

Molecular approach for identification of *Pangasianodon hypophthalmus* based on Mitochondrial Cytochrome b Oxidase Subunit I (COI) gene from Pakistan

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Abstract

The aquaculture industry is dependent on rich fish resources in water bodies. Human activities have led to a rapid decline of fish species. In Asia, the Pangasiidae family is highly valued for its potential for survival and its fillet meat. DNA barcoding is a taxonomic method using genetic markers in organisms mitochondrial DNA (mt DNA) for identification. The phylogeny and identification of *Pangasianodon hypophthalmus* in the subcontinent is of great concern. For species identification, a precise and rapid technique is DNA barcoding. This method is strongly effective for analyzing the divergence among species. DNA barcoding is more reliable as compared to external morphology. To avoid mislabeling and conservation of species, it is equally useful in juveniles as well as adult stages of fishes. As DNA bar-coding is a taxonomic method that uses small genetic markers in organisms' mitochondrial DNA (mt DNA) for identification of particular species. In recent study MAGA X and Kimura 2 Parameter was used to evaluate genetic distance and neighbor joining tree was constructed. BOLD and GenBank reveals the nearest identity matches. As mitochondrial *cyt-b* gene region was successfully used for identifying species and accepted as a standard region for DNA barcoding.

Introduction

Globally, fish account for more than half of all vertebrates, with more than 30,000 species. Additionally, fish provide humans with animal protein and are an important component of biodiversity. Classification and identification of fish are essential to fishery investigations, nature reserve assessments, and food and drug identification (Bingpeng et al., 2018). In order to study the huge variety of fish species, a proper taxonomical key is important and its molecular basis should be known (Vartak et al., 2015). Ontogenetic metamorphosis occurs in most fishes, resulting in a diverse range of morphological characteristics. On the path to ontogenetic development, many morphometric features changes. Species identification by Morphological characters poses great challenge and controversy to taxonomy as convergent or divergent evolution could cause changes in morphological characters (Bingpeng et al., 2018).

DNA barcoding is a tools that helps to identify the closely related species in diverse fish fauna that is based upon the isolation of DNA segments, as early studies have already recognized that sequence diversity in a 650-bp fragment of the mitochondrial-DNA, CO1 provides solid species level resolution for large variety of animal groups (Ramadan and Baeshen, 2012; Takahara et al., 2013). DNA barcoding technique is based upon the molecular approach of DNA isolation and tag them for a single species and stores them, later this data is used for identification purposes (Hebert and Gregory, 2005). From ovum to adult, barcoding eliminates the difficulties taxonomists face in identifying organisms (Ayesha et al., 2019).

The extraction of biomolecules, DNA, RNA, and protein, is of utmost importance in early days of experimental biology and for monitoring biological assaults (Wink, 2006; Armstrong and Ball, 2005). In contemporary biological sciences the isolation of DNA is a key step in many diagnostic kits for various diseases, DNA molecules can be extracted from various sample of dead tissues, bones, virus sample or dead organic matter for the purpose of study (Wink, 2006). DNA isolation is achieved by complete splintering of tissues that release cells and underlying organelles, denaturation of protein complexes, and activation of nucleases that release the DNA molecules (Doyle, 1996). Closely related intermixing of fish fauna is an important problem to solve, one species may evolve to many other species levels, and this requires a cheap and rapid method in a mass production environment (Darling and Blum, 2007).

DNA is relatively stable molecule at high temperature and usually provides far better hereditary (Pardo and Perez-Villarreal, 2004) and molecular identification of closely related species, that provides insight into diverse fish fauna, to keep a check on invasive and key varieties (Darling and Blum, 2007). It can be analyzed with a single piece of isolated gene to identify it again (Ram, et al. 1996). However, genetic information for less than 10% of the total species is yet documented (Bernardi et al., 2010). PCR (polymerase chain reaction) is an advance method of amplification of target sequence of gene and make copies for the desired DNA sequence, it is also noted that this process is very quick and specific but also very sensitive (Lockley and Bardsley, 2000).

The history of Lessepsian incursions demonstrates those species that becomes problematic to categorize commonly remain unrecognized or misidentified (Azzurro et al., 2015). In recent years the documentation of fish species has gained consideration due to the cumulative cases of deception in the fish processing industry (Espineira et al., 2009). DNA barcoding aims to provide a competent method for species-level identifications using a range of species-specific molecular tags derived from the five regions of the mitochondrial cytochrome c oxidase I (COI) gene (Hubert et al., 2008). As a result, scientists worldwide have established mitochondrial DNA databases and repositories for all animals, including fish (Ayesha et al., 2019). Striped catfish (*Pangasianodon hypophthalmus*) is presently the principal freshwater aquaculture species, more than 90% of it is marketed as fillet (Tung et al., 2001), out of which most is exported to over 127 countries worldwide and is often being mislabeled (Dung, 2008).

Materials And Methods

In recent work, for identification through DNA barcoding specimens of *P. hypophthalmus* were obtained from Tawakkal Fish Hatchery near Muzaffargarh, Punjab, Pakistan. As the standard taxonomic key was utilized to recognize the fish at morphological basis.

Phenol Chloroform extraction method was executed for isolation of DNA from fish tissues. The weight of tissues was approximately 50mg, they were grinded by adding extraction buffer. 12µl Proteinase-K was added and incubated at 37°C and 55°C for 60mins respectively. Then centrifuged at 5000rpm for 10mins. The supernatant was collected; Phenol, Chloroform and Isoamyl alcohol were added in 25:24:1 ratio and centrifuge at 12000rpm for 10min. Top aqueous layer was collected; Chloroform and Isoamyl alcohol in the ratio 24:1 were added, after gently mixing again centrifuge at 12000rpm for 10min. 0.1 volume of 3M sodium acetate and equal volume of ice cold ethanol(100%) was added to the supernatant and mixed thoroughly until DNA pallet was obtained. After incubation at -20°C for 1hr it was again centrifuged at 1000rpm for 10min and decant the supernatant. After washing with 70% ethanol air dry DNA pellets were suspended in 100µl of distilled water and stored at -20°C for further study. Finally sample was electrophoresed on 0.7% agarose gel at 80volt for 30mins. DNA concentration and purity was evaluated by Thermo-Scientific Nanodrops. DNA sequence was amplified from region of mitochondrial cytochrome b oxidase I (COI) gene using the primers pair as follows:

Cyt-b Forward primer: 5'AGCCTACGAAAAACCCACCC 3'

Cyt-b Reverse primer: 5'AAACTGCAGCCCCTCAGAATGATATTTGTCCTC 3'

SensQuest Labcycler was used for PCR amplification. For this processes 25µl of reaction mixture was used in PCR, containing distilled water 11.3µl, master mix 12.5µl, forward primer 0.1µl, reverse primer 0.1µl and DNA template sample 1µl. The PCR thermal cycling conditions includes an initial denaturation of 95°C (5 min), followed by denaturation at 95°C (30 sec; 40 cycles), annealing at 55°C (30 sec) and extension at 72°C (30 sec), with final extension at 72°C (7 min). After amplification, PCR products were run on 1.5% agarose gel for 50 min and then visualized using UV transilluminator to assess the quality of the amplified product. The most clarified samples were selected for the sequencing purpose.

Results

The purified PCR products was for sequencing. Blast results by NCBI helps to determine the best match homology. The barcode was deposited to GenBank under Accession number MT441543.1(*Pangasianodon hypophthalmus*) with 364bp and BLAST results in table.1.1 and pairwise genetic distance in table.1.2.

A distinctive electropherogram was formed after DNA sequencing to show nucleotides in colorful peaks(Fig. 1.1). By means of MEGA X software; genetic distances and phylogenetic relationship was analyzed as NJ trees and K2P genetics were produced Evolutionary analyses of the aligned sequences were conducted by program MEGA X (Fig. 1.3). The phylogenetic tree (Fig. 1.2) was rooted among *P hypophthalmus* Accession No: MT441543.1 and closely related KY586022.1,

KY586028.1, KY5860251, KY586008.1. The relationship was analyzed as NJ tree and K2P genetics were rooted among above (Fig. 1.2 and 1.3)

Table.1.1: Accession No. and BLAST results analysis of *Pangasianodon hypophthalmus*

Query ID	NCBI: taxonomy ID	Sequence Length	Accession No.	Percentage similarity	E Value	Max Score	Total Score	Accession No. of best match & % match
43205	310915	364	MT441543.1	100%	0.0	673	673	KY586022.1 (100%) KY586028.1 (99.73%) KY586025.1 (99.73%) KY586008.1 (100%)

Table.1.2: Pairwise Genetic Divergence between Species of *Pangasianodon hypophthalmus*

	MT441543.1 <i>P.hypophthalmus</i>	KY586022.1 <i>P.hypophthalmus</i>	KY586028.1 <i>P.hypophthalmus</i>	KY586025.1 <i>P.hypophthalmus</i>	KY586008.1 <i>P.hypophthalmus</i>
MT441543.1 <i>P.hypophthalmus</i>					
KY586022.1 <i>P.hypophthalmus</i>	0.0000				
KY586028.1 <i>P.hypophthalmus</i>	0.0028	0.0049			
KY586025.1 <i>P.hypophthalmus</i>	0.0028	0.0026	0.0000		
KY586008.1 <i>P.hypophthalmus</i>	0.0055	0.0099	0.0099	0.0077	

Discussion

For inferring fish phylogenetic relationships and understanding speciation, only morphometric characteristics were used before because *Pangasiidae* species are highly similar, it is difficult to distinguish them (Ahmed et al., 2018). Molecular analysis based on sequence of mitochondrial-DNA for is helpful in accurate recognition of unknown species (Dawnay et al., 2007 and Kerr et al., 2009). Cyt-b(cytochrome) gene sequence of 650 base pair are usually used in study for species identification (Lohman et al. 2009) and sequence of 649bp was used for identification of *Labeo bata* from Pakistan (Naeem and Hassan, 2019).

Present study on *Pangasianodon hypophthalmus* was conducted to examine the species by DNA barcoding. Amplified PCR product was sequenced, and complete barcode's fragments of 364 base pairs of *P. hypophthalmus* (Accession No: MT441543.1) were obtained. Earlier Gianmarco et al. (2009) reports fragments of base pairs extending 157 to 541 bp in humans, which lies close to recently identified base pairs(364bp). The most appropriate way of distinguishing the DNA depend diversity of intra and interspecific is barcode analysis used to distinguish diverse species, which correspond with neighbor joining tree. Besides that many standardized methods are still used for classification and distance measurement (Bhattacharjee et al. 2012). Comparison of quality and quantity of DNA were accessed by Nanodrop and absorbance were noted for 1.6 to 2 for purified DNA product after extraction (Cawthorn et al. 2011) as the value of absorbance were found 1.6–1.76 (Naeem and Hassan, 2019) study revealed that present extraction quantification were in general agreement.

Study of molecular biology by Cytochrome b also useful for molecular taxonomy of fish species (Luo et al., 2011). Identification sequence as Cytochrome b gene is significantly used for identification of animals and plants diversity (Khaliq, 2012). So the present study used Cytochrome b for the identification of fish species. DNA barcoding marked as very useful for evolutionist, taxonomist and phylogenetic study. Previous studies were in debate about the importance of these studies for taxonomists in DNA barcoding processes to identifying species and its bio-diversity (Hebert et al., 2003). Cyt b oxidase was successfully used for DNA barcoding of fishes, as it effectively mark the differences among specific species (Al-Zafiri et al., 2018). The barcoding could be useful in a taxonomic study but not as a whole; as it provides additional information to make the research significant (Ebach and Carvalho, 2010).

Species identification was accessed by using by BOLD and NCBI database for this study (Ha *et al.*,2018). Sometimes the results from Barcode of life database (NCBI and BOLD) had significant differences in identification percentage and phylogenetics. This database may also have some unknown species absent in record like *P. tenuispinis*. Recent results reveal that DNA barcoding is more specifically accessing fishes on basis of diversity (Takahara et al., 2013). Phylogenetic differences and species identification were noticeably explained on basis of cytochrome b genes, considered as the preeminent identification tool. The genetic divergence was found in their sequences due to variations. Those variation percentages are measured on the basis of knowing species similarity index (Falade et al., 2016).

Alignment of nitrogenous bases in present study was close to Pouyaud et al. (2009). Several countries require that species be identified when fish are supplied to souks without proper labeling (Marko *et al.*,2004). DNA-barcoding can be used for confirmation results of fish species that were under consideration, as previously studied by Panprommin et al. (2019). As these DNA bands clearly indicates the size of sequence that were under consideration for study. In many species of the catfish the specimen were mislabeled reasoned lacking in proper DNA based identification (Ha et al., 2018).

Species were observed to be interconnected, such analysis of closeness were assessed by measuring of distance by neighbor joining tree, as identical relationship was marked in present study where closely related species were grouped together to formulate a tree (Iyiola *et al.*,2018). As the general reference sequence, BOLD and NCBI are operative, and data-base helps in explaining sequences (Wong *et al.*,2011). As this study also mentioned it.

Conclusion

Identification of fishes on molecular bases, in Pakistan is not being considered an accustomed exercise. Numerous techniques practiced, for classifying documentation of fish species traditionally; are less authenticated in contrast to approach of techniques aligned at molecular roots. Recent study was an attempt to provoke the future of these modern approaches. Prior techniques utilized for eras were confined; as they do not show effective outcomes for mislabeling of fish-fillet and fish species, besides spoiled specimens. Substantiations among nucleic divergences amongst species; genera and family could be examined with molecular approaches.

Declarations

Disclosure statement

No potential conflict of interest was reported by the authors.

Prof. Dr. Muhammad Naeem designed and invigilate the work.

Tayyaba Malik performs and analyzed work and write the manuscript.

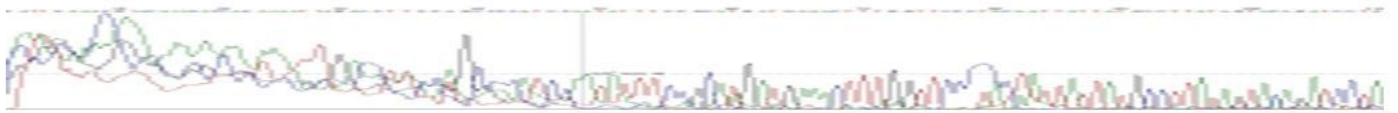
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Figures



Peaks of nucleotides Sequenced from 1-110 base pairs of forward primer.



Peaks of nucleotides Sequenced from 110-220 base pairs of forward primer



Peaks of nucleotides Sequenced from 220 to onward base pairs of forward primer



Peaks of nucleotides Sequenced from 1-100 base pairs of reverse primer



Peaks of nucleotides Sequenced from 100-200 base pairs of reverse primer



Peaks of nucleotides Sequenced from 200-300 base pairs of reverse primer



Peaks of nucleotides Sequenced from 300 to onward base pairs of reverse primer

Figure 1

1.1. Results of Electropherogram showing Peaks of nucleotides

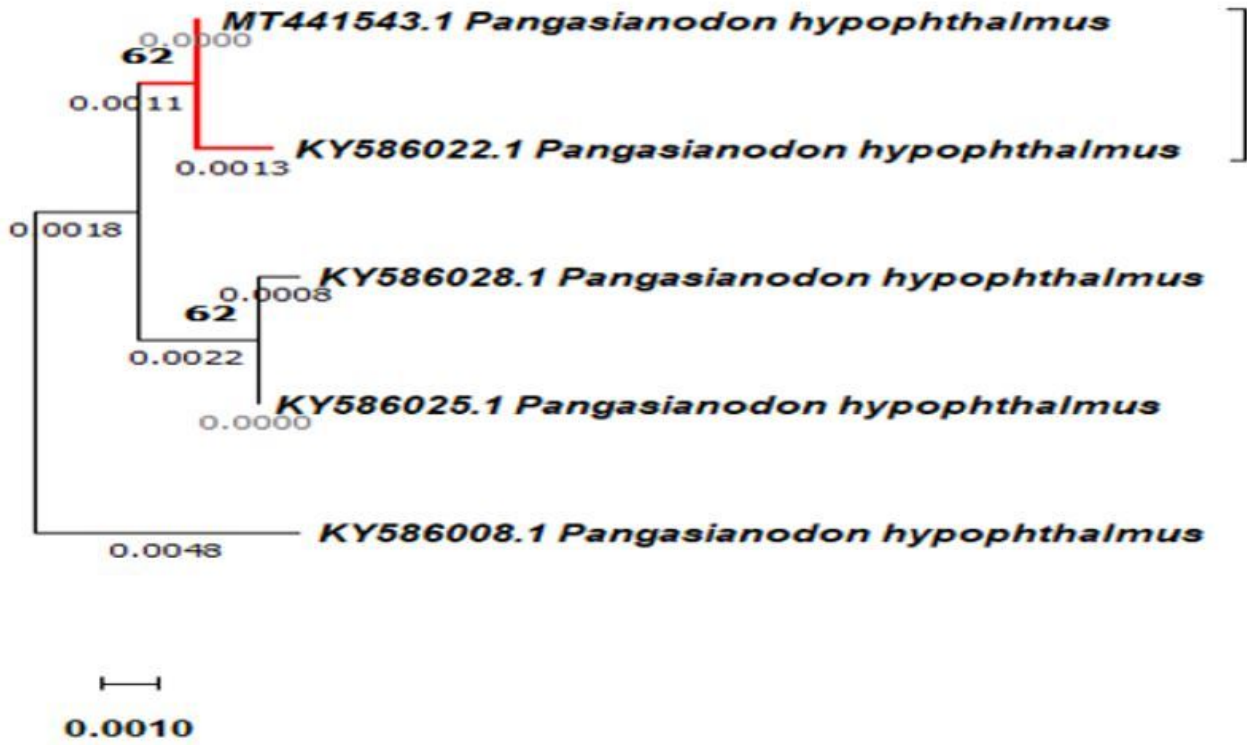


Figure 2

1.2. Phylogenetic distances

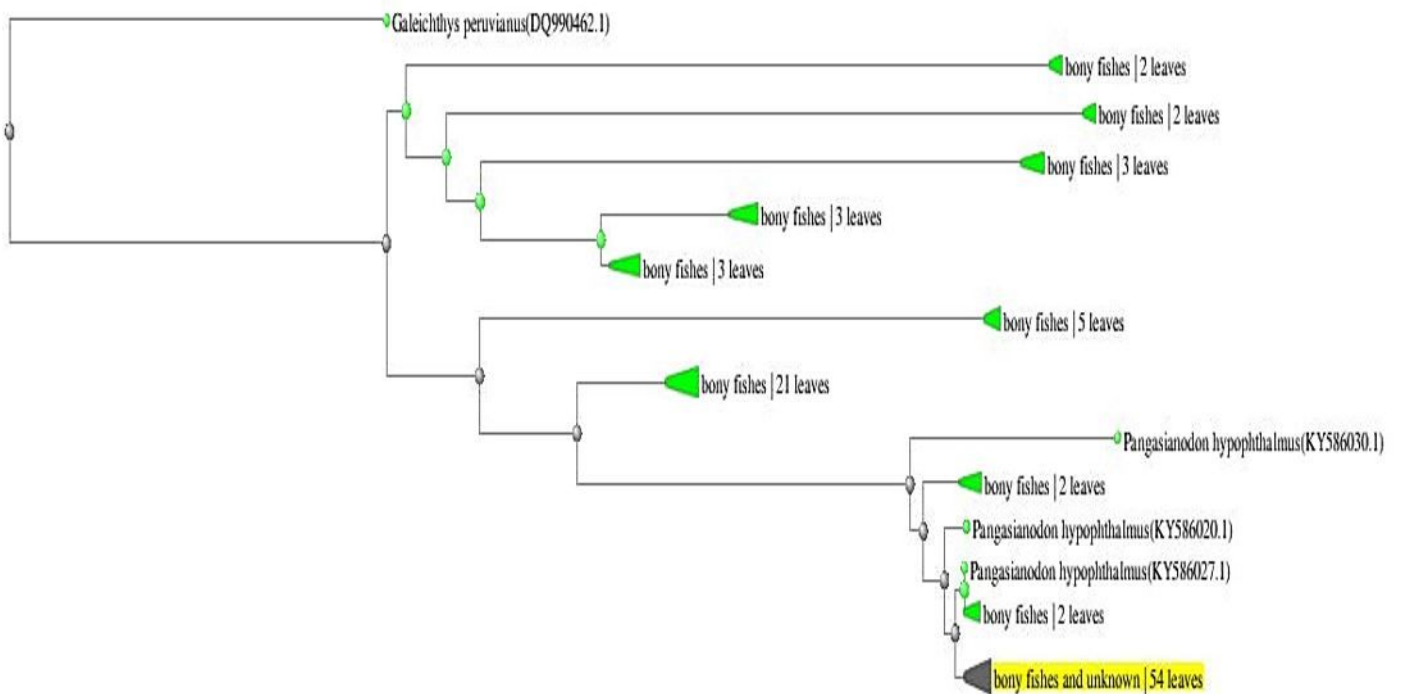


Figure 3

1.3. BLAST Neighbor joining tree by blast inquiry with Distance Evaluating of *P. hypophthalmus*