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Short Communication

A small leap toward DNA barcode library creation of ornamental fishes: development of 17 DNA barcodes from Manipur, India

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ABSTRACT

Proper identification is expected to result in proper conservation of a species. Morphology-based identifications are problematic in many cases and often time-consuming. DNA barcoding came out to be the problem solver in these cases. A researcher can easily identify a species by comparing generated barcode sequences with the barcode sequences from a DNA barcode library. We have developed 17 DNA barcodes representing 15 different species of ornamental fishes from Manipur, India, which are deposited in GenBank and BOLD. The present study will help future researchers to identify their ornamental fishes properly without confusion and ultimately may help in proper conservation of ornamental fishes that are threatened.

Introduction

Ornamental fishes usually mean eye-catching colorful and interesting fishes of various characteristics. Most of the small food fishes that are treated as unwanted for conventional farming have good potential as ornamental fishes and are popularly known as aquarium fishes (Khomdram et al 2014). Generally, ornamental fishes are selected based on their body color (preferably attractive), body shape, and aquarium suitability. Northeast India is the leading source of ornamental fishes in India. This region contributes 85% of the ornamental fish export from India (Devi et al 2013). Owing to the diversity of topographic and climatic features of Northeastern states of India, this region is rich in endemic fishes. These endemic species are attracting hobbyists both locally and globally. The up-to-date inventory of the fish species of the Northeastern hill region showed 250 potential ornamental fish species. Out of this, the highest number was recorded from Assam (187 species), followed by Arunachal Pradesh (165 species), Meghalaya (159 species), Manipur (139 species), Tripura (103 species), Nagaland (71 species), Mizoram (46 species), and Sikkim (29 species) (Mahapatra et al 2004). Manipur is endowed with a rich resource of native type of ornamental fish species. Ornamental fishery resources face a range of challenges: the need for their conservation and sustainable use, problems caused by habitat loss and degradation, harmful fishing practices (overfishing and destructive fishing, such as the use of cyanide), changes in international trade patterns, and concerns about the introduction of exotic species (fisheries and aquaculture department). One of the main problems faced by the aquarists is the proper identification of these fishes. If an ornamental fish species is properly identified, that very species could be properly conserved and all the related problems could be solved. Although many research works have been carried out for proper identification of the endemic fishes of Manipur (Vishwanath 2000; Vishwanath et al 1998; Vishwanath et al 2011; Vishwanath and Darshan 2006; Vishwanath and Devi 2005; Vishwanath and Dishma 2012; Vishwanath and Juliana 2004; Vishwanath and Linthoingambi 2007; Vishwanath and Shantakumar 2007; Vishwanath and Tombi 1985), the works were solely based on morphology. Morphology-based identifications of fishes are time-consuming and problematic for several reasons. For some fishes, it is difficult or impossible to identify juveniles. DNA barcoding protocol has been demonstrated as an effective fish identification tool in situations including consumer protection and fisheries management/conservation (Hebert et al 2003). Using barcodes for routine species identifications is the most widely accepted one of the potential applications of DNA barcoding (Rubinoff 2006). Several studies of Fish Barcode of Life initiative have generated a huge number of reference DNA barcode sequences from taxonomically authenticated fish species (Ratnasingham and Hebert 2007). Most of the endemic ornamental fishes of Manipur remain unexplored

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especially in the molecular level. Here, we present development of 17 DNA barcodes representing 15 species of ornamental fishes being sampled randomly from different regions of the state.

Material and methods

Sampling

A total of 17 ornamental fish samples were collected from wild habitats as well as from different markets of Manipur, India, after communicating with local fishermen. A small amount of muscle tissue was collected aseptically from each sample and stored in 500 μL of tris ethylene diamine tetra acetate salt buffer (50 mM TrisHCl, 25 mM ethylene diamine tetra acetate, and 150 mM NaCl) (Ghosh 2012).

Morphology-based identification

Before DNA extraction, a broad preliminary identification of the samples was done by consulting experts and referring to literature.

Genomic DNA isolation

For the isolation of genomic DNA, the standard protocol of phenol chloroform extraction method was followed. Once the nucleic acid complex has been purified, precipitation can be accomplished and stored (Ghosh 2012; Sambrook and Russel 2001). The purity and yield of the extracted DNA were checked in a spectrophotometer. The genomic DNA was then analyzed by agarose gel electrophoresis and was visualized in a ultraviolet transilluminator or Gel-DOC (BioRad).

Amplification and purification of polymerase chain reaction products

Primers published by Ward et al 2005 (Ward et al 2005) were used to amplify the barcode segment of the cytochrome oxidase subunit I (*COI*) gene in a Veriti Mastercycler (Applied Biosystems Inc., CA, USA). The polymerase chain reaction (PCR) was set with an initial denaturation temperature of 94°C host start for 3 minutes and subsequently, 94°C for 1 minute for denaturation, 50°C for 45 seconds 72°C for 45 seconds for extension primer annealing for 30 cycles followed by 72°C for 10 minutes for final extension. Aliquots for 10 μl of DNA products from PCR amplification were loaded in 1.5% agarose gel for electrophoresis in 1X tris acetic acid ethylene diamine tetra acetate. Gel was stained with ethidium bromide and observed under ultraviolet transilluminator and documented with Gel-DOC (BioRad).

The PCR-amplified products were analyzed in 1% low-melting agarose gel containing ethidium bromide stain (10 mg/mL). The single uniform band was excised and purified using QIA quick^{R} Gel extraction kit (QIAGEN, USA) following the manufacturer's instructions.

Sequencing

The purified amplicons of the *COI* were bidirectionally sequenced in an automated DNA sequencer (ABI 3500; Applied Biosystems Inc.,), obtained through GCC Biotech India Pvt. Ltd. (Kolkata, India).

Sequence annotation

Sequence annotation was performed using the software programs SeqScanner Version 1.0 (Applied Biosystems Inc.,), BLASTN program (Altschul et al 1990), ClustalX software (Thompson et al 2002), and ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Sequence submission

All the analyzed sequences were deposited in GenBank through the Banklt sequence submission tool (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) of GenBank and received valid accession numbers. The sequences were also submitted in BOLD following BOLD sequence submission protocol and received valid sequence IDs.

Species identification based on similarity match with database

Comprehensive barcoding identification results were generated by carrying out homology comparisons between two databases, GenBank and BOLD.

Results and discussion

Morphology-based identification of the ornamental fishes

In this study, a total of 17 specimens of ornamental fishes were collected from various locations in the state of Manipur, India. The morphological characters of these specimens were observed carefully and compared with described characters as mentioned in the leading taxonomic guide books, "The freshwater fishes of the Indian region" by KC Jayaram (1999) and "Inland fishes of India and adjacent countries" by PK Talwar and AG Jhingran (1991) (Jayaram 1999; Talwar and Jhingran 1991). Based on this study, following the standard family-level taxonomic keys, the specimens were categorized under eight families. The important aspects regarding the identification of the studied specimens under each family are explained below.

Cyprinidae

A total of nine different specimens (among our studied fishes) fall under this family. The specimens represented by the sample code SGBK-FO2B and SGBK-OF4 were identified as *Puntius sophore*. *Puntius sophore* was first described as *Cyprinus sophore* by Hamilton in 1822. They are readily and easily identifiable by the presence of 12 dorsal soft rays and eight anal soft rays and the presence of distinct reddish line in the mid body length.

The specimen being coded by the sample ID SGBK-AUFO5 was identified as *Puntius chola*. Its morphological characters perfectly match with the ones described by Talwar and Jhingran (Talwar and Jhingran 1991). It can be noted that the presence of deep body depth and no black dots or stripes are very helpful to diagnose this species from other congeners.

The specimen represented by the sample code SGBK-BF8 was identified as *Barbonymus gonionotus*. *Barbonymus gonionotus* is easily distinguishable from other congeners from the view that it is largest and its bright silver coloration in addition to body depth.

The specimens being represented by the sample codes SGBK-DF1, SGBK-DF2, SGBK-DF3, and SGBK-AUJF39 were identified as *Labeo bata*, *Labeo calbasu*, *Labeo gonius*, and *Labeo boga*, respectively. All these fishes have the identification key for the genus *Labeo*, i.e. they have spindle-shaped body, mouths looking too different, and a pronounced rostral cap, which covers the upper lip except when feeding. In case of *Labeo bata*, the body is elongate, and its dorsal profile is more convex than the ventral. The snout slightly projects beyond the mouth, often studded with pores

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