GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS OF THREE STOCKS OF *LABEO FIMBRIATUS* FROM INDIAN PENINSULA

Y. Basavaraju[1], A. Narasimha Reddy[1], K. B. Rajanna[2] & N. Chethan[2]
[1]Fisheries Research and Information Centre, Hesaraghatta, Bangalore, Karnataka, India
[2]Fisheries Research and Information Centre, Hebbal, Bangalore, Karnataka, India

ABSTRACT
The Random Amplified Polymorphic DNA (RAPD) was applied to analyze the genetic variation in three stocks of *Labeo fimbriatus* representing Cauvery, Vedavathi and Tungabhadra river streams of peninsular India. A total of 8 primers viz., OPA-07, OPP-11, OPB-17, OPF-01, OPF-03, OPF-12, OPD-19, OPP-16 were used to assay the polymorphisms among three stocks. The genetic relationships were studied based on polymorphisms generated by RAPD-PCR. The STATISTICA dendrogram based on the genetic distance matrix revealed a clear grouping of three stocks of fimbriatus into two clusters where in Cauvery and Vedavathi stocks together formed a cluster while Tungabhadra alone formed a separate cluster. It indicates that both Cauvery and Vedavathi are closely related whereas Tungabhadra was genetically away from other two stocks. The genetic distance within the stocks of Cauvery, Tungabhadra and Vedavathi stocks was 0.05, 0.12 and 0.11, respectively. It indicates that the Tungabhadra stock indicated higher proportion of heterozygous genotypes while Cauvery with more homogenate genotypes. Using this technique, only slight differences in genetic diversity were detected. Further, studying using mitochondrial DNA (mt-DNA) analysis, RFLP fingerprinting, or the sequencing of hyper variable regions of the mitochondrial genome may increase the validity of this conclusion.

KEYWORDS: RFLP fingerprinting, heterozygous genotypes, mitochondrial DNA (mt-DNA), molecular markers, primer, genetic diversity.

INTRODUCTION

Labeo fimbriatus (Bloch, 1795) 'fringed lip' peninsular carp is spread over rivers and reservoirs of peninsular India and forms an important capture and culture fishery in the south and central India. It has got good consumer demand due to its excellent meat quality, taste and it often fetches higher prices than Indian major carps. It is considered as a potential candidate species for freshwater aquaculture. It is a slow growing fish in wild but efforts have been made to induce breed and also to improve the genetic status through inter-specific and inter-generic hybridization (Basavaraju et al., 1990; 1993 and Narasimha Reddy et al., 2010). However, there is a limited knowledge on the genetic diversity of this species and therefore is of considerable importance in understanding the genetic structure of this species. A number of methods have been developed to measure genetic diversity within the species. Although, morphological and meristic traits are considered to discriminate the population of a species, these traits may not have a genetic basis and do not necessarily provide information on genetic and evolutionary relationships (Gall and Loudenslager, 1981). Further these traits are influenced by environment. To overcome these constraints, various 'molecular markers' for the detection of genetic variation have been devised. These 'markers' of genetic variation are generally independent of environmental factors and more numerous than phenotypic characters, thereby providing a clear indication of the underlying variation in the genome of an organism (Avise, 1994). RAPD (Random Amplified Polymorphic DNA) technique is one of the most frequently used molecular methods for taxonomic and systematic analyses of various organisms (Williams *et al.*, 1990). This technique has been widely used due to its rapidity, accessibility and high levels of polymorphism. RAPD has several advantages over other molecular techniques. In contrast to other types of analysis, a small amount of biological material is needed and can avoid sacrifice of the animals studied and previous knowledge of the genome of the species is not required. Further, fresh, frozen or alcohol preserved material can also be used for analysis. The present study deals with the genetic analysis of different stocks of *Labeo fimbriatus* from three peninsular rivers - Cauvery, Tungabhadra, and Vedavathi of Indian peninsula.

MATERIALS & METHODS

Fish stocks and sample collection

The *Labeo fimbriatus* stocks of Cauvery, Tungabhadra and Vedavathi rivers of peninsular India were used for the study. A total of 30 samples from each stock were obtained from the caudal fin clippings and preserved in 95% ethyl alcohol at -20^{o}C till use.

DNA Extraction

Genomic DNA was isolated from fin tissue samples by the phenol–chloroform (Sambrook *et al.*, 1989) procedure with little modifications. Approximately 50 mg of fin clippings was homogenized in 500 µl extraction buffer (8M Urea: 10mM Tris pH 8.0:10mM EDTA pH 8.0:125mM Nacl and 1% SDS), 20 µl Proteinase K (20 mg/ml) and 10 µl (10 mg/ml) RNAse A (Bangalore Genie, India) which was gently mixed and incubated in a water

bath at 55^0C for 3-4 hours with intermittent shaking. After incubation DNA was purified by successive extraction with buffered phenol, phenol – chloroform- isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1) respectively. DNA was precipitated with ice-cold ethanol and 3M sodium acetate (pH 5.2) and kept overnight at -20°C. The DNA was pelleted by centrifugation at 10,000 rpm, 10 minutes at 4°C and washed with 70 % ethanol. The pelted DNA was air dried and resuspended in 50 µl TE buffer (1M Tris HCl, pH 8.0: 0.5M EDTA, pH 8.0), stored at −20°C.

RAPD-PCR analysis

RAPD-PCR reactions were performed as described in Williams *et al.*, (1990). The following oligonucleotide decamer primers were tested in the present study: OPA-07, OPD-19, OPB-17, OPF-01, OPF-03, OPF-12, OPP-11, and OPP-16. RAPD reactions were run in a PCR thermocycler and RAPD products were separated on 2% agarose gels, containing 0.5% g/ml ethidium bromide. Band patterns were photographed under UV light.

Data analysis

RAPD patterns were physically scored on the presence or absence of clear, unambiguous and well separated distinct bands. The scores obtained from all primers in RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, genetic distance and constructing a dendrogram. Dendrogram was constructed by cluster analysis using STATISTICA software (ver. 6.0, India) and was computed based on Ward's method of clustering using minimum variance algorithm.

RESULTS

RAPD analysis was performed on the DNA extracted from fin tissue of three stocks *Labeo fimbriatus* viz., Cauvery, Tungabhadra and Vedavathi. A total of 90 samples (30 samples from each stock) were used for the RAPD study.

RAPD Analysis

Primers were evaluated on the basis of intensity or resolution of bands, consistency within individuals and potential to differentiate the populations. A total of 180 random decamer primers were screened for RAPD-PCR amplification, of which, 8 primers viz., OPA-07, OPP-11, OPB-17, OPF-01, OPF-03, OPF-12, OPD-19, OPP-16 resulted in amplification of more than five bands and were subsequently selected for the analysis (Table 1). The selected decamer primers exhibited good technical resolution with quality banding pattern, which were clear, consistent, and easy to score were used for estimating the genetic diversity and the RAPD profiles generated were used for systematic inference (Fig. 1).

TABLE 1. RAPD primers used for the study

Primer	Sequence (5' – 3')	G+C (%)	T_m (C)
OPA-07	GAAACGGGTG	60	32
OPD-19	CTGGGGACTT	60	32
OPB-17	AGGGAACGAG	60	32
OPF-01	ACGGATCCTG	60	32
OPF-03	CCTGATCACC	60	32
OPF-12	ACGGTACCAG	60	32
OPP-11	AACGCGTCGG	60	34
OPP-16	CCAAGCTGCC	70	34

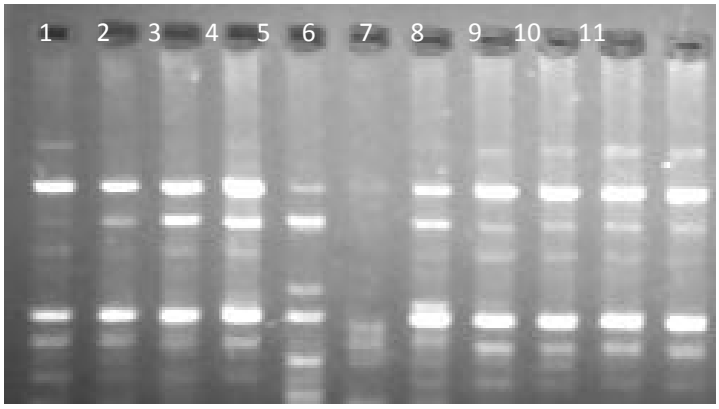

FIGURE 1. RAPD gel profile of *Labeo fimbriatus* with selected primer

The banding pattern in the RAPD profiles of *Labeo fimbriatus* stocks revealed that a total of 107 bands were recorded and out of which, 93 (85.82%) were polymorphic and 14 (13.08%) were monomorphic. The number of bands varied among the stocks and the primers as well but some cases were exceptional. For example, Cauvery and Vedavathi showed similar banding pattern (4 bands) for OPA-07 and OPF-12. The OPF-1 primer recorded similar banding pattern (4) in all the three stocks. In the present study, highest number of bands (7) was recorded in Tungabhadra with OPF-3, while the lowest of three bands was found in Cauvery with OPB-7 and OPP-12 and also Vedavathi with OPF-3. The polymorphism exhibited by different primers and fimbriatus stocks is given in Table 2.

TABLE 2. Total number of amplified fragments, number of polymorphic bands and percentage polymorphism generated by PCR using eight primers

Primers	Band pattern	*Labeo fimbriatus* stocks			Total (Mean ± SE)
		Cauvery	Tungabhadra	Vedavathi	
OPF - 12	P	4	6	4	14 (4.67 ± 0.94)
	M	0	0	0	0 (0.00 ± 0.00)
	%P	100.00[a]	100.00[a] *	100.00[a]	100.00 ± 0.00
OPD - 19	P	4	4	5	13 (4.30 ± 0.47)
	M	0	0	0	0 (0.00 ± 0.00)
	%P	100.00[a]	100.00[a]	100.00[a]	100.00 ± 0.00
OPA - 07	P	4	5	4	13 (4.33 ± 0.47)
	M	0	1	0	01 (0.33 ± 0.47)
	%P	100.00[a]	83.33[b]	100.00[a]	94.44 ± 0.08
OPB - 17	P	2	4	5	11 (3.67 ± 1.25)
	M	1	0	0	01 (0.33 ± 0.47)
	%P	66.67[b]	100.00[a]	100.00[a]	88.89 ± 0.16
OPF - 03	P	4	5	3	12 (4.00 ± 0.82)
	M	1	2	0	03 (1.00 ± 0.82)
	%P	80.00[b]	71.43[bc]	100.00[a]	83.81 ± 0.12
OPP - 16	P	2	5	5	12 (4.00 ± 1.70)
	M	2	0	0	02 (0.67 ± 0.94)
	%P	50.00 [bc]	100.00[a]	100.00[a]	83.33 ± 0.24
OPP - 11	P	1	6	4	11 (3.67 ± 2.05)
	M	2	0	0	02 (0.67 ± 0.94)
	%P	33.33[c]	100.00[a]	100.00[a]	77.78 ± 0.31
OPF - 01	P	0	3	4	07 (2.30 ± 1.70)
	M	4	1	0	05 (1.67 ± 1.70)
	%P	0.0[d]**	75.00[bc]	100.00[a]	58.33 ± 0.42
Average %P		66.25[bc] ± 0.34 (21)	78.85 [b] ± 0.32 (38)	100.00[a] ± 0.00 (34)	85.82 ± 0.13 (93)

Note: Numbers in a column with different alphabet superscripts are statistically significant (*P<0.05).*
P -Polymorphic bands; M - Monomorphic bands
* Primer showing highest percent polymorphism
** Primer showing lowest percent polymorphism

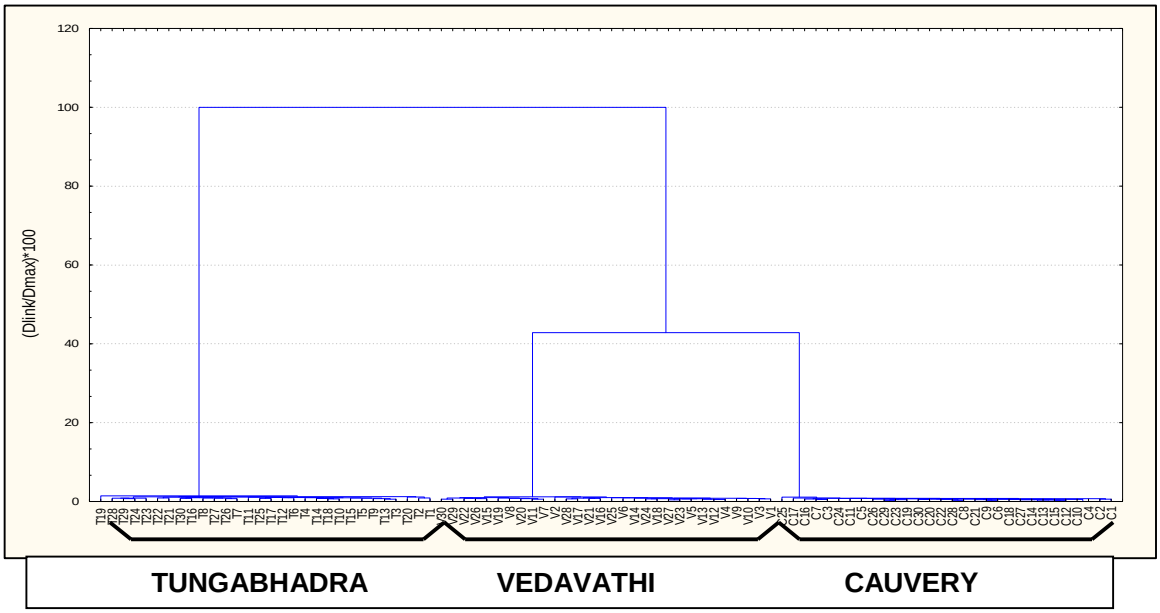

FIGURE 2. Dendrogram of three different stocks of *Labeo fimbriatus*

Genetic distance within the population/stock

Genetic distance was compared among the individuals within a population of *L. fimbriatus* (in all the three stocks). The genetic distance within the population of Cauvery, Tungabhadra and Vedavathi stocks was 0.05, 0.12 and 0.11, respectively. It is the genetic divergence between species or between populations within a species. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship.

DISCUSSION

Polymorphism among the primers

In the present study, eight primers were used of which OPF-12 exhibited high polymorphism (100%) while OPF-1 showed the lowest polymorphism (58.33%). The primers and percentage of polymorphism exhibited (in a descending order) in the analysis of *Labeo fimbriatus* stocks are as follows: OPF-12 (100.00%) > OPD-19 (100.00%) > OPA-7 (94.44) > OPB-17 (88.89%) > OPF-3 (83.81) > OPP-16 (83.33%) > OPP-11 (77.78%) > OPF-1 (58.33%). The percentage of polymorphism exhibited by eight primers for three *Labeo fimbriatus* stocks is given in Table 2.

Polymorphism among the fimbriatus stocks

The mean polymorphism exhibited by all the eight primers for each stock indicated that the Vedavathi stock exhibited the highest polymorphism of 100% and was followed by Tungabhadra stock with 78.85 %, while the lowest polymorphism of 66.25% was observed in Cauvery stock (Table 2). The STATISTICA dendrogram based on the genetic distance matrix for the RAPD markers showed two main clusters (Fig.2). Cluster-1 comprised of Tungabhadra, while cluster-2 consisted of Cauvery and Vedavathi. Results revealed the affinity between Vedavathi and Cauvery stocks besides the isolation of the Tungabhadra stock. However, the Tungabhadra stock was distantly related to other two stocks. However, the cluster-2 of the dendrogram failed to show a clear grouping of Cauvery and Vedavathi stocks according to their origin. It indicates that both Cauvery and Vedavathi are closely related. Genetic diversity or variation and its measurement have vital importance in interpretation, understanding and management of populations and individuals. Wild populations represent the primary source of genetic variability for aquaculture stocks. Reduction of genetic variability may cause greater sensitivity to environmental changes and eventually lead to extinction of a species. Moreover, it may affect growth and reproduction. Maintenance of genetic variability is very important for the conservation of a species. The study of the genetic variation in populations and its change, the following of allele frequencies in populations through time and space, is the main subject of population genetics. In view of the above, present study was under taken to assess the genetic variation in dwindling peninsular carp, *L. fimbriatus* as their density of population decreasing slowly in natural water bodies for various reasons. The genetic distance within the population of Cauvery, Tungabhadra and Vedavathi stocks was 0.05, 0.12 and 0.11, respectively. This means that the Tungabhadra populations indicated higher proportion of heterozygous genotypes while Cauvery had low level of heterozygous genotypes. Thorpe (1982) suggested that the genetic distance values over 0.3 differentiate most of the species. The study on genetic variation of *Labeo dussumieri* (an endangered and endemic cyprinid) from three riverine locations in the Western Ghats, India using allozyme, microsatellite and RAPD markers revealed low level of genetic variability (0.18) within the populations of different river samples (Gopalakrishnan et al., 2009). Similar study on *Eutropiichthys vacha* using RAPD markers indicated that the genetic distance of 0.20 and 0.18 in the population of river Kosi and Ganga, respectively. The low level of genetic heterogeneity in *L. fimbriatus* may be because of recent origin of populations. In effect, there may have been insufficient time for isolation and mutational events to give rise to new alleles and unique genotypes as reported in *C. mrigala* (Chauhan *et al*., 2007). The high level of homogeneity may also be due to limited number of individuals sampled as the population with similar genetic make-up needs more number of individuals and primers for precise differentiation or a significant gene flow within the each stock. Neigel (1997) suggested that the high levels of migration and gene flow between populations increase the similarity of populations. Tungabhadra, a tributary of Krishna, arises in Gangamoola of Varaha Parvatha (located near Kudremukh of Chickmagalore district in Karnataka) in the Western Ghats and mingles with Vedavathi near Sindhanur. It unites with the Krishna River at Gondimalla, near Alampur in Mahaboobnagar District in Andhra Pradesh and finally reaches the Bay of Bengal. Vedavathi, itself a tributary of the Tungabhadra, rises in the eastern part of the Sahyadri Hill range in Karnataka, and joins the Tungabhadra near Mantralayam in Andhra Pradesh. The Cauvery, arises at Talakaveri (in Kodagu District in Karnataka) in the Western Ghats mountain range, runs over a distance of 765 km and ultimately pours into the Bay of Bengal (Fig. 3.1). From the above information, the genetic similarity between Cauvery and Vedavathi observed is suspect, as mixing of these two populations is remote possibility at any location during the course of these two rivers. Though, Cauvery and Vedavathi originate from the same state in the Western Ghats, no linkage or inter connection exists between these rivers. Hence, reasons for the genetic similarity between these two stocks are difficult to discern. However, the probable reasons for mixing of these two populations could also be external and one such reason is restocking programme of reservoirs. In the recent years, ranching programmes have been carried out in rivers/reservoirs so as to boost population size of the dwindling fish species. In Vedavathi, restocking of fimbriatus seedlings had been carried out in the recent years at Vanivilasa Reservoir located at Hiriyur, Chitradurga district of Karnataka as population size of *L. fimbriatus* species was found considerably decreased at this reservoir. However, reliable data on source of the stocked population was not available and hence, reason is difficult to interpret. Another reason may be attributed to the similarity of these two populations: that these two populations probably originated from the same base population. However, no authenticated publications are available for supporting this

reason. In contrast to the above, Tungabhadra and Vedavathi rivers though, arising in the proximate regions of Karnataka, no genetic homogeneity was observed between the two stocks. The genetic makeup of Tungabhadra stands alone from other two stocks viz., Cauvery and Vedavathi. The site of collection of this stock was (Hospet, Tungabhadra reservoir) well isolated from the source of other two stocks. According to Bernardi et al., (2001), the genetic structure of organisms could be the result of habitat connectivity patterns, ecological conditions and dispersal potential. The influence of environmental factors also plays a decisive role in the differentiation of populations. If localized populations of fish inhabit similar environments or remain interconnected through migration and gene flow, they may display more or less homogenous arrays of phenotypic or genetic traits. However, if they are exposed to contrasting environmental conditions and/or only exchanges a few migrants, appreciable population differentiation may arise because of the accumulation of unique mutations in each population due to genetic drift or natural selection. Similar kind of results were noticed in the RAPD analysis of six common carp stocks from different geographical locations, such as India, Indonesia, Hungary and Vietnam (Basavaraju *et al.*, 2007). The four Asian stocks were grouped under one cluster and the two European stocks along with one Asian stock formed another cluster. Although, five stocks were grouped according to their geographical origin, one Asian stock was clustered with the European stocks and no definite conclusion was made on this stock. The RAPD analysis of six stocks of *Labeo rohita* from major hatcheries of different states grouped six *Labeo rohita* stocks into four distinct clusters. The results revealed that the hatcheries belonging to the same state (i.e. the Arrey, Khopoli in Maharashtra and Bhadra, TB dam in Karnataka) formed two separate clusters. Reasons suggested in the study was that the base populations in the hatcheries may have originated from the same source or there may have been an exchange of the stocks as these hatcheries are under one management control (Beena Kumari, 2005). Further, in many of the RAPD analysis, population diagnostic bands (That is, bands present in all individuals of one or more populations and absent in the remaining population of the same species) can be found. But, in the present study, no such bands were detected in any stock of fimbriatus.

CONCLUSION

The present study did not suggest any noticeable level of diversity among the three stocks of *Labeo fimbriatus*. The probable reason could be that all the stocks of fimbriatus may be of recent origin or these stocks may originate from the same base population. However, a continuation of this study using mitochondrial DNA (mt-DNA) analysis, RFLP fingerprinting, or the sequencing of hyper variable regions of the mitochondrial genome may increase the viability of this conclusion. The present study is probably the first step in the genetic characterization of *L. fimbriatus* species. As the population of the species is declining in the recent years for various reasons, the study is of considerable importance in the management of this scarce genetic resource and to protect its population.

ACKNOWLEDGEMENT

The study was carried out under the project financed by Indian Council of Agriculture Research, Govt. of India, New Delhi. We also acknowledge our sincere thanks to Karnataka Veterinary Animal and Fisheries Sciences University for logistic support.

REFERENCES

Avise, J.C. (2004) Molecular Markers, Natural History, and Evolution, 2nd Edition). Sinauer, Sunderland, MA. 684.

Barrero. (2006) Ecological and Evolutionary Responses to Recent Climate Change Annual Review of Ecology, Evolution, and Systematics, 37, 637-669.

Basavaraju, Y. (1993) Studies on the hybridization of Indian medium carp, Labeo fimbriatus (Bloch) with an Indian major carp and the evaluation of progeny in aquaculture. PhD thesis. Bangalore university.

Basavaraju, Y. (1990) Comparative growth of rohu-fimbriatus hybrid with parental species. Paper presented in the second Indian fisheries forum. May, 27-31.28.

Basavaraju, Y., Theertha Prasad., D., Kumuda Rani, Pradeep Kumar, S., Umesha, D., Shrinivas Jahagageerdar, Srivastava, P., Penman, D.J. and Mair, G.C. (2007) Genetic diversity in common carp stocks assayed by random-amplified polymorphic DNA markers. Aquaculture Research 38, 147-155.

Beena Kumari, (2005) Estimation of genetic variation in the hatchery bred stocks of rohu (*labeo rohita*) based on truss and RAPD analysis. M.F.Sc Dissertation. CIFE, Mumbai.

Bernardi, G., Holbrook, S. J. and Schmitt, R. J. (2001). Gene flow at three spatial scales in a coral reef fish, the three-spot dascyllus, *Dascyllus trimaculatus*. Marine Biology 138, 457-465.

Chauhan, T., Lal, K. K., Mohindra, V., Singh, R. K.,. Punia, P , Gopalakrishnan, A., Sharma P. C., and Lakra, W.S. (2007) Evaluating genetic differentiation in wild populations of the Indian major carp, *Cirrhinus mrigala* (Hamilton-Buchanan, 1882): evidence from allozyme and microsatellite markers. Aquaculture, 269, 135 – 149.

Cross, T., Dillane, E., and Galvin, P. (2004). Which molecular markers should be chosen for different specific applications in fisheries and aquaculture? http://www.ucc. ie/ucc/research/adc/molmark/index.

Gall, G.A.E., and Loudenslager, E.J. (1981) Biochemical genetics and systematics of Nevada trout populations. Final Report to Nevada Department of Wildlife, 53 pp.

Gopalakrishnan, A., Musammilu1, K. K., Basheer1, V.S., Lijo John1, Padmakumar3, K.G., Lal, K.K. Mohindra , V., Punia2, P. Dinesh, K. Hashim Manjebrayakath, Ponniah, A.G. and Lakra, W.S. (2009) Low genetic differentiation in the populations of the Malabar carp, *Labeo dussumieri*

as revealed by Allozymes, Microsatellites and RAPD. Asian Fisheries Science 22, 359-391.

Narasimha Reddy, A, Raghunath, M. R., Pradeep Kumar, S. and Basavaraju, Y. (2010) Production and evaluation of an intergeneric hybrid between Peninsular carp Fimbriatus *(Labeo fimbriatus)* and Common carp (*Cyprinus carpio*). Current Biotica, ISSN 0973-4031, 4(1), 1-9.

Niegel, J.E. (1997) A comparison of alternative strategies for estimating gene flow from genetic markers. Annu. Rev. Ecol. Syst. 28, 105-128.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular Cloning. CSH Laboratory Press, Cold Spring Harbor,NY, USA.

Thorpe, J.P. (1982) The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematics. Annu. Rev. Ecol. Syst. 13, 139–168.

Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J. A., and Tingey, S. V. (1990) DNA Polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18, 6531–6535.