Species Identification of 3 Hypsibarbus spp. (Pisces: Cyprinidae) Using PCR–RFLP of Cytochrome b Gene

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ABSTRACT

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) was used to identify 3 closely related Hypsibarbus spp: Hypsibarbus wetmorei, H. vernayi, and H. malcolmi. Mitochondrial cytochrome b gene (993 bp) in 3 Hypsibarbus spp. showed single PCR–product. The sequencing results of PCR–products in 3 Hypsibarbus spp. showed very low interspecific variation. However it could be used to discriminate these species by RFLP analysis. The combination of 2 restriction enzymes; Bsp143I and BclI were used to identify 3 Hypsibarbus spp. Bsp143I could discriminate H. vernayi from H. wetmorei and H. malcolmi, by generating 3 fragments (535 bp, 234 bp and 224 bp) in H. vernayi whereas 2 fragments of 769 bp and 224 bp in H. wetmorei and H. malcolmi. Thereafter, BclI was effectively discriminated H. wetmorei from H. malcolmi by generating 3 fragments (591 bp, 288 bp and 114 bp) in H. malcolmi and uncut fragment in H. wetmorei. There were intraspecific restriction polymorphism in H. vernayi using BclI which generated 2 patterns; an uncut fragment and 2 fragment of approximately 700 bp and 300 bp. Thus, PCR–RFLP technique could be used to identify 3 closely related Hypsibarbus spp.

Key words: Hypsibarbus spp, PCR–RFLP technique, identification, Cytochrome b gene

INTRODUCTION

Fish in the genus Hypsibarbus Rainboth, 1996 (family Cyprinidae) are an important in aquaculture. They are generally found in South East Asia. In Thailand, they distribute in the main stream of large rivers such as Chao Phraya basin, Mekong basin and Meklong basin. Rainboth (1996) reported that there were 6 species in Thailand; H. lagleri, H. malcolmi, H. salweenensis, H. suvattii, H. vernayi and H. wetmorei based on body proportion, gill and scale counting and geographic distribution. A recent study of Sunairattanaporn (2001), there were 6 species of Hypsibarbus in Thailand; H. lagleri, H. pierrei, H. salweenensis, H. tenasserimensis, H. vernayi and H. wetmorei based on mostly external morphology, body proportion and scale counting. According to this recent study, H. suvattii was a synonym of H. lagleri, H. malcolmi was a synonym of H. pierrei and H. tenasserimensis was a new species (unpublished).

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However, the synonym of these fishes were ambiguous due to the most characters in each species were similar. Furthermore, larval or juvenile stages within this genus are often morphologically similar and found in a large number. Hence it is difficult to accurately identify, and then it consequently leads to the hurdle for effective aquaculture, stock management and species conservation.

The purpose of this study is to enhance the reliable identification of 3 *Hypsibarbus* spp. that mostly distribute in river basin in Thailand and closely related intern of taxonomic and morphological similarities; *H. wetmorei*, *H. vernayi*, *H. malcolmi* using PCR–RFLP. This technique was used for fish identification such as freshwater eels (Lin *et al*., 2002), tuna fish (Pardo and Pérez–Villareal, 2004; Lin *et al*., 2005) and cod fish (Calo–Mata *et al*., 2003; Aranishi *et al*., 2005; Akasaki *et al*., 2006).

**MATERIALS AND METHODS**

**Sample collection**

From December 2002 to March 2006, the *Hypsibarbus* were obtained from Phetchaburi fishery station, Loei fishery station and were collected from Loei, Nakhon Phanom, Ubon Ratchathani and Nakhon Sawan provinces. All samples were labeled and photographed. The muscle tissue was dissected and preserved in 95% ethanol (ETOH) for DNA extraction.

**DNA extraction**

Prior to DNA extraction, the 95% ethanol (ETOH) preserved tissues were washed with distilled water to remove ethanol. Total genomic DNA was extracted from 0.10–0.15 g tissue samples. Samples were dissected and transferred into 1.5 ml microcentrifuge tube. The samples were digested in 500 µl of STE Buffer (0.1M NaCl, 50mM Tris–HCl pH 7.5, 1.0mM EDTA), 30 µl of 20% SDS and 30 µl of proteinase K (10 mg/ml in STE buffer). The samples were briefly vortexed and incubated at 55°C for 2 hours with occasional shaking. The homogeneous solution was then extracted with 500 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1), incubated for 5 min, and centrifuged at 7,000 g for 5 min at room temperature (RT) to separate the phenol and aqueous phases. The aqueous phase with DNA was transferred into the new microcentrifuge tube and was extracted once with 500 µl of chloroform: isoamyl alcohol (24: 1), centrifuged at 7,000 g for 3 minutes at RT. The aqueous phase was transferred into the new microcentrifuge tube and DNA was precipitated by adding 40–50 µl of 3M sodium acetate (pH 5.2) and 1 ml of cool absolute ETOH, kept at –20°C for 10–20 minutes, followed by centrifugation at 14,000 g 4°C for 3 minutes. After centrifugation, the precipitate was washed with 500 µl of 70% ETOH followed by centrifuged at 14,000 g 4°C for 2 minutes. Finally the supernatant was removed, the DNA pellet was dissolved in 100–200 µl of TE buffer (10 mM Tris–HCl pH 8.0 and 1.0mM EDTA pH 8.0). The DNA solution was stored at –20°C for long term or 4°C for short term using. The resulting DNA extracts were separated on 0.8% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transilluminator. The quantity of DNA was estimated by spectrophotometry (OD260 and OD280).

**Primer design**

The primers were designed based on 1140 base sequences of *cytochrome b* gene of 4 *Barbodes* species; *B. gonionotus*, *B. laticeps*, *B. heterostomus*, *B. schwannenfeldii* due to their morphological resemblance to *Hypsibarbus* (Kottelat, 1999; Sunairattanaporn, 2001). The base sequences were accessed from GENBANK (http://www.ncbi.nlm.nih.gov/). The sequences were aligned using CLUSTALW 1.82 from EMBL website (http://www.ebi.ac.uk/). The results from the multiple alignment were used for appropriate
 manually primers designation.

The 2 specific primers for *Hypsibarbus* spp. were designed from the best matching areas: the 58th–76th region for the forward primer (FWD primer; L–strand); 5' GACCTACCAGCACC ATCCA 3', and at the 1069th–1089th region for the reverse primer (REV primer; H–strand); 5' GAGGAATAGTGCGAAGTAG 3'.

**PCR amplification**

The cytochrome b gene fragment (993 bp) of 3 *Hypsibarbus* spp. were performed in a total volumes of 100 ml containing 100–300 ng of DNA template, 0.2 µM of each primer, 200 µM of each dNTPs, 3.0 mM of MgCl2, and 2.5 Units of *Taq* DNA polymerase. The PCR amplification were carried out to the following cycle program: initial denaturing step of 3 min at 92°C, followed by 35 cycles and each cycle with denaturation at 92°C for 1 min, annealing at 54°C, for 1 min, extension at 72°C for 1 min, and then a final extension step at 72°C for 1 min.

**DNA sequencing and selection of restriction enzymes**

The representative PCR–products of 3 *Hypsibarbus* spp. were sent to BSU (Bioservice unit) for DNA sequencing. The sequences were then edited with Chromas Lite version 2.01 program and aligned with the Webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html) to find out the species–specific restriction site for producing specific patterns for 3 *Hypsibarbus* spp. identification.

**PCR–RFLP**

For the restriction site analysis of the PCR–products of 3 *Hypsibarbus* spp. Two restriction enzymes (*Bsp*143I and *Bcu*I) were selected. The first enzyme, *Bsp*143I was used to discriminate *H. vernayi* from *H. wetmorei* and *H. malcolmi*. Thereafter, the second enzyme, *Bcu*I was used to discriminate *H. wetmorei* from *H. malcolmi*. The PCR samples were digested in 10–20 µ total volume reaction mix, containing 6 µl of PCR–product, 5 U of each enzyme and 1X enzyme buffer. Samples were digested for 1–2 hours at 37°C. DNA fragments were visualized on a 1.5% agarose gel electrophoresis.

**RESULTS AND DISCUSSION**

In this study the mitochondrial cytochrome b gene could be used to identify 3 *Hypsibarbus* spp. in all specimens because this gene has relatively high mutation rate and sufficient point mutation to enable discrimination of related species belonging to the same genera (Aranishi *et al.*, 2005). Therefore, the mitochondrial cytochrome b gene was useful for these species identification.

In this finding, the PCR-based technique was allowed the correct identification of 3 *Hypsibarbus* spp. in case of morphological ambiguity such as with larval stage as described by Olson *et al.* (1991). This technique have the advantage over morphological method because there was no need to sacrifice the organisms (White, 1993). The specific PCR–products of partial mitochondrial cytochrome b gene (993 bp) in 3 *Hypsibarbus* spp. were successfully amplification due to co–banding or non–specific product were not occurred. These specific PCR–products suggested that the primers sequence was suitable designed to the DNA template of these fishes. When each of the PCR–products of 3 morphological confirmed *Hypsibarbus* spp. were sequenced and analyzed, it showed that there were very low interspecific variation (Figure 1). This result was similar to those of 4 tuna species; *Thunnus* spp (Lin *et al.*, 2005). The representative PCR–product from 3 *Hypsibarbus* spp. were shown in Figure 2.

To discriminate 3 *Hypsibarbus* spp., PCR-RFLP had been used for species identification in this study due to the smaller
Figure 1  The multiple alignments of 3 Hypsibarbus spp. H. wetmorei, H. vernayi and H. malcolmi.
amounts of DNA samples used and no purity required (Peyachoknagul, 2002). This technique had been used in several fishes such as freshwater eels (Lin et al., 2002), tuna fish (Pardo and Pârez–Villareal, 2004; Lin et al., 2005) and cod fish (Calo–Mata et al., 2003; Aranishi et al., 2005; Akasaki et al., 2006). In this study, the combination of 2 restriction enzymes (Bsp143I and BcuI) were used. The first enzyme Bsp143I could discriminate *H. malcolmi* and *H. wetmorei* from *H. vernayi* by producing the same RFLP pattern in all specimens and generated 2 fragments of 769 bp and 224 bp. On the other hand, there were 3 fragments of 535 bp, 234 bp and 224 bp in *H. vernayi*. However, the 2 fragments of 234 and 224 bp from *H. vernayi* comigrated as a single broad band (Figure 3). Therefore, Bsp143I was useful to discriminate *H. vernayi* from *H. malcolmi* and *H. wetmorei*.

Thereafter; the second enzyme BcuI was used to discriminate remaining *H. malcolmi* from *H. wetmorei*. This enzyme produced 2 restriction sites in *H. malcolmi* by generated 3 fragments of 591 bp, 288 bp and 114 bp whereas an uncut fragment in *H. wetmorei* (Figure 4).

For BcuI, this enzyme produced 2 haplotypes in *H. vernayi*; by producing 2 patterns; one was an uncut fragment, another was 2 fragments approximately 700 bp and 300 bp (Figure 5). It may be the intraspecific variation in

![Figure 2](image2.png) The PCR–products of approximately 993 bp cytochrome b gene amplified from *H. wetmorei* (Hw), *H. vernayi* (Hv), *H. malcolmi* (Hm).

![Figure 3](image3.png) The RFLP patterns from PCR–products of *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with Bsp143I.

![Figure 4](image4.png) The RFLP patterns from PCR–products of *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with BcuI, Af was artifact from PCR reaction.

![Figure 5](image5.png) The RFLP polymorphic patterns (Hv1 and Hv2) in *H. vernayi* when cleaved with BcuI.
H. vernayi or it represented the different species. Therefore; to achieved the answer, the large number of specimens, the standard species sampling from various locations were needed (Calo-Mata et al., 2003; Akasaki et al., 2006). However; using both enzymes, it still permit the correct identification of these 3 Hypsibarbus spp. because the 2 haplotypes of H. vernayi were differ from those of H. wetmorei and H. malcolmi haplotypes.

Therefore; the PCR–RFLP by using 2 restriction enzymes; Bsp143I and BcuI could identify 3 Hypsibarbus spp. especially in juvenile stage and fragmentary specimens.

**CONCLUSION**

The specific PCR–product of partial mitochondrial cytochrome b gene (993 bp) in 3 Hypsibarbus spp. (H. wetmorei, H. vernayi and H. malcolmi) was useful in this identification. There were very low interspecific variation of partial cytochrome b gene among these species; however it could be used to discriminate by RFLP analysis. The first enzyme Bsp143I could discriminate H. vernayi from H. wetmorei and H. malcolmi. The second enzyme BcuI was used to discriminate H. malcolmi from H. wetmorei.

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**LITERATURE CITED**


