GENETIC DIVERGENCE OF *CYPRINUS CARPIO* (L.) IN NORTH EAST INDIA INFERRED FROM CYTOCHROME B GENE OF MTDNA

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ABSTRACT *Cyprinus carpio, an exotic fish was brought to India for aquaculture, but deliberately or accidentally, they have entered into the various natural water ecosystems. The present study has examined the genetic divergence of this fish in North East India by partial sequencing of mitochondrial DNA. DNA Extraction from tissue samples was done by QIAGEN kit procedure. Sequencing of 425bp Cytochrome b gene has revealed 6 haplotypes with 12 variable sites. The Neighbor-joining tree showed that two haplotypes clustered separately from the rest. Another haplotype has also clustered separately in the phylogenetic tree. Out of 6 haplotypes, one haplotype was seen as the common haplotype available almost all over the region. Two others divergent haplotypes were also recorded. To reconstruct the phylogenetic tree, Genbank sequences of Cyprinus carpio were used. The study reveals that the common carp population in North East India is genetically diverged.*

Keywords: *Cyprinus carpio, Cytochrome b, genetic divergence, haplotype.*

INTRODUCTION

Cyprinus carpio Linnaeus (1758), popularly known as common carp, is the oldest domesticated fish species *(FAO, 2004)* belonging to the family Cyprinidae of order Cypriniformes. It is the third most commonly introduced fish (Welcome, 1988) and the most important farmed freshwater fish in the world (*FAO, 2006)*. According to Balon (1995), the native range of common carp extends from Japan to the River Danube in Eastern Europe. The fish has been introduced into many water bodies throughout Asia, Africa, the Americas, Oceania, Australia and New Zealand (Koehn, 2004) and it is included in the list of invaders species of IUCN (Lowe *et al*., 2000). A large number of strains of *Cyprinus carpio* have been developed in different countries of the world for different purposes. In India, the Prussian strain of common carp was first introduced to India in 1937 (Chacko, 1945). Later, in 1957 the Chinese stock (Bangkok stock) was introduced, and it has been distributed throughout India including the north eastern region (Alikunhi, 1966; Sen, 1985).The *C. carpio* var. *communis* of Chinese stock, popularly called scale carp, is found in all over India. The exotic *C. carpio* have escaped from the culture system and entered into the natural water bodies and becomes available in rivers, ponds and wetlands (Goswami, 2000; 2006). The possible cause of rapid distribution of the fish is that it can tolerate a variety of habitat types which facilitate them to distribute in a different geographical regions (Lubinski, 1986). The fish has become popular in India due to good return in harvesting and easily adaptable nature. Today *Cyprinus carpio* is the most extensively cultivated species in India and their population has been increasing alarmingly and has occupied considerable water bodies including wetlands and major rivers in India including the north eastern region which bear the biodiversity hot spots zone (Goswami *et al*., 2007; Choudhury & Goswami, 2012). They have been invading natural water bodies due to frequent floods in the country. The invasive nature of common carp is well documented from different countries of the world and the invasion of common carp also causes several disturbances to indigenous fish species in India (Lakra *et al*., 2006).

In India, the *Cyprinus carpio communis* strain is the most available and consumer preferred fish. However, it is a matter of investigation whether the genetic structure of this particular strain of common carp, is the same everywhere since its introduction in 1957 or certain micro-mutations have created any changes in their genetic constitution. To ascertain this hypothesis cytochrome b gene of mitochondrial genome was investigated, because mtDNA have been widely used to study genetic structure, phylogeny and mutation rate among individuals of a species (Ward and Grewe, 1994; Zhou *et al*., 2003).

MATERIALS AND METHODS

Study area and Sample Collection

A total of 129 tissue samples of *Cyprinus carpio* communis were collected from 75 different places of North East India (Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland and Tripura). Fish samples

were collected both from natural ecosystem (such as rivers and wetlands), culture system (pond) and markets of different places of NE India. Taxonomical studies were performed as per Talwar and Jhingran (1991) and Jayaram (1999). A large number of samples were collected from Assam where the fish is now available in all rivers, tributaries and wetlands. Wetlands (locally known as *beels*) in Assam are the important reservoirs of different kind of fish from where a considerable sampling was carried out. Most of the fish samples were caught by indigenous methods, commonly by using gill nets and cast net from different rivers, rivulets, wetlands and ponds (fish farm etc) with the help of local fishermen and were caught alive. The collected samples were immediately dipped in ice, kept and transported in polystyrene boxes to sustain freshness and then brought to the laboratory. Few tissue samples were also collected and preserved in absolute alcohol because of large sized fish. Upon arrival in the laboratory, fish were individually measured for their body weight and length. Then tissue samples were carefully preserved in absolute alcohol without any contamination. Individual ID and date was written on each sample bottles.

DNA extraction and sequencing

Total genomic DNA was extracted from muscle by QIAGEN kit procedure. For each sample approximately 25 mg of tissues was cut into small pieces and genomic DNA was extracted by Proteinase K digestion. The extracted DNA was stored at -20^{0}C for PCR amplification. Amplification of cytochrome b gene of mtDNA was performed in 15µl reaction containing 3.75µl water, 1.5µl taq buffer, 1.5µl MgCl2, 1.5µl dNTPs, 1.5µl primers, 1.5µl of BSA, 0.75µl of *Taq* DNA polymerase enzyme and 3µl of DNA. The cycling profile of the PCR was 94^{0}C for 5 minutes followed by 35 cycles of 94^{0}Cfor 30 sec, 53.4^{0}C for 30sec, 72^{0}C for 50 sec and finally 72^{0}C for 10 minutes. The primers L14724 (5'-CGAAGCTTGATATGAAAAACCATCGTTG-3') and H15149 (5'-AAACTGCAGCCCCTCGAATGATATTTGTCCTCA-3') used in this study were selected from Irwin *et al.* (1991) for the PCR amplification and sequencing of a segment of the mitochondrial Cytochrome B gene. The PCR products were then resolved on 2% agarose gel in 1XTBE buffer containing 2µl of ethidium bromide (Fig-1). After electrophoresis the PCR products were purified by adding 1µl of Exo-SAP (Shrimp Alkaline Phosphatase) per 10µl reaction. The cycling profile for the purification of DNA was 37^0 C for 70 minute, 80^0 C for 25 minute and finally at 4^{0}C. After this, the purified PCR products were used for sequencing using both the amplifying primers by the ABI Prism BigDye Terminator Cycle Sequencing kit according to the manufacturer's instructions (Applied Biosystems).

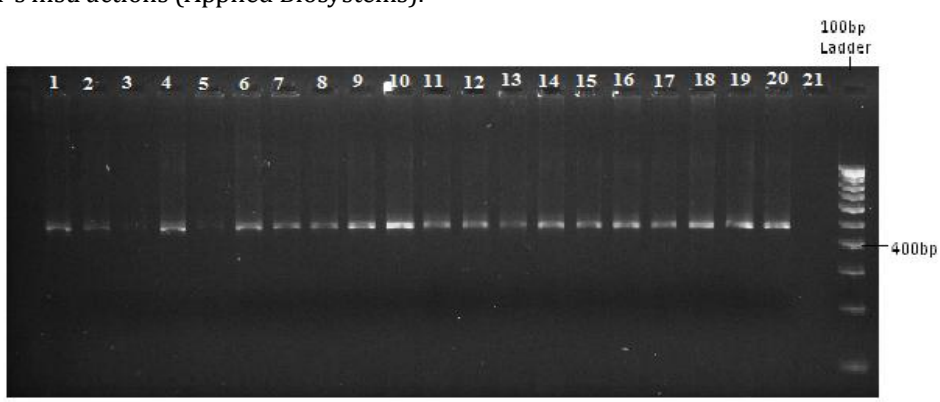

Fig.- 1: Ethidium bromide stained gel photograph of PCR products of 425bp. 100 bp ladder was used to find out the amplicon size.

Data Analysis

Amplified cytochrome b regions were sequenced in both the directions to check the validity of the sequence data. Alignment of sequences was done using using CLUSTALW program (Thompson *et al*., 1994), inbuilt in the genetic analysis package MEGA 7 (Tamura *et al*., 2011). The sequences were checked with the Chromas software (Technelysium, Australia), comparing both forward and reverse sequence. The sequenced data were analyzed with the help of BLAST (www.ncbi.nlm.nih.gov.) for homology search. Neighbor-joining phylogenetic tree (Saitou & Nei, 1987) was constructed from the haplotypes obtained 129 samples to resolving the relationships among closely related haplotypes. The levels of divergence of the haplotypes of this study were calculated and reconstructed the divergence by phylogenetic trees from few GenBank sequences. Bootstrap analysis (1000 data sets) was used to assess confidence in the branching order into the dendrogram. Genetic distances among different mtDNA sequences were calculated by Kimura 2 parameter method and a matrix was made to calculate pair wise genetic distance within the population using MEGA7.0. DnaSP5.0 (Librado and Rozas, 2009) was used to define the haplotype diversity and

polymorphic sites from the aligned data sets. The Nucleotide diversity of the haplotypes, mean number of pairwise differences and the molecular diversity indexes were calculated with ARLEQUIN version 3.5 (Excoffier and Lischer, 2010).

RESULTS

A total of 425 bp of cytochrome b gene fragments of *Cyprinus carpio* were successfully sequenced from 129 samples from North east India. The six different haplotypes (AsDhu1, MizKo1, TriGum, AsBpt1, TriKol, and AsGhy5) were obtained from the sequences. Out of the 12 polymorphic sites detected, ten are singleton sites and two are parsimony informative sites. The polymorphic sites of 425bp cytochrome b sequence of 6 haplotypes of *Cyprinus carpio* are shown in Table 1. The Neighbor-joining phylogenetic tree (Fig 2) was constructed from the 6 haplotypes obtained from all 129 samples collected from NE India. A common haplotype (AsDhu1) was found in many places of North east India which perhaps the introduced stock or dominant stock population. The Neighbor-joining tree showed that two haplotypes AsBpt1 and AsGhy5 clustered separately from the rest. Another haplotype TriKol has also clustered separately in the tree. The molecular diversity indexes calculated in the Arlequine show that there are 9 transitions and 3 transversions combining into a total 12 of substitutions in the six haplotype sequences. The nucleotide diversity, haplotype diversity (Hd) and the mean number of pair wise differences are (Pi) of the haplotypes are 0.01035, 1.000 ± 0.0962 and 4.400000 ±2.493090, respectively. The pair-wise genetic distance values among haplotypes were also calculated in MEGA where the distance between haplotype AsBpt1 and TriKol was found 0.020, the highest genetic distance.

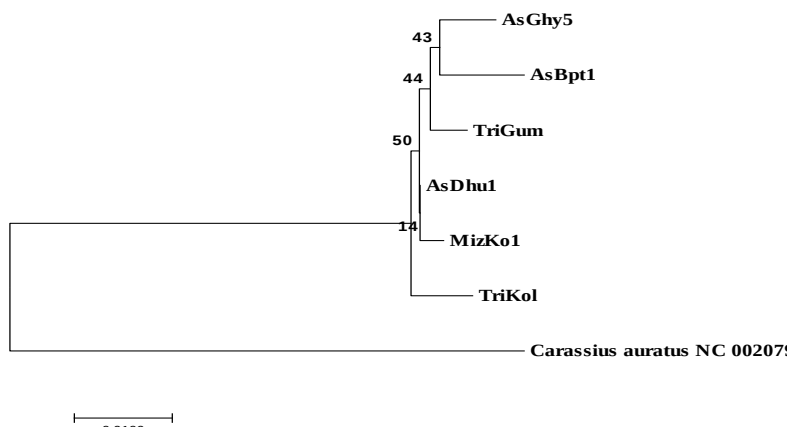

Figure 2: Neighbor-Joining tree displaying relationships among the 6 haplotypes obtained from 129 sequences of common carp samples from India with *Carassius auratus* as an outgroup. The evolutionary distances were computed using the Kimura 2-parameter method based on 1000 replications.

Table 1: Polymorphic sites within 6 haplotype sequences of *Cyprinus Carpio*. Dots denote the identical nucleotides.

Haplotypes	Nucleotide positions											
	95	100	140	200	285	375	391	392	395	401	422	424
AsDhu1	G	A	A	T	T	G	T	C	A	A	C	C
MizKo1	•	•	•	•	•	•	•	•	•	•	•	A
TriGum	•	•	•	C	•	•	•	•	•	C	•	•
AsBpt1	A	•	•	C	•	A	•	•	G	•	T	•
TriKol	•	•	•	•	C	•	C	A	•	•	•	•
AsGhy5	•	G	G	•	•	•	•	•	G	•	•	•

Phylogenetic reconstruction and comparison with the Genbank sequences

It has been found that 6 different haplotypes were obtained from 129 samples collected from different locations of India. To reconstruct the phylogenetic tree, 30 Genbank sequences of *Cyprinus carpio.* These are: X61010, AP009047, EU676848, HQ443697, AY347284, AY347285, AY347286, AY347287, AY347276, AY347277, AY347278, AY347279, AY347280, AY347290, AY347282, AB158803, DQ464970, DQ464969, DQ985031, JN105352, JN105354, JX188253, JX188254, JN105357, FJ655301, FJ655300, ACM91664, JN105353, ACH56448, and FJ655294 were downloaded and are used in this study. The cytochrome b

sequences thus obtained were compared with the sequences of other countries. The Neighbour-joining tree showed that the 5 haplotypes from India cluster separately which are grouped with a sequence of China. Surprisingly, in the phylogenetic tree, one haplotype (hap 5, AsBpt1) from India clusters with few Genbank sequences (Fig 3). These samples were from Greece, Vietnam and USA as per the address of the authors. Interestingly, the haplotype AsBpt1, found to cluster with GenBank sequences Acc. No. EU676848 which is from Oregon, USA.

Figure 3: The Neighbour-joining tree of *Cyprinus carpio* based on the cytochrome b sequences depicting the genetic relationship among the haplotypes from this study (Black triangles) and some GenBank sequences of different geographic locations. The values on the branch are bootstrap support based on

DISCUSSION

In the present study altogether 6 haplotypes of *Cyprinus carpio* communis were identified in NE India. One haplotype (AsDhu1) was found in different places of study area which perhaps accounts for the introduced stock of common carp. Three haplotypes (TriKol, TriGum and MizKo1) are found to confine in two states of North east India besides the common haplotype. The haplotype MizKo1 is differ from the haplotype AsDhu1by one substitutions and TriGum by two substitutions, which may be a natural genetic variability that may occur due to the evolutionary process. Another haplotype (TriKol) also showed considerable variation from the most common haplotype AsDhu1. Surprisingly, the haplotype AsBpt1, obtained from four fish samples showed a high mutation rate bearing 5 substitutions and have similarity with GenBank sequence Acc. No. EU676848. Most of the samples obtained from different places were included in the haplotype AsDhu1, which implies that AsDhu1 is the widely distributed haplotype of *Cyprinus carpio* in these regions. When the sequences of this study are compared with the sequences of south-east Asian and other countries (assumed from address of authors), it has been found that almost all sequences were clustered separately in the phylogenetic tree which detect the presence of several genetically variable strain

of common carp. The present findings have also shown that the common carp population in NE India has similarity with the strains of other countries. However in another review based on genetic variability in carp populations throughout their distribution range, using three marker systems such as allozyme, microsatellite DNA, and mitochondrial DNA markers, reported two distinct groups of carp viz. Europe/Central Asia and East/ South-East Asia (Kohlmann *et al*., 2003). Now a good number of strains and stocks of common carp are available due to different forces like geographic isolation, adaptation, accumulation of mutations and natural as well as human selection pressures (Hulata, 1995). The seven-decade long farming of exotic common carp in Indian freshwater ecosystems has been following repeated breeding through artificial methods and in natural ecosystem the fish has been reproducing successfully. This creates scope for the variability of the common carp population in India. The cytochrome b sequencing analysis showed that in Indian geographical conditions, the introduced population of common carp has been undergoing mutation and forming diverged populations. Thus the findings revealed presence of different haplotype distribution in the North East India. This may be due to connection of water bodies or business with neighbouring countries like Bangladesh, Myanmar and Bhutan.

Genetic variation provides the basis for selection, adaptation and speciation (Amos & Harwood, 1998). The genetic variation of common carp could help to the species in finding out the adaptation for new habitat niche utilization capability. But this may create niche overlapping between indigenous fish species in India where Indian Major Carp share same habit and habitat, whereby the sharing of habitat may become a threat to indigenous fish species of India. According to Singh *et al.*(2010), the common-carp population all over the world was able to establish itself everywhere, replacing the indigenous fish species. The natural distribution of wild common carp has been expanding to new areas other than their original home range and has occupied diverse habitats all over the world. From this study it can be said that after its introduction in India the common carp strain was mutated, forming many genetic variants that are available in lotic and lentic water bodies of India. The formation of haplotypes (strains) seems to be a genetic adjustment with the new environment or it may be a normal mutation process as the protein coding region contained less synonymous sites and more non synonymous sites. Various studies have indicated that the evolution of genetic variants of common carp would have a deleterious effect on endemic fish groups in north east India which is the home of 422 diversified fish species (Goswami *et al*., 2012). Though the increased fish production from common carp farming has fulfilled the requirements of the fish eating population in India, yet its invasiveness has started to cause disturbances to other fish species in aquaculture as well as in beel fisheries. In some places it has brought about changes in population structure and has replaced indigenous fish species in wild waters (Singh & Lakra, 2026). Biological invasions in freshwater systems are more dangerous than invasion in terrestrial ecosystems because they cause rapid biodiversity changes (Sala *et al*., 2000; Goswami, 2006). Conclusively, the present study clearly shows that the introduced population of common carp found to be mutated in India and the different microhabitat and diverged ecological niche could influence the fish for greater mutation which results in genetic variation.

CONCLUSION

The study reveals that the exotic common carp population in North East India is genetically diverged. When the sequences of this study are compared with the sequences of other countries (assumed from address of authors), it has been found that almost all the present sequences were clustered separately in the phylogenetic tree which detect the presence of genetically variable strain of common carp in NE India.

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