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Note

Mitochondrial DNA markers for population studies in *Labeo rohita*

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ABSTRACT

For the identification of population genetic markers in rohu *Labeo rohita*, universal mitochondrial DNA primers of ND1/16s rRNA and ND5/6 genes were tested and found to amplify particular fragments. Restriction digestion of the amplified products showed polymorphism suggesting its potential as a genetic marker for population studies.

Labeo rohita is widely distributed in freshwater rivers. It has been successfully transplanted to almost the whole of peninsular India as well as to many other continents (Chondar, 1999). *L. rohita* is an essential component of the Indian polyculture system and grown with other major carps. Given its importance, information on its population structure is essential for supporting programmes on domestication and genetic upgradation, as well as management of fishery stocks. It is essential to characterise and quantify the genetic variability of wild stocks. Identification of genetic markers suitable for quantification of genetic variability in this species is the first step in this direction. The present study is a part of a larger programme being carried out on *L. rohita*.

Molecular genetic markers have been used for studies related to conservation, genetic diversity, determination of population structure in wild and cultured population, identification of species and hybrids and genetic impact of intro-

duction of cultured fish on natural population (Foreguson *et al.*, 1995). The mitochondrial and nuclear markers prove invaluable in mapping and linkage analysis, for discriminating between strains and stock discrimination for assessing relationships between genetic variability and fitness. Due to its highly variable nature in most of the species, mtDNA has been extensively utilized as a genetic marker to provide a sensitive means of detecting intraspecific variations useful in quantifying population genetic structure and discrimination of genetic stocks among different geographic populations (Hynes *et al.*, 1996; Hansen and Loescheke, 1996; Chubb *et al.*, 1998). Mitochondrial DNA has a faster rate of evolution, does not recombine, and a smaller effective size (1/4) than that of the genome being haploid and maternally inherited. Thus information on male and female mediated gene flow as well as genetic bottlenecks can be better obtained from its analyses than by using only nuclear markers (Avice, 1994). On the ba-

sis of neutral theory (Kimur, 1983) the mtDNA analysis would detect greater heterogeneity among the study populations. These characteristics lead mitochondria to have a longer retention of the history of past isolation events (Mortiz, 1994). Thus the amount of differentiation among the populations based on mtDNA data would be four times of that of the value calculated by allozyme data (Crease *et al.*, 1990). Values differing from this expectation may reflect differences in dispersal between the sexes because mtDNA is indicative of only female lineages (Awise, 1994).

With the development of polymerase chain reaction (PCR), amplified restriction fragment polymorphism (ARFLP) analysis of mtDNA has become a commonly used technique for population genetic studies. As compared to complete digestion of whole mtDNA molecule or sequencing or use of a probe, this technique is less time consuming and therefore enables screening of a large part of mitochondrial genome and many populations. For this purpose, the elements of NADH complex of the respiratory pathway, ND1/16srRNA, ND5 and 6 of mtDNA have been extensively studied in fishes (Hansen and Loescheke, 1996; Rognan and Guyomard, 1997; Elliot *et al.*, 1998; Chubb *et al.*, 1998; Nielson *et al.*, 1998). Restriction fragment length polymorphism has been observed for farm and riverine populations using whole mtDNA in *L. rohita* (Padhi and Mandal, 1998). Till date, there has been no literature on mtDNA ARFLP genetic markers in *L. rohita*.

The aim of this study was to investigate ND1/16s rRNA and ND5/6 genes of mtDNA regions using universal primers to examine the possibility of using these as genetic markers for studying genetic variations among *L. rohita* populations.

Samples of *L. rohita* were collected from natural populations. Livers were dissected out from the live fishes and stored in liquid nitrogen till DNA isolation. Total DNA was isolated from frozen livers following the method of Stevens *et al.* (1993).

For amplification of ND1/16srRNA and ND5/6 mtDNA segments, universal primers (Cronin *et al.*, 1993) were used. The base sequences of these primers were:

ND1/16s rRNA :-

Forward 5' ACCCCGCCTGTTTACCAAAAACAT 3'

Reverse 5' CGTATGAGCCCGATAGCTTA 3'

ND5/6:-

Forward 5' AATAGTTTATCCAGTTGGTCTTAGG 3'

Reverse 5' TTACAACGATGGTTTTTCATAGTCA 3'

Total genomic DNA (approximately 25-100 ng) was used as template for amplification. PCR reaction was set up in a volume of 50 µl and consisted of 0.05-0.2 µM primer, 0.2mM of dNTPs mix, IX assay buffer (10X = 100Mm TAPS (pH8.8), 500Mm potassium chloride, 0.1% gelatin), 1-3 U of Taq DNA polymerase. Taq polymerase was added after the initial denaturation step during PCR. The reaction mix was overlaid with 25 µl of sterile mineral oil. Amplification cycle conditions were (1) 95°C for 5 min for initial denaturation (1 cycle) (2) 94°C for 30 sec., annealing temperature (at 55°C for Nd5/6 and 50°C for ND1/16s rRNA) for 1 min. 72°C for 1 min 30 sec (30 cycles) (2) 72°C for 10 min (3) soak at 4°C in Perkin Elmer cetus thermal cyler 480. Four microlitre of reaction mix was used to determine the quality and quantity of the amplified product, separated by 1% TAE-Agarose gels incorporated with ethidium bromide and visualized under ultraviolet light.

TABLE 1. Amplified restriction fragments of mtDNA (ND1/16srRNA and ND5/6 of *Labeo rohita*)

Locus	Restriction enzymes												
	Alul		Bsh1	Cfr 131	Hha I	Hinf I		Mbo I	Msp I	Mva I	Rsa I	Taq I	Tru I
	A	B	A	A	A	AB	AB	A	A	A	A	A	A
ND1/ 16s rRNA (n=12)	800	750	1200	750	800	750	750	1250	1400	800	500	1300	600
	600	600	800	550	800	320	320	290	500	700	400	700	500
	350	350		400	400	300	260	240	180	600	350		350
	300	320		300		260	240	220			270		300
							260				260		250
											250		
	EcoRI		Hae III	HinfI	Hha I	Taq I							
	A	B	III	I	I								
ND5/6 (n=4)	1955	2462	708	548	1463	1256							
	507		708	548	919	660							
			441	404	80	568							
			300	329									
			191	260									
			111	224									
				154									

A - most common haplotype
B- polymorphic haplotype

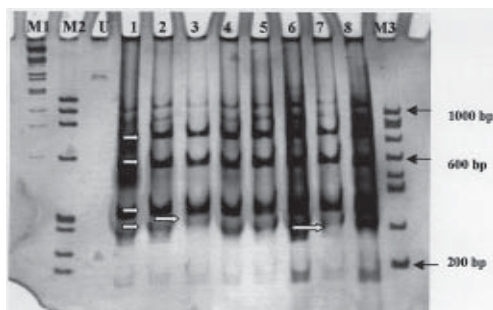


Fig.1. Mitochondrial amplified restriction fragment length polymorphism in ND1 & 16s rRNA genes of *L. rohita* mtDNA with restriction enzyme Alul. M1, M2, M3 - Molecular weight markers. U- Uncut PCR amplified fragment 1-8 different individuals showing four restriction fragments (⇒). 4th and 8th individuals showing polymorphism (⇒).

The PCR amplified mtDNA fragments were used as such for digestions with the restriction endonuclease en-

zymes : 17 (Alu I, Bsh I, Cfr 131, Dra I I, Hae III, Hha I, Hinf I, Kpn I, Mbo I, Msp I, Mva I, Rsa I, Taq I, Tru I, Xba I, Xho I, Bgl I) for ND1/16s rRNA and 11 (Bam HI, Eco RI, Hind III, Hpa II, Hinf I, Hae III, Hha I, Msp I, PVU II, Taq I, Xba I) for ND5/6. Restriction digestion reactions were performed in 100 ul volumes with 100-200 ng of PCR amplified DNA, 1-3 U of enzymes and 1:10 dilution of the manufacturer recommended 10X digestion buffer (Bangalore Genei). Digestion reactions were incubated at 37°C for 3 h except for Taq I and Tru I, which were incubated at 65°C. The digested products were precipitated with 2 volumes of absolute alcohol and 0.2 volume of sodium acetate overnight at -20°C. Restricted DNA was pelleted and washed with 70% alcohol, air dried and redissolved in 10 µl of TE, pH 7.4 (10mM Tris-HCl, pH 7.4; 1 mM EDTA). Restriction digestion products of ND1/16s rRNA were separated by using 6% polyacrylamide gel buffered in IX TAE at 70 V for 2.5-3 h. following elec-

TABLE 2. Composite haplotypes based on amplified restriction fragments of mtDNA (ND1/16srRNA and ND5/6) in *Labeo rohita*

mtDNA segment	Composite haplotypes	% individuals
ND-1/16s rRNA (N=12)	1. AAAAAAAAAAAAAAAAAA	58.3
	2. AAAAAAAAAAAAAAAAAA	25.0
	3. BAAAAAAAAAAAAAAAAA	16.7
ND-5/6 (n=4)	1. AAAAAAAAAA	75.0
	2. BAAAAAAAAA	25.0

Sequence of restriction enzymes :

ND-116srRNA : Alu I, Bsh I, Cfr 13I, Dra I, Hae III, Hha I, Hinf I, Kpn I, Mbo I, Msp I, Mva I, Rsa I, Taq I, Tru I, Xba I, Hha I, and Bgl I.

ND-5/6 : Eco RI, Bam HI, Hind III, Hpa II, Hinf I, Hae III, Hha I, Msp I, Pvu II, Taq I, and Xba I

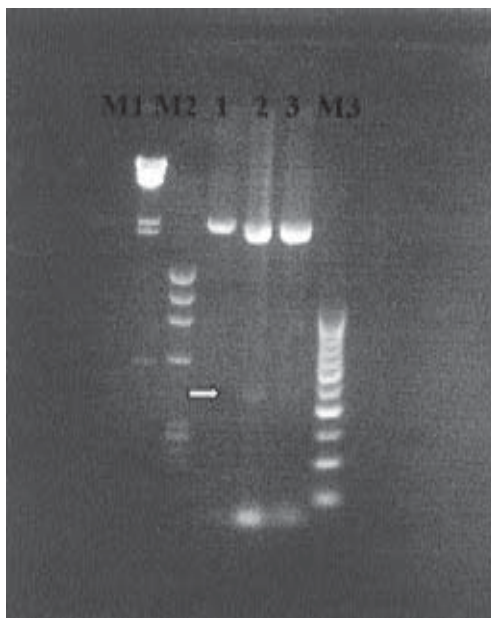


Fig.2. Mitochondrial amplified restriction fragment length polymorphism in ND5/ND6 genes with restriction enzyme EcoRI.

1-3 Different individuals; 1 haplotype A, 2 & 3 haplotype B, arrow (\Rightarrow) showing 507 bp fragment (see text). M1, M2, M3 Molecular weight markers.

trophoresis, restriction patterns were visualized by silver staining (Silver plus one, Amersham Pharmacia, U.S.A). For ND5/6 gene, digested products were ana-

lysed using 1.2% TAE agarose with ethidium bromide incorporated. The images captured with CCD camera were stored in computer and scored for restriction digestion patterns. The sizes of the bands were estimated by interpolation on a standard curve estimated from standard molecular weight markers (λ DNA Hind III/Eco RI cut and 100 bp ladder) with the Biovis 1D Software (Expert Vision Labs Pvt. Ltd., Bombay, India).

PCR products obtained from the amplification of ND1/16srRNA & ND5/6 was approximately 2.0 kb & 2.5 kb in size, respectively. Table 1 shows size estimates for all fragment patterns resulting from the restriction analysis of *L. rohita* mtDNA segment ND1/16srRNA & ND5/6 genes. The fragments smaller than 200 bp were not included in the restriction pattern. The differences in the approximate molecular weight of the undigested PCR product and the sum of restriction fragments may be due to this reason.

In ND1/16s rRNA PCR amplified fragment, out of seventeen enzymes tested, six (Dra-I, Hae-III, Kpn-I, Xba-I, Xho-I) have no restriction site. Banding patterns of ALu-I (Fig.1), and Hinf-I detected polymorphic individuals. Total three types of haplotypes were recognized (Table 2) in this region. For ND5/6, out of 11 restriction enzymes used no site was

present for Bam HI, Hind III, Hpa II, Msp I, Pvu II and XbaI. Two haplotypes with EcoRI, A : 1955 and 507 bp and B : 2462 bp (Fig. 2) were observed.

Previous studies have involved RFLP as measurement of genetic variation in genetically discrete populations (Hall and Nawrocki, 1995, Hynes *et al.*, 1996, Hansen and Loescheke, 1996). In the present study, we have identified markers of amplified restriction fragment length polymorphism in ND1/16s rRNA and ND5/6 mtDNA region. As mtDNA supplies information, which could not have been obtained using nuclear markers, this study indicates possibility of using this in measurement of genetic variation in a particular population and stock identification, in addition to the nuclear markers, such as allozymes and microsatellites already identified in *L. rohita*. The combined use of nuclear and mitochondrial genetic markers may provide a more comprehensive view of population structure.

Acknowledgements

Authors acknowledge the technical assistance rendered by Mr. M. Ponnuraj, Mr. Akhilesh Mishra and Mr. Rajesh Kumar for this work.

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