MERIT RESEARCH JOURNALS
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Merit Research Journal of Agricultural Science and Soil Sciences (ISSN: 2350-2274) Vol. 6(3) pp. 024-031, March, 2018
Available online http://meritresearchjournals.org/asss/index.htm

Original Research Article

CO1 gene based identification and phylogenetic analysis of two fresh water fish species *Labeogonius* and *Cirrhinusmrigala* (family: Cyprinidae) from River Ravi, Punjab, Pakistan

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Abstract

Taxonomy has always been a challenging job for the scientists. Traditional methods for identification of fishes are based on morphological features which sometimes create great confusion due to great morphological similarities in closed species. DNA barcoding is a modern species identification method in which a short standardized mitochondrial DNA region is used. In this method sequence diversity in a 658 base pair fragment near the 5′ end of the mitochondrial Cytochrome *C* Oxidase 1 (*CO1*) gene is employed as a tool for species identification. This method is capable enough for identification of processed, preserved and damaged fishes as compared to traditional morphometric methods and is also appropriate for rapid identification of juveniles as well as adult stages of fishes. Present study is conducted to identify two fresh water fishes, *Labeogonius* and *Cirrhinusmrigala* on basis of *CO1* gene.Partial sequences of *CO1* gene of these fishes were amplified by conventional PCR method and BLAST analysis confirmed that genetically identified fishes are the same fishes which were identified morphologically at the beginning of the study with the help of identification key. The phylogenetic tree analysis confirmed the evolutionary relationships between *L.gonius* and *C.mrigala* from Pakistan and fishes from other parts of the world. From these findings, it is concluded that the partial *CO1* gene sequence, may serve as a potential marker for identification of most of fish species at molecular-level and their phylogenetic analysis.

Keywords: DNA barcoding, *CO1* gene, *Cirrhinusmrigala, Labeogonius,* MEGA 7, dnaSP5

INTRODUCTION

Precise identification of fishes is very important for ecological checks, evaluation of environmental effects, fish industry profit, organization of fishery resources, stopping illegal trafficking and establishing protective areas for fishes (Moura et al*.,* 2008; Valdez et al., 2010). It is also important to identify fish species for preservation of biodiversity of a region (Frankham, 2005). Fishes in Pakistan are generally identified on traditional morphology based methods which depend on a particular set of morphological characters mainly found in adult specimen. Accurate identification of fishes becomes very challenging where morphological characters are not identifiable like in trade practices, where fish is in the form of fish fillets or during developmental stages where

morphology is not complete. Such situations demand an alternative method for fish identification. DNA barcoding has proved so far a promising alternate identification tool.

Morphological limitations are further complicated by variation in ploidy levels within genera (Agnèse et al*.,* 1990). The limitations of morphological characters have been improved with technological advances in molecular biology, which have led to the use of allozymes, DNA arrays, multiplex polymerase chain reactions (PCR) and recently DNA barcoding has been employed to complement or refine morphological species identification (Chen et al*.,* 2015; Kochzius et al*.,* 2010; Pereira et al*.,* 2011). The DNA barcoding technique in particular is based on the use of a short standardized cytochrome c oxidase subunit I (*COI*) sequence to distinguish between animal species (Hebert et al*.,* 2003/2004). It has gained worldwide support because it is rapid, cost effective (Stein et al*.,* 2014), and applicable to species identification across the animal kingdom (e.g. Hebert et al*.,* 2003; Nigro et al*.,* 2016; Sethusa et al*.,* 2014; Ward et al*.,* 2005; Van der Bank et al*.,* 2013).

Mitochondrial DNA (mt-DNA) has been widely used in phylogenetic studies of animals because it evolves much more rapidly than nuclear DNA (Brown et al*.,* 1979). In fact, the rapid pace of sequence changes in mt-DNA results in differences between populations that have only been separated for brief periods. CO1 gene is considered as one of the widely used markers in the studies of population genetics and evolution, because it is among the most conservative protein-coding genes found in the mitochondrial genomes of animals.

The mitochondrial cytochrome oxidase 1 gene has been proposed to be the most effective barcoding gene for metazoans. The total length of *CO1* gene in vertebrates is about 1545 bp in length and the region nominated as “barcode” comprises of a standardized 648 bp segment start from 58 to 705 bp region from 5′ terminus. The segment has been defined using mouse mitochondrial genome as reference. The DNA barcoding is gaining widespread popularity. Molecular tools facilitate rapid evaluation. Species identification through DNA barcoding is based upon the principle that interspecific divergence sufficiently beat intra specific divergence and the biological species can be clearly established by onset value, which corresponds to the divergence between the nearest neighbors within a group (Hebert et al., 2003)

Pakistan possesses significantly large natural inland water resources including rivers with their tributaries, many canals and lakes both artificial and natural. If we look at the flowing pattern of rivers and streams, three major drainage systems can be found in Pakistan including, the Indus drainage, the Baluchistan coastal drainage and landlocked drainage systems. Indus drainage is the largest river system of the country, consisting of the main Indus River and all its associated rivers and streams in Gilgit-Baltistan, Khyber Pakhtunkhwa, Punjab, northern and eastern Baluchistan and Sindh. These areas create a transient zone between the Oriental, Palearctic and Ethiopian zoogeographical regions resulting in the variation of fish fauna (Mirza, 1994). The fish fauna of water bodies located in the areas under Pakistan is known through a number of comparatively recent studies conducted at different places and times (Mirza, 1975, 1978, 1980, 1990, 2003, Rafique and Qureshi, 1997; Rafique, 2000; Rafique, 2001; Rafique et al., 2003). Although these studies offer starting point information on dispersal and variety of species in different areas, but still these studies do not cover the species of special importance and their conservation status totally.

The freshwater fish fauna of Pakistan is represented by a minimum of 193 fish species. These species are divided into 3 main groups containing 86 genera (Rafique, 2007; Rafique&Mian, 2012). This diversity also includes the exotic species introduced in wild or fish farming system of Pakistan during the recent past. Among the total fish fauna of Pakistan, 86 species (8 exotic and 78 indigenous) have been identified as “species of special importance” on the basis of endemism, IUCN status, economic importance and rarity. Among the indigenous species of special importance, 43 species have been identified as endemic to Pakistan and Kashmir.

During present study two fresh water fish species were selected due to their high commercial values (Rafique and Mian, 2012). *Labeogonius* commonly known as “Kurialabeo” is an indigenous species found in two provinces Punjab and Sindh with least Concern status by IUCN. Its maximum length reaches 70 cm and their weight reaches 2.5 kg. *Labeogonius* has high commercial value. The second species *Cirrhinusmrigala* commonly known as “Mrigal/Mori” is found in Balochistan, KPK, Punjab and Sindh. It is also an Indigenous fish species with least Concern IUCN status. Its maximum length reaches to 100 cm and weight 12.7 kg and its commercial value is also very high.

Aims of this study were to use the DNA barcoding technique for genetic identification of two fresh water fish species by using short sequence of *CO1* gene from mitochondrial DNA. The DNA barcoding technique is relatively new in Pakistan and the pioneer work was done on three farm fish species *Ctenopharyngodonidella, Cyprinuscarpio and Cirrhinusmrigala* (karim et al., 20015). Phylogenetic analysis was also done using the partial *CO1* sequences of both fishes and between sequences of other species from different geographical regionsto evaluate the genetic distances among them.

MATERIALS AND METHODS

Sampling

Fish samples were collected from River Ravi at Head

Balloki region in district Kasur, Punjab, Pakistan. Fishes were captured by using cast net with the help of fisher man from Punjab fisheries department. Captured specimens were photographed according to the protocol given on www.boldsystem.org. One of the pelvic fins of captured fishes was removed and transferred immediately to autoclaved 1.5 ml Eppendorf tubes. The fishes were then preserved as voucher specimen in the laboratory of Zoology Department of Govt. College of Science Wahdat Road, Lahore. DNA Extraction Kit (TIAN ampg) was used to extract DNA from fins of fishes. Extracted DNA was run on the agarose gel to check quality of the extracted nucleic acid. For this purpose, 5ul DNA sample along with 3ul loading dye (3X bromophenol blue) was run on 0.8% agarose gel prior to its amplification. Total 11 DNA samples were run on gel to determine the success of DNA extraction. DNA bands were seen by passing UV light through the gel in Gel Documentation System. The bands in the gel documentation indicated the presence of DNA. All the DNA samples were amplified by using FishF1 and FishR2 primers.

Fish F1**:** 5'TCAACCAACCACAAAGACATTGGCAC3'
Fish R2**:** 5'ACTTCAGGGTGACCGAAGAATCAGAA3'

A total of 25µl PCR reaction mixture was used for each of the 11 DNA samples with following ingredients 2 µl of DNA template, 5 µl of master mix (containing buffer, dNTPs, Taq polymerase, Magnesium Chloride), 1 µl of each primer and 17 µl of de-ionized water. The thermal conditions of PCR consisted of an initial step of 5 minutes at 95^{o}C (initialization) followed by 35 cycles of 0.5 minutes each at 94^{o}C (denaturation), 0.5 minutes at 60.5^{o}C (annealing) and 1 minute at 72^{o}C (extension/elongation), followed by 10 minutes at 72^{o}C (final extension/elongation) and then cooled at 4^{o}C (termination/final hold). PCR products were run on agarose gel and the products with gene of interest (*CO1*gene) were selected for sequencing.

Genetic Analysis

For comparative purposes, *CO1* additional sequences of the genera *Cirrhinus and Labeo* and other fishes from same and different families were downloaded from data bases of GenBank and BOLD System (table S1). Sequences were aligned using MUSCLE found in MEGA7 (Molecular Evolutionary Genetic Analysis). After alignment, Phylogenetical analysis was done for tabulating Kimura two-parameter (K2P) distances (Kimura, 1980).

Analysis was done on the basis of generation of Neighbor-joining tree. Neighbor – joining analysis reveals the genetic distances in *CO1* among individuals into genetically distinct lineages which in teleost fishes, correspond well with species (Baldwin and Weigt, 2012, Weigt *et al.* 2012b). GC content percentage in the sequences was also studied through MEGA7. Haplotype numbers in each sequence, its diversity and Polymorphic sites were also studied by DNA SPv5.

RESULTS

A total of 11 fishes belonging to two generas and two species were used in the present study. These fishes were from the order cypriniformes, family cyprinidae and subfamily labeoninae. The fish species contributed in this study were *Cirrhinusmrigala* (n=6) and *Labeogonius* (n=5). The partial *CO1* sequences of these fishes were submitted in Gen Bank with Accession numbers MF468121, MF468122, MF468123, MF468124, MF468125, MF468126, MF468127, MF468128, MF468129, MF468130 and KY569370.

Cirrhinusmrigala barcodes analysis (dnaSP5.10) showed that they had an average read length of 615 base pairs. There were 55 sites with alignment gaps or missing data, 577 invariable or monomorphic sites, 16 variables or polymorphic sites, 15singleton variable sites, 17 mutations and 1 parsimony informative site found in the barcodes. While barcodes of *Labeogonius* had an average read length of 597 base pairs. There were 147 sites with alignment gaps or missing data, 485 invariable or monomorphic sites, 5 variable sites or polymorphic sites, 3 singleton variable sites, 7 mutations and 2 parsimony informative sites found in the barcodes.

Sequences were also analyzed for presence of haplotypes and haplotype diversity. Haplotypes were found in both experimental fish species, showing that besides having genetic variations among them, all of these form a haplo-group and share same ancestor with a single-nucleotide polymorphic mutation. In our present work three haplotypes were found in barcodes of *C.mrigala* and 5 haplotypes were found in partial sequences of *L.gonius.* The haplotype diversity in our sequences of fish is 0 .600 and haplotype diversity in 5 sequences of *Labeogonius* was calculated to be 1.00.

Kimura 2 parameter method was chosen as a best method for intra- and interspecific variations. The overall mean K2P intraspecific distance was calculated using sample sequences of *Labeogonius* and *Cirrhinusmrigala* and downloaded sequences from other geographical regions for calculation of conspecifics and congeneric divergences. Sequences were downloaded from the public data portal of the data bases of BOLD System and Gen Bank (table S1). Analysis of barcode sequences permitted clear discrimination of taxonomic status of all the species examined. The mean intraspecific K2P genetic distances of *Labeogonius* and *Cirrhinusmrigala* are shown in Table 1 and 2.

The overall mean K2P intraspecific distance was calculated using sample sequences of *Labeogonius* and

Table 1. K2P pairwise distances for *Labeogonius*

Taxonomic level	K2P DISTANCES % Comparison within				K2P DISTANCES % Comparison between			
	Min	Max	Mean	S.E	Min	Max	Mean	S.E
SPECIES n=9	0.000	0.043	0.012	0.003	0.02	2.197	0.903	0.123
GENERA n=19	0.00	1.318	0.374	0.026	0.051	1.371	0.380	0.028
FAMILY n=63	0.00	2.258	0.744	0.058	_	_	_	_

Table 2. K2P pairwise distances for *Cirrhinusmrigala*

Taxonomic level	K2P DISTANCES % Comparison within				K2P DISTANCES % Comparison between			
	Min	Max	Mean	S.E	Min	Max	Mean	S.E
SPECIES n=7	0.000	1.623	0.474	0.076	0.03	2.197	0.966	0.211
GENERA n=27	0.00	2.474	0.653	0.116	0.070	1.821	0.345	0.034
FAMILY n=113	0.00	3.420	0.657	0.073	_	_	_	_

Table 3. Haplotype data of *Labeogonius* and *Cirrhinusmrigala*

Species name	No. of haplotypes	Haplotype diversity	Haplotype distribution
Labeogonius	5	1.00	Hap_1: 1 MF468121 Hap_2:1 MF468122 Hap_3:1 MF468123 Hap_4: 1 MF468125 Hap_5: 1 MF468126
Cirrhinusmrigala	3	0,600	Hap_1: 4 KY569370,MF468127 MF468128 ,MF468129 Hap_2: 1 MF468124 Hap_3: 1 MF468130

Table 4. GC content at 1st, 2nd and 3rd codon positions of *Labeogonius* and *Cirrhinusmrigala*

Fish Species	n	1st	2nd	3rd
Labeogonius	5	33.2%	33.3%	33.4%
Cirrhinusmrigala	6	33.4%	33.3%	33.2%

Cirrhinusmrigala along with downloaded sequences. An increase in the mean %age distances was observed with increase in taxonomic level i.e., species, genus and family (Table 1 and 2). The average base composition for bases T,C,A and G was 25.2%, 18.8%, 28.6% and 27.4% in *L.gonius* and 25.7%, 18.8%, 28.2% and 27.3% in *Cirrhinusmrigala.*

DISCUSSION

Species identification based on morphological characters is boring due to high morphological similarities between species (Yang et al*.,* 2015). These morphological limitations are further complicated by variation in ploidy levels within genera (Agnèse et al*.,* 1990). The limitations of morphological characters have been improved with technological advances in molecular biology. Recently DNA barcoding has been employed to complement or refine morphological species identification (Chen et al*.,* 2015; Kochzius et al*.,* 2010; Pereira et al*.,* 2011). The DNA barcoding technique is based on the use of a short standardized cytochrome c oxidase subunit I (*COI*) gene sequence to distinguish between animal species (Hebert et al*.,* 2003 and 2004).

The limited sample size in this study required addi-

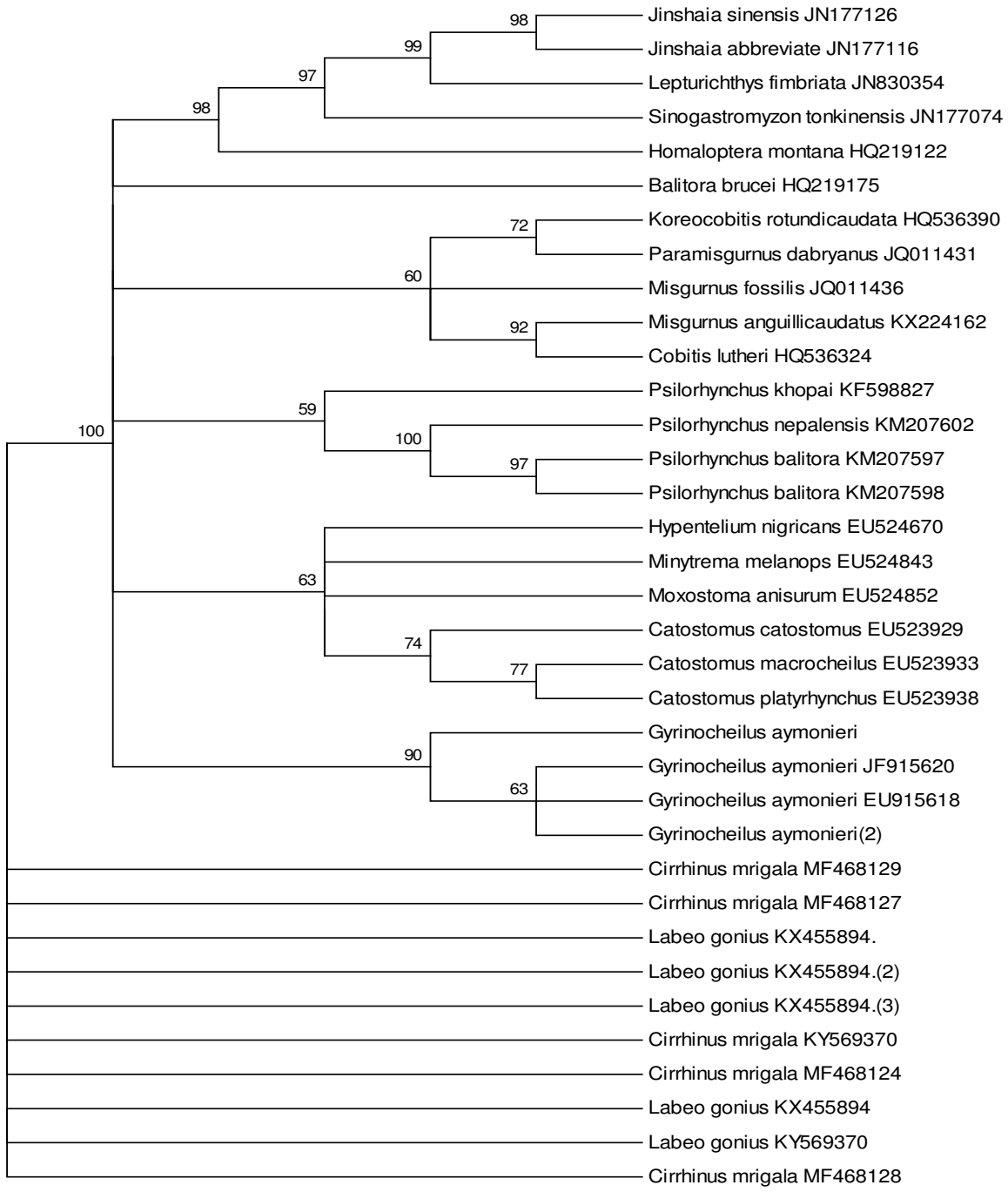

Figure 1. N-J tree showing boots trap values of our experimental fishes and other fish species from other regions of world

tional *COI*gene sequences of *C.mrigala* from other geographical regions for calculation of conspecifics and congeneric divergences, which were obtained from the public data portal of the data base of BOLD system.org and GenBank in order to proceed for the nucleotide diversity analysis of our barcode sequences. Subsequent analysis of barcode sequences permitted clear discrimination of taxonomic status of all the species examined.

In general, the average conspecific, congeneric, and confamilial K2P distances calculated in our experimental *C.mriagala* and *L.gonius* were 0.45%, 0.65% 0.66% and .012%, 0.374%, 0.744% respectively (Table 1 and 2). Hence with the raise of taxonomic level, a steady increase in the genetic distance is observed. Such type of steady increase is consistent with the range observed among the Indonesian fresh water fishes 0.15%, 2.53%, 5.63%, Canadian freshwater fishes 0.27%, 8.37%,

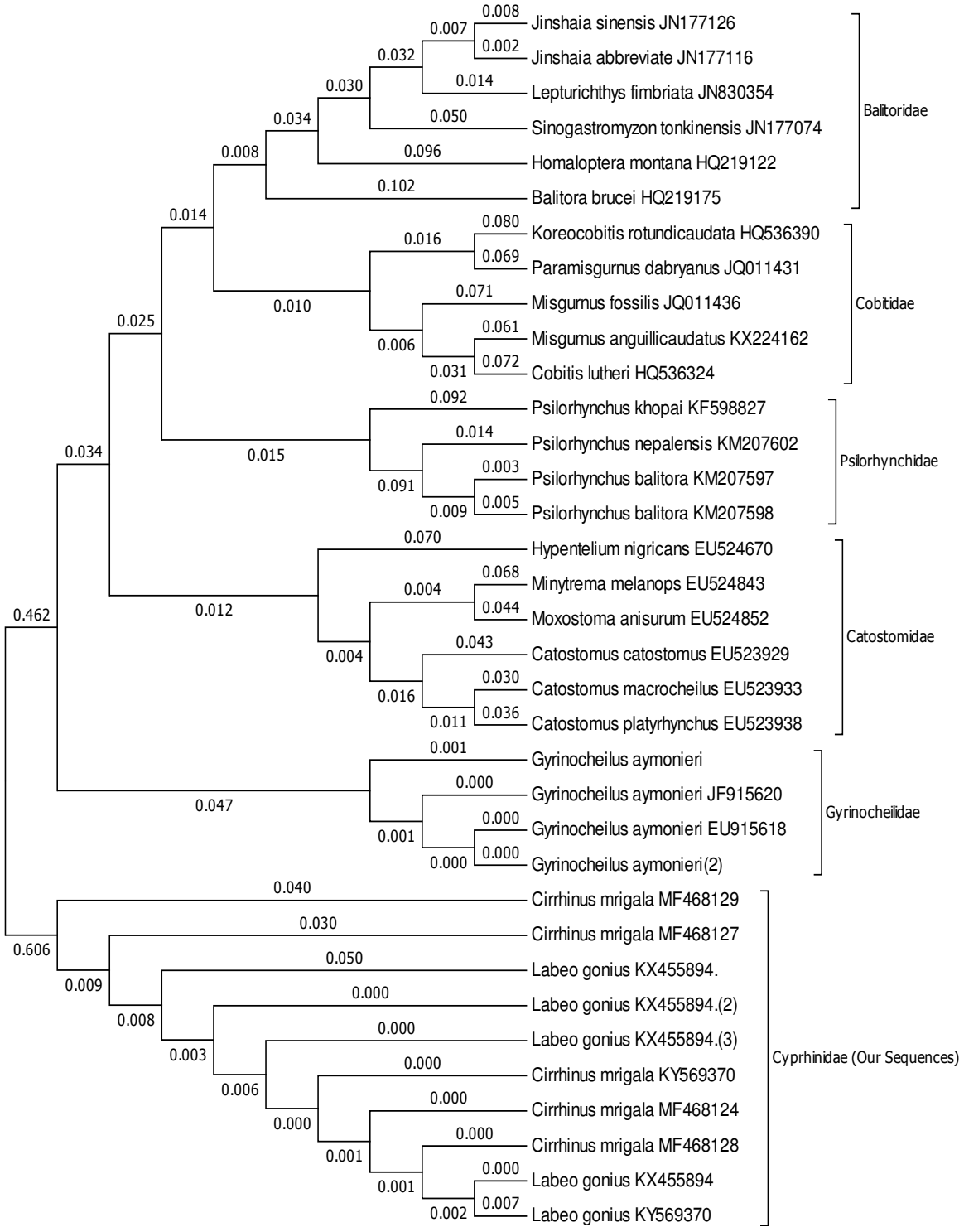

Figure 2. Neighbour joining tree showing relationship among different families of Cypriniformes

15.38%, and Australian marine fishes 0.39%, 9.93%,15.46%, respectively (Hubert et al.,2008; Muchlisin et al., 2013; Ward et al., 2005). K2P results of our present work are in accordance with the reported literature which indicates that nucleotide divergence increases with an increase in the taxonomic status, mainly due to substitutional saturation (Lakraet al., 2011; Mat Jaafar et al., 2012; Ward et al., 2005).

The NJ tree clearly showed the phylogenetic relationships among the species, with same species clustering under the same node forming one clan, while divergent species clustered under separate nodes in

different clans. The nodes in K2P distance-based NJ trees were supported by high bootstrap values (50–100%). Although the sole purpose behind barcode analysis is to differentiate among different species, even those with high morphological resemblance, the beauty of this analysis is that it also contains some clues about phylogenetic relationships in COI sequence data. Congeneric species always clustered together and in most cases so did the confamilial species (Figure 1 and 2).

Mitochondrial DNA passes along a maternal lineage that can date back thousands of years. A host of early studies suggested that relatively few (2–6) haplotypes may serve to describe the genetic variation along extended stretches of DNA (Philip et al*.,* 2013). 3 haplotypes were found in 6 barcodes of *C.mrigala* while 5 haplotypes were found in *L.gonius* sequences. Presence of haplotypes in our sequences clearly suggests presence of genetic variations in our fish. *C.mrigala* with 3 haplotypes shows fewer variations in its 6 barcodes as compared to the presence of 5 haplotypes in *L.gonius* (Table 3).

According to a report haplotype diversity which lies in the range 0.75 to 0.92, is considered high, when compared to many other species. Haplotype diversity isalso considered high when it is greater than 0.98 as studied in a research project (Jean et al*.,* 2013). Haplotype diversity in *L.gonius* and *C.mrigala* was calculated as 1.00 and 0.600 respectively. These results show that the haplotype diversity in *L.gonius*is high while in *C.mrigala* it is relatively low.

Phylogenetic tree based on nucleotide sequence of *CO1* genes from *L.gonius*and *C.mrigala* was constructed based on NJ method using K2P model. Each of six families Psilorhyncidae, Cyprinidae, Catostomidae, Gyrinocheilidae, Balitoridae and Cobitidae belonging to order Cypriniformes considered for construction of NJ tree was found to form a separate clade.

L. gonius and *C.mrigala* aligned in a small cluster within the Cyprinidae. Gyrinocheilidae was found to be the closest relative of family Cyprinidae, though it showed more affinity towards Catostomidae than to Cyprinidae. Cobitidae represented the most distant relative of family Cyprinidae. NJ tree seems to suggest that Cobitidae was the first family to have diverged from the common Cyriniformes ancestor. Balitoridae was the next in line. Cyprinidae then diverged from Gyrinoccheilidae and Catostomidae. Analysis of the NJ tree confirmed that *labeogonius* belong to Cyprinidae family and share a common lineage with other Cyprinidae fishes like *C.mrigala* (Figure 2).

Saccone et al*.,* 1999 suggested that there is variation in level of GC content of entire genome, within and among major groups of organisms. Total GC content also varies taxonomically within mitochondrial genomes; poor GC content is reported in all mitochondrial genomes sequenced to date. The lowest concentration is found in insects and nematodes which ranges 15–35% while somewhat higher concentration is found in molluscs as it ranges 29–40% while the highest values are reported in birds, mammals (particularly primates) and teleost fish with ranges 32–46%. Despite the commonly held view that invertebrate mitochondria are AT-rich, while chordate mitochondria are GC-rich (Hebert et al*.,* 2003, Clare et al*.,* 2008; Mooers et al*.,* 2000). In case of our study the GC composition range is about 33% in both experimental fishes (Table 4), which falls within the range of teleost fishes described by Saccone *et al.* (1999).

CONCLUSION

It is concluded that DNA barcoding is an effective and modern molecular technique for documentation of different fish species as compared with traditional method of identification based on morphological characters. Due to limitations in traditional method of identification of fish larvae, damaged specimen, processed fish, and fish fillet, with incomplete morphology or no morphology at all . DNA barcoding is based on a short sequence of *CO1*gene of *mt* DNA which has enough variability to distinguish among species. Besides distinguishing among species, it can also estimate the nucleotide divergence among species, genera, and families and infer the evolutionary history of various species.

ACKNOWLEDGEMENTS

Special thanks to Dr. MasroorElahi Babar, Registrar, Virtual university of Pakistan, Lahore for giving permission and providing full lab facilities for present experimental work.

Declaration of Interest

All the authors of this paper declare that they have no conflicts of interest. Moreover the authors alone are responsible for the content and writing of the paper.

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