DNA barcoding of *Cynoglossus arel* using mitochondrial COI and 16S rRNA genes

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ABSTRACT

DNA barcoding is not a substitute for taxonomy; however, it does provide a powerful tool to aid species identifications and focus on future taxonomic research efforts. In the present study, an attempt is made to identify and validate *Cynoglossus arel* collected from the Mangalore coast by DNA barcoding using mitochondrial COI and 16S rRNA genes. The primer pairs used in the study could successfully amplify 646 bp segment of COI and 616 bp segment of 16S rRNA gene in *C. arel*. The K2P average genetic distance calculated among species in the Cynoglossidae family was 0.22 and 0.09 among COI and 16S rRNA sequences respectively. This study makes an essential contribution to the NCBI database as this work provides the first 16S rRNA gene sequence for *C. arel* in the database. The COI and 16S rRNA gene sequences of *C. arel* and related species in phylogenetic analysis segregated Family Cynoglossidae into one clade and the outgroup (Family Tetraodontidae) as another clade.

**Keywords:** COI gene, *Cynoglossus arel*, DNA barcoding, 16S rRNA gene

The biological diversity of each country is a valuable and vulnerable natural resource. Thus, sampling, identifying, and studying biological specimens are among the first steps towards protecting and benefiting from biodiversity. Species recognition is a basic need for large scale biodiversity monitoring and conservation. Classical taxonomy falls short in this race to catalogue biological diversity before it disappears. This slow rate of progress in fish identification is mainly due to the lack of trained taxonomists in these regions, which contribute to the ‘taxonomic impediment’ (Swartz *et al.* 2008). Thus it was felt that there is a need for an easy approach of species identification, which helps in the documentation of biodiversity.

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism’s DNA to identify it as belonging to a particular species. This technique is working under the principle that inter-species variations are more significant than the intraspecies variations (Ward *et al.* 2005), allowing one to distinguish the species using nucleotide sequences. Mitochondrial (mt) DNA analyses using conserved genes like 16S rRNA, Cyt b, or cytochrome oxidase subunit I (COI) have emerged as powerful approaches to answer questions of fish taxonomy, species identification, and population genetics (Hebert *et al.* 2003). Rapid barcoding and comparison with the growing databases of gene sequences will increase the speed of identification of newly collected or unknown specimens.

Flatfish comes under the order Pleuronectiformes, which include families Bothidae (flounders), Psettodidae (Indian halibut), Soleidae (soles), and Cynoglossidae (tongue soles). Eleven genera and 25 species of flatfishes contribute to the fisheries along the Indian coast (Vivekanandan *et al.* 2003). The present work was carried out for developing DNA barcodes of *Cynoglossus arel* species belonging to the family Cynoglossidae. This study provides the baseline data for further research on DNA barcoding of *Cynoglossus arel* as there is a lack of information on this aspect in the National Center for Biotechnology Information (NCBI).

**MATERIALS AND METHODS**

Sample collection: *Cynoglossus arel* samples were collected from the Mangalore fish landing center, Karnataka. Following collection, the specimens were digitally photographed and identified by using conventional taxonomic methods with suitable reference materials (Munroe 2001). Fin and muscle tissue from each sample was aseptically removed and preserved in absolute alcohol at −20°C for further molecular study. The voucher specimen of *Cynoglossus arel* were preserved for future references.

Genomic DNA isolation, amplification, cloning, and sequencing: DNA was extracted from fin and muscle tissue by following the standard phenol-chloroform method (Sambrook *et al.* 1989). DNA concentration and purity in the sample was estimated by using a spectrophotometer (NanoDrop 2000c Thermo Scientific, USA). Extracted DNA was subjected to PCR amplification using gene-specific universal primers (Table 1) targeting 658 bp fragment of COI and 583 bp fragment of 16S rRNA. Amplification of genes was carried out in a programmable
thermocycler (Bio-Rad Laboratories, USA) with slight modifications of published data of Mohanty et al. (2013). The optimized PCR programme consisted of initial denaturation at 95°C for 4 min, followed by 34 cycles of 1 min denaturation at 94°C, 30 sec of annealing at 54°C and 62°C for COI and 16S rRNA primers respectively, 1 min of extension at 72°C and the final extension at 72°C for 15 min. The PCR amplified products were visualized in 1.5% agarose gel by electrophoresis and analyzed using the Gel documentation system (Herolab, Germany).

The amplified PCR products were purified by using the GenElute PCR Clean-Up Kit (Sigma–Aldrich). The amplified PCR products of COI and 16S rRNA genes were ligated into the Genei T vector for cloning by following the manufacture’s protocol (GeNei INSTANT cloning kit). The frozen competent E. coli strains (DH5α cells) were used for the transformation of COI and 16S rRNA, followed by the screening of recombinant transformants. The purified recombinant plasmids having COI and 16S rRNA gene inserts were sequenced to determine the nucleotide sequences by outsourcing them to Chromous biotech, Bengaluru using gene-specific primers.

**Sequence analysis:** The sequences obtained were subjected to BLAST analysis and aligned using ClustalW (Thompson et al. 1994). The sequences determined were submitted to the GenBank, NCBI. The phylogenetic analysis was performed using MEGA version 4.0 (Tamura et al. 2007). The nucleotide composition and the number of transition and transversion between species were determined by DnaSp ver3. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura et al. 2004). The Neighbour Joining tree was constructed using the Kimura -2 parameter model with 1000 bootstrap replicates to test the efficacy of barcodes in discriminating the species for analysis using a model with 1000 bootstrap replicates to test the efficacy of joining tree was constructed using the Kimura -2 parameter substitutions per site (Tamura method and are in the units of the number of base substitutions per site (Tamura et al. 2007). The average number of substitutions for the COI and 16S rRNA genes were determined by using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura et al. 2004). The Neighbour Joining tree was constructed using the Kimura -2 parameter model with 1000 bootstrap replicates to test the efficacy of barcodes in discriminating the species for analysis using a model with 1000 bootstrap replicates to test the efficacy of joining tree was constructed using the Kimura -2 parameter substitutions per site (Tamura et al. 2007).

**RESULTS AND DISCUSSION**

**Morphometric identification of Cynoglossus arel:** Morphometric characters are significant key aspects of conventional taxonomical identification. So it is imperative to identify the fish through the classical taxonomical approach and further use DNA barcoding as a confirmative molecular tool. Morphometric evaluation of C. arel was done by using taxonomic keys in the FAO species identification sheet for fishery purposes. The diagnostic characters of C. arel (Fig. 1) species collected include an elongated body with dorsal and anal fins joined to the caudal fin.

Eyes on the left side of the body, two lateral lines on the eyed side, and lips which are not fringed confirmed it as Cynoglossus spp. Two lateral lines on the eyed side but none on the blind side and eyes with a small scaly interorbital space confirmed the species as C. arel. The total length of species collected ranged between 17.5 cm to 23 cm. In the present study, taxonomic keys were used systematically for confirmation of all the species since it is essential to identify the fish through the Linnaean taxonomy and then proceed with molecular tools. The meristic and morphologic classification should be revised using molecular techniques (Infante et al. 2004). The trend which appears to be that DNA barcoding needs to be used alongside traditional taxonomic tools and alternative forms of molecular systematics so that taxonomic ambiguities can be resolved (Emery et al. 2009).

**Amplification of COI and 16S rRNA gene by using PCR and cloning:** Sequencing of the mitochondrial COI gene is an established methodology with an international campaign directed at barcoding of all fishes (Puckridge et al. 2013), as it helps not only faster identification of species, but also in identifying eggs, larvae or fragments of a species which could not be identified by conventional taxonomic approach. DNA concentration measured showed a purity range from 1.74 to 1.91 in the samples, which were in conformity with the purity range of ~1.8 in 260/280 ratio. The size of the COI amplified PCR product was about 650 bp for C. arel, and the amplification of 16S rRNA for Cynoglossus arel yielded a product size of about 580 bp.

The purified PCR products of the gene coding for COI and 16S rRNA C. arel were ligated into the Genei T vector for cloning. The positive clones were confirmed by PCR using gene-specific primers, and all the positive clones were archived at −80°C in the glycerol stock solution.

**Sequencing and analyses of COI and 16S rRNA genes of C. arel:** The generated sequences were submitted in Genbank, NCBI under the accession numbers KT323970, KT323971. The sequences determined were submitted to the GenBank, NCBI under the accession numbers KT323970, KT323971.
Sequence alignment of the COI gene in *C. arel* produced 646 nucleotide base pairs, and the 16S rRNA gene in *C. arel* yielded 616 nucleotide base pairs. Since most of the global biodiversity remains unknown, molecular barcoding can only hint at the existence of new taxa, but not delimit or describe them (DeSalle 2006, Rubinoff 2006). Multiple sequence alignment of COI gene sequences of *C. arel* and 16S rRNA gene sequences of *C. arel* with other related species from NCBI were done to find the nucleotide substitutions.

**Nucleotide composition, frequency and substitutions:** The COI and 16S rRNA gene sequence analysis revealed the nucleotide composition of *C. arel*, as shown in Table 2. Min and Hickey (2007) reported a strong correlation between the GC composition of the COI gene and the entire mitochondrial genome. The GC content of COI in *C. arel* is 42.4%, which is in conformity with the expected range of 42.2–47.1% in COI (Ward et al. 2005, Lakra et al. 2011, Viswambharan et al. 2013).

In this study, considering the nucleotide pair frequencies, the average number of transitional and transversional pairs was calculated. The average transitional pairs (si=71) were more than transversional pairs (sv=51) with an average ratio of 1.4 in the COI gene of *C. arel*. The nucleotide pair frequencies of the 16S rRNA gene of *C. arel* revealed an average transitional pair (si=52), which are more than transversional pairs (sv=35) with an average ratio of 1.49. The average number of transitional pairs outnumbered the transversional pairs in accordance with the previous reports on mtDNA in fish (Vinson et al. 2004, Chakraborty and Ghosh 2014). Generally, for teleost mtDNA, a more significant excess of transitions related to transversion is typically observed (Ward et al. 2005). The maximum composite likelihood model shows the probability of nucleotide substitution from one base (row) to another base (column) instantaneously. The values are given in the tabular format, where only entries within a row should be compared. Rates of different transitional substitutions are shown in bold, and those of transversional substitutions are shown in italics (Table 3).

| Accession | Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|-----------|---------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|
| EU541319  | *Cynoglossus Macrolepidotus* | – |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JQ639062  | *C. itinus* | 0.99 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| FJ347912  | *C. macrostomus* | 0.23 0.21 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JQ349003  | *C. puncticeps* | 0.21 0.19 0.16 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JX983279  | *C. cynoglossus* | 0.22 0.22 0.20 0.19 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JQ738572  | *C. puppareomaculatus* | 0.23 0.21 0.19 0.21 0.25 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| HM180553  | *C. robustus* | 0.23 0.21 0.19 0.21 0.24 0.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| KT323970  | *Cynoglossus arel* | 0.23 0.22 0.20 0.22 0.24 0.14 0.13 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| GQ380410  | *C. abbreviatus* | 0.23 0.24 0.23 0.21 0.23 0.19 0.19 0.21 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| GQ380409  | *C. semilaevis* | 0.24 0.23 0.20 0.19 0.23 0.22 0.22 0.22 0.12 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| EU513630  | *C. canariensis* | 0.24 0.22 0.23 0.22 0.23 0.21 0.21 0.21 0.21 0.20 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| FJ347908  | *C. dubius* | 0.31 0.29 0.27 0.25 0.29 0.31 0.31 0.30 0.28 0.25 0.23 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JQ349000  | *C. bilineatus* | 0.25 0.24 0.23 0.20 0.24 0.25 0.26 0.25 0.23 0.20 0.19 0.15 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| EU513627  | *C. browni* | 0.26 0.25 0.23 0.22 0.25 0.22 0.22 0.25 0.24 0.22 0.21 0.23 0.17 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| JQ348998  | *C. sinicus* | 0.25 0.25 0.25 0.25 0.25 0.24 0.24 0.26 0.22 0.24 0.24 0.25 0.21 0.20 – |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

Table 2. Nucleotide composition of *Cynoglossus arel*

| Gene bank Accession No. | Percentage base composition | GC content (%) | Total length |
|------------------------|----------------------------|----------------|-------------|
| KT323970               | 25.2 32.4 17.8 24.6        | 42.4           | 646         |
| KT323971               | 30.7 25 19.6 24.7          | 44.3           | 616         |

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution

| Base | COI      | 16S rRNA |
|------|----------|----------|
| A    | – 6.1    | – 5.36   |
| T    | 5.10 – 17.90 | 6.54 – 10.29 |
| C    | 5.10 22.3 – 3.55 | 6.54 11.43 – 4.21 |
| G    | 12.11 6.1 4.89 – | 5.36 4.83 14.26 |

Table 4. Pair-wise genetic distances (Kimura 2-parameter) of *Cynoglossus arel* and related species based on COI gene sequences
transversion bias (R) value obtained with Maximum Composite Likelihood estimate is 1.412 and 1.11 for the COI and 16S rRNA gene of *C. arel* respectively.

Genetic distance: The comparison of pairwise genetic distance for COI and 16S rRNA gene sequences of the Cynoglossidae family are given in Table 4 and Table 5, respectively. The average genetic distance among species in the Cynoglossidae family was 0.22 and 0.09 among COI and 16S rRNA sequences, respectively. In case of the COI gene, *Cynoglossus arel* had the highest intraspecies distance (0.24) with *C. cynoglossus* and lowest intraspecies distance (0.11) among *C. sinicus* and *C. bilineatus* and lowest intraspecies distance (0.08) between *C. puncticeps*. The barcoding studies conducted by the usage of K2P genetic distance for analyzing the data exposed that barcode variations keep on increasing from species to genera, family (Ward et al. 2005, Hajibabei et al. 2007 and Lakra et al. 2011). Usually, interspecific divergence should be about ten times higher than intraspecific divergence (Hebert et al. 2004). The mean K2P distance values obtained in the case of 16S rRNA in *C. arel* was 0.09, which is relatively low was likely attributable to the highly conserved nature of the mitochondrial 16 S rRNA gene in vertebrates (Di Finizio et al. 2007, Kitano et al. 2007).

Evolutionary relationships using phylogram: The phylogenetic analysis provides the most accurate reconstruction of evolutionary relationships and distances between nucleotide sequences. The phylogram generated using MEGA 4 through the Neighbour-Joining method (Saitou and Nei 1987) was used to study the evolutionary relationship between related species. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The Neighbour Joining (NJ) tree illustrated COI based genetic divergence among related species present in NCBI as different branches in *Cynoglossus arel*. Phylogenetic relationships based on morphological and molecular characters are mostly concordant (Ward et al. 2005). The ability of the 16S rRNA gene to differentiate two genera was evident through the phylogram as different clades in *C. arel*. The phylogram generated through the neighbour joining method using K2P distance was highly reliable as the outgroup used was clearly segregated in the phylogram. The evolutionary relationship between the COI gene sequence of *C. arel* and other species in the same family (Fig. 2) were represented using Tetradon ocellatus (Family Tetraodontidae) as an outgroup. The 16S rRNA gene sequences of *C. arel* and related species (Fig. 3) were segregated into one clade and the outgroup Tetradon abei (Family Tetraodontidae) into another clade. Phylogenetic analyses indicated the evolutionary distance between species in the various family such as Cynoglossidae, Soleidae, Bothidae, Paralichthyidae, Citharidae, Pleuronectidae, and Psettodidae belonging to the same order Pleuronectiformes. Pleuronectiformes and Tetrodontiformes are derived orders from Perciformes, and

![Fig. 2. Neighbour joining tree of COI gene sequences of Cynoglossus arel and related species with Tetradon ocellatus as outgroup constructed with bootstrap value 1000, scale bar: 0.02 substitution per site.](image-url)

Table 5. Pair-wise genetic distances (Kimura 2-parameter) of *Cynoglossus arel* and related species based on 16S rRNA gene sequences

| Accession Number | Species            | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  |
|------------------|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| JQ348998         | Cynoglossussinicus | -   |     |     |     |     |     |     |     |     |     |     |     |     |
| JQ349000         | *C. bilineatus*    | 0.04|     |     |     |     |     |     |     |     |     |     |     |     |
| JQ349002         | Paraplagusiablochii| 0.11| 0.12|     |     |     |     |     |     |     |     |     |     |     |
| JQ639066         | *P. japonica*      | 0.11| 0.11| 0.03|     |     |     |     |     |     |     |     |     |     |
| JQ349001         | *P. bilineata*     | 0.11| 0.11| 0.04| 0.04|     |     |     |     |     |     |     |     |     |
| JQ349003         | Cynoglossuspuncticeps | 0.10| 0.10| 0.06| 0.06| 0.06|     |     |     |     |     |     |     |     |
| KP057581         | *C. trigrammus*    | 0.11| 0.12| 0.09| 0.09| 0.09| 0.08|     |     |     |     |     |     |     |
| JQ639062         | *C. tinus*         | 0.11| 0.11| 0.11| 0.11| 0.11| 0.10| 0.10| 0.11| 0.10| 0.10| 0.10| 0.10| 0.10|
| KT323971         | Cynoglossusarel    | 0.11| 0.11| 0.09| 0.10| 0.10| 0.08| 0.10| 0.10| 0.10| 0.10| 0.10| 0.10| 0.10|
| GQ380410         | *C. abbreviatius*  | 0.09| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09|
| DQ112680         | *C. puppureomaculatus* | 0.09| 0.09| 0.10| 0.09| 0.09| 0.09| 0.10| 0.09| 0.09| 0.09| 0.09| 0.09| 0.09|
| GQ380409         | *C. semilaevis*    | 0.08| 0.09| 0.10| 0.10| 0.10| 0.09| 0.09| 0.09| 0.09| 0.33| 0.33|     |     |
| DQ112683         | *C. lighti*        | 0.10| 0.10| 0.09| 0.09| 0.10| 0.08| 0.11| 0.10| 0.09| 0.07| 0.07| 0.07| 0.06| -  |
Formes and Tetrodontiformes are derived orders from Perciformes, and thus a relationship between species of family Tetrodontidae was visible in the phylogram.

This study provides the first 16S rRNA gene sequence for C. arel. Chakraborty et al. (2006) indicated the usefulness of the 16S rRNA gene sequence for accurate identification of species. Partial sequences of both COI and 16S rRNA genes provided sufficient phylogenetic information to identify C. arel, indicating the usefulness of the mtDNA-based approach in species identification. The DNA barcodes developed in this study could be useful for assessing flatfish diversity, and the sequences reported in this study will supplement the previous reports for estimating phylogenetic diversity among flatfishes.

India being a maritime country, its rich fishery resources, hold the key to food security and economic development. Species recognition is a basic need for large scale biodiversity monitoring and conservation. The present study revealed that morphological characteristics and DNA barcoding using COI and 16S rRNA gene clearly distinguish the identified fish species from the Mangalore coast as C. arel. This work provides the first 16S rRNA gene sequence for C. arel. Partial sequences of both COI and 16S rRNA genes provided sufficient phylogenetic information to identify C. arel, indicating the usefulness of the mtDNA-based approach in species identification. This study has supported the claim of the robustness of universal primers for 16S rRNA and COI genes. The phylogenetic position of C. arel was analyzed based on COI and 16S rRNA gene sequences to find out the evolutionary relationship and exhibited identical phylogenetic resolution. Pleuronectiformes and Tetrodontiformes are derived orders from Perciformes, and thus a relationship between species of family Tetrodontidae was visible in the phylogram.

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REFERENCES

Chakraborty A, Aranishi F and Iwatsuki W. 2006. Genetic differences among three species of the genus Trichius (Perciformes: Trichiuridae) based on mitochondrial DNA analysis. Ichthyological Research 53(1): 93–96.

Chakraborty M and Ghosh S K. 2014. An assessment of the DNA barcodes of Indian freshwater fishes. Gene 537(1): 20–28.

DeSalle R O. 2006. Species discovery versus species identification in DNA barcoding efforts: Response to Rubinoff. Conservation Biology 20(5): 1545–47.

Di Finizio A, Guerriero G, Russo G and Ciarcia G. 2007. Identification of gadoid species (Pisces, Gadidae) by sequencing and PCR RFLP analysis of mitochondrial 12S and 16S rRNA gene fragments. European Food Research and Technology 225(3–4): 337–44.

Emery V J, Landry J F and Eckert C G. 2009. Barcoding Arthropods Combining DNA barcoding and morphological analysis to identify specialist floral parasites (Lepidoptera: Coleophoridae: Momphinae: Mompha). Molecular Ecology Resources 9(1): 217–23.

Hajibabaei M, Singer G A, Hebert P D and Hickey D A. 2007. DNA barcoding: How it complements taxonomy, molecular phylogenetics, and population genetics. TRENDS in Genetics 23(4): 167–72.

Hebert P D, Cywinska A, Ball S L and DeWaard J R. 2003. Biological identification through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences 270: 313–19.

Hebert P D, Penton E H, Burns J M, Janzen D H and Hallwachs W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. Proceedings of the National Academy of Sciences 101(41): 14812–17.

Infante C, Catanese G and Manchado M. 2004. Phylogenetic Relationships Among Ten Sole Species (Soleidae, Pleuronectiformes) from the Gulf of Cadiz (Spain) based on mitochondrial DNA Sequences. Marine Biotechnology 6(6): 612–24.

Kitano T, Umetsu K, Tian W and Osawa M. 2007. Two universal primer sets for species identification among vertebrates. International Journal of Legal Medicine 121(5): 423–7.

Lakra W S, Verma M S, Goswami M, Lal K K, Mohindra V, Punia P, Gopalakrishnan A, Singh K V, Ward R D and Hebert P. 2011. DNA barcoding Indian marine fishes. Molecular Ecology Resources 11(1): 60–71.

Min X J and Hickey D A. 2007. DNA barcodes provide a quick preview of mitochondrial genome composition. PLoS ONE 2(3): e325.

Mohanty M, Jayasankar P, Sahoo L and Das P A. 2013. A Comparative study of COI and 16 S rRNA genes for DNA barcoding cultivable carps in India. Mitochondrial DNA 26(1): 83–7.
Munroe T A. 2001. Cynoglossidae. Tonguesoles. FAO species identification guide for fishery purposes, pp. 3890–3901. The living marine resources of the Western Central Pacific. Vol. 6. Bony fishes part 4 (Labridae to Latimeriidae), estuarine crocodiles. (Eds.) Carpenter K E and Niem V. FAO, Rome.

Palumbi S R. 1996. PCR and molecular systematics. Molecular Systematics, 2nd edition. (Eds) Hillis D, Moritz C and Mable B. Sinauer Press, pp. 205–47.

Puckridge M, Andreakis N, Appleyard S A and Ward R D. 2013. Cryptic diversity in flathead fishes (Scorpaeniformes: Platyccephalidae) across the Indo-West Pacific uncovered by DNA barcoding. Molecular Ecology Resources 13: 32–42.

Rubinoff D. 2006. Utility of mitochondrial DNA barcodes in species conservation. Conservation Biology 20(4): 1026–33.

Sambrook J, Fritsch E F and Maniatis T. 1989. Molecular Cloning: A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Saitou N and Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4): 406–25.

Swartz E R, Mwale M and Hanner R. 2008. A Role For Barcoding in the study of African fish diversity and conservation. South African Journal of Science 104: 293–98.

Tamura K, Nei M and Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences 101(30):11030–35.

Tamura K, Dudley J, Nei M and Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24(8): 1596–99.

Thompson J D, Higgins D G and Gibson T J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22(22): 4673–80.

Vinson C, Grazielle G, Schneider H and Sampaio I. 2004. Sciaenidae fish of the Caete river estuary, Northern Brazil: mitochondrial DNA suggests explosive radiation for the Western Atlantic assemblage. Genetics and Molecular Biology 27(2): 174–80.

Viswambharan D, Pavankumar A, Singh D P, Jaiswar A K, Chakraborty S K, Nair J R and Lakra W S. 2013. DNA barcoding of gobidi fishes (Perciformes, Gobioidi). Mitochondrial DNA (0): 1–5.

Vivekanandan E, Zachariah P U, Feroz Khan M and Rekha J N. 2003. Status of exploited marine fishery resources of India, pp. 164–170. (Eds.) Mohan Joseph M and Jayaprakash AA. Central Marine Fisheries Research Institute, Kochi, India. .

Ward R D, Zemlak T S, Innes B H, Last P R and Hebert P D N. 2005. DNA barcoding Australia’s fish species. Philosophical Transactions of the Royal Society of London. Series B. Biological Sciences 360(1462): 1847–57.