DNA Barcoding and Phylogenetic Relationships of Selected South Indian Freshwater Fishes Based on mtDNA COI Sequences

Manickam Raja and Pachiappan Perumal
Department of Biotechnology, Periyar University, Periyar Palkalai Nagar, Salem - 636 011, Tamil Nadu, India

Corresponding author: Manickam Raja, Department of Biotechnology, School of Biosciences, Periyar University, Periyar Palkalai Nagar, Salem- 636 011, Tamil Nadu, India, Tel: +91 9894277036; E-mail: wetlandraja@gmail.com

Receiving date: Aug 22, 2017; Acceptance date: Sep 12, 2017; Publication date: Sep 15, 2017

Copyright: © 2017 Raja M. et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

DNA barcoding is an effective tool for the identification of species representing diverse taxa especially through the sequence analysis of mitochondrial cytochrome c oxidase subunit I (COI) gene. In the present study, DNA barcodes were generated from 46 species of freshwater fishes covering the Orders Cypriniformes, Siluriformes, Synbranchiformes and Perciformes representing 30 genera under 9 families. All the samples were collected from diverse sites which also includes some endemic species. A total of 47 COI sequences were generated. After amplification and sequencing of 678 base pair fragment of COI, primers were trimmed which invariably generated a 635 base pair barcode sequence. The average Kimura two-parameter (K2P) distances within-species, genera, families, and orders were 0.32%, 8.40%, 14.50%, and 18.65%, respectively. DNA barcode discriminated congeneric species without any confusion. The present study strongly supports the efficiency of COI as an ideal marker for DNA barcoding of selected freshwater fishes.

Keywords: Barcoding; COI; Indian freshwater fishes; mtDNA; Phylogeny

Introduction

India is rich in fishery resources as it inhabits about 2508 fish species [1] of which the 856 are freshwater occupants [2,3]. The fishes are the most diverse vertebrate in world and about 40% of them live in freshwater. India is one of the mega biodiversity countries in the world and occupies the ninth position in terms of freshwater mega biodiversity and contributed 11.72% of the globe fish biodiversity [4]. However, the actual number of fish species found in India is still not accurately documented because of prevailing taxonomic confusion [5] due to inadequate exploration, indiscernibility among cryptic forms coupled with species ambiguity in the taxonomic keys [6]. As a result, many species have been considered as cryptic and some of which may also be dormant [6,7]. Therefore, for legible characterization of Indian freshwater fishes, there is a vital need of species scrutiny using advanced molecular methods. Hence, there is an urgent need for the assessment of Indian freshwater fish species through DNA barcoding.

DNA barcoding is a promising technique for species identification using a short mitochondrial DNA sequence of COI gene [8]. This technique involves the analysis of the sequence diversity of a 50 segment mitochondrial COI gene to identify species [9]. Of late, DNA barcoding method has been extensively followed for species identification as well as species discovery in various groups of organism [10,11]. Effectiveness of DNA barcoding has now been validated for many groups of animals [12] and among them fishes being one of the most extensively studied groups [13,14].

In recent years, several such molecular studies have been conducted on members of this group to better understand their relationships and to develop more accurate taxonomic classifications based on phylogeny [15-25]. Use of COI gene for barcode is considered to be suitable marker to discriminate the closely related species of fishes [26-29]. But the challenge in use of small DNA barcode (only 655bp) based phylogenetic study is selection of a nearly perfect nucleotide substitution model for the dataset, so that the weakest evolutionary signal can be correctly detected [30]. However, a comprehensive assessment of DNA barcodes of Indian freshwater fishes is limited, though a similar study has been done for the selected Indian freshwater fishes by Lakra [8]. Therefore, the present study reported additional DNA barcoding of 46 commercially important Indian freshwater fish species belonging to 30 genera under 9 families and 4 orders.

Materials and Methods

Sample collection and morphological identification

The tissue and voucher specimens of 46 species (9 families) were collected from different riverine systems of south India namely from Cauvery and Bhavani river systems. Approximately 100 mg of muscle tissue and fin clips from two to five individuals of each species were preserved in 95% ethanol until used. The species identification and confirmation were carried out using standard literature [31,32]. The valid nomenclatural names were adopted as per the Catalogue of Fishes of the California Academy of Sciences [1,33]. The live specimens were photographed with Canon 1100 Digital SLR Camera and later preserved in 7% formalin solution for future reference. Table 1 represents specimen details and GenBank accession numbers.
| S. no. | Order            | Family    | Species                                | Voucher No. | Accession no. |
|-------|------------------|-----------|----------------------------------------|-------------|---------------|
| 1     | Cypriniformes    | Cyprinidae| Salmophasia bacaila (Hamilton, 1822)   | PUMNH 08/2013 | KX266823     |
| 2     |                  |           | Barilius canarensis (Jerdon, 1849)     | PUMNH 30/2013 | KX230848     |
| 3     |                  |           | Barilius gatensis (Valenciennes, 1844) | PUMNH 31/2013 | KX230845     |
| 4     |                  |           | Barilius bendelisis (Hamilton, 1807)   | PUMNH32 /2013 | KX230846     |
| 5     |                  |           | Barilius bakeri (Day, 1865)            | PUMNH01 /2013 | KX230847     |
| 6     |                  |           | Danio rerio (Hamilton, 1822)           | PUMNH76 /2013 | KX266821     |
| 7     |                  |           | Danio rerio (Hamilton, 1822)           | PUMNH77 /2013 | KX266822     |
| 8     |                  |           | Devario aequipinnatus (McClelland, 1839)| PUMNH 04/2013 | KX289313     |
| 9     |                  |           | Devario malabaricus (Jerdon, 1849)     | PUMNH02 /2013 | KX529835     |
| 10    |                  |           | Rasbora daniconius (Hamilton, 1822)    | PUMNH34 /2013 | KX239494     |
| 11    |                  |           | Esomus danricus (Hamilton, 1822)       | PUMNH06 /2014 | KX266826     |
| 12    |                  |           | Amblypharyngodon mola (Hamilton, 1822) | PUMNH 28/2013 | KX266827     |
| 13    |                  |           | Tor khudree (Sykes, 1839)              | PUMNH 47/2013 | KX550003     |
| 14    |                  |           | Neolissochilus hexagonolepis (McClelland, 1839) | PUMNH 48/2013 | KX266828     |
| 15    |                  |           | Systomus sarana (Hamilton, 1822)       | PUMNH 49/2013 | KX239499     |
| 16    |                  |           | Dawkinsia filamentosae (Valenciennes, 1844) | PUMNH 50/2013 | KX230844     |
| 17    |                  |           | Dawkinsia arulius (Jerdon, 1849)       | PUMNH 06/2013 | KX239496     |
| 18    |                  |           | Puntius amphibius (Valenciennes, 1842) | PUMNH 07/2013 | KX529836     |
| 19    |                  |           | Puntius sophore (Hamilton, 1822)       | PUMNH03 /2014 | KX289308     |
| 20    |                  |           | Haludaria fasciata (Jerdon, 1849)      | PUMNH 51/2013 | KX550002     |
| 21    |                  |           | Pethia narayani (Hora, 1937)           | PUMNH 07/2014 | KX289310     |
| 22    |                  |           | Barbodes carnaticus (Jerdon, 1849)     | PUMNH 52/2013 | KX239492     |
| 23    |                  |           | Hypselobarbus dubius (Day, 1867)       | PUMNH 53/2013 | KX266817     |
| 24    |                  |           | Hypselobarbus curmuga (Hamilton, 1807) | PUMNH 13/2014 | KX266819     |
| 25    |                  |           | Hypselobarbus kurali (Menon & Rema Devi, 1995) | PUMNH 23/2014 | KX266820     |
| 26    |                  |           | Hypselobarbus kos (Sykes, 1839)        | PUMNH 39/2014 | KX266818     |
| 27    |                  |           | Hypselobarbus micropogon (Valenciennes, 1842) | PUMNH 72/2014 | KX266813     |
| 28    |                  |           | Hypselobarbus periyarensis (Raj, 1941) | PUMNH 93/2014 | KX266814     |
| 29    |                  |           | Osteochilichthys nashii (Day, 1868)    | PUMNH 62/2013 | KX239498     |
| 30    |                  |           | Osteochilichthys thomassi (Day, 1877)  | PUMNH 19/2014 | KX239497     |
| 31    |                  |           | Osteobrama cotio (Hamilton, 1822)      | PUMNH 23/2014 | KX550004     |
Genomic extractions were taken from fin clips, preserved in >95% ethanol using Invitrogen's "Pure Link Genomic DNA Mini Kit" following the manufactures instructions. COI amplification was carried out in 25-μL reaction mixtures containing 1 μl template DNA, 1X reaction buffer, 2.5 mM MgCl₂, 2.5 mM dNTPs, 0.5 μl of each primer, and 0.2 U TaqDNA polymerase in a PTC-200 (Bio-Rad, USA) PCR machine. The reaction mixtures were preheated at 94°C for 5 min, followed by 50 cycles of amplification (94°C for 45 sec, 48°C for 45 sec, and 72°C for 60 sec), and a final extension at 72°C for 6 min. The COI gene was amplified using the universal primer set: The primers used for the amplification of the COI gene were: Fish F1-5′-TCAACCAACCACAAAGACATTGGGCAC-3′ and Fish R1-5′-TAGACTTCTGGGTGGCCAA AGAATCA-3′ [34]. Sequencing was performed using Big Dye Terminator on ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) and sequenced bidirectionally using an ABI 3730 capillary sequencer following instructions of the manufacturer. One individual of each species was used for the nucleotide sequence analyses.

**Table 1:** List of freshwater fish species barcoded along with GenBank accession numbers.

| Number | Species                          | GenBank Accession Numbers |
|--------|----------------------------------|---------------------------|
| 32     | _Laboe bata_ (Hamilton, 1822)    | PUMNH 12/2013 KX289314    |
| 33     | _Laboe rohita_ (Hamilton, 1822)  | PUMNH 14/2013 KX266835    |
| 34     | _Garra mullya_ (Sykes, 1839)     | PUMNH 57/2013 KX239490    |
| 35     | _Garra bicomuta_ (Narayan Rao, 1920) | PUMNH 15/2013 KX289309  |
| 36     | _Garra mclellandii_ (Jerdon, 1849) | PUMNH 56/2013 KX239495   |
| 37     | _Cobitidae_                      |                           |
| 38     | _Lepidocephalichthys thermalis_ (Valenciennes, 1846) | PUMNH 54/2013 KX266825   |
| 39     | _Lepidocephalichthys guntea_ (Hamilton, 1822) | PUMNH 18/2014 KX266824   |
| 40     | _Botia striata_ Narayan Rao, 1920 | PUMNH 13/2014 KX575850    |
| 41     | _Bhavana australis_ (Jerdon, 1849) | PUMNH 12/2014 KX289311   |
| 42     | _Balitoridae_                    |                           |
| 43     | _Bagridae_                       |                           |
| 44     | _Siluridae_                      |                           |
| 45     | _Sisorididae_                    |                           |
| 46     | _Nandidae_                       |                           |
| 47     | _Gobidae_                        |                           |

**Amplification and sequencing**

Genomic extractions were taken from fin clips, preserved in >95% ethanol using Invitrogen's "Pure Link Genomic DNA Mini Kit" following the manufactures instructions. COI amplification was carried out in 25-μL reaction mixtures containing 1 μl template DNA, 1X reaction buffer, 2.5 mM MgCl₂, 2.5 mM dNTPs, 0.5 μl of each primer, and 0.2 U TaqDNA polymerase in a PTC-200 (Bio-Rad, USA) PCR machine. The reaction mixtures were preheated at 94°C for 5 min, followed by 50 cycles of amplification (94°C for 45 sec, 48°C for 45 sec, and 72°C for 60 sec), and a final extension at 72°C for 6 min. The COI gene was amplified using the universal primer set: The primers used for the amplification of the COI gene were: Fish F1-5′-TCAACCAACCACAAAGACATTGGGCAC-3′ and Fish R1-5′-TAGACTTCTGGGTGGCCAA AGAATCA-3′ [34]. Sequencing was performed using Big Dye Terminator on ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). The PCR products were visualized on 1.2% agarose gels and the most intense products were selected for sequencing. Products were labeled using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) and sequenced bidirectionally using an ABI 3730 capillary sequencer following instructions of the manufacturer. One individual of each species was used for the nucleotide sequence analyses.

**Sequence analysis**

Sequences were aligned using Clustal W [35] and then submitted to GenBank. The extent of sequence difference between species was calculated by averaging pair-wise comparisons of sequence difference across all individuals. Pair-wise evolutionary distance among haplotypes was determined by the Kimura 2-Parameter method [36] using the software program MEGA 3 (Molecular Evolutionary Genetics Analysis, MEGA Inc., Englewood, NJ) [37]. The Neighbor-Joining (NJ) tree was constructed using MEGA 3 and to verify the robustness of the internal nodes of NJ tree, and bootstrap analysis was carried out using 1000 pseudo replications.

**Results**

**Genetic divergence and phylogenetic analysis**

A total of 47 sequences were generated from 46 freshwater fish species. Sequence alignment of COI gene after trimming of primers yielded 635 nucleotide base pairs per taxon. All the sequences showed simplicity and un-ambiguity, and no insertions, deletions, or stop codons were observed in any of the sequences. The sequence analysis revealed average nucleotide frequencies as A=26.00%, T=29.80%, G=26.40%, C=17.90% and C=17.90% respectively. The average K2P distances in percentage among different taxonomic levels were analyzed (Table 2). The average transversional pairs (sv=56) with an average ratio of 1.30. The average genetic distances within order, family, genus and species were 18.65%, 14.50%, 8.40%, and 0.32% respectively. The overall average genetic distance among all the species was 23.90%.
The phylogram was divided into two main clades with high bootstrap support (>50%) (Figure 1). The clade I was subdivided into six separated subgroups: subgroup 1.1 includes 11 species belong to 6 genus (Hypselobarbus, Osteochilichthys, Barbodes, Neolissochilus, Tor and Osteochilus) of the family Cyprinidae.
belonging to the order Synbranchiformes. Subgroup 2.2 consists of 3 species belonging to 3 genera (Garra, Danio, Mesonemacheilus, Botia) of the order Siluriformes. Subgroup 1.4 includes 9 species belonging to 5 genera (Barilius, Salmophasia, Rasbora, Amblypharyngodon, Lepidocephalichthys) of the family Cyprinidae. Subgroup 1.5 includes 3 species belonging to 2 genera (Pethia, Devario) of the family Cyprinidae. Subgroup 1.6 consists of 2 species belonging to 2 genera (Esomus, Bhavania). At the genus level all the species showed monophyly with high BT support. The clade II was divided into two subgroups. The Subgroup 2.1 consists of 3 species from two orders, Nandus nandus and Glossogobius giurus belonging to the order Perciformes and Mastacembelus aramatus belonging to the order Synbranchiformes. Subgroup 2.2 consists of 3 species of the genera; Ompak, Mystus, Glyptothorax belonging to the order Siluriformes.

**Discussion**

During DNA barcoding by Hebert et al. [38], the sequencing of a ~650bp region of the mitochondrial cytochrome oxidase I gene (COI), has been proven to be extremely an effective method for discriminating fish species [28,34,39]. Interestingly, recent research has illustrated some straightforward benefits from the use of standardized species-specific molecular tags derived from COI gene for species-level identifications [40]. DNA barcoding analysis has clearly discriminated freshwater fish species from India [8] Canada [28] and Mexico and Guatemala [39]. Presently, we have effectively used partial COI genes as DNA barcode in 46 freshwater fish species from south Indian waters representing 4 orders (Cypriniformes, Siluriformes, Perciformes and Synbranchiformes) representing 9 families and 30 genera. The universal primers amplified the target region in all 46 species, thus generating 47 COI barcodes of 635 bp and no insertions, deletions, stop codons or NUMTs were observed in any sequence, which support the hypothesis that all the amplified sequences derive from a functional mitochondrial COI sequences. And the present findings are in line with the previous reports [34]. Although the primary objective of DNA barcoding is to identify species, phylogeographic structure among COI sequences within species became evident. DNA barcoding pursues to provide a convenient, accurate and valid tool for species identification, and any candidate gene must suit this qualification. Use of a single, universal gene has many advantages, especially as barcoding applications expand to ecological questions and in the identification of illegally imported parts of organisms [41]. The study indicates that the standard barcoding marker, COI, can identify fish species [42,43,44]. The barcode sequences clearly discriminated all the studied freshwater fish species along with the apparent phylogenetic resolution. Although intra- and inter-specific genetic divergences overlap, tree-based methods can distinguish species in unidentified samples. For the ecologist and taxonomist alike, DNA barcoding would provide a powerful tool for the correct species identification, biodiversity assessments and locating the occurrence of cryptic species [45,46].

**Acknowledgements**

The first author (M.R) is grateful to the SERB - DST (Government of India), for providing the Startup Research Grant under Young Investigator Scheme (File No. DST No. SB/YS/LS-36/2013) and UGC, New Delhi for the grant of Dr. DSK Postdoctoral Fellowship (File No.F. 42/2006 (BSR)/BL/ 1516/0408), to carry out this study.

**References**

1. Eschmeyer WN, Fricke R (2012) Catalog of fishes electronic version (20 December, 2013). Electronic database accessible at http://research.calacademy.org/research/ichthyology/catalog/ fishcatmain.asp.
2. Menon AGK (1999) Check list-Fresh water fishes of India. Records of the Zoological Survey of India. Misc Publ 175: 1-366.
3. Froese R, Pauly (2012) Fish Base. World Wide Web Electronic Publication Available: www.fishbase.org Accessed: 2012 Dec 28.
4. Leveque C, Oberdorff T, Paugy D, Sitasamy MLJ, Tedesco PA (2008) Global diversity of fish (Pisces) in freshwater. Hydrobiologia 198: 545-567.
5. Hoagland KE (1996) The Taxonomic Impediment and the Convention on Biodiversity. ASC News 24: 66-67.
6. Pethyagoda R, Kottelat M (1994) Three new species of fishes of the genera Osteochilichthys (Cyprinidae), Travancoria (Baltaridiae) and Horabagrus (Bagridae) from the Chalakudy River Kerela India. J South Asian Nat Hist 1: 97-116.
7. Darshan A, Anganthoib N, Vishwanath W (2010) Redescription of the striped catfish Mystus carpio (Hamilton) (Siluriformes: Bagridae). Zootaxa 2475: 48-54.
8. Lakra WS, Singh M, Goswami M, Gopalakrishnan A, Lal KK (2015) DNA barcoding Indian freshwater fishes. Mitochondrial DNA 1-8.
9. Hebert PD, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten single-copy DNA barcode reveals cryptic species in the neotropical skipper butterfly Astrapylus fuligerus. Proc Natl Acad Sci USA 101: 14812-14817.
10. Hajibabaei M (2012) The golden age of DNA meta systemsatics. Trends Genet 28: 535-537.
11. Mendonca A, Cunha A, Chakrabarti R (2012) Natural Resources, Sustainability and Humanity: A Comprehensive View, Springer.
12. Waugh J (2007) DNA barcoding in animal species: progress, potential and pitfalls. Bioessays 29: 188-197.
13. Becker S, Hanner R, Steinke D (2011) Five years of FISH-BOL: brief status report. Mitochondrial DNA 22: 3-9.
14. Ward RD (2012) FISH-BOL: a case study for DNA barcodes. Methods Mol Biol 858: 423-439.
15. Liu H, Chen Y (2003) Phylogeny of the East Asian cyprinids inferred from sequences of the mitochondrial DNA control region. Can J Zool 81: 1938-1946.
16. Saitoh K, Sado T, Mayden RL, Hanzawa N, Nakamura K (2006) Mitogenomic evolution and interrelationships of the Cypriniformes (Actinopterygii: Ostariophysi): the first evidence toward resolution of higher-level relationships of the world's largest freshwater fish clade based on 59 whole mitogenome sequences. J Mol Evol 63: 826-841.

**Table 2**: Summary of genetic divergences of different taxonomic levels (based on the K2P distance model).

| Taxa          | Min dist (%) | Mean dist (%) | Max dist (%) | Standard error (SE) |
|--------------|--------------|---------------|--------------|---------------------|
| Within species | 0            | 0.32          | 0.77         | 0.003               |
| Within genus  | 0.12         | 8.4           | 14.32        | 0.007               |
| Within family | 0.73         | 14.5          | 25.6         | 0.01                |
| Within order  | 5.2          | 18.65         | 29.5         | 0.016               |

Citation: Raja M, Perumal P (2017) DNA Barcoding and Phylogenetic Relationships of Selected South Indian Freshwater Fishes Based on mtDNA COI Sequences. J Phylogenetics Evol Biol 5: 184. doi:10.4172/2329-9002.1000184
17. Ruber L, Kottelat M, Tan HH, Ng P, Britz R (2007) Evolution of miniaturization and the phylogenetic position of Paedocypris, comprising the world’s smallest vertebrate. BMC Evol Biol 7: 38.

18. Slechta V, Bohlen J, Tan HH (2007) Families of Cobitoidea (Teleostei: Cypriniformes) as revealed from nuclear genetic data and the position of the mysterious genera Barbucca, Paliorhynchus, Serpenticobitis and Vaillantella. Mol Phylogenet Evol 44: 1358-1365.

19. Conway KW, Chen WJ, Mayden RL (2010) The "celestial pearl danio" is a miniature Danio (ss) (Ostariophysi: Cyprinidae): evidence from morphology and molecules. Zootaxa 1686: 1-28.

20. Chen WJ, Mayden RL (2009) Molecular systematics of the Cyprinoidea (Teleostei: Cyprinidae), the world’s largest clade of freshwater fishes: further evidence from six nuclear genes. Mol Phylogenet Evol 51: 544-549.

21. Mayden RL, Chen WJ, Bart HL, Doosey MH, Simons AM (2009) Reconstructing the phylogenetic relationships of the earth’s most diverse clade of freshwater fishes-order Cypriniformes (Actinopterygii: Ostariophysi): a case study using multiple nuclear loci and the mitochondrial genome. Mol Phylogenet Evol 51: 500-514.

22. Mayden RL, Chen WJ (2010) The world’s smallest vertebrate species of the genus Paedocypris: a new family of freshwater fishes and the sister group to the world’s most diverse clade of freshwater fishes (Teleostei: Cypriniformes). Mol Phylogenet Evol 57: 152-175.

23. Yang L, Mayden RL, Sado T, He SP, Saitoh K (2010) Molecular phylogeny of the fishes traditionally referred to Cyprini sensu stricto (Teleostei: Cyprinidae). Zool Scr 39: 527-550.

24. Tang KL, Agnew MK, Chen WJ, Hirt MV, Sado T (2010) Systematics of the subfamily Danioninae (Teleostei: Cypriniformes: Cyprinidae). Mol Phylogenet Evol 57: 189-214.

25. Tang KL, Agnew MK, Chen WJ, Hirt MV, Raley ME (2011) Phylogeny of the gudgeons (Teleostei: Cyprinidae: Gobioninae). Mol Phylogenet Evol 63: 103-124.

26. Frati F, Simon C, Sullivan J, Swofford DL (1997) Evolution of the mitochondrial cytochrome oxidase II gene in Collembola. J Mol Evol 44: 145-158.

27. Steinke D, Vences M, Salzburger W, Meyer A (2005) A software tool for DNA barcoding using distance methods. Phil Trans Roy Soc London Series B 360: 1847-1857.

28. Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E (2008) Identifying Canadian freshwater fishes through DNA barcodes. PLoS One 3: e2490.

29. Lakra WS, Verma MS, Goswami M, Lal KK, Mohindra V (2011) DNA barcoding Indian marine fishes. Mol Ecol Resour 11: 60-71.

30. Sullivan J, Markert JA, Kilpatrick CW (1997) Phytophagography and molecular systematics of the Peromyscus aztecus species group (Rodentia: Muridae) inferred using parsimony and likelihood. Syst Biol 46: 426-440.

31. Talwar PK, Jhingran AG (1991) Inland fishes of India and adjacent countries. Oxford & IBH Pub Co.

32. Jayaram KC (2010) The freshwater fishes of the Indian Region. Narendra Publishing House, New Delhi India 616.

33. Pethiyagoda R, Meeqaskumbura M, Maduwage K (2012) A synopsis of the South Asian fishes referred to Puntius (Pisces: Cyprinidae). Ichth Explo Fresh 23: 69-95.

34. Ward RD, Zemlak TC, Innes BH, Last PR, Hebert PD (2005) DNA barcoding Australia’s fish species. Philos Trans R Soc Lond B Biol Sci 360: 1847-1857.

35. Thompson JD, Gibson TJ, Pfenwiak E, Jemamougin E, Higgins DG (1997) The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876-4882.

36. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120.

37. Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5: 150-163.

38. Hebert PD, Cywinska A, Ball SL, de Waard JR (2003) Biological identifications through DNA barcodes. Proc Biol Sci 270: 313321.

39. Valdez-Moreno M, Ivanova NV, Elias-Gutierrez M, Contreras-Balderas S, Hebert PD (2009) Probing diversity in freshwater fishes from Mexico and Guatemala with DNA barcodes. J Fish Biol 74: 377-402.

40. Pradhan V, Kamble Y, Ladiya V, Mogul M (2015) An overview of species identification by DNA barcoding. Int J Curr Microbiol App Sci 4: 127-140.

41. Xia Y, Gu HJ, Peng R, Chen Q, Zheng YC (2012) COI is better than 16S rDNA for DNA barcoding Asiatic salamanders (Amphibia: Caudata: Hynobiidae). Mol Ecol Resour 12: 48-56.

42. Smith M, Poyarkov NA, Hebert PD (2008) DNA barcoding: COI DNA barcoding amphibians: take the chance, meet the challenge. Mol Ecol Resour 8: 235-246.

43. Vences M, Thomas M, Bonett RM, Vieites DR (2005) Deciphering amphibian diversity through DNA barcoding: chances and challenges. Philos Trans R Soc Lond B Biol Sci 360: 1859-1868.

44. Vences M, Thomas M, Vander Mejden A, Chiari Y, Vieites DR (2005) Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Front Zool 2: 5.

45. Vences M, Chiari Y, Teschke M, Glaw F (2008) Which frogs are out there? Underestimation of Madagascar’s biodiversity evidenced by an integrative amphibian inventory. Proc Natl Acad Sci USA 106: 8267-8272.