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Genetic divergence in natural populations of bronze featherback, *Notopterus notopterus* (Osteoglossiformes: Notopteridae) from five Indian rivers, analyzed through mtDNA *ATPase6/8* regions ☆

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ABSTRACT

The present study characterized 842 bp fragment of mitochondrial ATP synthase 6 and 8 (*ATPase6/8*) genes in *Notopterus notopterus*. In all, 97 samples of *N. notopterus* were collected from five distant rivers; viz Satluj, Gomti, Yamuna, Brahmaputra and Mahanadi representing 4 river basins in India. The analysis of variation revealed presence of 23 haplotypes in *ATPase6/8* gene with haplotype diversity (Hd) of 0.899 and nucleotide diversity (π) of 0.00336. The within population variation which was 41.78% of the total variation of 58.22% was found among population. The Fst value of 0.582 ($P < 0.05$) of the total population was found significant. The results concluded that the polymorphism in *ATPase6/8* gene is a potential marker that is important for determining genetic divergence of wild *N. notopterus* populations. The findings reveal common ancestry of mahanadi population with the populations in rivers of Indo-Gangetic region. However, long evolutionary isolation must be responsible for the high

Abbreviations: ATPase, adenosine tri phosphates; DNA, deoxyribonucleic acid; dent, deoxyribonucleoside triphosphate; mtDNA, mitochondrial DNA; FAO, Food and Agriculture Organization; IUCN Red List, International Union for Conservation of Nature; CAMP, Conservation Assessment and Management Plan

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genetic divergence between *N. notopterus* in Mahanadi and other regions.

1. Introduction

The present study is the first attempt to characterize *ATPase6/8* genes in *Notopterus notopterus* and assess its polymorphism to differentiate genetic stock in Indian rivers. The *N. notopterus*, commonly known as bronze featherback, is a teleostean fish of the order Osteoglossiformes and family Notopteridae. The order Osteoglossiformes is considered primitive (Taverne, 1975) with 19 fossil genera found in upper Jurassic to lower Cretaceous deposits (Nelson, 1994; Otero and Gayet, 2001). The featherback, *N. notopterus* is widely distributed in South and Southeast Asia (Talwar and Jhingran, 1991). The fish has been categorized as an important commercial fish by FAO (Casavas et al., 1996) for food as well as for ornamental trade. Populations of bronze featherback in wild are declining and categorized as one of the threatened species of the country (CAMP, 1998). However, the species is listed on the IUCN Red List as Least Concern (LC) due to ambiguity in taxonomy and suggested to be a species complex (Ng, 2010). The *N. notopterus* is a local migrant and prefers stagnant pools, wetlands than fast moving waters, coupled with its breeding preference (Rainboth, 1996); the fish may possibly have limited scope of dispersal. Therefore the species is likely to have high genetic heterogeneity. Though, to address the need to maintain wild populations and also for aquaculture, artificial propagation of the *N. notopterus* is pursued with encouraging results (Haniffa et al., 2004), however, such interventions without stock structure assessed through molecular markers, may not translate into effective means to conserve natural genetic variation.

There is a need of specific knowledge of intraspecific genetic variations to plan rehabilitation and management strategies for populations of the species which are declining in their natural habitat. The *ATPase8* and *ATPase6* regions of mitochondrial DNA have been successfully analyzed for both phylogeny as well as phylogeography in many fish species (Chow and Ushiama, 2004; Dammannagoda et al., 2008; Vergara-Chen et al., 2009; Xin-Hong et al., 2004; Yan et al., 2009). However, the information on polymorphic molecular markers and their applications is limited to a small part of vast distribution (Takagi et al., 2006a,b, 2010) and genetic relatedness with another notopterid fish, *Chitala chitala* (Lal et al., 2006). The present study characterized complete sequences of mitochondrial *ATPase8* and *ATPase6* regions in *N. notopterus* collected from 5 distant major rivers namely, Satluj, Ganga, Yamuna, Brahmaputa and Mahanadi, and analyzed the polymorphism and identify evolutionary significant subpopulations.

2. Materials and methods

2.1. Sample collection

Details of sampling sites for *N. notopterus* across different rivers are given in Table 1. The rivers are distant and represent different river basins (Fig. 1). The specimens of *N. notopterus* were obtained from commercial riverine catches and tissue (blood and muscle) samples were collected at site. The blood was collected through caudal puncture and fixed in 95% ethanol in 1:5 (blood: ethanol) ratio and dorsal white muscles (50 mg) as an alternate source of DNA, were stored in 95% ethanol at 4 °C until use.

Table 1
Sample size, location and year of collections of *Notopterus notopterus* from five different rivers in India.

River basin	River	Location/collection sites	Longitude & latitude	Year of collection	*n*
Indus	Satluj	Harike patan, Punjab	31°09′N, 74°56′E	May 2000	23
Ganges	Gomti	Sultanpur, U. P	26°15′N, 82°04′E	Oct. 2008	16
	Yamuna	Yamuna nagar, Haryana	30°16′N, 77°17′E	March 2001	19
Brahmaputra	Kalang	Kalangpar, Assam	26°11′N, 91°47′E	Feb. 2002	12
Mahanadi	Mahanadi	Cuttack, Orissa	20°27′N, 85°52′E	Jan. 2002	27
Total					97

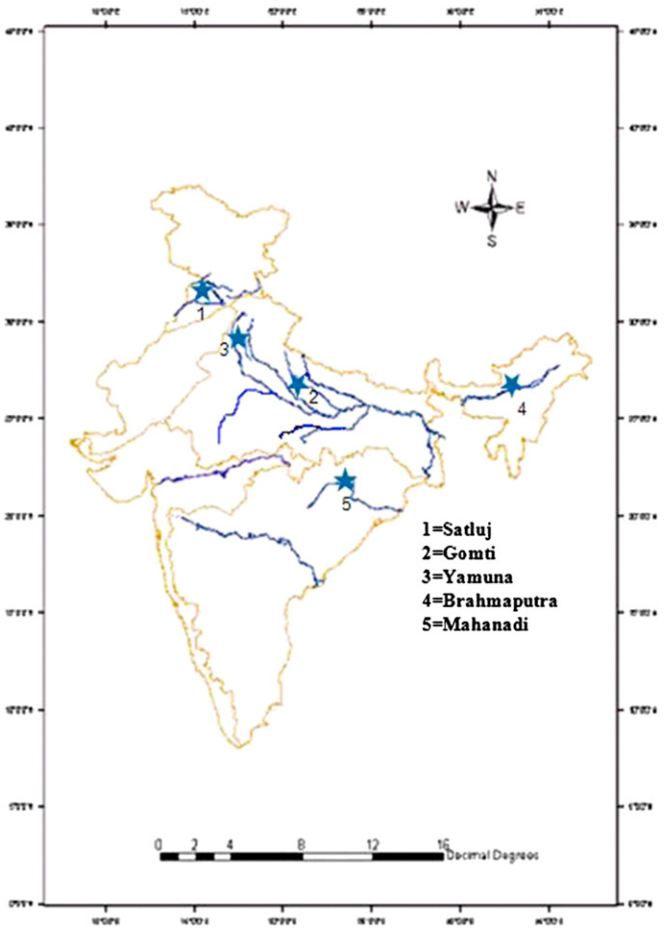

Fig. 1. Map showing collection sites *Notopterus notopterus* samples.

2.2. DNA extraction and PCR amplification

The total genomic DNA was extracted from blood and soft muscle tissue using Phenol–Chloroform methods of Bentzen et al. (Bentzen et al., 1990) as described in Ruzzante et al. (Ruzzante et al., 1996). DNA was amplified using universal primers for mtDNA *ATPase8* and *ATPase6* region; ATP8.2 L8331and CO111.2H9236 (Sivasundar et al., 2001). The amplification conditions consisted of initial denaturation at 94 °C for 5 min, 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 90 s and final extension at 72 °C for 10 min. PCR amplification was carried out in 50 μl reaction volume (5 μl 1× PCR buffer (Tris with 15 mM $MgCl_2$), 200 μM of each dNTP, 10 pmoles of each primer, 2.5 μM $MgCl_2$, 3U Taq DNA polymerase and 50–100 ng template DNA). The size and quality of the PCR product was checked against a 100-bp DNA ladder in 2% agarose gel and amplicons were purified using gel elution method. With the same sets of primers purified amplicons were sequenced bidirectional on ABI337 according to the manufacturer's recommendations.

2.3. Analysis of DNA sequences

The DNA sequences were aligned using CLUSTAL W (Thompson et al., 1994), and sequence composition was estimated using software MEGA 4.1 (Tamura et al., 2007). Analysis for genetic

differentiation; AMOVA, molecular diversity indices, genetic differentiation values and Fst values were determined using the software ARLEQUIN 3.11 (Excoffier and Schneider, 2005), haplotype and nucleotide diversity was estimated using DNASP 4.5 (Rozas et al., 2003) and mtDNA parsimony cladogram of haplotypes was constructed (at 95% level connectivity) using TCS version 1.18 (Clement et al., 2000). The neighbor joining tree was constructed to depict genetic relatedness between the samples using software MEGA 4.1 (Tamura et al., 2007) and molecular clock was calibrated using nucleotide substitution rate of 1.3% per million years (My) as suggested for ATPase 6 and 8 genes in animals (Bermingham et al., 1997).

3. Results

3.1. Characteristics of ATPase6/8 genes in N. notopterus

There were 168 bp fragments of *ATPase8* and 684 bp of *ATPase6*, with an overlapping region of 10 bp from 159 to 168 bp. These two fragments were analyzed together to determine genetic variation. The average frequencies of four nucleotides for all the samples of *N. notopterus* were A: 30.4%; T: 30.4%; C: 27.5%, G: 11.7% and were found to be A + T rich (60.8%). The greater part of nucleotide variation was due to transition, with an average transition to transversion ratio (Ts:Tv) of 10.45. The alignment of the sequences revealed 23 different haplotypes (Table 2), defined by 23 divergent nucleotide sites. It was also found that out of 842 bases, 819 were constant, 16 bp variables with parsimony informative and 7 were singletons. Mean haplotype diversity (h) was 0.698 while nucleotide diversity (π) was 0.00336.

3.2. Genetic differentiation and phylogenetic analysis

The haplotype diversity ranged from 0.581 (Satluj) to 0.772 (Brahmaputra) and nucleotide diversity from 0.0008 (Satluj) to 0.0021 (Brahmaputra) (Table 3). Analysis of Molecular Variance (AMOVA) revealed that out of total variation, only 41.78% was attributed to variation within the population whereas 58.22% was due to differentiation among the groups (Table 4). However, without Mahanadi samples

Table 2
Relative haplotype frequencies for *ATPase6/8* region in five natural populations of *Notopterus notopterus*.

Haplotype	Satluj	Gomti	Yamuna	Brahmaputra	Mahanadi	Accession no.
	(23)	(16)	(19)	(12)	(27)	
h01	0.609	0	0.421	0	0	KC249583
h02	0	0	0.053	0.167	0	KC249594
h03	0	0.5	0.211	0	0	KC249578
h04	0.044	0	0	0	0	KC249526
h05	0.261	0	0	0	0	KC249545
h06	0.044	0	0	0	0	KC249535
h07	0.044	0	0	0	0	KC249546
h08	0	0.063	0	0	0	KC249549
h09	0	0.125	0	0	0	KC249555
h10	0	0.125	0	0	0	KC249559
h11	0	0.063	0	0	0	KC249560
h12	0	0.063	0	0	0	KC249562
h13	0	0.063	0	0	0	KC249563
h14	0	0	0.211	0	0	KC249582
h15	0	0	0.053	0	0	KC249572
h16	0	0	0.053	0	0	KC249573
h17	0	0	0	0.25	0	KC249595
h18	0	0	0	0.417	0	KC249592
h19	0	0	0	0.167	0	KC249588
h20	0	0	0	0	0.407	KC249622
h21	0	0	0	0	0.481	KC249619
h22	0	0	0	0	0.074	KC249620
h23	0	0	0	0	0.037	KC249606

Table 3
Intra-population haplotype diversities (h) and nucleotide diversities (π) for *ATPase6/8* region from five natural populations of *Notopterus notopterus*.

Location	Haplotype diversity (h)	Nucleotide diversity (π)
Satluj	0.581 ± 0.093	0.000836 ± 0.000729
Gomti	0.750 ± 0.107	0.001890 ± 0.001326
Yamuna	0.766 ± 0.070	0.001820 ± 0.001275
Brahmaputra	0.773 ± 0.083	0.002105 ± 0.001474
Mahanadi	0.618 ± 0.054	0.001705 ± 0.001194

differentiation among the groups was found to reduce to only 24.68%. The pairwise Fst values ranged from 0.082 to 0.738 ($P < 0.05$) with an overall population Fst value of 0.582 ($P < 0.05$) (Table 4).

The haplotype network (Fig. 2) based on statistical parsimony indicated that all individuals from five populations clustered into one clade. The haplotype (h01) represented that the individual from Yamuna and Satluj was ancestral and formed the center of the network. Each haplotype was connected with others by 1 to 4 mutational steps and suggests that there was no deep branching among halotypes for *N. notopterus*. The haplotype h02 was presented in samples from Yamuna and Brahmaputra and haplotype h03 was shared between Yamuna and Gomti. The population specific haplotypes were found in all the populations. A maximum of 6 population specific haplotypes were found in samples of river Gomti, followed by river Satluj with 4 haplotypes. The Neighborhood Joining (NJ) tree (Fig. 3) depicted that populations of river Gomti and Yamuna form a single group while three populations from Satluj, Brahmputra and Mahanadi formed individual clusters.

4. Discussion

During the study, caution was used in assessment of the specimens, as *N. notopterus* is suspected to be a complex of species (CAMP, 1998), though there is no empirical evidence available as such. All the sequences were thoroughly confirmed to assess that all the sequence belonged to conspecific individuals, such as no deviation in amino acid translation. The haplotype network also doesn't suggest that the individuals studied are of heterogeneous group of species.

The *N. notopterus* populations from the rivers studied, hold substantial haplotype diversity and are highly structured genetically, which is in agreement with the biology of this species (Talwar and Jhingran, 1991). AMOVA results in congruence with significant pairwise and overall Fst values, supported the presence of significant genetic divergence between populations of Satluj, Gomti, Yamuna, Brahmaputra and Mahanadi rivers.

A total of 23 haplotypes were observed, however, only 3 haplotypes were shared between different populations and thus high genetic differentiation could be attributed to the high number of population of specific haplotypes observed. Nevertheless, all the haplotypes originated through mutations in haplotypes h01, as it is evident from the haplotype network, and h01, a dominant haplotype in Satluj and Yamuna possibly represents ancestral lineages. The study suggests that the *N. notopterus* in the rivers belonging to four basins might have originated from common ancestor before radiating and range expansion. The origin

Table 4
Pair wise Fst (below diagonal), their p values (above diagonal) and population specific Fst (at diagonal) between five natural populations of *Notopterus notopterus*.

	Satluj	Gomti	Yamuna	Brahmaputra	Mahanadi
Satluj		0.000	0.000	0.000	0.000
Gomti	0.287**		0.036	0.000	0.000
Yamuna	0.152**	0.082*		0.000	0.000
Brahmaputra	0.375**	0.356**	0.270**		0.000
Mahanadi	0.738**	0.693**	0.681**	0.699**	

* $P < 0.05$.
** $P < 0.001$.

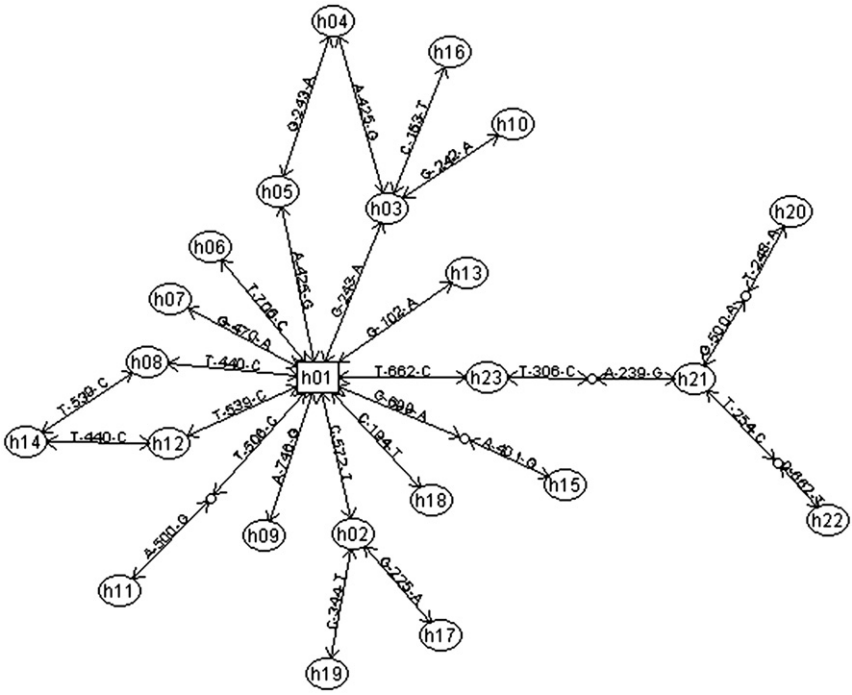

Fig. 2. Haplotype network for *ATPase6/8* region obtained from five natural populations of *Notopterus notopterus*.

of new haplotypes as observed from high haplotype diversity, is possible as *ATPase6/8* gene is reported to have high mutation rate of 1.3% per million years (My) (Bermingham et al., 1997). The haplotype diversity which was higher than that of nucleotide diversity, possibly indicates that the population of *N. notopterus* could have undergone a population bottleneck followed by sudden expansion and formation of new haplotypes which are found in low frequencies (Grant and Bowen, 1998). The star like haplotype network observed in *N. notopterus* mtDNA data also supports the possibility that population experienced a sudden expansion. Mandal et al. (Mandal et al., 2009, 2012) also reported sudden population expansion in *C. chitala*, another notopterid fish from India.

AMOVA results revealed that most of the genetic variation in *N. notopterus* is among the population (58.22%) which is higher than that reported (32.4%) for non-migratory species (Vrijenhoek, 1998). It is hypothesized that high level of genetic differentiation is the characteristic of species *N. notopterus*, which indicates restricted gene flow between populations. The gene flow could be influenced due to poor dispersal capability of the species, either through movement of adult fish or the passive transportation of

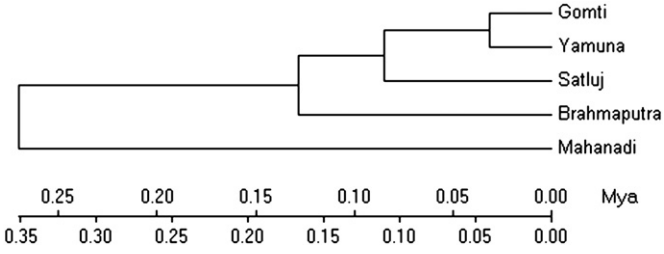

Fig. 3. Neighbor Joining (NJ) tree depiction for genetic relatedness on the basis of *ATPase6/8* regions in five natural populations of *Notopterus notopterus*.

eggs. The adult bronze featherbacks have poor swimming abilities and inhabit sluggish water in wetlands and floodplains which may or may not be fed with rivers (Rainboth, 1996) and lay eggs in small clumps on submerged vegetation (Talwar and Jhingran, 1991) and thus are not easily dispersed. Takagi (Takagi et al., 2006a, 2006b, 2010) also observed high genetic differentiation between the populations of *N. notopterus* from Mekong basin.

The populations after fragmentation experience genetic drift and restricted gene flow can lead to divergence (Hartl and Clark, 1997). In most of the ancient populations, more mutations are accumulated and populations get diversified. Such divergent populations will also have opportunity to share haplotypes with other populations, however such sharing will depend upon gene flow between them (Ward et al., 1993). However, Mahanadi river samples were not found to have any common haplotypes with any of the other four populations from Indo-Gangetic basins. This suggests lack of connectivity of Mahanadi population for a long time. The palaeogeographic evidences also indicated a possible fragmentation of Mahanadi populations during early Pleistocene (Menon, 1951). Population genetic variation (AMOVA) without Mahanadi samples is highly reduced (24.68%) as compared to all the 5 populations i.e. 58.22% including Mahanadi samples, further proving contribution of Mahanadi samples to high genetic divergence in the population. The samples from river Mahanadi were significantly diversified from those of the Satluj, Gomti, Yamuna and Brahmaputra.

The rivers Yamuna and Gomti are part of Ganga river system. Though the Brahmaputra river is an independent river basin but it joins Ganga river system, at present. Th ganga river system is reported to have shared fauna with river Yamuna and possibly separated only 3000 years ago (Chauhan et al., 2007). Therefore, it can be conceived that these rivers have connectivity which may be able to render some amount of gene flow as possibly explained in stepping stone model of migration (Hartl and Clark, 1997). This probably explains the genetic proximity of Yamuna, Gomti, Brahmaputra and Satluj populations as compared to Mahanadi population.

Therefore, the observed pattern of genetic divergence between *N. notopterus* from the 5 rivers appears to be consequence of not only the restricted gene flow between populations but also phylogeographic events resulting in long independent histories or evolutionary isolated populations like Mahanadi. These populations can be important for their adaptive significance and should be a priority for conservation programmes.

5. Conclusion

The findings demonstrated that 842 bp *ATPase6/8* gene is a promising tool to estimate genetic variation both within as well as among populations in *N. notopterus*. Population sub-structure of *N. notopterus* described here clearly reveals at least five genetically distinct stocks found in rivers of Indus, Ganges, Brahmaputra and Mahanadi basins within India. The potential of *ATPase6/8* mtDNA gene recognized for population genetics and baseline information on genetic divergence in wild *N. notopterus* populations will be useful for making fine scale exploration of genetic structure of this fish in its range of native distribution which is required for conservation assessment of this species.

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