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Molecular Characterization and Genetic Classification of Pakistani Freshwater Fishes through DNA Barcoding

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Authors' Contribution
LR conducted this research. MT and MA helped in sampling and basic DNA work. RMA, YM and SF supported in data analyses and manuscript preparations. MW and MA helped in research work as supervisory committee. ARA supervised the complete research project.

Key words
Freshwater fishes, DNA barcoding, Mitochondrial DNA, Molecular characterization, *Cytochrome b* gene, Molecular taxonomy

ABSTRACT

Pakistan is endowed with an extensive network of fisheries resources. Approximately 193 fish species are present in fresh waters of Pakistan, majority of which are edible. Around 310 thousand metric tons of fish production is appraised from inland waters of Pakistan. In recent years, adulteration and mislabeling of raw and processed meat has become a major problem for food safety and security. In this regard, fish recognition and identification is considered as a challenging tasks by using its taxonomic characterization. Such limitations encourage the adoption of genetic identification system for the fishes. The present study deals with mitochondrial gene marker *Cytochrome b (cyt b)* based identification and molecular characterization of 23 major fresh water fish species of Pakistan belonging to 09 families and analyzing their evolutionary trends. A total of 115 fish samples (5 for each 23 species) were collected from different fresh water bodies. The DNA was extracted through organic method for amplification of different primer sets of *Cyt b* gene. The amplicons were sequenced and then utilized for the BLAST, homology and phylogenetic analysis. The study proved authenticity of *cyt b* gene as a successful marker for identification of intraspecific variations on basis of which DNA barcodes have been developed and can be used as a reference for genetic identification and molecular classification of Pakistani fresh water fish species. This report of molecular classification will facilitate scientific community to understand phylogenetic lineage of Pakistani fresh water fishes and also help in addressing the problem of mislabeling fish meat and its adulteration.

INTRODUCTION

Pakistan has been bestowed with colossal natural water bodies that are in the form of rivers, tributaries and canals. In different areas of Pakistan there is a wide range of abiotic variations like soil types, topography, land usage, and presence of lakes and other wetlands. This all results in a variety of water quality conditions, which in turn have great effect on distribution of wide variety of fish species (Koel, 1997). The fresh water fish species in Pakistan belong to class Actinopterygii and sub-class Teleostei. In this class there are 3 cohorts, 6 super orders, 11 orders, 30 families and 86 genera (Rafique, 2007; Rafique and Khan, 2012). Some of these fishes are exotic species i.e., introduced in waters of Pakistan either in the wild or in fish farming system (Rafique and Khan, 2012). The increasing interaction of natural biodiversity with the human society and its understanding leads to a major task in science and technology i.e., the molecular identification of these species (Kuksa and Pavlovic, 2009).

Biological properties of mitochondrial DNA (mtDNA) make it a competent marker for molecular biodiversity studies. As it inherits from mother side, the process of recombination is absent in it. If mitochondrial divergence level is known, the divergence time can be calculated (Gissi *et al*., 2008; Galtier *et al*., 2009). For the species identification and delimitations different mitochondrial single genes are employed such as cytochrome b (*Cyt b*) (Hebert *et al*., 2004; Pacheco *et al*., 2011). DNA barcoding

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is a technique that is gaining popularity with every passing day. It works as a taxonomic tool for identification of species using partial sequences of standard mtDNA (Hebert *et al*., 2003; Awan *et al*., 2013). These particular regions are monophyletic in association: The details of mtDNA remain stored and intact within a species and shows greater variations and divergence between species than within species. Particularly, a partial region of *Cyt b* gene is employed as barcoding markers owing such properties (Hebert *et al*., 2003).

More particular, mitochondrial DNA allows the more quick discrimination of closely related taxa due to the high mutation rate in it which is 10 times greater than nuclear DNA as point mutations accumulate very fast in it (Jorde *et al*., 1998). Cytochrome b gene location within mtDNA is between two genes that produce tRNAGlu and tRNAThr. They encode partial cytochrome c oxidoreductase, a complex enzyme in oxidative phosphorylation (Leonard and Schapira, 2000). The studies of *cyt b* gene deal with evolution and inheritance. It is believed that species evolution occurs as a result of accumulation of mutations, if it is so then the common ancestor of two genomes can be identified by the nucleotide sequence divergence between the two. This principle forms the basics of the molecular classification and molecular phylogenetics.

Hence main objectives set forth for this study is the genetic characterization and molecular identification of economically important fresh water fish species of Pakistan using DNA barcoding.

MATERIALS AND METHODS

Selection of fishes

A total of 23 fish species that belong to nine families were selected for this study. All fishes were identified before experimentations based on morphological characters with the help of key to the Fisheries of the Punjab (Mirza and Sharif, 2003). The species belong to family Cyprinidae include (*Catla catla, Cirrhinus mirgala, Labeo rohita, Labeo calbasu, Cyprinus carpio, Labeo gonius, Puntius sophore, Amblypharygodon mola, Osteobrama cotio*), family Channidae (*Channa marulius, Channa punctatus*), family Notopteridae (*Notopterus notopterus*), family Bagridae (*Mystus cavasius, Mystus teengara, Rita rita*), family Siluridae (*Ompok pabda, Ompok bimaculatus, Wallagu attu*), family Schilbeidae (*Clupisoma garua, Gagata caenia*), family Belontidae (*Colisa fasciatus*), family Ambassidae (*Chanda nama*), family Clupeidae (*Gudusia chapra).*

Five intact fish specimens of each species had been obtained from different sampling sites across different sites of the rivers running across Pakistan including Head Qadir Abad River Chenab (District Mandi Bahauddin), Head Balloki River Ravi (District Kasur), Chashma barrage River Indus (District Mianwali), fish farms from Pattoki (District Kasur) and Manawan Fish Farms, Lahore. A detailed list of fish samples was prepared. After that the fish specimens were transported to the laboratory for molecular investigation.

DNA extraction and PCR amplification of Cyt b *genes*

The 0.5-2 mL of blood or meat sample was taken from each specimen. Genomic DNA from each sample of blood or meat was extracted following standard method of phenol-chloroform extraction (Sambrook and Russell, 2001).

Cyt b genes of fishes were PCR amplified by using the primers according to Kocher *et al*. (1989).
L14841 5` CCATCCAACATCTCAGCATGATGAAA 3`;
H15149 5` GCCCCTCAGAATGATATTTGTCCTCA 3`

The PCR conditions for gene amplification were optimized. The amplicon mixed with gel loading dye (bromophenol blue) was electrophoresed on 1.2% agarose gel mixed with ethidium bromide along with molecular weight ladder on 120 volts and products were visualized on gel documentation system. The PCR product were purified and dissolved in nuclease free water.

DNA sequencing

Purified amplicons were used for sequencing in forward and reverse directions using AB1 3100 XL Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA) at CAMB (Centre of Applied Molecular Biology) Lahore following standard protocol.

Phylogenetic analysis

National Center for Biotechnology and Information (NCBI) was used to retrieve the member species, their accession number and geographical distribution that became the part of the phylogenetic analysis (BLAST: Basic Local Alignment Search Tool (nih.gov). To construct phylogenetic trees theorem of minimum evolution algorithm was employed (Desper and Gascuel, 2004).

Statistical analysis

The statistical characters of the sequence like conserved sites, variable sites, parsimony active sites and single tone sites were identified through Molecular Evolutionary Genetic Analysis (MEGA, V 6.0).

RESULTS

The data proved cytochrome b region as an important phylogenetic marker for the phylogeny and molecular

characterization of the fishes. It is the significant step towards barcode and diagnostic test development for identification of Pakistani fresh water fish species that will help in addressing the problem of mislabeling the fish meat and its adulteration. The results of the genetic characterization and molecular classification of the nine studied fish families are given below.

Family Cyprinidae

There are total nine individual species selected, belonging to Family Cyprinidae for this analysis. The homology analysis of *Catla catla* and *Cirrhinus mrigala* showed the maximum homology 100% with references (Supplementary Figs. 1A, B). The *Labeo rohita* samples showed homology (99%) with other isolates of *Labeo rohita* (Supplementary Fig. 1C). *Cyt b* sequences of all the samples of *L. calbasu* showed 100% homology with references (Supplementary Fig. 1D). The five unrelated sequences of gene of species *Cyprinus carpio* employed for homology through BLAST showed maximum identity 100% with reference species showing no variations (Supplementary Fig. 1E). The homology analysis of *Cyt b* gene sequence of *Labeo gonius* and *Puntius sophore* showed 100% identity to their reference species (Supplementary Figs. 1F, G). The homology analysis of *Cyt b* gene sequence of *Amblypharyngodon mola* showed 97% homology with reference species while *Osteobrama cotio* showed 100% identity to its reference species (Supplementary Figs. 1H, I).

Family Channidae

Two sample species of Family Channidae were included in the study. The BLAST homology analysis of *Channa marulius* showed the maximum identity 100% to its reference species while *Channa punctatus* showed 99 % homology (Supplementary Figs. 1J, K).

Family Notopteridae

The particular sample species of this family selected for this study is *Notopterus notopterus* and homology analysis of all five samples of *Notopterus notopterus* through BLAST showed the maximum identity with its reference species (Supplementary Fig. 1L).

Family Bagridae

The sample species of this family selected for this study are *Mystus cavasius*, *Mystus teengara* and *Rita rita.* The analysis of all five samples of *Mystus cavasius* showed 97% identity. In addition, *Mystus teengara and Rita rita* showed the maximum 100% homology with their reference species (Supplementary Figs. 1 M, N, O).

Family Siluridae

Homology analysis of all five samples of *Ompok pabda confirms* 97% homology. *Ompok bimaculatus* and *Wallagu attu* confirmed 96% and 97% identity, respectively with their reference species (Supplementary Figs. P, Q, R).

Family Schilbidae

There are two important fish species that belong to family Schilbidae, *Clupisoma garua* and *Gagata caenia* are included in the study. The first one shows 97% while second one shows 96% homology (Supplementary Figs. 1S, T).

Family Belontidae, family Ambassidae and family Clupeidae

The species under study that belonged to family Belontidae is *Colisa fasciatus* and its *Cyt b* gene analysis through BLAST showed 97 % identity (Supplementary Fig. 1U). Further, Family Ambassidae *species (Chanda nama) Cyt b* gene analysis through BLAST showed 100% (Supplementary Fig. V) while family Clupeidae species (*Gudusia chapra)* showed 97% identity with their reference species (Supplementary Fig. 1W).

DISCUSSION

Fish is an important meat source for human consumption and animal meal (Ryan *et al*., 2011). Among 193 fresh water fish species, 30 are considered economically important (Rafique and Khan, 2012). The visible morphology is helpful for characterization of fish species and it is performed with the help of different morphological keys (Ward *et al*., 2009). However, inadequate morphological diagnostic characters hinder identification of every species.

DNA Barcoding is a new molecular technique which focuses analysis of short consistent portions of mtDNA (Moftah *et al*., 2011). The cytochrome b region of mtDNA is polymorphic across orders and is helpful in discovering intraspecific variation in several species (Abol-Munafi *et al*., 2007). The study was designed to reveal secrets of phylogeny and molecular classification of fresh water fish species through *Cyt b* gene marker. It was intended to formulate DNA barcodes and methodology to clearly identify fish species of Pakistan.

The members of economically important and major nine families of Pakistani fresh water fish species had been selected and analyzed for their molecular classification. The phylogenetic tree showed that all samples of *Catla catla* from Pakistan formed a single clade with *Catla catla* (KF574602.1) which was reported from Indian waters representing monophyletic and same line of evolution.

Labeo barbatulus (KC631289.1) had been seen to be fallen in same link as *Catla catla* from Pakistani and Indian waters (Supplementary Fig. 1A). Pakistani *Cirrhinus mrigala* is forming a monophyletic relation with Indian *Cirrhinus mrigala* showing similar evolutionary history (Supplementary Fig. 1B). *Labeo rohita* from Pakistani waters formed a monophyly representing same line of evolution whereas the *Labeo rohita* (KR185963.1) from Indian waters formed a separate link with 1% divergence (Supplementary Fig. 1C). The *Labeo calbasu* of Pakistan showed complete similarity with Indian *Labeo calbasu* (KF574601.1) and formed a single clade showing similar pattern of evolution (Supplementary Fig. 1D). The *Cyprinus carpio, Cyprinus melanes* and *Pseudorasbora parva* are close relatives evolving with similar evolutionary patterns (Normark *et al*., 1991). Our results coincide with results of Thai *et al*. (2007) who found very low divergence levels (1-1.9%) between two species *Cyprinus carpio* and *Cyprinus melanes* (Supplementary Fig. 1E). *Labeo gonius* showed monophyletic origin for all samples from Pakistan but reference species (KX245008.1) showed distant evolution (Supplementary Fig. 1F).

Puntius sophore and *Puntius chola* both are close relatives (Supplementary Fig. 1G). *Puntius chola* might be considered as recent ancestor of *Puntius sophore* according to evolutionary patterns revealed (Pethiyagoda *et al*., 2012). One study described *Puntius* as a paraphyletic genus (Moghaddam, 2012). They used different data analyses and found unstable position of different species of *Puntius* in phylogenetic tree on the basis of *RAG* gene. These findings coincide with other molecular phylogenetic analyses (Wang *et al*., 2007). Phylogenetic analysis of *Amblypharyngodon mola*, showed it as a recently radiated species as compared to other species of same subfamily (*Rasbora rasbora* subfamily Danioninae). Moreover, *Amblypharyngodon mola* of Pakistani water system made a single clade with *Amblypharyngodon mola* of Nepali waters (Supplementary Fig. 1H). The *Cyt b* analysis of Pakistani *Osteobrama cotio* revealed that it is originating similarly with Indian *Osteobrama cotio* having common ancestors (Supplementary Fig. 1I).

Phylogenetic analysis of both species (*Channa maruloides* and *Channa punctatus)* showed *Channa punctatus* as closest relative of *Channa marulius* (Supplementary Figs. 1J, K). The both share a common ancestor where *Channa marulius* is more primitive than the other (Zhu *et al*., 2013). According to Adamson *Channa marulius* forms a sister group with *Channa striata*. Moreover, Li *et al*. (2006) reported *Channa marulius* as sister species of *Channa maruloides*.

In addition, *Notopterus notopterus* showed monophyletic origin of all samples from Pakistani waters but their reference species (AY504822.1) from USA showed distant evolution (Supplementary Fig. 1L). This is first report from this gene marker of this species in Pakistan and the surrounding waters of Pakistan. *Mystus cavasius* formed a single clade whereas reported sequences (HQ257292.1) of same species from Thailand appeared to be in a separate link showing distant evolution of both but having common ancestors (Supplementary Fig. 1M). The *Mystus teengara* from Pakistan and from Bangladesh (KF809934.1) showed 100% DNA sequence identity and appeared to be present in a single linkage showing similar line of evolution (Supplementary Fig. 1N). The studied *Rita rita* showed complete concordance with the Indian *Rita rita (*EU490921.1) representing 100% identity with monophyletic origin and similar evolutionary trends (Supplementary Fig. 1O).

The *Ompok pabda* showed monophyletic origin for all samples from Pakistan but reference species (FJ711250.1) showed distant evolution (Supplementary Fig. 1P). The *Ostiobrma cotio* has 100% homology with the reference representative (KF574721.1) (Supplementary Fig. 1Q). The all five analyzed samples of *Wallagu attu* from the rivers of Pakistan have no complete homology with the any other representative of the *Wallagu attu* or its related species (Supplementary Fig. 1R). *Clupisoma garua* formed a link separate to Indian *Clupisoma garua* (KC464430.1) showing distant evolutionary patterns (Supplementary Fig. 1S). *Clupisoma sinense* (JN020090.1) and *Clupisoma prateri* (HM236378.1) are closest relatives according to the cladogram. In addition, neutrality test suggested that *Clupisoma garua* may have undergone a population expansion. It is thus also established that *cyt b* is polymorphic and a probable gene marker which is helpful in determining the population structure of this species (Saraswat *et al*., 2014). *Gagata cenia* showed the mono formation for Pakistani species but the one from Chinese water (DQ192468.1) appeared to be present in a separate group showing a distinct pattern of evolution but have diverged from same common ancestors (Supplementary Fig. 1T).

The analyzed *Colisa fasciatus* showed the monophyletic origin of Pakistani *Colisa fasciatus* but reference species from Chinese water is present in a different clade and is being evolved more primitively than Pakistani *Colisa fasciatus* (Supplementary Fig. 1U). *Chanda nama* phylogenetic analysis represents it in complete agreement with the Indian species (KC774691.1) having 100 % identity (Supplementary Fig. 1V). They form a mono clade and found to have monophyletic origin The *Gudusia chapra* forms a distant clade from the one from USA whereas a mono calde is revealed by Pakistani *Gudusia chapra* (Supplementary Fig. 1W).

CONCLUSION

The present study revealed *Cyt b* region as an important phylogenetic marker because trees that are obtained with its data set coincides with pre-established phylogeny of fresh water fish species. This comprehensive picture of molecular characterization of Pakistani fresh water fish species using *cyt b* gene marker would help scientists to know about their phylogenetic and taxonomic status. DNA barcode used in this study can be formulated into a genetic test to identify fish species of Pakistan. Moreover meta-analysis of inferred DNA sequences of Pakistani fish species can elucidate interesting results and reveal the new taxonomic features of these species.

DECLARATIONS

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20240414043202

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

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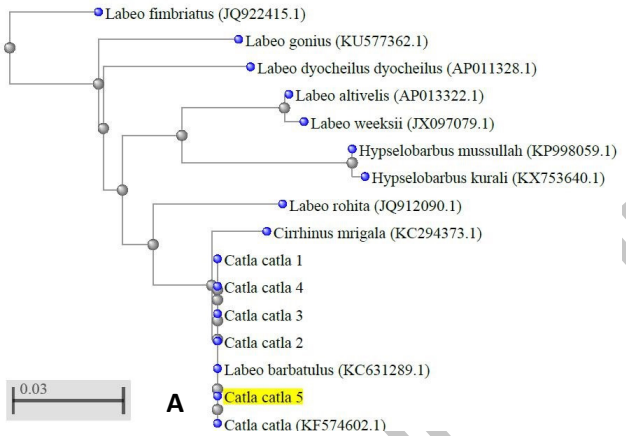

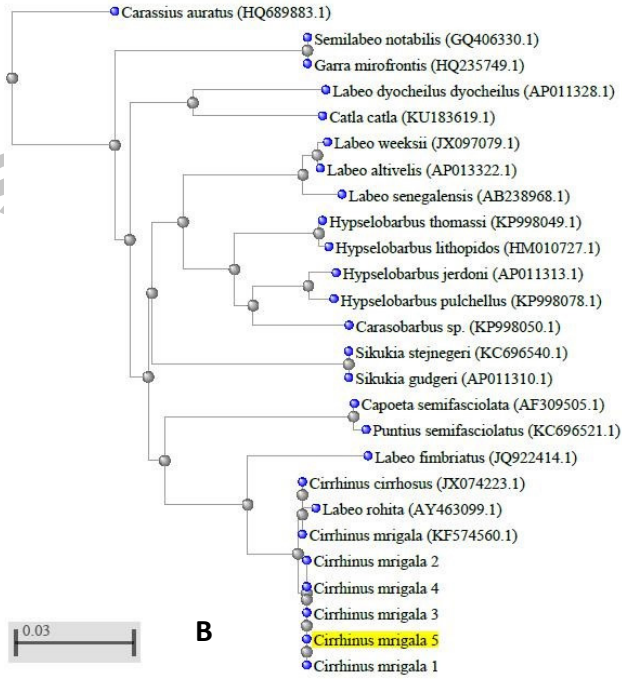

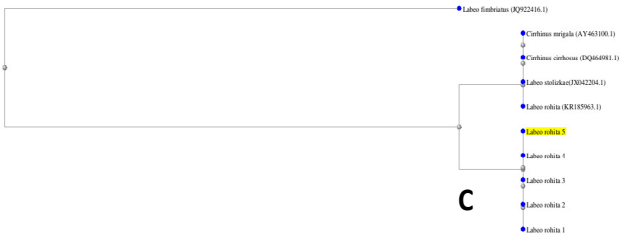

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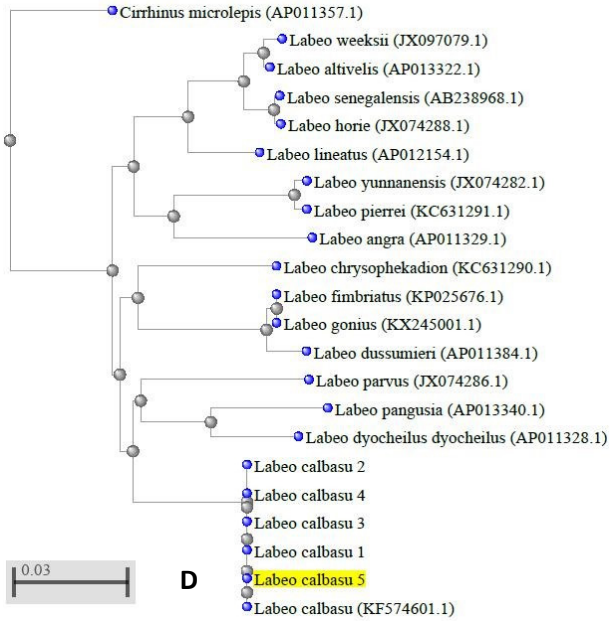
0030-9923/2024/0001-0001 $ 9.00/0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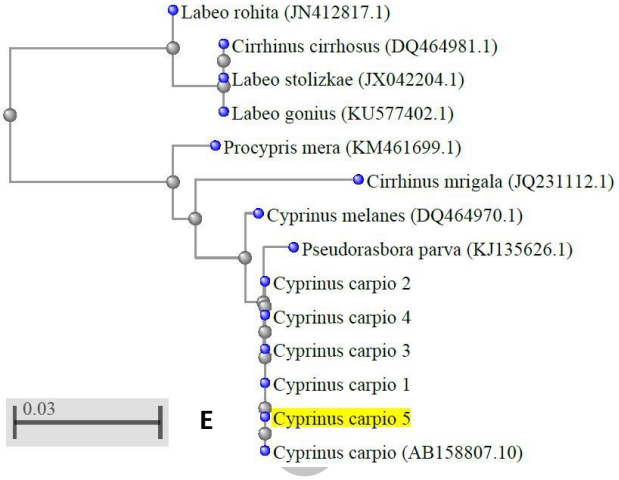
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Cirrhinus mrigala (JQ231112.1)
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Pseudorasbora parva (KJ135626.1)
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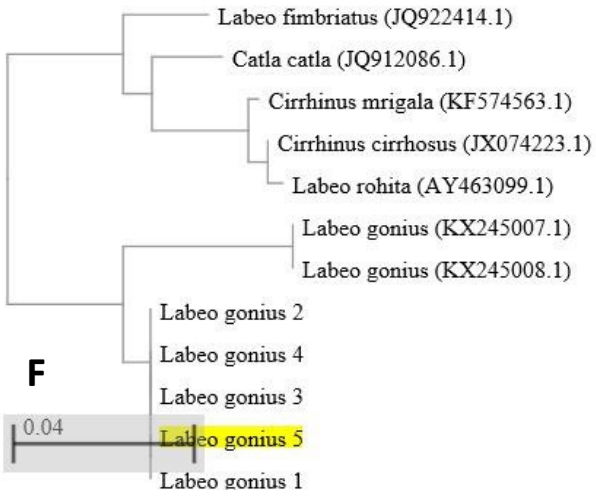
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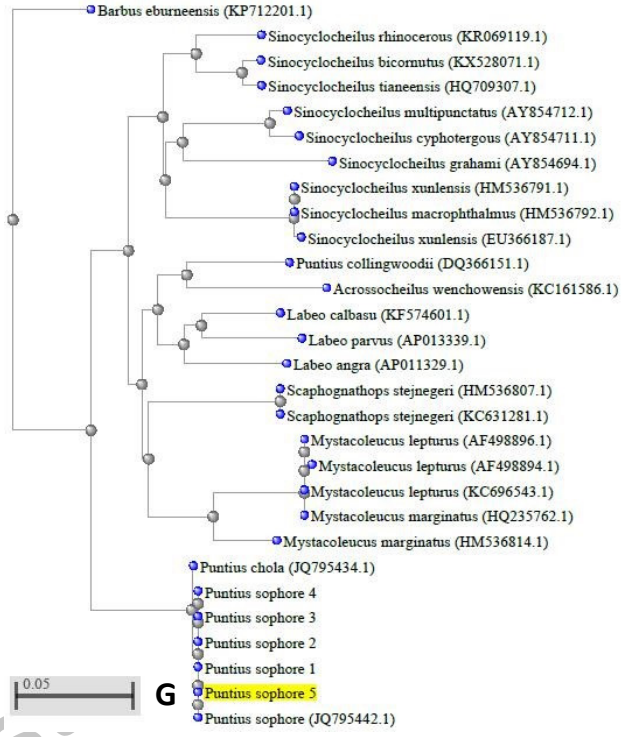
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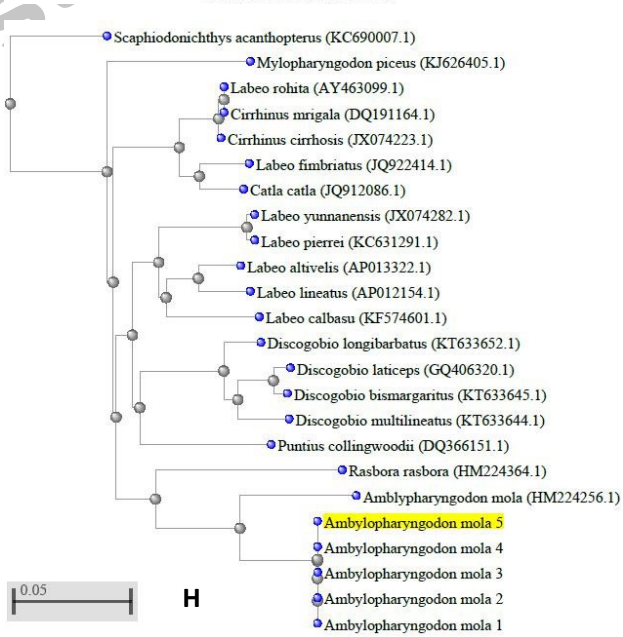
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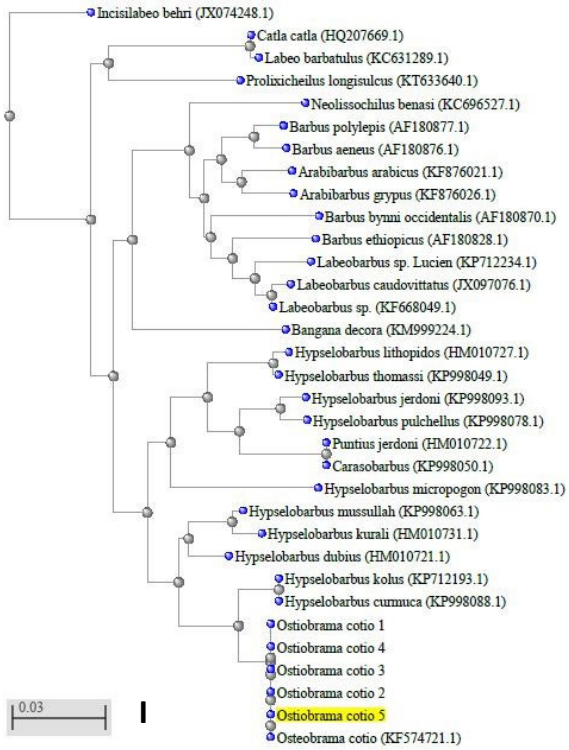
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Hypselobarbus lithopidos (HM010727.1)
Hypselobarbus thomassi (KP998049.1)
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Puntius jerdoni (HM010722.1)
Carasobarbus (KP998050.1)
Hypselobarbus micropogon (KP998083.1)
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Hypselobarbus kolus (KP712193.1)
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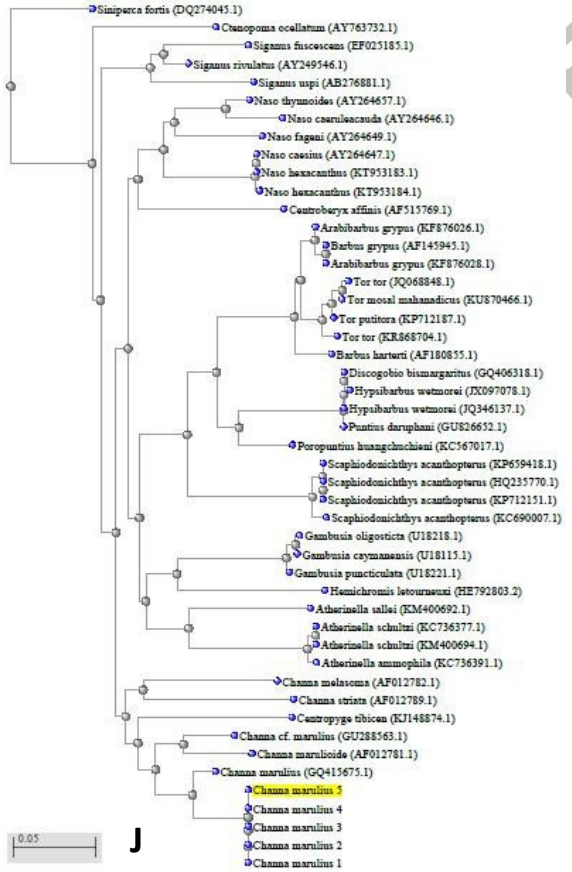
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Atherinella schultzi (KC736377.1)
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Atherinella ammophila (KC736391.1)
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Centropyge tibicen (KJ148874.1)
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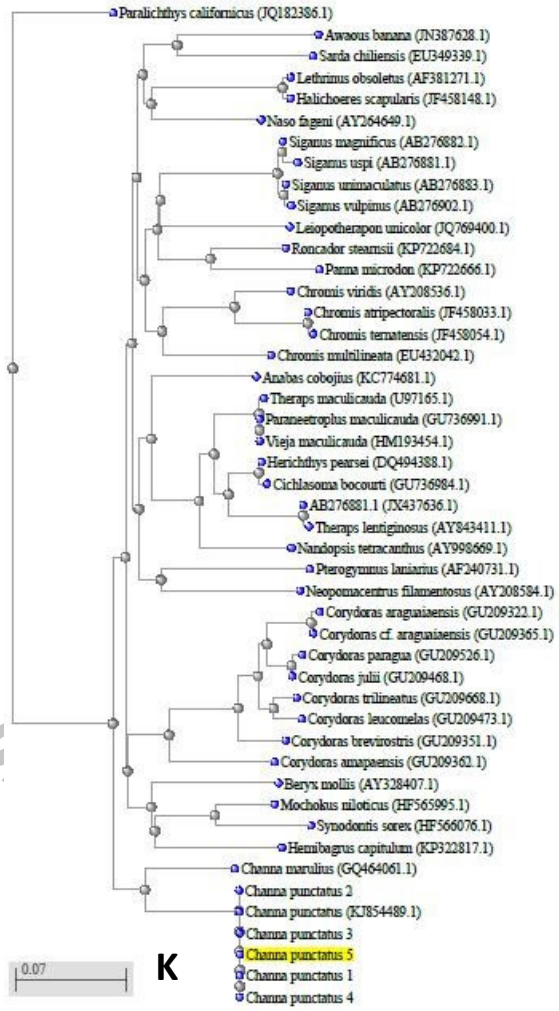
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Roncador stearnsii (KP722684.1)
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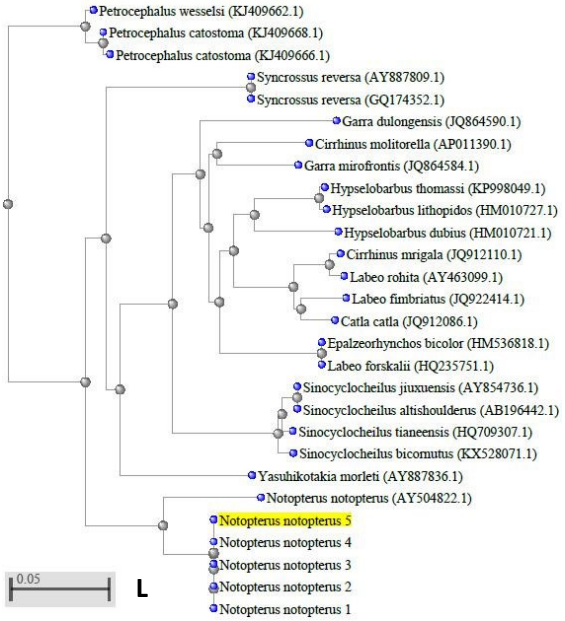
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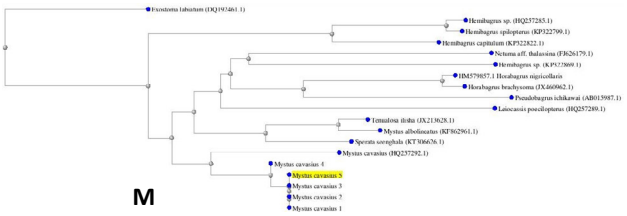
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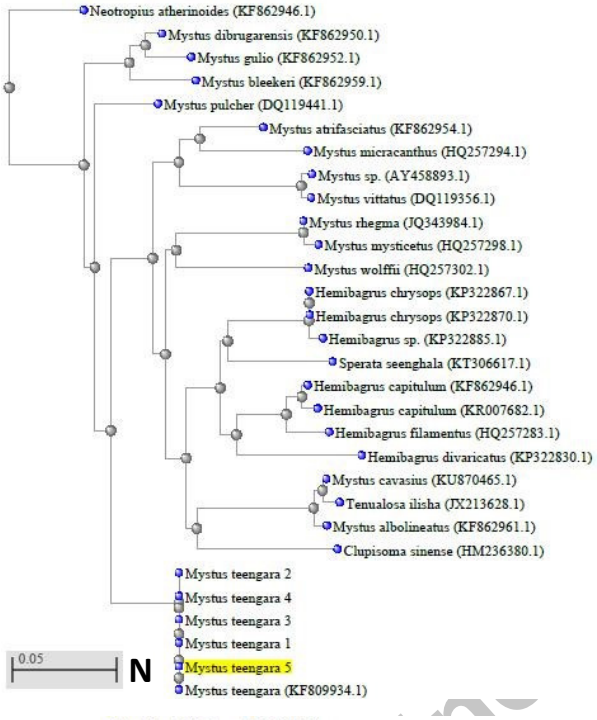
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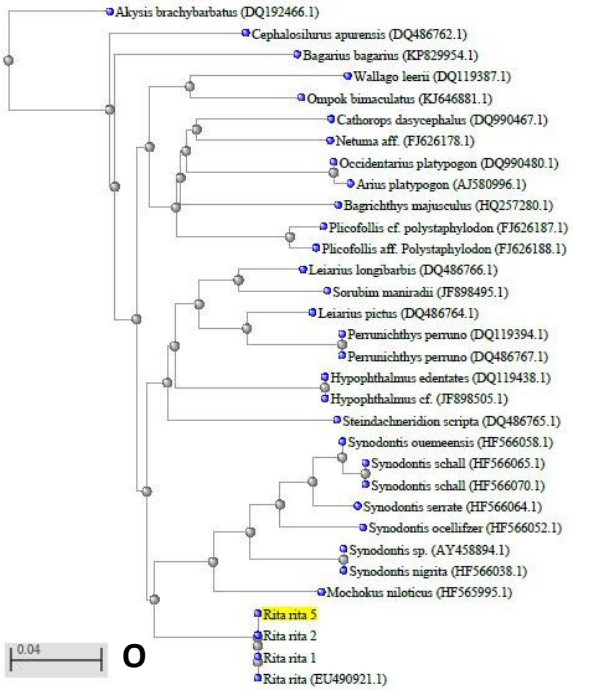
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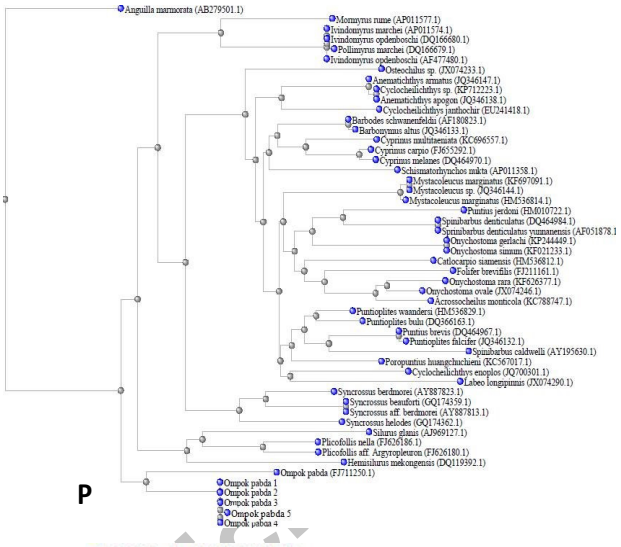
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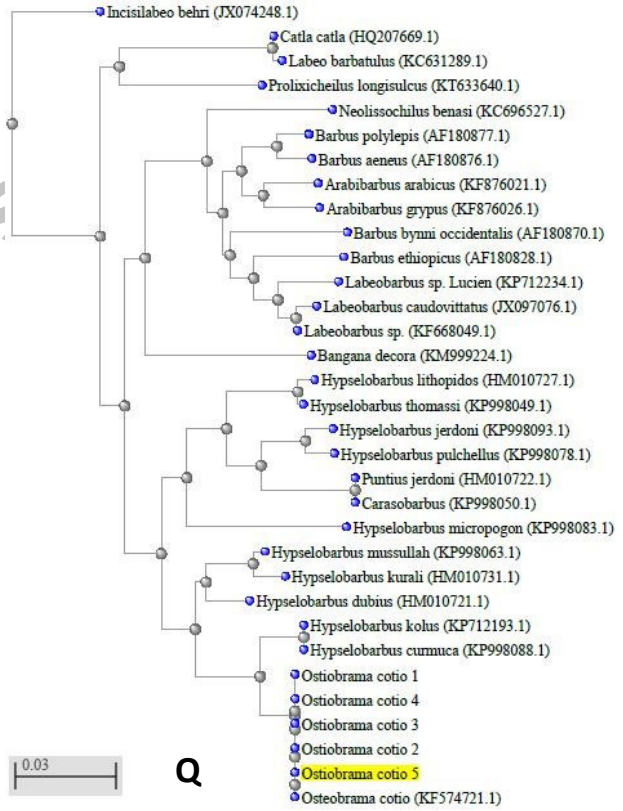
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Ostiobrama cotio 1
Ostiobrama cotio 4
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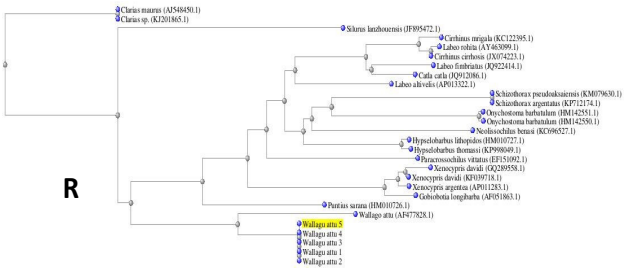
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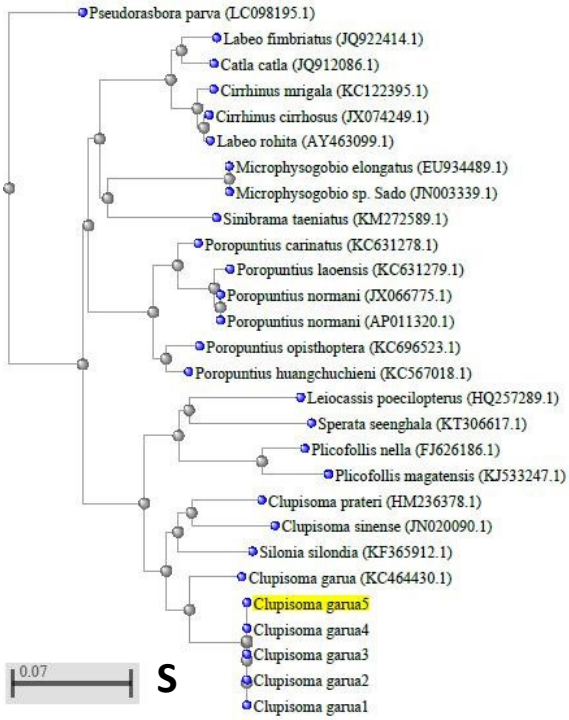
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Poroputius huangchuchieni (KC567018.1)
Leiocassis poecilopterus (HQ257289.1)
Sperata seenghala (KT306617.1)
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Clupisoma prateri (HM236378.1)
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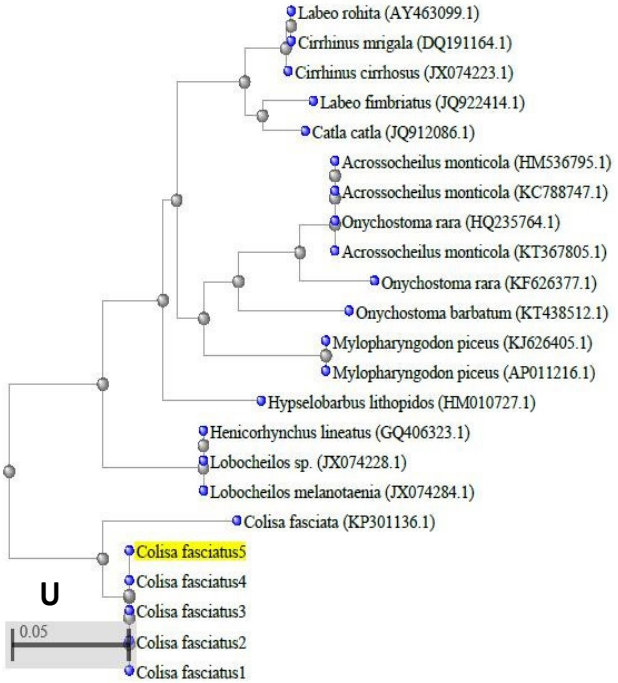
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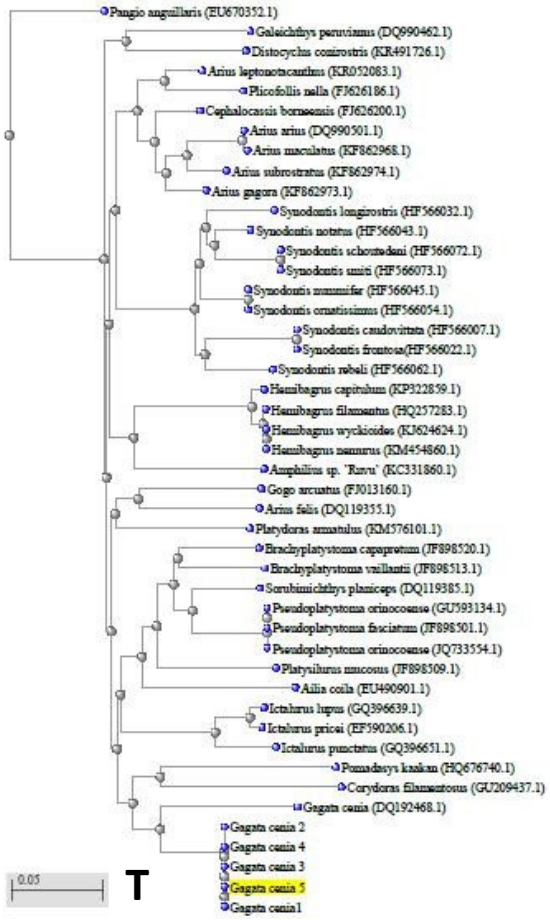
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Plicofollis nella (FJ626186.1)
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Arius arius (DQ990501.1)
Arius maculatus (KF862968.1)
Arius subrostratus (KF862974.1)
Arius gagora (KF862973.1)
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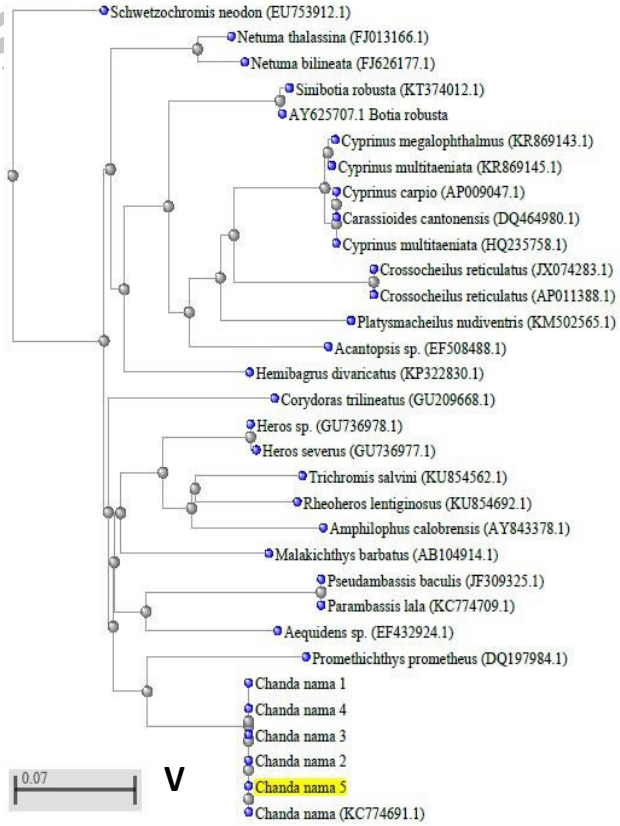
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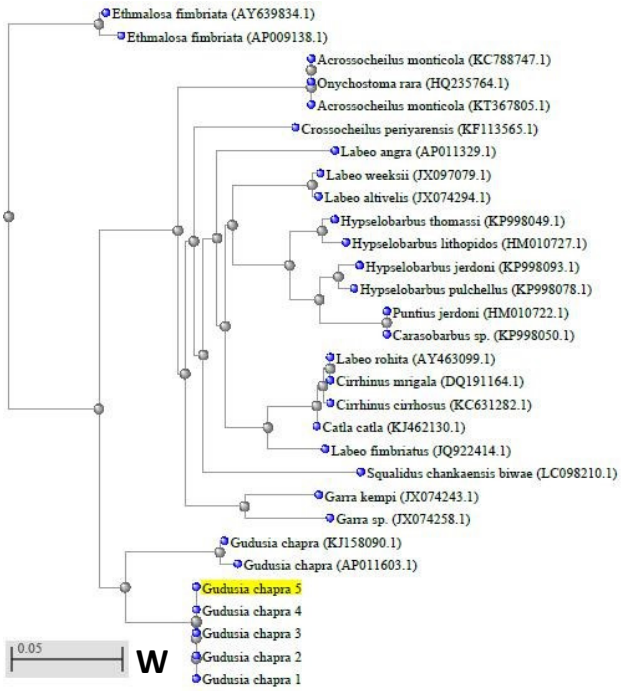

Supplementary Fig. 1. Phylogenetic analysis of based on *Cyt b* gene.
A, *Catla catla*; B, *Cirrhinus mrigala*; C, *Labeo rohita*; D, *Labeo calbasu*; E, *Cyprinus carpio*; F, *Labeo gonius*; G, *Puntius sophore*; H, *Amblypharyngodon mola*; I, *Osteobrama cotio*; J, *Channa marulius*; K, *Channa punctatus*; L, *Notopterus notopterus*; M, *Mystus cavasius*; N, *Mystus teengara*; O, *Rita rita*; P, *Ompok pabda*; Q, *Osteobrama cotio*; R, *Wallagu attu*; S, *Clupisoma garua*; T, *Gagata caenia*; U, *Colisa fasciatusa*; V, *Chanda nama*; W, *Gudusia chapra*.