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Genetic distance and phylogenetic studies of two Indian snakehead and one carp fish species

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Abstract

Aim : To study genetic distance and phylogenetic relationship of two different stock of three important fish species, i.e., *Channa stewartii*, *C. striatus* and *Labeo gonius* of Assam.

Methodology : Three fish species, namely *Channa stewartii*, *C. striatus* and *Labeo gonius* were collected from two beels, i.e., Thekera and Samaguri beels of Assam, India. Standard procedures were followed for isolation of their DNA and sequencing. Thereafter, genetic distance and diversity and phylogenetic studies of three species were calculated using computer based softwares ClustalW and MEGA6.

Results : In the present study, the pairwise genetic distance of three fish species ranged from 0 to 20.436. No pairwise distance was found between two stocks at different locations Thekera and Samaguri beels of Assam for each of three species *C. stewartii*, *C. striatus* and *L. gonius*. The highest values (20.436) of pairwise distance were found between *C. striatus* and *L. gonius* population of Thekera beel; Thekera and Samaguri beels; and *L. gonius* population of the Thekera and Samaguri beels. The overall average distance for the two populations of three fish species was 15.387. No significant intraspecific difference was observed in the phylogenetic studies of two stocks of three fish species.

Interpretation : The overall average distance (15.387) of three fish species could be attributed to genetic distance from each other. The high value of coefficient of differentiation (1.000) for three fish species from two stocks indicated that all the species were different from each other. Overall genetic and phylogenetic studies revealed that *C. stewartii*, *C. striatus* and *L. gonius* were less differentiated from each other.

Key words: Barcoding, *Channa stewartii*, *C. striatus*, *Labeo gonius*, Phylogenetic relationship

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Introduction

In order to meet the challenges of changing environment, genetic variation in the species is essential (Fisher, 1930) and for survival adaptation to the changing environment is must. This genetic diversity vary nonrandomly among populations, species and higher taxonomic groups (Nevo and Beiles, 2011). Data on genetic variation has many and varied application in species conservation, management and their genetic improvement programmes. Generally, individuals with greater genetic variability have higher growth rates, developmental stability, viability, fecundity and resistance to environmental stress and diseases (Carvalho, 1993). Application of molecular techniques for species identification, development of specific breeding plan for conservation, etc., is increasingly popular because of its reliability (Mishra *et al.*, 2017; Kednapat *et al.*, 2018; Ayyappan *et al.*, 2014; Pope *et al.*, 2010). Application of molecular techniques for species barcoding and analysis of nucleotide sequences using computer based softwares have revolutionized the analytical power that is necessary to explore the genetic diversity of a species (Chauhan and Kumar, 2010).

The North Eastern Region of India comprising eight states including Assam provide unique ecological variations. The freshwater ecosystems of this region, forms from a part of the Himalayas and Indo-Burma plateau, which are highly diverse and have regional importance in terms of livelihood and economic importance of the people living there in (Allen *et al.*, 2010). This part of India has been recognized as a global hot spot of freshwater fish biodiversity. Sen (2000) enlisted 266 species of fishes belonging to 114 genera under 38 families and 10 orders. A perusal of literatures reveal that the North Eastern Region possess more than 100 fish species having potential ornamental value (Viswanath, 2000). Goswami *et al.* (2012) listed 422 fish species from North East India, belonging to 133 genera and 38 families; the highest 183 species of ornamental fish has been recorded from Manipur and lowest 98 from Tripura, while Assam possess 180 species of such fishes. Nearly 200 species of ornamental fishes are exported from India, of the freshwater species 85% are from the North Eastern States (Nair, 2004).

The ornamental fishes exported from the region are mainly from wild collection. Based on IUCN categories, the Conservation Assessment and Management Plan (CAMP) Workshop (Molur and Walker, 1998) for freshwater fishes identified that certain fish species have attained the threatened /endangered status. Though the North Eastern India is rich in freshwater fish biodiversity, however, due to export of ornamental fishes from wild collection many of the fish species of the region are becoming threatened. Therefore, a study was conducted to investigate the diversity, phylogenetic relationships and genetic distance, etc. applying RAPD- polymerase chain reaction on three selected fish species comprising two food fishes (*Channa striatus* and *Labeo gonius*) and one ornamental fish (*Channa*

stewartii) collected from two populations of two water bodies of Assam, India and sequencing their nucleotides using DNA Barcoding Sequencing Module of Bio-Rad (Catalog#166-5115EDU).

Materials and Methods

Collection of samples : For the present study, fresh fish samples of two stocks of three fish species, namely *Channa stewartii*, *C. striatus* and *Labeo gonius* were collected from two *beels* (flood plain wetland), i.e., Thekera and Samaguri *beels* of Assam, India.

DNA Isolation: Approximately, 100 mg of fin tissue from samples of three fish species were preserved in 95% ethanol. DNA was isolated from preserved samples following the method of Ruzzante *et al.* (1996) with minor modifications.

PCR amplification : PCR amplification of isolated fish DNA samples were done using BIO-RAD Fish DNA Barcoding Kit (Catalog #166-5100EDU). The PCR amplifications were carried out using a Bio-Rad's T100™ thermal cycler in a reaction volume of 40 µl. The 40 µl PCR reaction volume for each fish sample contained 35 µl of COI master mix and 5 µl fish DNA sample. The COI master mix contained 10x PCR buffer, dNTP, fish primer mix (It contains two sets of forward and reverse PCR primers instead of the single set used in typical PCR reaction). This composed of a universal primer sequence and a gene specific primer sequence. This resulted in PCR products with the universal prime sequence at 5' and 3' of finished PCR products. This allows for easy sequencing of PCR products using standard sequencing primers, Taq DNA polymerase and sterile water. The amplification condition were 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 2 min and 72°C for 1 min with final extension of 72°C for 10 min and holding at 4°C till electrophoresis and sequencing of PCR products.

Agarose gel electrophoresis and visualization of bands : After PCR amplification, 5 µl PCR products were resolved in 1.5% agarose gel containing UVView 6x loading dye in 1x TBE buffer. As UVView 6x dye contains a fluorescent compound that binds to DNA and allow DNA to be visualized with UV light, so no additional staining of gel was required for visualization of PCR amplified DNA bands. A molecular weight marker (DNA standard) provided in the Bio-Rad kit was used as a reference sample containing several DNA molecules of known molecular weights. The expected band size that corresponds to successfully amplified COI gene PCR product was approximately 650 bp.

Sequencing : A 30 µl of PCR products derived from each selected six fish DNA samples using DNA Barcoding Sequencing Module of Bio-Rad (Catalog#166-5115EDU) were used for sequencing. Prior to sequencing, PCR products were cleaned to remove the unused reagents (unreacted nucleotides, primers, Taq polymerase, and buffer) in the PCR reaction. Amplicon sequencing was done by using both sequencing primers M13 forward (TGTAACGACGGCCAGT) and M13 reverse

(CAGGAAACAGCTATGA) for two direction sequencing to ensure having reliable results.

Statistical analysis : After getting the DNA sequences from the selected PCR amplified six fish DNA samples, the sequences were aligned by Clustal W. Genetic distance and phylogenetic tree were calculated and constructed using the software MEGA6.

Results and Discussion

Fish samples of two stocks of three selected fish species, namely *Channa stewartii*, *C. striatus* and *Labeo gonius* were

collected from Thekera and Samaguri beels of Assam, India. High quality DNAs (260/280=1.8) were extracted from the collected fish samples using BIO-RAD Fish DNA Barcoding Kit. After PCR amplification and PCR product sequencing, expected size of sequences were obtained (Table 1). The estimate of genetic distance to discriminate the fish species studied under the present investigation are summarised under pairwise distance, within group and net between group mean distance. Bhat *et al.* (2011) observed the positive correlation of genetic distance with geographical distance for eight Indian Snakehead species using RAPD markers. In the present study, the pairwise genetic distance of three fish species ranged from 0.000 to 20.436. The

Fig. 1 : (a) UPGMA Tree dendrogram of two stocks of three fish species; (b) Neighbour joining tree of two stocks of three fish species and (c) Minimum evolutionary tree of two stocks of three fish species.

lowest pairwise distance, which was 0 or no distance, was found between samples collected from Thekera and Samaguri beels of Assam for each species of *C. stewartii*, *C. striatus* and *L. gonius*. No pairwise distance between samples within species could be attributed to same origin of those stocks of *C. stewartii*, *C. striatus* and *L. gonius*. They may be isolated by natural calamities like flood which is annual phenomenon of the region. The highest value (20.436) was observed between the *L. gonius* populations

of Thekera and Samaguri beels. The greatest distance between *C. striatus* and *L. gonius* based on DNA sequences, confirmed that these two species showed high genetic divergence however the significant pairwise distance of *L. gonius* population of the Thekera and Samaguri beels indicated that they were belonged two different stocks. The overall average distance was 15.387 for three fish species of two populations of *C. stewartii*, *C. striatus* and *L. gonius*. The presence of variability among populations as

Table 1 : Mitochondrial COI gene sequences of three fish species collected from two different stocks

Species	DNA sequence
<i>Channa stewartii</i> 1 (Thekera beel)	CTCTTCTAGGCAACGACCAAAATTTATAATGTAATTGTTACGGCACACGCCTTCGTACATAATCTTCTTCAT GGTTATGCCAATAATAATTGGGGGCTTTGGAACTGACTGGTGCCACTCATGATTGGTGCCCTGACA TAGCTTTCCCTCGAATAAACAATATAAGTTTTGACTCCTTCCCCCTTCTTCTCCTCCTCCTGGCCTC TTCTGCAGTAGAAGCCGGAGCTGGGACAGGCTGAACAGTCTACCCACCTCTAGCTGGTAATCTGGCC CACGCAGGAGCATCCGTAGACCTAGCCATCTTCTTTACATCTTGACGGTGTCTCTTCAATTTTAGGG GCAATTAACCTTCATCACAAATCATTAAACATGAAACCCCGAGCCATCTCTCAGTACCAGACACCTCTG TTTGTGTGGGCTATTCTAATCACTGCTATCCTTCTACTTCTTCTCTTCCGCTTCTAGCTGCCGGTATCA CAATACTATTAACAGACCGAACTTAAACACAACCTTCTTTGACCCTGT
<i>C. stewartii</i> 2 (Samaguri beel)	TACTGATCCGGGCGGAGCTTAGCCAGCCCGGCGCTCTTCTAGGCAACGACCAAAATTTATAATGTAATT GTTACGGCACACGCCTTCGTACATAATCTTCTTCATGGTTATGCCAATAATAATTGGGGGCTTTGGAAAC TGACTGGTGCCACTCATGATTGGTGCCCTGACATAGCTTTCCCTCGAATAAACAATATAAGTTTTGA CTCCTTCCCCCTTCTTCTCCTCCTCCTGGCCTCTCTGCAGTAGAAGCCGGAGCTGGGACAGGCT GAACAGTCTACCCACCTCTAGCTGGTAATCTGGCCACGCAGGAGCATCCGTAGACCTAGCCATCTTC TCTTACATCTTGACGGTGTCTCTTCAATTTAGGGGCAATTAACCTTCATCACAAATCATTAAACATGA AACCCCGAGCCATCTCTCAGTACCAGACACCTCTGTTGTGTGGGCTATTCTAATCACTGCTACCTTC TACTTCTTCTTCCGCTCTAGCTGCCGGTAT
<i>C. striatus</i> 1 (Thekera beel)	GCCAACCTGGCGCTCTCCTCGGAGACGATCAAAATTTATAATGTAATTGTAACAGCACACGCCTTTGTAA TAATCTTTTCATGGTTATGCCAATAATAATTGGAGGCTTCGGAAATGACTTATTCTCTTATGATTGGC GCCCCGACATGGCCTTCCCCGAATAAATAACATGAGCTTCTGATTGCTCCCTCCATCATTCTCCTT TTACTAGCCTCTTCTGCTGTAGAAGCCGGGGCCGGAACCGGGTGAACAGTTTACCCGCCCTAGCCA GCAATTTAGCCACGCAGGGGCTCCGTCGATCTAACCATTTCTCCCTACACCTGGCTGGCGTATCC TCAATTTAGGGGCCATTAACCTTTTACTACTATTATTAACATAAAACCCCTGCTATTTCTCAATATCAA ACCCCTATTGTATGGGCTATCTTAATTACAGCCGTACTACTTTTACTTTCCCTCCAGTATTAGCTG CAGGCATTACAATGCTACTCACAGACCGAAATCTAACACCACCTTCTTCGACCCTGCTGGCGGAGGT GACCCCATCTTTATCAACACTTATTCTGATTCTCGGTACCCCTGAAGTG
<i>C. striatus</i> 2 (Samaguri beel)	TCTCCTCGGAGACGATCAAAATTTATAATGTAATTGTAACAGCACACGCCTTTGTAATAATCTTTTCATGG TTATGCCAATAATAATTGGAGGCTTCGGAAATGACTTATTCTCTTATGATTGGCGCCCGGACATGG CCTTCCCCGAATAAATAACATGAGCTTCTGATTGCTCCCTCCATCATTCTCTTTTACTAGCCTCTTC TGCTGTAGAAGCCGGGGCCGGAACCGGGTGAACAGTTTACCCGCCCTAGCCAGCAATTTAGCCCA CGCAGGGGCTCCGTCGATCTAACCATTTCTCCCTACACCTGGCTGGCGTATCCCAATTTAGGGG CCATTAACCTTTTACTACTATTATTAACATAAAACCCCTGCTATTTCTCAATATCAAACCCCTATTGT TATGGGCTATCTTAATTACAGCCGTACTACTTTTACTTTCCCTCCAGTATTAGCTGCAGG
<i>Labeo gonius</i> 1 (Thekera beel)	GGTGACGACCAAAATTTATAATGTTATCGTAACGCTCAGCCTTCGTAATAATTTCTTTATAGTAATGCC AATCCTCATTGGAGGATTTGGGAAGTACTCGTACCATAATGATCGGAGCCCCAGACATGGCATTCC CTCGTATAAATAATAAGCTTCTGACTCCTACCTCCATCATTCTACTACTATTAGCCTCTTCTGGTGTC GAAGCTGGAGCTGGGACAGGATGAACAGTATACCCACCTCTTGCGGGTAACCTAGCCACGCAGGAG CATCAGTAGACCTAACAATTTTCTCATTACACTTAGCAGGATTTTCATCAATCCTAGGAGCTATTAACCT CATTACTACAATATTAATATGAAACCTCCAGCCATCTCACAATATCAAAACCCCTATTCTGTTGATCCG TACTTGTAACCGCGTACTACTTCTCTATCACTACCAGTACTGGCTGCTGGCA
<i>L. gonius</i> 2 (Samaguri beel)	CGCTTCTAGGTGACGACCAAAATTTATAATGTTATCGTAACGCTCAGCCTTCGTAATAATTTCTTTATA GTAATGCCAATCCTCATTGGAGGATTTGGGAAGTACTCGTACCATAATGATCGGAGCCCCAGACAT GGCATTCCCTCGTATAAATAATAAGCTTCTGACTCCTACCTCCATCATTCTACTACTATTAGCCTCTT CTGGTGTGAAAGCTGGAGCTGGGACAGGATGAACAGTATACCCACCTTTCGCGGTAACTTAGCCCA CGCAGGAGCATCAGTAGACCTAACAATTTTCTCATTACACTTAGCAGGAGTTTCATCAATCCTAGGGG TATTAACCTCATTACTACAATATTAATATGAAACCTCCAGCCATCTCACAATATCAAAACCCCTATTCTG TTTGATCCGTACTTGTAACCGCGTACTACTTCTCTATCACTACCAGTACTGGCTGCTGGCA

well as individuals within a population is essential for their ability to survive and successfully respond to environmental changes (Ryman *et al.*, 1995). Bhat *et al.* (2014) found a significant correlation between genetic closeness with geographical distance in Indian snakehead specie, *C. striatus*. However, the present findings on the distance within the group of each species. *i.e.*, one from Thekera and another from Samaguri beel ranged from 0 to 0.002, which was almost negligible. The result indicated that the species wise genetic variation within two groups for each fish specie was very less and it could be assumed that they have same origin, though presently living in two water bodies isolated by several kilometres. The present findings of species wise low genetic variability of three fish species of two populations may also be attributed to their low population size which corroborates the findings of Pinsky and Palumbi (2014).

The net group mean distance analysis for three species of two populations showed that the group net group distance of *C. stewartii* and *C. striatus* (0.245) were more closer compared to the net group distance of *C. stewartii* and *L. gonius* (0.269) and *C. striatus* and *L. gonius* (0.274). This is because *C. stewartii* and *C. striatus* come under same taxonomic group whereas *L. gonius* belongs to different taxonomic group.

Genetic diversity with geographical isolation of populations and individuals within the population bear a positive correlation. However, Tewari *et al.* (2013) found a low genetic divergence in *L. gonius* stocks from three reservoirs of Uttarakhand using RAPD marker. Zhou *et al.* (2019) also observed low genetic diversity in the northern snakeheads *Channa argus*. In the present study, the mean genetic diversity value of three fish species from two different stocks was very low, ranging from 0 to 0.001. The lowest value of 0 was found in *C. stewartii* and *C. striatus*. The same taxonomic group of the above species may be the reason for their low genetic diversity. Low genetic diversity of three species of two populations could also be attributed to intense fishing and poor management practices followed in two water bodies. Pinsky and Palumbi (2014) also reported low genetic diversity in overfished population. The highest value of 0.001 in *C. stewartii* and *L. gonius* and *C. striatus* and *L. gonius* may be attributed to the fact that two belong to different species.

Phylogenetic studies, based on fish species DNA sequences, were carried out to estimate UPGMA tree dendrogram, Neighbour joining tree and Minimum evolutionary tree for two stock of three species from two water bodies. The UPGMA dendrogram (Fig. 1a) based on Nei's unbiased estimate of genetic identity and distance estimated for two stocks of three fish species revealed that the two stocks of *C. stewartii*, *C. striatus* and *L. gonius* were separately grouped but they were in the same group within same species. The groups of *C. stewartii* and *C. striatus* were closer, however, *L. gonius* group was different from *C. stewartii* and *C. striatus*. This confirms the unique identity of *L. gonius* from *C. stewartii* and *C. striatus*. Tewari *et al.* (2013) and Conte-Grand *et al.* (2017) reported that genetic distance value increases with an increase in geographical distance. However,

the findings of the present study did not match with the latter authors, as the two stocks of three fish species are isolated geographically but their origin may be from the same stock. The neighbour joining tree (Fig. 1b) also revealed that the two stocks of each fish were close neighbour. This indicates there was no significant genetic variation between the two stocks of three fishes. The minimum evolutionary tree (Fig. 1c) showed that they were descendents of same progenitor, therefore *C. stewartii* and *C. striatus* and *L. gonius* cannot be separated. Ahmed *et al.* (2018) and Zhou *et al.* (2019) also observed the similar findings of phylogenetic relationship in *Channa* spp.

The present study on genetic distance and phylogenetic relationship of *Channa stewartii* and *C. striatus* and *Labeo gonius* fish species of two stocks yield moderate to low genetic diversity among the stocks of intraspecies, however, a comparable difference was obtained among the interspecies stocks.

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