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Evaluation of genetic variation in *Labeo fimbriatus* (Bloch, 1795) populations using heterologous primers

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ABSTRACT

The fringe lipped peninsula carp, *Labeo fimbriatus,* is native to lower peninsular region of India. It is a commercially important species widely fished and consumed throughout much of the tropical and subtropical region of south India. Knowledge of genetic diversity of this species is important to support management and conservation programmes which will subsequently help in sustainable production of this species. DNA markers, mostly microsatellites are excellent tools for evaluation of genetic variation of populations. However, development of these markers from each species is time consuming and costly. Therefore, we studied the usefulness of heterologous markers by assessing the genetic variation of three populations of *L. fimbriatus*. We examined population structure of *L. fimbriatus* from different riverine locations in India using rohu microsatellite loci. Out of 30 rohu microsatellite loci, 15 produced amplified products having unambiguous band pattern with clarity in allele scoring. Therefore, these 15 loci were used in 110 samples of *L. fimbriatus* collected from river Krishna (Vijayawada; n= 30), river Kaveri (Bengaluru; n=50) and river Mahanadi (Sambalpur; n=30). Out of 15 loci, only 3 loci were polymorphic in all the populations and remaining 12 loci were found to be monomorphic. The range of expected heterozygosity values for these polymorphic loci was 0.597 to 0.602. One locus did not satisfy Hardy–Weinberg equilibrium (p=.004) in Kaveri population. Negative inbreeding coefficients (F_{is}) were found across populations with no significant population structuring (F_{st}) indicating existence of panmictic populations. As only 3 loci (20%) out of 15 were polymorphic, use of heterologous primers may not be a choice for population studies in *L. fimbriatus*.

Keywords: Heterologous primers, *Labeo fimbriatus*, Microsatellite markers

Introduction

Aquaculture is the fastest growing primary production sector in the world. Being home for more than 10% of global fish biodiversity, India ranks third in the world in total fish production (Ayyappan *et al.,* 2009). Indian aquaculture production comprises mainly three Indian major carps (*Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*). Common carp and other medium and minor carps constitute about 5% of the total freshwater aquaculture production. With the increase in demand for aquaculture foods, there is need for more efficient production systems. Therefore, emphasis is being given to species diversification. Several species of *Labeo,* other than rohu such as *L. fimbriatus, L. calbasu*, *L. bata* and *L. gonius* are being introduced in the culture system as potential candidate species (Jena *et al.,* 2011). *L. fimbriatus*, locally known as 'podosi' is gaining importance for commercial aquaculture. This species is widely distributed in South-East Asia including Indian River systems. Published work on *L. fimbriatus* include evaluation of growth in tanks provided with solid substrates (Mridula *et al.,* 2003), impact of endosulfan (Saravanan *et al.,* 2011) and on life cycle trait pattern like age, growth, maturity and fecundity (Rao, 2011). Limited information is available on the genetic aspect of this species, except for preliminary studies on karyotyping (Biswal *et al.,* 2010).

Knowledge of genetic parameters of the species cultured is vital for sustainability of the production system. More emphasis is being given on genetic improvement of aquacultured species, following the success story of salmon, tilapia and rohu selective breeding programmes (Gjedrem and Baranski, 2009). Owing to high market value, excellent flavor and meat quality, *L. fimbriatus* is being targeted for its genetic improvement in order to increase production efficiency which necessitates proper evaluation of genetic stock that will greatly help to create base population.

Microsatellite markers have been developed for selected Indian fish species such as rohu (Das *et al.,* 2005; Patel *et al.,* 2009), catla (McConnell *et al.,* 2001), chitala (Punia *et al.,* 2006) and mrigala (Lal *et al.,* 2011). Simple sequence repeat (SSR) markers are preferable because they are codominant and highly polymorphic. In addition, microsatellites have a wide distribution in the genome and can be efficiently identified, which is essential in studies on the genetic variability of populations (Balloux and Lugonae, 2002). However, the use of microsatellite markers requires the development of specific primers, *i.e.,* the unique DNA sequences flanking SSR loci. Given that the development of primers can be costly, several studies have tested for possible heterologous amplifications in a target species using primers already described for evolutionarily related species or even for evolutionarily related genera (Zhan and Fu, 2008). Many studies have successfully demonstrated heterologous amplification (Barbosa *et al.,* 2006) thereby lowering the cost of future projects. However, the loci detected by successful heterologous amplification should also be tested for their ability to assess polymorphism in the genome of the target species. Therefore, the aim of this study was to investigate the heterologous amplification of microsatellite loci originally developed from rohu and to evaluate genetic variability in 3 riverine stocks of *L. fimbriatus*.

Materials and methods

Individuals of *L. fimbriatus* were collected from river Krishna (n=30), Mahanadi (n=30) and Kaveri (n=50) between 2009 to 2011 (Fig. 1). DNA was isolated from fin tissues following standard phenol chloroform method (Sambrook *et al.,* 2001) and quantified using 0.8% agarose gel. PCR amplification was done employing heterologous primers (n=30) which were originally isolated from rohu, taking *L. fimbriatus* DNA (25 ng μl^{-1}, each) as template. A touchdown PCR protocol was adopted and amplifications performed in a GeneAmp 9700 thermocycler (Applied Biosystems, USA). PCR was performed in a 10 µl reaction volume containing 25 ng μl^{-1} genomic DNA, 2.5 pmol of each forward and reverse primers, 200 µM of each *d*NTP, 2 mM $MgCl_2$ and 0.2 units *Taq* DNA polymerase (Bangalore Genei, India). Amplification was carried out with the following temperature profiles: one cycle at 94 °C for 5min, 35 cycles at 94 °C for 45 sec, 55 °C for 1.5 min and 72 °C for 2 min and a final extension of 72 °C for 7 min. The PCR products were concentrated on a vacuum concentrator and then added with 5 µL loading dye each (98% deionised formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 95 °C for 5 min and immediately chilled on ice. 4 µl aliquots of each PCR products were analysed on a 6% denaturing PAGE Gel. The samples along with DNA standard, ϕx174/*Hind*III (Finnzymes, Finland) were subjected to electrophoresis at constant power supply of 25 watt for 2 h approximately at a constant temperature of 50 ºC. Gels were silver stained and documented by visual counting.

Descriptive statistics like average number of alleles per locus (A), expected proportion of heterozygotes (H_e), observed proportion of heterozygotes (H_o) and test of Hardy-Weinberg equilibrium (HWE) for each population were estimated using the software GDA, version 1.0, (Lewis and Zaykin, 1999) . The estimates of H_e was unbiased for sample size (Weir and Ott, 1996). Pairwise linkage disequilibrium (LD) among loci was tested using the allele frequency for loci that were in HWE and genotypic frequencies were used to calculate LD of loci that deviated from HWE to prevent interference from within-locus disequilibrium. Assessment of genetic structure was done using the infinite allele model (Kimura and Crow, 1964) and F_{st} values were estimated using co-ancestry identity (Weir and Cockerham, 1984). F-statistics were estimated by bootstrap analysis using 1000 replicates, with a 95% confidence interval, using same software.

Fig. 1. Map showing the rivers and the spots from where the samples were collected (star marks indicate the sampling sites).

Results and discussion

In *L. fimbriatus* only 15 out of the 30 microsatellite markers of rohu were successfully cross amplified (Table 1) and 3 markers showed polymorphism in all the 3 riverine populations. In total, only 9 alleles were found in 110 individuals representing 3 natural populations of *L. fimbriatus*. The average number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e) and p value for HWE per locus per population are presented (Table 2). Further, populationwise genetic diversity measures are also presented (Table 3). For each population, expected heterozygosity (H_e) was generally lower than the observed heterozygosity (H_o) leading to negative inbreeding coefficients (F_{is}). Exact test for Hardy Weinberg Equilibrium (HWE) showed Lro-26 (p=0.004) in Kaveri population to be significantly deviating from HWE at 5% significance level (Table 2). No significant (p>0.05) linkage disequilibrium was detected between different genotypes at each of the different microsatellite loci. Bootstrapping over loci to obtain confidence intervals for F statistic analysis showed no significant levels (the 95% confidence region of these estimates span 0.0) of these estimates (Table 4).

In order to know how the genetic variation is distributed within and among populations, the following hypotheses were tested. The first two null hypotheses tested were that the allelic distribution is identical across populations and between population pairs for each locus. The second two null hypotheses tested were that the genotypic distribution is identical across populations and between population pairs. Though, Mahanadi population was found to be genetically more different from Kaveri and Krishna (Fig. 2; Table 5) the F_{st} values among populations were not significantly different from 0.0.

Table 1. List of rohu microsatellite loci that are amplified in *L. fimbriatus*.

Locus name	Accession no.	Repeat types	Primer 5'-3'		*L. rohita* (product sizes)	*L. fimbriatus* (product sizes)
Lr-03	AJ507520	(TG)19	F	ATC TGG CTG CCT ATT CAC C	155	145
			R	CAT CGG CGA CTG CAC TGG A		
Lr-10	AJ507523	(CA)13	F	GAT CTT CAG CGC CAG CGT G	245	240
			R	GAG GAC CTG CCC AGC ATG		
Lr-24	AJ831438	(TG)17	F	CAA GGC GAA AAG TGT CCA T	165	160
			R	AGG AAA TTG GTA AAG TGT TTC		
Lr-26	AJ831439	(TG)8	F	CCA GGG AGC TGC TAA GAA T	145	140
			R	AGC GCT TCA TGC AGT CTA C		
Lr-28	AM231177	(AC)18	F	TTCACGGACAGATTTGACCCAG	180	170
			R	AGTCTTTTCAGGAGATTAGCAG		
Lr-30	AM231179	(AC)15	F	ACGCGCTAGGGTCGTACAGTG	185	180
			R	CAGCATCATGTTAAGCGCTGTC		
Lr-35	AM269525	(CA)13	F	TGT GAA CAT GCA AGC TCT CAG	155	145
			R	CTA GTC CCA CTC TAG TCA GCA		
Lr-36	AM269526	(CA)10	F	AGC GTG TCT GAT GTG TGA AAG G	175	170
			R	TCA GAT GCC TCC TGC ATT CTG		
Lr-38	AM269528	(GT)12	F	ATA GCA TCA CCA TCT GTT GGT G	150	145
			R	TCT GCT TCA GTC ACT CAG CAC		
Lr-39	AM269529	(GT)11	F	GTT TTT ATT CAG ACA GTC AGA C	160	155
			R	GTG AGA ATC CAG AGT GTC AC		
Lro-6	AM184133	(GT)29	F	TCT CCA CCC TCC ATA CCA CGA	185	180
			R	TGC CCT GCA TTT CTC CCA TCC		
Lro-26	AM184144	(GT)17	F	AGA TCA TTG CTG GGG AGT GTT TAT	205	200
			R	GAC CTG CCT GTG CCA TCT GTA		
Lro-31	AM184147	(GT)22	F	CAT AAT AGC AGT GGC GAG CAG	180	185
			R	AAC CAC CAG CAC ACC TTT CAC		
Lro-37	AM184153	(CA)16	F	ATG TTG TGG TCA TCA TGT AAA TC	170	175
			R	C AGT TTC CTC CCT TCA TAG TTT		

Table 2. No. of individuals (n), no. of alleles (A), alleles per locus (A_p), expected heterozygosity (H_e), observed heterozygosity (H_o), F_{is} value (f) and p value for testing Hardy–Weinberg equilibrium in three populations of *L. fimbriatus*.

Locus name	Parameters	Krishna	Mahanadi	Kaveri
Lr-36	n	24	30	27
	A	3	3	3
	A_p	3	3	3
	H_e	0.642	0.472	0.645
	H_o	0.833	0.433	0.814
	f	-0.304	0.083	-0.268
	p	0.115	0.079	0.265
Lro-26	n	25	27	28
	A	3	3	3
	A_p	3	3	3
	H_e	0.649	0.561	0.592
	H_o	0.520	0.370	0.214
	f	0.203	0.344	0.642
	p	0.064	0.060	0.004
Lr-30	n	28	28	26
	A	3	3	3
	A_p	3	3	3
	H_e	0.575	0.607	0.631
	H_o	0.678	0.892	0.846
	f	-0.182	-0.483	-0.349
	p	0.500	0.078	0.080

Table 3. Descriptive statistics (by population) of *L. fimbriatus,* where n= no. of individuals, A = no. of alleles, A_p = alleles per locus, H_e = expected heterozygosity, H_o = observed heterozygosity, f = F_{is} value (inbreeding coefficient)

River	n	A	A_p	H_e	H_o	f	Bootstrap confidence intervals for f (1000 replications, at nominal confidence interval = 95%)
Krishna	30	3	3	0.622	0.677	-0.089	Upper 0.203
							Lower -0.304
Mahanadi	30	3	3	0.546	0.565	-0.034	Upper 0.344
							Lower -0.483
Kaveri	50	3	3	0.623	0.625	-0.002	Upper 0.642
							Lower -0.349
Mean		3	3	0.597	0.622	-0.042	

Table 4. F-statistics and its bootstrap values of overall populations and loci; where f=F_{is}

Locus name	f	F_{it}	F_{st}
Lr-36	-0.173562	-0.106508	0.057137
Lro-26	0.400378	0.392183	-0.013668
Lr-30	-0.340769	-0.349937	-0.006838
Mean	-0.035292	-0.022204	0.012641
Bootstrap confidence intervals for F- statistics (1000 replications, at nominal confidence interval = 95%)	Upper 0.400 Lower -0.340	Upper 0.392 Lower -0.349	Upper 0.057 Lower -0.013

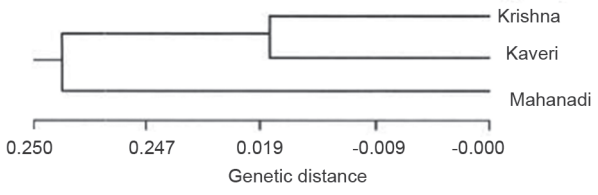

Fig. 2. Dendogram based Nei's (1972) genetic distance summerising variations among 3 populations of *L. funbriatus* using heterologus microsatellite markers

Despite the popularity of microsatellites in the study of population genetics, their development requires substantial time, financial as well as technical resources. Cross-species amplification is an alternative strategy to extend the utilisation of microsatellites across related species. Microsatellite loci generally show considerable evolutionary conservation, suggesting that primers developed for any one species may often be useful across a

Table 5. Pairwise comparison of F_{ST} values (below diagonal) and their bootstrap values of 1000 replications at nominal confidence interval = 95% (above diagonal) among three riverine populations of *L. fimbriatus*.

River	Krishna	Mahanadi	Kaveri
Krishna	0	Upper 0.066	Upper - 0.003
		Lower -0.008	Lower -0.015
Mahanadi	0.019	0	Upper 0.104
			Lower -0.025
Kaveri	-0.009	0.025	0

wide range of taxa. However, one drawback of heterologous primers is that mutations in the flanking sequences, to which PCR primers are designed to anneal, can result in non-amplifying PCR null alleles (Hoffman and Amos, 2005; Selkoe and Toonen, 2006). Null alleles produce an apparent heterozygote deficiency in a sample due to mis-scoring of heterozygotes as homozygotes. Heterozygote deficiency can also reflect various biological processes such as inbreeding, Wahlund effects and selection (Van Oosterhout *et al.,* 2004). It is very important to be able to determine the source of heterozygote deficiency observed in a sample. However, in the present study, only one locus deviated from HWE at 0.05% significance level (Lro-26; Kaveri population).

Present study was conducted with one of the objectives being, evaluation of usefulness of rohu microsatellite loci in population genetic study of *L. fimbriatus*. Though *L. fimbriatus* belongs to the same genus as *L. rohita*, only 15 loci (50%) out of 30 were successfully cross amplified in this species. This finding is inconsistent with the earlier observation of cross species amplification conducted in same genus where nearly 90% loci were cross amplified (Das *et al.,* 2005). Number of polymorphic loci of 3 (20%) found in this study was also very low as compared to rohu loci when checked in *L. bata* (47%) (Patel *et al.,* 2010). This indicates that genome complexity of *L. fimbriatus* is quite diverse from other species of *Labeo* genus. However, large numbers of loci may be needed to support this.

The second objective of this study was to estimate genetic variability of riverine stocks of this species. Obligate freshwater fishes are expected to display greater levels of genetic differentiation and population subdivision than marine species due to the isolating nature of river systems and small effective population sizes (Ward *et al.,* 1994). In the present study, data reveals that the levels of genetic differentiation among populations of *L. fimbriatus* were not significant (Table 4) and estimates of F_{is} for each population was also found to be negative indicating excess heterozygosity. Overall F_{st} for all samples combined was found to be 0.012 indicating approximately 1% genetic variation caused by genetic differentiation of *L. fimbriatus*. Similar finding (2%) was reported in *L. dero* (Chaturvedi *et al.,* 2011) while studying Himalayas at central plateaue of riverine populations in India and Queensland population of *Hypseleotris compressa* (F_{st}=0.014) (McGlashan and Hughes, 2001), but significantly different (F_{st}=0.158) from *L. rohita* populations of Indian rivers when analysed using MtDNA cytochrome b region (Luhariya *et al.,* 2011). Negative F_{is} value and no significant F_{st} among populations of *L. fimbriatus* may be attributed to less aquaculture practice of this species as compared with commonly cultivated species like rohu and catla.

Several evolutionary forces like random genetic drift, migration, mutation and their mutual interactions act on the wild populations and influence the pattern of genetic differentiation (Avise, 1994). Random genetic drift tends to cause genetic differentiation, after subpopulations are fragmented and gene flow between them is either reduced or absent. *L. fimbriatus* is primarily known to be a fringed lip peninsular carp inhabiting parts of rivers in foothill regions that have substrata having rocky bed. It is highly unlikely that in the present geographical scenario, *L. fimbriatus* in the rivers have the chance of intermixing. The rivers studied are more than 200 km apart and no water connection exists among them. Therefore, possible explanation for no significant genetic differentiation among populations, is that *L. fimbriatus* sampled from different river basins in the present study could have common ancestral gene pool. It appears that there has been at least historical connectivity between populations now inhabiting different rivers. Secondly, use of only three polymorphic markers may be highly insufficient to draw valid conclusions. The study suggests that stocks of *L. fimbriatus* are panmictic and genetic variation exists within and between populations.

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