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Phylogenetic Analysis of Channa Species of Manipur Based on Mitochondrial *Cytochrome b* Gene Sequences.

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ABSTRACT

Molecular phylogeny of five species of genus *Channa* belonging to Channidae family of Manipur, Northeast India was investigated based on the *cytochrome b* gene sequences. The nucleotide sequences length ranges from 307-349 bp(basepair). Here 84 characters were included out of which 44 were conserved sites (monomorphic) and 27 were variable sites (polymorphic), 7 were parsimony informative sites and 19 were singleton. The transition /transversion bias (R) is 1.21. Substitution patterns and rates were estimated under the Kimura (1980) 2-parameter model. The average nucleotide frequencies are 29.9% (A), 31.0% (T), 29.0% (C), and 13.2% (G). The overall mean distance is 0.241. Molecular phylogenetic analysis is performed in MEGA (ver. 7) software. Sequence data was subsequently analyzed for UPGMA method (*Unweighted Pair-Group Method with Arithmetic Mean*). Phylogenetic analysis showed the interspecies genetic divergence between *C. marulius*, *C. orientalis*, *C. gachua*, *C. punctata* and *C. striata*. Finally, *C. punctata* is the most distantly related compare to the aforementioned species indicated by the distinct branch. The results indicate that *cytochrome b* gene is useful in analyzing genetic variation as well as in unraveling phylogenetic relationship among species level.

KEYWORDS: Cytochrome b gene, *Channa*, Molecular phylogeny.

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INTRODUCTION

Genus *Channa* of the family *channidae* is one of the most widespread families of freshwater fish. *Channa* are primary freshwater fishes inhabiting African and Asian continents ¹. They are commonly called Snakeheads and are distinguished from other genera of the family in having peculiar morphological features, such as elongated cylindrical body, long and entirely soft-rayed dorsal and anal fins, a large mouth with well-developed teeth on both upper and lower jaws, and an accessory air-breathing apparatus known as the suprabranchial organ ^{2,3}. They have flattened heads; possess large scales on their heads and eyes being located in the dorsoventral position on the anterior part of the head. The *channidae* are represented by 38 valid species across its natural range from Africa to Asia ⁴. Nine species are known from Northeastern India out of which five species (*Channa marulius*, *channa orientalis*, *channa punctata*, *channa gachua* and *channa striatus*) are found in Manipur.

Manipur is a small hill girt state in the north-eastern corner of India. It lies between 23.83°N and 25.68°N and between 93.03°E and 94.78°E. The state is having rich freshwater resources as it is included in the freshwater biodiversity hotspot map of the world ⁵. So far Snakeheads have been identified conventionally based on morphological and anatomical characters ⁶. However, there are ambiguities due to morphological closeness and changing colour patterns from juvenile to adult stage. In spite of their economic and scientific importance to date there is very limited information available on the extent of molecular genetic structure in these species ^{1,7}. Molecular techniques have become major tools for systematic ichthyologists and may also be useful to fishery biologists for ratification of taxonomic problems at species and population levels ⁸⁻¹⁰.

Mitochondrial DNA analysis is extensively used for inferring phylogenetic relationship among organisms because they have the properties of fast evolutionary rate, maternal inheritance, smaller molecular weight and a lack of introns ^{11, 12}. Among the various mitochondrial genes, the mitochondrial cytochrome b (cyt b) gene has been widely used to identify genetic variation in many fish species ¹³. The aim of the present study is to establish molecular phylogeny of the *cytochrome b* sequences of five species of *Channa*.

MATERIALS AND METHODS

Collection of fish samples: A total of forty five live fish specimens presenting five species of *Channa* were collected from different water areas of Manipur from February 2016 to December 2018. The morphological identification was done according to Vishwanath (2014) ¹⁴. For molecular identification, muscle tissue samples were collected and preserved in 70% ethanol at -20°C until used.

Table 1: Study materials for the present studies

SI No.	Species	Collection site	No. of specimen
1	<i>Channa marulius</i>	Lamlong, Bamon kampu, Sugnu, Thoubal	14
2	<i>Channa orientalis</i>	Imphal west, sugnu, Moreh Thoubal	10
3	<i>Channa punctata</i>	Imphal west, Lamlong, Sugnu, Thoubal	12
4	<i>Channa gachua</i>	Tamelong, Jiribam, Imphal west	4
5	<i>Channa striatus</i>	Moreh, Chandel	5

Genomic DNA isolation and amplification: The total genomic DNA was isolated from the muscle tissue samples using DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturers protocol. The DNA was kept at -20°C until use.

Cytb gene analysis: The mitochondrial cytb gene was amplified using the primer

CytBF AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA

CytBR AAAGTGCAGCCCCTCAGAATGATATTTGTCCTCA

The PCR amplifications were performed in 50- μl reaction volume containing 10X *Taq* polymerase buffer, 2 mM MgCl_2 , 0.2 mM dNTP mix, 10 pmol of each primer, 1U *Taq* DNA Polymerase and 50 ng genomic DNA. PCR consisted of the following cycling condition: initial denaturation for 4 min at 95°C ; 40 cycles of denaturation at 30 s for 95°C , annealing for 30s at 47°C , and extension for 1.5 min at 72°C ; final extension for 10 min at 72°C ¹⁵. The amplified products were resolved in 1.1% agarose gel stain with ethidium bromide. The PCR amplicon was purified with ExoSap enzymatic purification Kit as per the manufacturer instruction.

DNA sequencing: The products were subjected to Sanger sequencing using ABI, 3730XL DNA analyzer using BdT v3.1 chemistry. Each forward and reverse reaction of PCR amplified products were sequenced separately.

Sequence alignment and Phylogenetic Analysis

The mitochondrial cytb gene sequences in FASTA format were imported into the sequence alignment application of MEGA 7 software package and multiple sequence alignments were performed using the default parameters Clustal W¹⁶. Molecular phylogenetic analyses were performed in MEGA (ver. 7) software¹⁶. The phylogenetic analysis was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model with bootstraps of 1000 replicates¹⁷. The evolutionary distances were calculated using the maximum composite likelihood method which was shown by the units of the number of base substitutions per site. All position containing gaps and missing data were eliminated from data set (complete deletion). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of

pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

RESULTS

Mitochondrial DNA sequence: The nucleotide sequences of the partial mitochondrial cytochrome b gene (*cytb*) of *Channa marulius*, *Channa gachua* and *Channa striatus* were downloaded from the NCBI (Genbank) for present study and two sequences of *Channa orientalis* and *Channa punctata* were generated (Table 2).

Table 2: Details of sequences and source, species names and codes of the sequences in the present studies.

SI no	Species	Scession No	Source
1	<i>Channa marulius</i>	GQ464065.1	NCBI
2	<i>Channa orientalis</i>	This study	NCBI
3	<i>Channa punctata</i>	This study	NCBI
4	<i>Channa gachua</i>	Z30271.1	NCBI
5	<i>Channa striatus</i>	KU245353.1	NCBI

Nucleotide diversity: The nucleotide sequence length range from 307-349 bp. Here 84 characters were included out of which 44 were conserved sites (monomorphic) and 27 were variable sites (polymorphic), 7 were parsimony informative sites and 19 were singleton. The transition/transversion bias (R) was 1.21. Substitution patterns and rates were estimated under the Kimura (1980) 2-parameter model. The average nucleotide frequencies are 29.9% (A), 31.0% (T), 29.0% (C), and 13.2% (G). The overall mean distance is 0.241.

Table 3: Nucleotide composition of *cytb* b sequence of *Channa* species

Name of Species	T(U)	C	A	G
<i>Channa marulius</i>	31.4	30.0	24.3	14.3
<i>Channa orientalis</i>	37.1	27.1	24.3	11.4
<i>Channa punctata</i>	30.0	28.6	28.6	12.9
<i>Channa gachua</i>	37.1	27.1	24.3	11.4
<i>Channa striata</i>	23.5	32.4	27.9	16.2
Avg.	31.0	29.0	25.9	13.2

Phylogenetic analysis:

The phylogenetic analysis was inferred by using the UPGMA (*Unweighted Pair-Group Method with Arithmetic Mean*) method. The optimal tree with the sum of branch length = 0.53537939 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 5 nucleotide sequences. Codon positions

included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 56 positions in the final dataset.

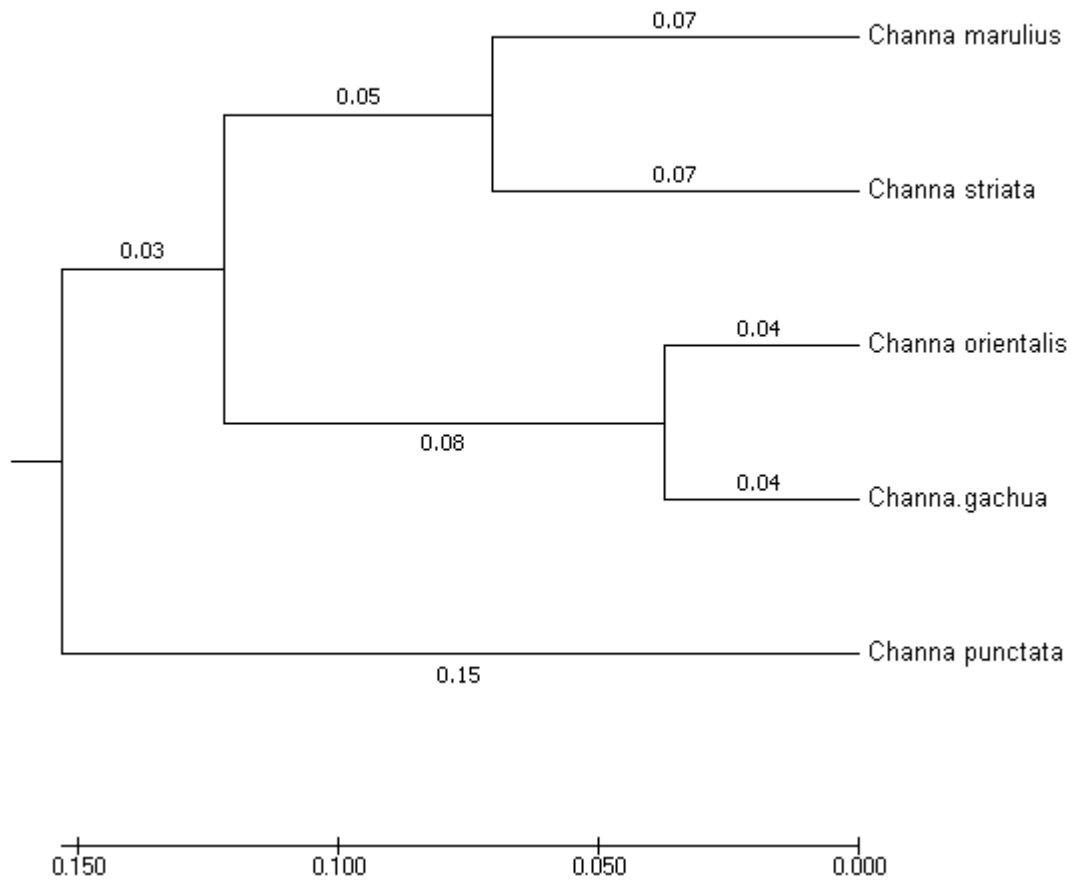


Fig 1: Phylogenetic tree of cytochrome b gene sequences of Channa species.

Phylogenetic analysis showed the interspecies genetic divergence between *C. marulius*, *C. orientalis*, *C. gachua*, *C. punctata* and *C. striata*, were clustered together in agreement with their taxonomic classification at the species level (Fig. 1). The Maximum Likelihood tree showed that *C. striata* shares the same node with *C. marulius*, this means that they share variations obtained from a common ancestor. *Channa gachua* shares a node with common ancestor of *C. orientalis*, indicating it diverged at an earlier time. Finally, *C. punctata* is the most distantly related compare to the aforementioned species indicated by the distinct branch (Fig. 1).

DISCUSSION:

In the past, morphometric and meristic characteristics were the only tools used for inferring fish phylogenetic relationship, and to understand speciation (Musikasinthorn 2000). But it is difficult to differentiate the fishes, especially Channidae species because of the similarity in their external morphology (Khanet al. 2013; Miyan et al. 2014). Therefore, the construction of phylogenetic trees

based on only morphology is controversial due to the complex evolutionary changes in their morphological and physiological characters.

In the present study, *Channa* species were initially identified based on morphological characteristics and for resolving the taxonomic problems, a molecular study has also been done. Therefore, this study shows that the result obtained from the morphometric, meristic and molecular analysis is similar and thus *C. marulius*, *C. orientalis*, *C. gachua*, *C. punctata* and *C. striata* are different species that can be differentiated under the same genus. Phylogenetic relationship among *Channa* species observed in this study was quite similar as described by the previous authors (Barman et al. 2018; Conte-Grand et al. 2017).

Thus molecular genetics methods are a powerful tool to identify, re-confirm or describe species, along with traditional taxonomy. Hence, molecular identification will help fish experts to resolve taxonomic ambiguities between closely related species with overlapping morpho-meristic characters and produce hybrid species in the near future.

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