International Journal of Zoology Studies
ISSN: 2455-7269
Impact Factor: RJIF 5.14
www.zoologyjournals.com
Volume 2; Issue 5; September 2017; Page No. 100-105

DNA barcoding of endangered fish *Dawkinsia tambraparniei* for species identification

Vijay Velu, *Ramesh Uthandakalaipandian
Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

Abstract
Dawkinsia tambraparniei were captured using mosquito nets during period of mid-October of 2015. Species were less found in period of February to May and rich availability at period of August to December. Fishes of length: 6±0.2cm and weight: 4±0.3g were used for study. Hence it was always misjudged as *D. arulius* further characterization was carried out. The main objective of this study is to determine species identification to avoid false identification. Genetic characterization of *D. tambraparniei* with mitochondrial Cytochrome c oxidase I (COI) genes revealed that *D. tambraparniei* has 99% similarity with *D. arulius* followed by *D. filamentosus, P. denisonii,* and *P. conchonius* with 96, 88 and 86% similarity, respectively. Phylogenetic analysis also showed that *Dawkinsia tambraparniei* is closely related to *D. arulius*. A discrete Gamma distribution was used to model evolutionary rate differences among these species were done (5 categories (+G, parameter = 0.6335). The obtained COI sequences of *D. tambraparniei* have been submitted to NCBI database with the reference of GenBank accession number KM978936.1. The present study concludes that the *D. tambraparniei* obtained from Tamiraparani river shows species variation against *D. arulius.* It was accurately and rapidly identified by database provided by bold.

Keywords: *D. tambraparniei, D. arulius,* COI sequences, barcode, bold

1. Introduction
Aquatic biodiversity was getting lost its quality and its ecosystem stability due to many serious threats in the aquatic environment [1]. The global extermination amount of fish has been unproven to be higher than it would have been naturally predictable [2]. The fish diversity in the river ecosystem is mainly declined due to habitat loss of fishes, the introduction of invasive species, water depletion, importantly pollution of water and changes in Globe [3].
Therefore, appropriate conservatory events are to be implemented to lessen the impact of the pressures laid down by the environmental as well as anthropogenic strains so that ichthyo-diversity of the endemic fish species in India may be endangered [4]. The endemic fishes noted from Tamil Nadu, it's IUCN Red list status and district wise distribution which may be useful to adopt specific conservation approach at the area level. This record also contains the IUCN, not assessed fishes which are recorded in India since 2015 [5]. A gathering of data on the endemic fish species in Tamil Nadu along with their taxonomic status could be of informative to the academicians and young researchers all over the regions of the country.
Mitochondrial Cytochrome C Oxidase Subunit I (COI) was considered as a tool for effective identification of animals and fishes [6]. It was demonstrated COI as an effective fish proof of identity tool in circumstances including consumer protection [7, 8] and maintenance of fisheries**.** Barcoding was also implied in marine ornamental fish trading results in success of identification of particular species [9, 10]. In addition, many ornamental aquarium fishes are difficult to identify and it has a potential risk of identification of invasive species. It may claim to deliver invasive species to native [11].
Most of the Indian freshwater fishes are poorly understood and classified. Many hidden species complexes become endemic. Many limitations in traditional taxonomic review end up with only on morphological approaches. Thus, molecular approaches like DNA barcoding helps in diagnosing some mysterious species [12].
Dawkinsia tambraparniei [13] is limited to a particular area and it won't be exceeding more than 100 km^2 surroundings of Tamiraparani River, southern Western Ghats, India. The fish populations have declined recently due to anthropogenic activities, yielding the fish as endangered one.
The present study investigates genetic variation between and barcoding of *D. tambraparniei* obtained from Tamiraparani river against *D. arulius.*

2. Materials and Methods
2.1 Collection of fish
Live samples of *Dawkinsia tambraparaniei* were collected from Tamiraparani River in different locations such as Vannarapetai, Ambasamudram and Agasthiar falls respectively during mid-October of 2015. Longitudes and latitudes of the site area were given in Table 1. The fishes were collected from the locations using mono filamentous, multi filamentous gill nets and cast nets of different mesh sizes from 8-29mm. Few individuals were preserved in 10% formalin for morphometric studies. These fishes were acclimatized in laboratory condition for further research. Specimens were conserved in Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai.

Table 1: Longitude and Latitude positions of sites where fish were captured.

Place	Latitude	Longitude
Agasthiyar Falls	8.7045601	77.3642489
Ambasamudram	8.693540	77.462746
Vannarpettai	8.728790	77.715420

2.2 Fish mortality

During transportation, there are many factors involved in survivability of fish. It includes an adequate amount of oxygen, water quality, temperature, travel distance etc., Due to these factors the rate of mortality was in the ratio of 3:10.

It was acclimatized in laboratory conditions in glass tanks for a minimum period of 14 days. It was maintained under temperature conditions of 25±2°C, 12 h/12 h light/dark cycle. At the time of the experiment, the fish were fed with Artemia.

2.3 Isolation of Total Genomic DNA

Total genomic DNA was extracted *Dawkinsia tambraparaniei.* Most frequently used the method of DNA isolation is the phenol-chloroform method [14], which removes proteins and other cellular components from nucleic acids and relatively pure DNA can be obtained for further analysis.

Incubation buffer was prepared that contains 0.5ml lysis buffer +0.5mg SDS per sample, which should be kept at 37ºC until tissue dissolves completely. 5µl of proteinase K per sample was added to above lysis buffer (it should be added before adding it to sample). 0.5ml incubation buffer was added to each tube. Samples were incubated at 42ºC for overnight in a water bath. 2ml Tris-saturated phenol was added in each tube. Samples were shaken (by repeatedly inverting tubes) slowly for 10min on a shaker. 2ml of chloroform: isoamyl alcohol (24:1v/v) was added in each tube. Tubes were centrifuged at 10,000rpm for 10min at 20ºC. Using 1ml cut tips pipette out supernatant, avoiding white layer at the interface. 2ml of chloroform: isoamyl alcohol (24:1) was added to supernatant in each tube. Tubes were centrifuged at 10,000 rpm for 10min at 20ºC. 1/10 (150µl) volume of 3M sodium acetate (pH 5.2) was added and 2.5 times (3.5ml) of ice cold absolute ethanol also added to the supernatant. All tubes were kept on ice for one hour. Tubes were centrifuged at 10,000rpm for 10min at 4ºC. Ethanol was discarded carefully. After complete drying, 20µL TE (pH 8.0) buffer was added. DNA samples were stored at 4ºC [15].

2.4 Amplification and sequencing of cytochrome c oxidase I (COI) gene

Standard polymerase chain reaction (PCR) procedures were applied to amplify cox1 fragments. Primers complementary to the cox1 region encoding for the cox1 subunit of the cytochrome c oxidase were designed. The following primer pairs

F-5' TCA ACC AAC CAC AAA GAC ATT GGC AC 3'

R-5' TAG ACT TCT GGG TGG CCA AAG AAT CA 3'

were used to amplify COI gene. PCR reactions were carried out in an Agilent thermal cycler [16].

2.5 Visualization of PCR products

To check the amplification of mt DNA products, load 10µl of resulting PCR products were on 2% agarose gels for one hour in 1x TBE buffer (pH 8). Subsequently, gels were stained with ethidium bromide and visualized on a UV Transilluminator. If the band was observed, then the samples were purified using Quiagen quick spin columns in a microcentrifuge and utilized for COI gene sequencing. The amplified PCR products were sequenced using Big Dye on ABI 3700 automated DNA sequencer.

2.6 Statistical analysis

The CLUSTAL programs were used for sequence editing and alignment purpose respectively. The maximum likelihood phylogenetic tree was constructed using the program MEGA7 and reflects the phenotypic similarities between sequences.

3. Results and Discussion

This study represents the molecular survey of *Dawkinsia tambraparaniei* using COI barcode data of Tamiraparani River system in Tamil Nadu.

Dawkinsia tambraparaniei and *Dawkinsia arulius* are two different cyprinid species outstandingly similar in looks and in the naked eye it is difficult in identification. DNA barcodes reveal the similarities between the species.

When the obtained sequence was aligned by CLUSTAL W [17] and Pairwise alignment, the similarity difference between *Dawkinsia tambraparaniei* and *D. arulius* is less than 1%. The obtained sequence was interlinked with *D. arulius* COI sequence to obtain maximum likelihood tree along with bootstrap value. The obtained tree is shown in Fig 4.

It shows how these two species are closely related. However, there was low inter-species sequence deviation originating with some additional species of fish which are morphologically identified as distinct species.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [18]**.** Maximum likelihood tree shows the relationship between the *Dawkinsia tambraparniei* and other closely related species viz., *Dawkinsia arulius* along with significant information. The tree with the highest log likelihood (-1231.4169) is shown in fig 4.

Fig 1: Illustrating the importance of DNA barcoding A) *Dawkinsia tambraparaniei* B) *Dawkinsia arulius.* Two different species blindingly similar in appearance; morphological alterations are particularly difficult to distinguish when these are in juveniles.

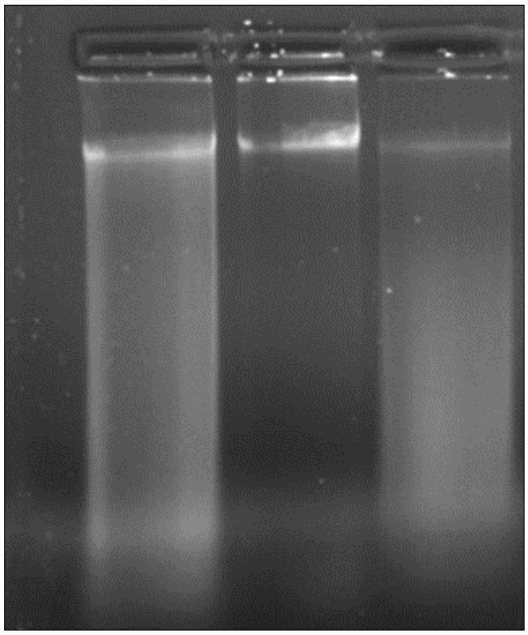

Fig 2: Genomic DNA was purified and electrophoresed on a 0.8% agarose gel, stained with ethidium bromide. Observed high-quality genomic DNA that ranges from ~30 to 50kb in size. Lanes 1-3 are genomic DNA samples obtained from fish fins.

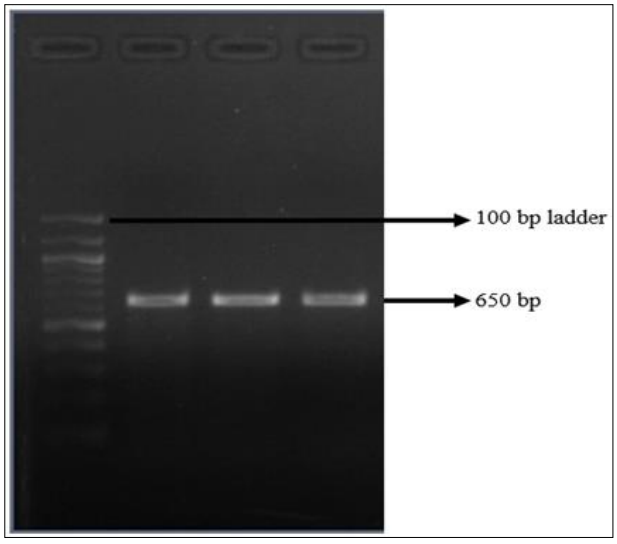

Fig 3: Agarose gel electrophoresis of Cytochrome Oxidase Subunit I (Lane 2, 3 and 4) on a 1% agarose gel. Lane 1 in contains 100bp ladder, with base pair sizes indicated on the right. Arrows show a single band each that was of the expected size for COI subunit (650 bp)

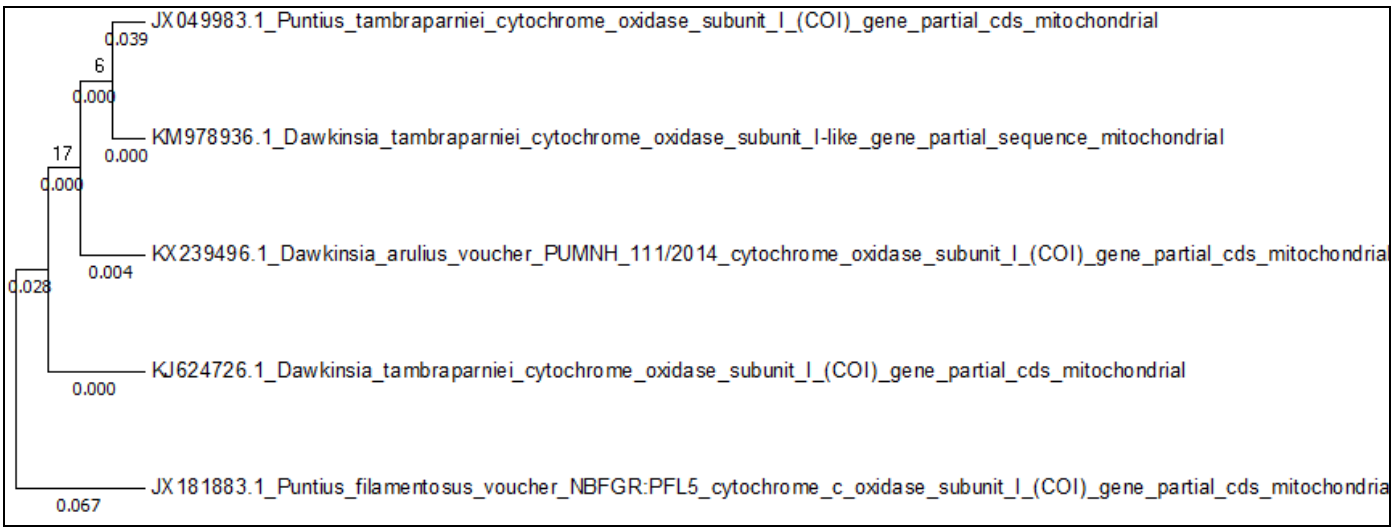

Fig 4: The Phylogenetic tree was constructed based on nucleics

equence data of COI gene fragment of approximately 650 bp. The percentage of trees in which the associated taxa clustered together was shown next to the branches. 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. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6335)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 72.8703% sites). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 5 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}+$ Noncoding. There was a total of 700 positions in the final dataset. These analyses were conducted by means of MEGA7.

The branch length of the tree tends to represent distribution variation between species individuals by a gap. These variations were called as barcoding gap [19].

The relationship between different individuals was clearly mentioned and pictured on the phylogenetic tree. The nodes were used to separate species. The species which are under the same category were under single node and species which remain not similar to each are classified in a different node. The chief nodes were sustained by great bootstrap standards from 70 to 100%.

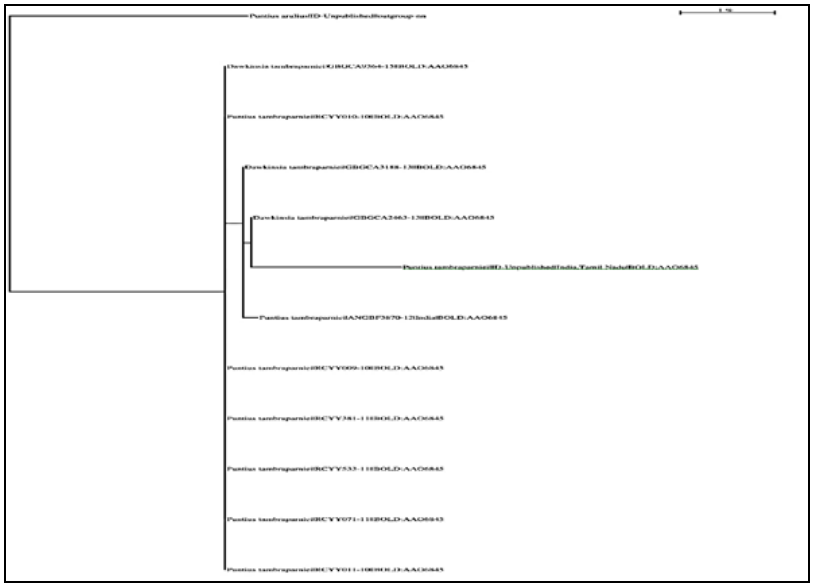

Fig 5: Neighbour-Joining (NJ) tree for BOLD Taxon ID developed by means of a K2P distance of COI sequences of *Dawkinsia tambraparaniei.* Specific ID was given for species.

The BOLD tree was constructed by country, process ID and Barcode Index Number. Till now 12 individuals of *D. tambraparniei* was submitted on the BOLD database. Since there was no specific species variation no separate BOLD id was given. The BOLD Barcode Index Number (BIN) assigned for *D. tambraparniei* was AAO6845.

More than years *D. tambraparniei* was considered as *D. arulius* due to the similarity among these fishes. Without any reason, *D. arulius* was considered as a low-grade substitute [20]. By noticing minor morphological changes such as lateral line scales and fin rays *D. tambraparniei* was again morphomeristically characterized [16,17,18]. Finally, *D. tambraparniei* was considered as an effective species and no more compared with *D. arulius.* The dorsal fin rays in males differed from *D. arulius*. It has terminal mouth whereas *D. tambraparniei* subterminal mouth and the maxillary barbels of *D. tambraparniei* half of high diameter of the eye. In the case of *D. arulius,* the barbell stands quarter time lesser than *D. tambraparniei.* In *D. tambraparniei* there was a filament like extension was seen at the dorsal rays of males and it was absent in *D. arulius.* Since it consists of filaments at a juvenile stage earlier it was considered under the group of *Dawkinsia filamentosa.* Later on, it was characterized by different bands on the juveniles. The first band saw on the body side secondly was beneath dorsal fin base, third above anal fin base and at lastly one at caudal fin.

The main application of BOLD database was to provide conservation data on fish resources via through trustworthy catches and estimation of manipulation rate. DNA barcoding has a foremost goal line to construct barcode libraries of all species consequently that it would be valuable for molecular aspects [21]**.** Since India remains rich in fish diversity both marine and fresh, it would be needful to document and characterize fish diversity for fisheries. The principal goal of DNA barcoding is to generate reference DNA-barcode libraries for recognized species to be used as DNA-identifiers. Hence barcoding of *D. tambraparniei* provides a valid research on its classification.

4. Conclusion

The present survey confirms that employing Cytochrome Oxidase I gene as a barcoding tool can help in the identification of the fish species in numerous river systems. Misjudgement of *D. tambraparaniei* with *D. arulius* was eradicated by these findings.

5. Acknowledgement

The author would like to thank DST-INSPIRE Fellowship Scheme for the funding and Madurai Kamaraj University UGC-NRCBS for providing instrumentation facility.

6. References

1. Lakra WS, Sarkar UK, Gopalakrishnan A, Kathirvelpandian A. Threatened freshwater fishes of India. National Bureau of Fish Genetic Resources, 2010.
2. Mishra SK, Sarkar UK, Gupta BK, Trivedi SP, Dubey VK, Pal A. Pattern of freshwater fish diversity, threats and issues of fisheries management in an unexplored tributary of the Ganges basin, Northern India. Journal of Ecophysiology and Occupational Health. 2011; 11(3/4):149.
3. Gibbs JP. Wetland loss and biodiversity conservation. Conservation biology. 2000; 14(1):314-7.
4. Sarkar UK, Pathak AK, Lakra WS. Conservation of freshwater fish resources of India: new approaches, assessment and challenges. Biodiversity and conservation. 2008; 17(10):2495-2511.
5. Chakraborty A, Rumki S, Koushik G. An inventory of endemic fish species in India with notes on state-wise distribution and conservation measures. International Journal of Fisheries and Aquatic Studies. 2017; 5(1):253-264.
6. Ward RD, Hanner R, Hebert PD. The campaign to DNA barcode all fishes, FISH-BOL. Journal of fish biology. 2009; 74(2):329-356.
7. Hebert PD, Cywinska A, Ball SL. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B: Biological Sciences. 2003; 270(1512):313-21.
8. Lowenstein JH, Burger J, Jeitner CW, Amato G, Kolokotronis SO, Gochfeld M. DNA barcodes reveal species-specific mercury levels in tuna sushi that pose a health risk to consumers. Biology Letters. 2010; 6(5):692-5.
9. Wong EH, Hanner RH. DNA barcoding detects market substitution in North American seafood. Food Research International. 2008; 41(8):828-37.
10. Steinke D, Zemlak TS, Hebert PD. Barcoding Nemo: DNA-based identifications for the ornamental fish trade. PLoS one. 2009; 4(7):e6300.
11. Rahel FJ. Biogeographic barriers, connectivity and homogenization of freshwater faunas: it's a small world after all. Freshwater Biology. 2007; 52(4):696-710.
12. Chakraborty M, Ghosh SK. An assessment of the DNA barcodes of Indian freshwater fishes. Gene. 2014; 537(1):20-8.
13. Silas EG. New fishes from the Western Ghats, with notes on Puntius arulius (Jerdon). Records of Indian Museum. 1953; 51(1):27-38.
14. Wasko AP, Martins C, Oliveira C, Foresti F. Non-destructive genetic sampling in fish. An improved method for DNA extraction from fish fins and scales. Hereditas. 2003; 138(3):161-5.
15. Madisen L, Hoar DI, Holroyd CD, Crisp M, Hodes ME, Reynolds JF. The effects of storage of blood and isolated DNA on the integrity of DNA. American Journal of Medical Genetics Part A. 1987; 27(2):379-90.
16. Bhattacharjee MJ, Laskar BA, Dhar B, Ghosh SK. Identification and re-evaluation of freshwater catfishes through DNA barcoding. PloS one. 2012; 7(11):49950.
17. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H *et al.* version 2.0. bioinformatics. 2007; 23(21):2947-8.
18. Srivathsan A, Meier R. On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. Cladistics. 2012; 28(2):190-4.
19. Meyer CP, Paulay G. DNA barcoding: error rates based on comprehensive sampling. PLoS biology. 2005; 3(12):e422.
20. Talwar PK. Inland fishes of India and adjacent countries. CRC Press, 1991.
21. Jerdon TC. On the freshwater fishes of southern India. Madras Journal of Literature and Science. 1849; 15:141-149.
22. Pethiyagoda R, Kottelat M. A review of the barbs of the Puntius filamentosus group (Teleostei: Cyprinidae) of

southern India and Sri Lanka. The Raffles Bulletin of Zoology. 2005; 12:127-44.
23. Kerr KC, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, Hebert PD. Comprehensive DNA barcode coverage of North American birds. Molecular Ecology Resources. 2007; 7(4):535-43.
24. Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, *et al.* Identifying Canadian freshwater fishes through DNA barcodes. PLoS one. 2008; 3(6):2490.