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Genetic analysis of cyprinid species from Kashmir Valley based on PCR-RFLP (Restriction fragment length polymorphism)

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Abstract
DNA material was collected from the three cyprinid species viz. *cyprinus carpio var. communis*, *cyprinus carpio var. specularis* and *Carassius carassius,* found in different water bodies of Kashmir valley. To analyze the population level genetic variations molecular approach viz restriction digestion of mitochondrial *Cyt b* (PCR-RFLP) was used for 25 samples of each species. Further Experiments were conducted to assess the efficiency of PCR-RFLP marker systems as molecular tools for genetic variation of three cyprinid species. Total genomic DNA was extracted and used for PCR amplification of the *Cyt b* region. The purified PCR products were subjected to single digestion with *Mbo*I, *Msp*I, *BsuR*I and *Hin6*I enzymes and visualized on 1.5% agarose gel. The phylogenetic analysis demonstrated that scale carp (*Cyprinus carpio communis*) is the closest to mirror carp (*Cyprinus carpio specularis*) than from crucian carp (*Carassius carassius*).

Keywords: *Cyt b*, PCR-RFLP, Polymorphism, Phylogenetic

Introduction
DNA methods based on polymerase chain reaction (PCR) amplification have been successfully used to characterize genetic variability in the white-clawed crayfish (Amouret *et al*., 2015) [2]. PCR-restriction fragment length polymorphism (PCR-RFLP) consists of the amplifi- cation of a DNA fragment, followed by restriction enzyme treatment and electrophoretic separation. Specific band profiles allow detecting variation at the DNA level without costly DNA sequencing. PCR-RFLP analysis of mitochondrial DNA (mtDNA) has been used to assess genetic diversity in several crustacean species, such as lobsters (García-Rodríguez *et al*., 2006) [9], mitten crabs (Cho *et al*., 2014) [7].
In fishes RAPD and RFLP (Restriction Fragment Length Polymorphism) markers are among the molecular markers used to analyze genetic diversity. The development of molecular methods has permitted genomic analysis and made it possible to analyze existing variation, both in regions that encode genes, as well as in regions with unknown functions (Regitano, 2001) [14]. Genetic monitoring is ideal for use in a reproduction program with the aim of genetic conservation (i.e., stocking). Molecular markers are a realistic and useful tool for the investigation and monitoring of genetic conditions both in native populations and in captive lots (Alam and Islam, 2005) [1]. PCR-RFLP of Cytochrome *b* gene is an efficient marker for identification of fishes and size of *Cyt b* fragment is 1140bp (Durand *et al*., 2002) [8].
Since the fishes have been introduced into the Kashmir and their ancestry and origin is not known. Therefore, it is imperative to study their phylogenetics as there is no data with regard to phylogenetics of common and crucian carp, the present study will be undertaken to study the genetic diversity in *Cyprinus carpio* & *Carassius carassius* species of Kashmir using PCR-RFLP of mitochondrial Cytochrome *b* gene with the following objectives: To study the genetic variability of two cyprinid species by restriction fragment length polymorphism (PCR-RFLP) of Cytochrome *b* gene.

Materials and Methods
Species and phenotypes studied: Species and phenotypes of family *Cyprinidae* used in this study.

DNA extraction: DNA was extracted from fine tissue (muscle) of each genus following the method described by (Bardakci and Skibinski, 1994) [4].

PCR amplification of mitochondrial genes

For phylogenetic studies published on mitochondrial genes (Cyt *b*) was used. The following set of primers was used.
Cyt b (Briolay *et al.,* 1998) [6]:
Forward- (5' GTT TGA TCC CGT TTC GTG TA 3')
Reverse- (5' AAT GAC TTGAAGAACCACCGT 3')

Purification of PCR Products

Purification of amplified PCR products was done by low melting agarose gels or by commercially available kits.

PCR- RFLP of *Cyt b* gene

Purified products of *Cyt b* gene was used for restriction digestion analyses. Purification of the PCR products was done by eluting it from low melting agarose gel as discussed above. Four different Restriction endonuclease enzymes viz., MboI, BsuI, MspI, Hin6I (Fermentas, Germany) was employed for the digestions of amplified *cyt b* gene Table 1.

Table 1: Following Restriction endonuclease enzymes was used

Restriction enzyme	Type	Source	Reaction conditions
MboI	Tetra-cutter	Moraxella bovis	37 °C
Hin6I (HinP1I)	-do-	Haemophilus influenzae RFL6	37 °C
BsuRI (HaeIII)	-do-	Bacillus Sutilis	37 °C
MspI (HPaII)	-do-	Moraxella species	37 °C

Detection of PCR products by Agarose gel electrophoresis

The PCR products were electrophoresed in 1.5% Agarose gel was stained in ethidium bromide (conc. 0.5µg/ml) and photographed using Gel Doc system (Alpha-Innotech, U.S.A).

Results

The 680bp PCR product of partial mitochondrial cytochrome gene was individually amplified by using primers *Cyt*F1 and *Cyt*R1 (Briolay *et al*., 1998) [6]. To exclude the intra specific diversity, 25 specimens in each species were investigated. Four different Restriction endonuclease enzymes viz., MboI, BsuI, MspI, Hin6I, DdeI, and AluI (Fermentas, Germany) was employed for the digestions of amplified *Cyt b* region presented in Fig 1, 2, 3.

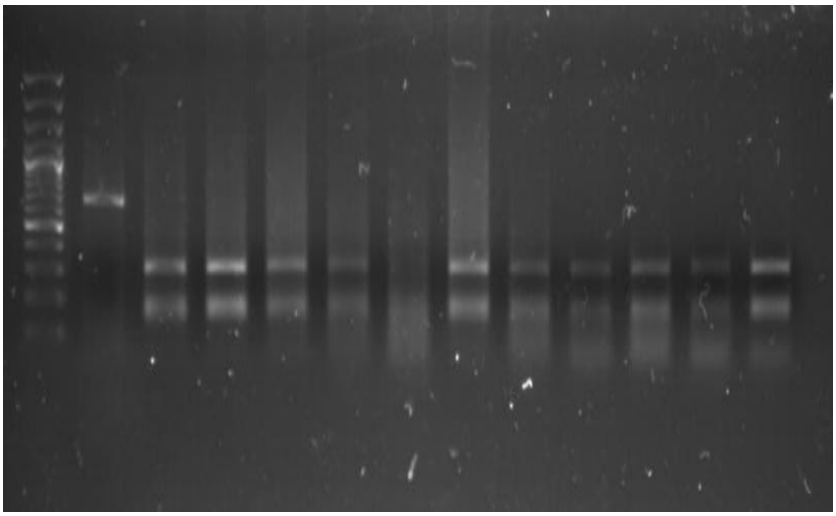

Fig 1: 1.5% Agarose gel, DdeI, 100bp ladder, uncut, SC-1, SC-2, SC-3, SC-4, SC-6, SC-7, SC-8, SC-9, SC-10, SC-11, SC-15

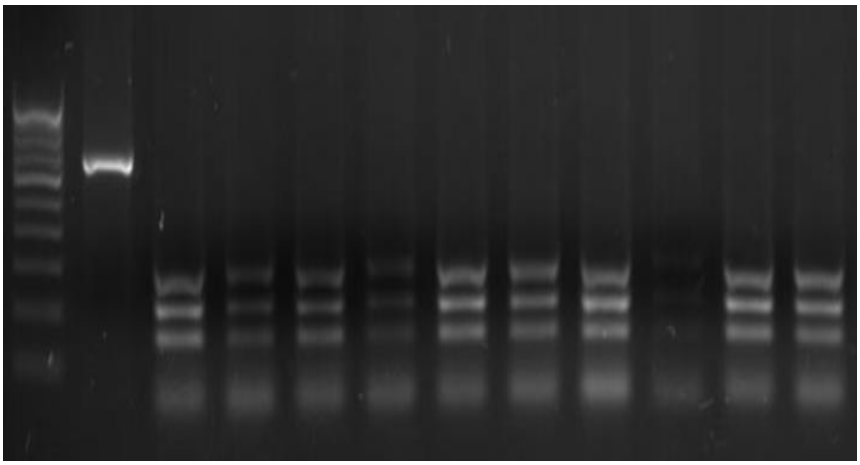

Fig 2: 1.5% Agarose gel, AluI, 100bp ladder, uncut, MC-16, MC-17, MC-18, MC-19, MC-20, MC-21, MC-22, MC-23, MC-24, MC-25

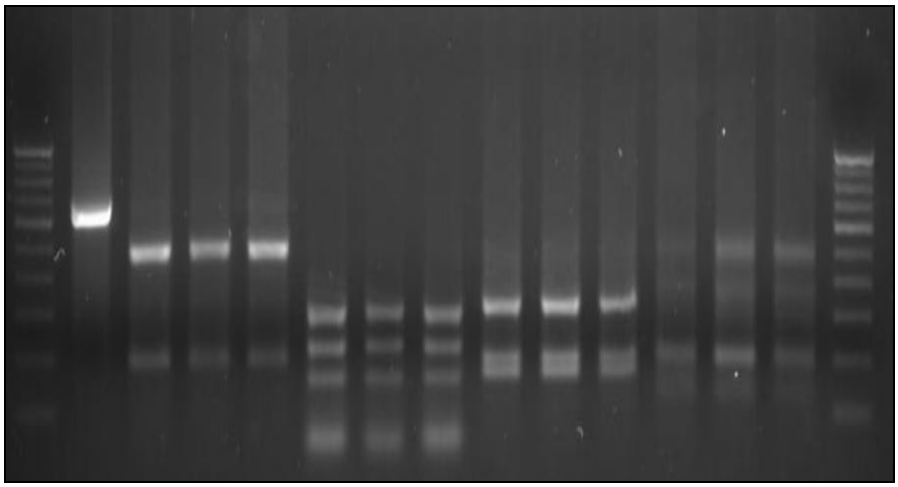

Fig 3: 1.5% Agarose gel, MboI, Bsu, Msp, Hin, 100bp ladder, uncut, CC-17, CC-18, CC-20

Diversity based on Shannon's index (H) of combined fragment size patterns averaged with four restriction enzymes was 0.58 with values ranging from 0.23 to 0.29 which is attributed to differences among the populations. For further analysis, one way ANOVA was performed for all the three species. The results after compairing total no of bands by four restriction enzymes, revealed 100% of variation between the species while no variation was found within the species Table 2. Also, the result obtained by Arlequin 3.1 software package (Nei, 1987) [11] was analysed for haplotype diversity of the three species. The mean nucleotide diversity of the three species was 1.0000±0.0113 and the value of mean haplotype diversity was 0.0000±0.0000. As expected the value for nucleotide diversity were similair (1.0000±0.0113) for all the three species. It is evident that the low number of nucleotide substitutions between the haplotypes determined the low indices of genetic diversity (1.0000±0.0113) in the pooled sample of cyprinid species as shown in Table3.

Table 2: ANOVA Table for RFLP analysis

Source of variation	DF	Sum of squares	Variance components	Percentage of variation	
Between species	2	316.667	6.33333Va	100.00	Fst = 1.00000
Within species	72	0.000	0.00000Vb	0.00	P-value =0.00000±0.00000
Total	74	316.667	6.33333		

Table 3: Values of Haplotype diversity and Nucleotide diversity

Sample	Haplotype	Sum of square frequencies	Gene diversity
Scale carp	110010100000010101011	0.0400	1.0000 ± 0.0113
Mirror carp	111000011010010110000	0.0400	1.0000 ± 0.0113
Crucian carp	110101100101101000110	0.0400	1.0000 ± 0.0113

For cluster analysis UPGMA dendrogram revealed that Crucian carp i.e.Cc-1 to Cc-25 distributed in III cluster displayed maximum dissimilarity coefficient of 0.38 with other individuals, while, other two species i.e. Scale carp and Mirror carp clearly distributed in two clusters I & II resp. shown in Fig. 4. The distribution of different individuals revealed that there were 25 individuals in cluster I i.e., Sc-1 to Sc-25 in cluster II i.e., Mc-1 to Mc-25 as shown in Table2. The PCR-RFLP results showed no polymorphism within the species. However, the marker clearly helps in diversifying the species.

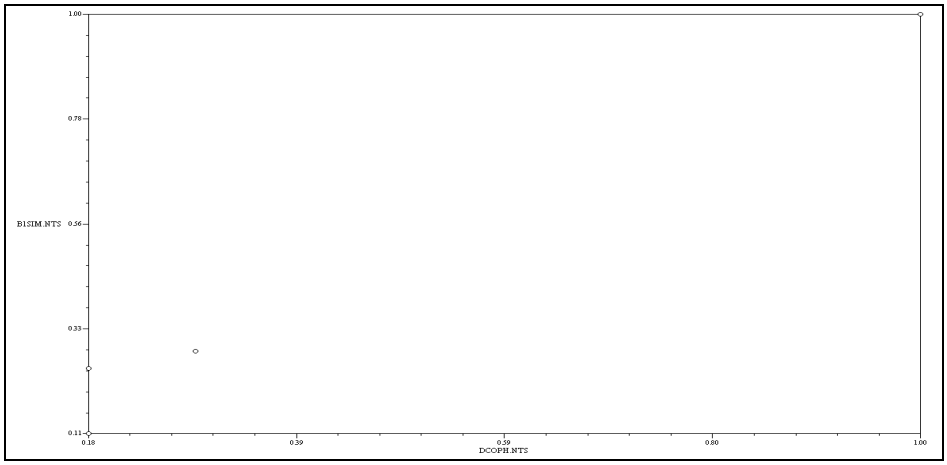

Fig 4: Scatterplots: a coordinate graph of data points for PCR-RFLP analysis

Discussion

PCR-RFLP of cytochrome b gene has been employed efficiently for the study of genetic variations, identification and resolving taxonomical ambiguity of closed related fish species. The same study was done by (Wen *et al.* 2005) [15] used Sequence analysis and polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) technique to identify the species of *Thunnus thynnus*, *T. alalunga*, *T. obesus* and *T. albacares*. In our study, comparable levels of genetic polymorphism (50.0, 72.7, 87.5 and 87.5%) were obtained by using four restriction endonucleses (MboI, Hin6I, Bsu1 and MspI).

Our results do showed intraspecific variations using the PCR-RFLP of *Cyt b* gene. The mean nucleotide diversity of the three species was 1.0000±0.0113 and the value of mean haplotype diversity was 0.0000±0.0000. As expected the value for nucleotide diversity were similar (1.0000±0.0113) for all the three species. The result also reported by (Nei,1973) [10] evaluated interpopulation polymorphism through the calculation of means of the differences between the haplotype pairs in the sample and of nucleotide (π) and haplotype (h) diversity (Nei, 1973, 1987) [10, 11]. It is evident that the low number of nucleotide substitutions between the haplotypes determined the low indices of genetic diversity (1.0000±0.0113) in the pooled sample of cyprinid species. The diversity was comparable with those obtained earlier based on RFLP analysis of mtDNA (Oleinik and Polyakova, 1994) [12]. Although PCR-RFLP of *Cyt b* cannot help us to resolve the exact phylogeny of species but there is a considerable intraspecific variation in the *Cyt b* regions of cyprinid species. mtDNA variation can resolve relationships of species that have diverged as long as 8-10 million years before present (Peacock *et al*., 2001) [13].

Restriction enzymes MboI and MspI showed higher *Cyt b* variations than that by Hin6I *and* BsuRI. In our study Mitochondrial DNA analysis has proven a powerful tool for assessing intraspecific phylogenetic patterns in cyprinid species as also reported by (Bernatchez *et al*. 1992; Avise, 1994) [5, 3].

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