measures of genetic identity and genetic distance in four different stocks of grass carp

The dendogram constructed using genetic distance data clearly differentiated grass carp stocks according to their geographical locations. Only Thanjavur stock fell in a separate cluster, whereas rest stocks formed second cluster. The cluster two was sub-divided into 2 with BRP stock in one sub-cluster and Kolkata and Kaikaluru stocks in other sub-cluster (Fig. 2). Thus, cross breeding Kolkata stock × Thanjavur stock and Kaikaluru stock × Thanjavur stock; which are in different clusters in dendogram appear to be more beneficial. The study indicates that RAPD can effectively be employed to study the genetic variation in different grass carp stocks collected from variable geographic locations/environments.

Acknowledgments: The financial support for present research received through projects funded by RKVY (Rashtriya Krishi Vikas Yojana) and JSYS (Jala Samvardhane Yojana Sangha) in Fisheries Research and Information Centre (KVAFSU) at Hebbal and Hessarghatta, Bangalore is duly acknowledged.

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