
MITOCHONDRIAL DNA VARIATION IN FOUR SPECIES OF SNAKEHEADED FISH CHANNA

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Hindu College, Moradabad**ABSTRACT**

We assessed genetic variation in D-loop region of mtDNA of four species of *Channa* (Gronovius) viz., *Channa punctatus*, *C. gachua*, *C. striatus* and *C. marulius*. The primer sequence F- TTF: 5' GCC TAA GAG CAT CGG TCT TGT AA 3' and R- 12R: 5' GTC AGG ACC ATG CCT TTG TG 3' was used for amplification. We got a lot of size variation in the length of PCR product ranges 789-1369bp. RFLP analysis was done with Tsp 5091, Nla III and Rsa I. The RFLP patterns obtained for each enzyme show large amount of inter-specific genetic variation. All the species show species specific RFLP patterns with minimal amount of similarity.

Key Words- D-loop, *Channa*, PCR, heteroplasmy, RFLP.

INTRODUCTION

Snakeheads, are primary freshwater edible fishes, belong to the family *channidae* and commonly known as murrels. In India, the genus *Channa* (Gronovius) is represented by nine valid species. Most of them are locally distributed in northeastern and southern region of India (Day, 1876). Four species namely *Channa punctatus*, *C.gachua*, *C.striatus* and *C.marulius* are commonly found throughout the India and commonly known as Sowl or Sowly. The study in the research field of taxonomy and systematics is very useful and significant for various aspects of fish and fisheries. Taxonomically, all the channid species are very distinct to each other and are good example of phenotypic homoplasticity. Recently, various studies on evolutionary interrelationships among Indian channid species based on protein polymorphism, and morphological variations have been attempted by different workers (Singh *et al.*, 2002 and Vishwanath *et al.*, 2009).

Several Mitochondrial DNA regions have been well studied in fishes, as the knowledge of universal primer sequences (Kocher *et al.*, 1989; Meyer *et al.*, 1990; Cronin *et al.*, 1993; Palumbi, 1991) for amplification and sequencing has made them very accessible. We selected control (D-loop) region of mitochondrial DNA to study genetic variations by using the technique Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Earlier studies have been made on this region suggest that it shows a great degree of genetic divergence in most of the fish species like *Gymnocephalus cernus*, lake ruffle (Carol Stepien, 2003); paddlefish, *Polyodon spathula* (Szalanski, 2000); European brown trout (Bernatchez *et al.*, 1992), and found it suitable for high-resolution analysis of evolutionary processes and population genetic structure to evaluate genetic relatedness at the lower rank of hierarchy. The objective of the study was focused on the assessment of inter-specific variation in mtDNA control region of snakehead fish and to investigate the utility of PCR-RFLP method in population and evolutionary studies.

MATERIALS AND METHODS

Sample Preparation: Fin tissue samples were collected from 56 individuals of four species of *Channa*, *C. punctatus*, *C. gachua*, *C. marulius* and *C. striatus* from various fishing sites of Meerut district, Uttar Pradesh. Fin tissues were stored in 90% ethanol.

Isolation of Total Genomic DNA: Total genomic DNA was extracted from the fin tissue using phenol-chloroform method (Sambrook *et al.*, 2001). DNA quantity was measured using UV spectrophotometer (Thermoscientific). The quality of DNA was checked out by electrophoresis on 0.8 % agarose gel. Comparatively good quality DNA samples were used for amplification.

Amplification of Mitochondrial D-loop Region: We used degenerative primers with DNA sequence “F- TTF: 5’ GCC TAA GAG CAT CGG TCT TGT AA 3’ and R- 12R: 5’ GTC AGG ACC ATG CCT TTG TG 3’” (Sivasundar *et al.*, 2001 and Batista *et al.*, 2006) for amplification of D-loop region. PCR reaction was carried out in 50.0 µl reaction volume containing 1X PCR buffer with 1.5 mM MgCl₂, 200 µM dNTP mix, 10 pM of each Primer, 1 unit of Taq Polymerase, and 50 ng of genomic DNA. All the PCR reagents were procured from Bangalore Genei, India. The PCR was carried out in master cycler gradient PCR machine (Eppendorf). The reaction was programmed first for denaturation at 92°C for 2.0 min. followed by 35 cycles of 92°C for 1 min., 57°C for 1.5 min., 72°C for 1min. and the final extension step at 72°C for 10 min.

RESTRICTION ENZYME ANALYSIS: RsaI, NlaIII and Tsp5091 restriction enzymes (Fermentas) were used for digestion in all four species of *Channa*. The digestion was carried out according to the instructions of manufacturer.

DATA ANALYSIS: The size of PCR and RFLP fragments were studied and determined by the comparison with DNA marker (1 kb ladder and 100 bp ladder) with the help of the program given in the Gel doc. System (Alpha infotech computation). The frequency of Length variants in each species was determined. The number of haplotypes for each enzyme and composite haplotypes for all three enzymes and their frequency was estimated for each species in each species.

RESULTS AND DISCUSSION

The PCR amplification of D-loop region of mtDNA has been carried out in *C. punctatus*, *C. gachua*, *C. marulius* and *C. striatus*. In the present study, we obtained PCR products with in channid species with size range 789- 1369bp, different from the expected size as reported, 1277bp in the freshwater fish *Prochilodus* (Sivasunder *et al.*, 2001) and 1037bp in pimelodid catfish, *B. rousseauxi* (Batista *et al.*, 2006). The smallest fragment was obtained in *C. gachua* and the longest fragment was there in *C. punctatus*. We found extensive length variation in the size of PCR products not only among the species but also within the each species. The frequency and the rate of length variants in each species are shown in the (table 1).

The D-loop region is the most variable region of fish mitochondrial DNA (Meyer, 1993). Because of the reduced functional constraints, some portions of the control region evolve much faster than the average mitochondrial sequence (Brown, 1985). The term heteroplasmy explains this kind of length variations and can be defined as a state in which the number of different mitochondrial genomes i.e., mtDNA D-loop types, as distinguished by the length of PCR product, are found. It can occur at both inter-and intra-individual level (Ludwig *et al.*, 2000). In fish, length variation is not a rare phenomenon, despite the small size range of the mtDNA molecule (Billington and Hebert 1991; Meyer, 1994) and has been widely studied (Bentzen *et al.*, 1988; Mulligan and Chapman 1989; Wirgin *et al.*, 1989; Buroker *et al.*, 1990; Arnason and Rand 1992; Rand 1993, Brown *et al.*, 1992). In the present study heteroplasmy is clearly shown by the PCR profile of D-loop region species of snakehead fish (fig. 1).

RFLP analysis has been done by using three restriction enzymes, NlaIII, RsaI and Tsp5091 which recognize the 4bp restriction site. We obtained species specific RFLP pattern of each species for each restriction enzyme. The enzyme Tsp 5091 revealed total nine digestion patterns A, B, C, D, E, F, G, H and I in all species. This enzyme shows high degree of polymorphism in *C. punctatus* with five different haplotypes. In *C. gachua* no polymorphism was observed while in *C. striatus* two different haplotype were found. In *C. marulius* amplified products were remain undigested (Table 2, Fig.2). The enzyme Nla III revealed total six RFLP patterns A, B, C, D, E and F in all four species of *Channa*. In *C. marulius* and *C. gachua* no polymorphism was observed (Table 2, Fig.3). The enzyme Rsa I revealed low degree of polymorphism in the Channid species. Total five haplotypes were observed in three species. Only *C. marulius* show polymorphism in the digestion pattern with this enzyme. The PCR products of *C. gachua* were not available for the digestion with this enzyme. No digestion has occurred in *C. striatus* (Table 2, Fig.4).

Total 11 composite haplotypes were observed on digestion of PCR products of D-loop region by three enzymes, Tsp 5091, Nla III and Rsa I. *C. punctatus* was observed to be highly polymorphic in comparison to other species of *Channa*. Maximum six composite haplotypes were observed in *C. punctatus*, while in *C. gachua* only one haplotype was observed. Both *C. striatus* and *C. marulius* contain four haplotypes, two in each. The frequency and rate of the composite haplotypes are shown in table 3.

The sum of fragment sizes of all haplotypes was found to exceed the size of PCR product (789-1369 bp) in all the species of *Channa*, studied which is compatible with partial digestion and/ or heteroplasmy. Our experiments support the presence of heteroplasmy in Channid species. The further sequence studies can provide the valuable information about the evolutionary dynamics of D-loop region as well as evolutionary trends among the species of *Channa*. In 2005, Xia li *et al.*, reported molecular phylogeny of 20 species of freshwater fishes of family Channidae, Nandidae, Badidae and Osphronemidae on the basis of mtDNA sequences of approximately 1.5 kbp including the NADH dehydrogenase subunit 2 (ND 2) gene and its flanking gene regions.

To the best of our knowledge, this study is the first attempt to assess genetic variation in D-loop region among four channid species not only in the India but in the world. In the present study we found a large amount of genetic variation in the control region (D-loop) by using PCR-RFLP method of analysis among the four channid species. The detection of total 23 bands in PCR-RFLP pattern of four species of *Channa* with only three enzymes suggests usefulness of the method in studying genetic variations among the species. Species specific RFLP pattern have the potential to be used in identification. In the present study the amplification of D-loop region yields different sizes of fragment in the PCR reaction with same primers which clearly shows the usefulness of this region in identification of the species of *Channa*.

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Table 2. Fragment size estimates (in base pairs) of different haplotypes observed in RFLP pattern of mtDNA D-loop region digested with restriction enzymes (Tsp5091, Nla III and Rsa I) among four species of *Channa*.

	Tsp 509I									Nla III						Rsa I					
	A	B	C	D	E	F	G	H	I	A	B	C	D	E	F	A	B	C	D	E	F
1200	-	-	-							1339				-		1159		-	-		
931	-							-		931		-		-	-	1105	-	-		-	
824			-			-				800		-	-			1093				-	-
750	-	-	-	-	-					750		-				1000	-				
700									-	600			-	-	-	680	-	-		-	-
600	-	-	-	-						550	-	-				450			-		
500	-	-	-	-	-			-		500	-					400	-			-	
450						-				430			-	-		389				-	-
350	-					-				400				-	-						
260	-		-		-			-		300	-	-			-						
250								-	-	250	-	-									

Table 3. Composite haplotypes, haplotype frequency, rate of occurrence and sample size of four species studied.

Haplotype	Tsp 5091	Nla III	Rsa I	<i>C. punctatus</i>	<i>C. gachua</i>	<i>C. striatus</i>	<i>C. marulius</i>
Type1	C	F	E	1	0	0	0
Type2	E	F	F	1	0	0	0
Type3	C	E	E	7	0	0	0
Type4	B	E	F	1	0	0	0
Type5	A	E	F	3	0	0	0
Type6	D	E	F	1	0	0	0
Type7	F	D	-	0	14	0	0
Type8	I	C	D	0	0	1	0
Type9	H	B	C	0	0	13	0
Type10	G	A	A	0	0	0	7
Type11	G	A	B	0	0	0	7
Sample Size				14	14	14	14
No. of Haplotype				6	1	2	2
Rate of Haplotypes				0.4285	0.0714	0.1428	0.1428

Table1. Frequency and rate of occurrence of length variants in PCR products in *Channa* sps.

Frequency of length variants	Size of PCR product	<i>Channa gachua</i>	<i>Channa marulius</i>	<i>Channa striatus</i>	<i>Channa punctatus</i>
9	789	0.6428			
5	824	0.3572			
4	1004		0.2857		
5	1053		0.3573		
2	1105		0.1428		
3	1159		0.2142		
6	1078			0.4285	
3	1102			0.2142	
5	1126			0.3573	
3	1310				0.2144
5	1339				0.3571
6	1369				0.4285

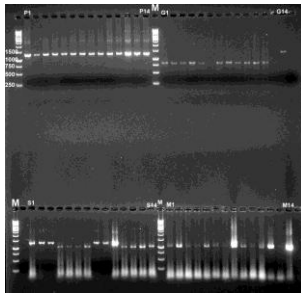


Fig. 1 PCR profile of amplification of D-loop region
 In upper row: Lane 2-14, *C. punctatus*, Lane16-32, *C.gachua*
 In lower row: Lane 3-16, *C. striatus*, lane 18-33, *C.marulius*

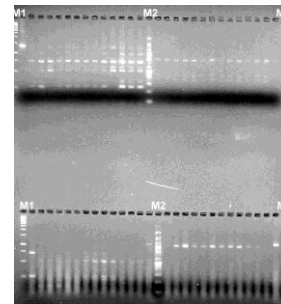


Fig.2 RFLP profile of D-loop PCR products digested with **Tsp 509**. In upper row: Lane4-16, *C.punctatus* Lane16-32, *C. gachua*. In lower row: Lane 3-16 *C.striatus*, Lane18-33, *C.marulius*.
 M1: 1Kb ladder, M2:100bp ladder

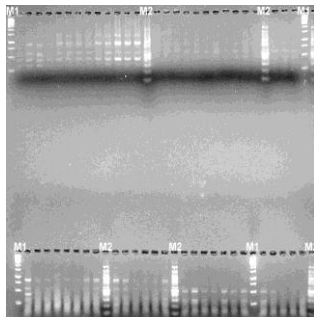


Fig. 3 RFLP profile of D-loop PCR products digested with **NlaIII**. In upper row: Lane 2-14, *C. punctatus* Lane16-32, *C. gachua*. In lower row: Lane 2-16, *C.striatus*, Lane 18-33, *C.marulius*.

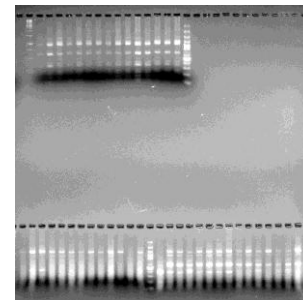


Fig. 4 RFLP profile of D-loop PCR products digested with **Rsa I**. In upper row: Lane 2-14, *C. punctatus*, In lower row: Lane 2-16, *C.striatus*, Lane18-33, *C.marulius*. M1:1Kb ladder, M2:100bp ladder

REFERENCES

1. **Arnason, E. and Rand, D. M. (1992).** Heteroplasmy of short tandem repeats in mitochondrial DNA of Atlantic cod, *Gadus morhua*. **Genetics** **132**: 211-220
2. **Batista, J. S. and Alves-Gomes, J. A. (2006).** Phylogeography of *Brachyplatystoma rousseuxii* (Siluriformes Pimelodidae) in the Amazon basin offers preliminary evidence for the first case of “Homing” for an Amazonian migratory fish. **Genet. Mol. Res.** **5** (4): 723-740
3. **Bentzen, P., Legget, W. C. and Brown, G. G. (1988).** Length and restriction site heteroplasmy in the mitochondrial DNA of American shad (*Alosa sapidissima*). **Genetics** **118**: 509-518
4. **Bernatchez, L. Guyomard, R. and Bonhomme, F. (1992).** DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout (*Salmo trutta*) populations. **Mol. Eco.** **1**: 161-173
5. **Billington, N. and Hebert, P. D. N. (1991).** Mitochondrial DNA diversity in fishes and its implications for introductions. **Can. J. Fish Aquat. Sci.** **48**: 80-94
6. **Buroker, N. E., Brown, J. R., Gilbert, T. A. et al., (1990).** Length heteroplasmy of sturgeon mitochondrial DNA: an illegitimate elongation model. **Genetics** **124**: 157-163
7. **Brown, J. R., Beckenbach, A. T. and Smith, M. J. (1992).** Mitochondrial DNA length variation and heteroplasmy in populations of white sturgeon (*Acipenser transmontanus*). **Genetics** **132**: 221-228
8. **Brown, W. M. (1985).** The mitochondrial genome of animals. In *molecular Evolutionary Genetics* (ed. R. J. Macintyre). **Plenum Press, NewYork** pp. 95-130
9. **Cronin, M. A., Spearman, W. J., Wilmot, R. L., Patton, J. C. and Bickham, J. W. (1993).** Mitochondrial variation in chinook (*Oncorhynchus tshawytscha*) and chum salmon (*O. keta*), detected by restriction enzyme analysis of polymerase chain reaction (PCR). **Can. J. Fish. Aquat. Sci.** **50**: 708-715
10. **Day, F. (1879).** The fishes of India; being a natural history of the fishes known to inhabit the seas and freshwaters of India, Burma, and Ceylon. **Fishes India Part 2**: 169-368, pls 41-78
11. **Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pabo, S., Villablanca, F. X. and Wilson, A. C. (1989).** Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. **Proc. Natl. Acad. Sci. U.S.A.** **86**: 6196-6200
12. **Li, X., Musikasinthorn, P. and Kumazawa, Y. (2006).** Molecular phylogenetic analysis of snakeheads (Perciformes: Channidae) using mitochondrial DNA sequences. **Ichthyol. Res.** **53**:148-159
13. **Ludwig, A., May, B., Debus, L. B. and Jenneckens, I. (2000).** Heteroplasmy in the mtDNA Control Region of Sturgeon (Acipenser, Huso and Scaphirhynchus). **Anim. Genet.** **31**: 153-154
14. **Meyer, A. (1993).** Evolution of mitochondrial DNA in fishes, In *Biochemistry and Molecular Biology of Fish*. Vol. 2 (eds P. W. Hochachka and P. Mommsen), **Elsevier Press**. pp 1-38
15. **Meyer, A. (1994).** Molecular phylogenetic studies of fish. In *Genetics and Evolution of Aquatic Organisms* (Beaumont, A.R., ed.), pp. 219-249. **London : Chapman and Hall**.
16. **Meyer, A. and Wilson, A. C. (1990).** Origin of tetrapods inferred from their mitochondrial DNA and affiliation to lungfish. **J. Mol. Evol.** **31**: 359-364
17. **Mulligan, T. J. and Chapman, R. W. (1989).** Mitochondrial analysis of Chesapeake Bay white perch, *Morone Americana*. **Copeia** 679-688
18. **Palumbi, S. R., Martin, A., Romano, S. et al., (1991).** Simple fool's guide to PCR. Department of Zoology. **University of Hawaii, Honolulu**.
19. **Rand, D. M. (1993).** Endotherms ectotherms and mitochondrial genome-size variation. **J. Mol. Evol.** **37**: 281-295
20. **Sambrook, J. and Russell, D. W. (2001).** Molecular cloning: a laboratory manual. 3rd ed. New York: **Cold Spring Harbor Laboratory Press**.
21. **Singh, H.S., Arya, P. V., Sharma, V., Rastogi, R. and Tyagi, Y. K. (2002).** *J. Aquatic Biology* **17** (2): 59 – 62
22. **Sivasundar, A., Bermingham, E. and Ortí, G. (2001).** Population structure and biogeography of migratory freshwater fishes (*Prochilodus*: Characiformes) in major South American rivers. **Molecular Ecology** **10**: 407-418
23. **Stepien, C. A. (2003).** The Project Report- Ohio Sea Grant On *Gymnocephalus* in the north American Great Lakes and Euresia.
24. **Szalanski, A.L., Bischog, R. and Mesti, G. (2000).** Population genetic structure of Nebraska Paddlefish based on mitochondrial DNA Variation. **Trans. Am. Fish. Soc.** **129**: 1060 - 1065.
25. **Vishvanath, W. and Geetakumari, K. (2009).** Diagnosis and interrelationships of fishes of the genus *Channa* Scopoli (Teleostei: Channidae) of northeastern India. **J. Threatened Taxa** **1**(2): 97-105|
26. **Wirgin, I. I., Proenca, R. and Grossfield, J. (1989).** Mitochondrial DNA diversity among population of striped bass in the southeastern United States. **Can. J. Zool.** **67**: 891-907