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

Pattanee Jantrarotai[1]*, Surapop Sutthiwises[1], Wongpathom Kamonrat[2], Surin Peyachoknagul[3] and Chavalit Vidthayanon[4]

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; *Bsp*143I and *Bcu*I were used to identify 3 *Hypsibarbus* spp. *Bsp*143I 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, *Bcu*I 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 *Bcu*I 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).

1 Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
2 Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand.
3 Department of Genetic, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
4 World Wildlife Fund Thailand, Bangkok 10200, Thailand.
* Coreesponding author: e-mail: fscipnj@ku.ac.th

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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 interm 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 transfered into the new microcentifuge 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 (OD_{260} and OD_{280}).

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' GAGGAATAGTGCGAAGTA TAG 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 $MgCl_2$, 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

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H.wetmorei      GGCCTATTCCTAGCCATACACTACACTTCAGACATCTCAACTGCATTCTCATCAGTAACC 60
H.malcolmi      GGCCTATTCCTAGCCATACACTACACTTCAGACATYTCACCCGCATTCTCATCAGTAACC 60
H.vernayi       GGCCTATTCCTAGCCATACACTACACCTCCGATATTTCSACCGCATTCTCATCAGTGACC 60
                ************************** ** ** ** **  * ************** ***

H.wetmorei      CATATCTGCCGAGACGTAAACTACGGGTGACTAATTCGTAATATACACGCCAATGGGGCA 120
H.malcolmi      CATATCTGCCGAGACGTAAACTACGGGTGACTAATTCGTAATATACACGCCAATGGGGCA 120
H.vernayi       CACATCTGTCGAGACGTAAACTACGGATGACTAATTCGTAATATACACGCTAACGGGGCA 120
                ** ***** ***************** ********************** ** ******

H.wetmorei      TCATTCTTCTTCATCTGTATTTACATACACATCGCCCGAGGCCTATATTACGGGTCATAC 180
H.malcolmi      TCATTCTTCTTCATCTGTATTTACATACACATCGCCCGAGGCCTATATTACGGGTCATAC 180
H.vernayi       TCATTCTTCTTCATCTGTATTTATATACATATCGCCCGAGGCCTATATTACGGGTCATAC 180
                ********************** ***** ******************************

H.wetmorei      CTCTACAAAGAAACCTGAAATATCGGAGTAGTCCTCCTACTATTAGTTATAATAACAGCC 240
H.malcolmi      CTCTACAAAGAAACCTGAAATATCGGAGTAGTCCTCCTACTACTAGTTATAATAACAGCC 240
H.vernayi       CTCTACAAAGAAACCTGAAACATCGGAGTTGTCCTTCTACTACTGGTCATAATAACAGCC 240
                ******************** ******** ***** ****** * ** ************

H.wetmorei      TTCGATGGCTACGTTCTCCCATGAGGACAAATGTCCTTCTGAGGCGCCACAGAAATTACA 300
H.malcolmi      TTCGTTGGCTACGTTCTCCCATGAGGACAAATGTCCTTCTGAGGCGCCACAGTAATTACA 300
H.vernayi       TTCGTTGGTTACGTCCTCCCATGAGGACAAATGTCCTTCTGAGGTGCCACAGTAATCACA 300
                **** *** ***** ****************************** ******* *** ***

H.wetmorei      AACCTCCAATCTGCCGTGCCATACATAGGGGACATATTAGTCCAATGAGTTTGAGGTGGG 360
H.malcolmi      AACCTCCTATCTGCCGTCCCATACATAGGGGACATACTAGTCCAATGAATTTGAGGTGGG 360
H.vernayi       AACCTCCTATCTGCCGTCCCATACATAGGAGACATGCTAGTCCAATGAATCTGAGGCGGA 360
                ******* ********* *********** *****  *********** * ***** **

H.wetmorei      TTCTCGGTAGACAACGCAACRTTAACACGATTGTTTGCATTCCACTTCCTGCTACCATTC 420
H.malcolmi      TTCTCGGTAGACAACGCAACATTAACACGATTCTTTGCATTCCACTTCCTGCTACCATTC 420
H.vernayi       TTCTCAGTAGACAACGCGACGCTGACGCGGTTCTTTGCATTCCACTTCCTACTACCATTT 420
                ***** *********** **  * ** ** ** ***************** ********

H.wetmorei      GTTATTGCTGCAGCAACCGTCCTACACCTACTATTCCTCCATGAAACAGGGTCAAATAAC 480
H.malcolmi      GTTATTGCTGCAGCAACCGTCCTACACCTACTATTCCTCCATGAAACAGGGTCAAATAAC 480
H.vernayi       GTTATTGCCGCAGCAACAATTCTACACCTACTATTCCTCCACGAAACAGGATCAAACAAC 480
                ******** ********  * ******************** ******** ***** ***

H.wetmorei      CCAATTGGCCTAAACTCAGATGCAGACAAAATCTCATTCCACCCATACTTCACGTACAAA 540
H.malcolmi      CCAATTGGACTAAACTCAGATGCAGACAAAATCTCATTCCACCCATACTTCACGTACAAA 540
H.vernayi       CCAATCGGACTAAACTCAGACGCAGATAAAATCTCATTCCACCCATACTTTACATACAAA 540
                ***** ** *********** ***** ********************** ** ******

H.wetmorei      GACCTCCTTGGATTCGTAATTATACTCGTAGGTCTTACACTACTAGCGCTATTCTCCCCT 600
H.malcolmi      GACCTCCTTGGATTCGTAATTATACTCCTAGGTCTTACACTACTAGCGCTATTCTCCCCC 600
H.vernayi       GACCTCCTCGGATTCGTAATTATACTACTAGGCCTTACACTACTAGCACTATTCTCCCCC 600
                ******** *****************  **** ************** ***********

H.wetmorei      AACCTATTAGGAGACCCAGAAAACTTCACCCCTGCCAACCCTCTAGTTACCCCACCACAC 660
H.malcolmi      AACCTATTAGGAGACCCAGAAAACTTCACCCCTGCCAACCCTCTAGTTACCCCACCACAC 660
H.vernayi       AACCTGCTGGGAGAGCCAGAAAACTTCACCCCCGCCAACCCCCTAGTTACCCCACCACAC 660
                *****  * ***** ***************** ******** ******************

H.wetmorei      ATCAAACCAGAATGATATTTCCTATTTGCCTATGCCATTTTACGATCCATCCCAAATAAA 720
H.malcolmi      ATCAAACCAGAATGATATTTCCTATTTGCCTATGCCATTTTACGATCCATCCCAAATAAA 720
H.vernayi       ATCAGGCCAGAATGATACTTCCTATTTGCCTACGCCATTCTACGATCCATTCCAAATAAA 720
                ****  *********** ************** ****** ********** *********

H.wetmorei      CTAGGAGGCGTCCTTGCACTACTATTCTCCATCCTAATTCTAATAGTAGTCCCCCTATTA 780
H.malcolmi      CTAGGAGGCGTCCTTGCACTACTATTCTCCATCCTAATTCTAATAGTAGTCCCCCTATTA 780
H.vernayi       CTAGGAGGCGTCCTCGCACTACTATTCTCCATCCTAGNCCTAATAGTAGT---------- 770
                ************** ********************   ***********

H.wetmorei      CATACCTCAAAGCAACGAGGACTAACATTCCGCCCAATTACCCAATTCCTATTCTGAACC 840
H.malcolmi      CATACCTCAAAGCAACGAGGACTAACATTCCGCCCAATTACCCAATTCCTATTCTGAACC 840
H.vernayi       ------------------------------------------------------------

H.wetmorei      CTAGTAGCAAGCATAATTA---------------------------------------- 859
H.malcolmi      CTAGTAGCAAACATAATTATTCTAACATGAATCGGAGGTATGCCAGTAGAACACCCATTC 900
H.vernayi       ------------------------------------------------------------

H.wetmorei      ---------------------------------------------
H.malcolmi      ATTATCATCGGACAATTGCATCCATCTATACTTCGCACTATTCCTA 946
H.vernayi       ---------------------------------------------
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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 (*Bsp*143I and *Bcu*I) were used. The first enzyme *Bsp*143I 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.

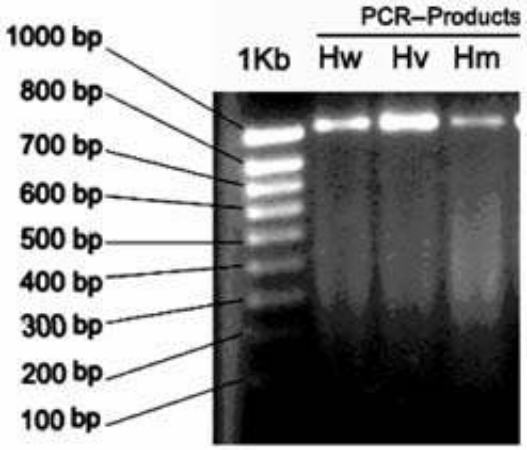

Figure 2 The PCR–products of approximately 993 bp *cytochrome b* gene amplified from *H. wetmorei* (Hw), *H. vernayi* (Hv), *H. malcolmi* (Hm).

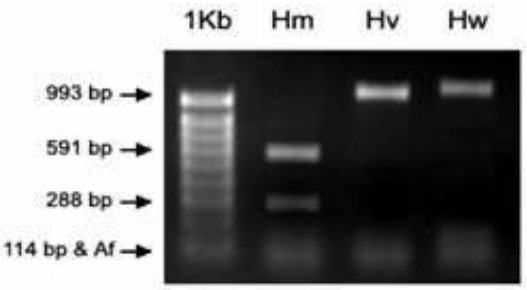

Figure 4 The RFLP patterns from PCR–products of *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with *Bcu*I, Af was artifact from PCR reaction.

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, *Bsp*143I was useful to discriminate *H. vernayi* from *H. malcolmi* and *H. wetmorei*.

Thereafter; the second enzyme *Bcu*I 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 *Bcu*I, 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

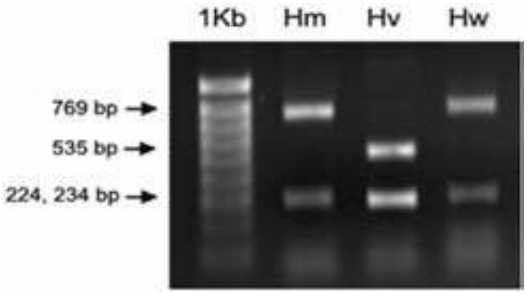

Figure 3 The RFLP patterns from PCR–products of *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with *Bsp*143I.

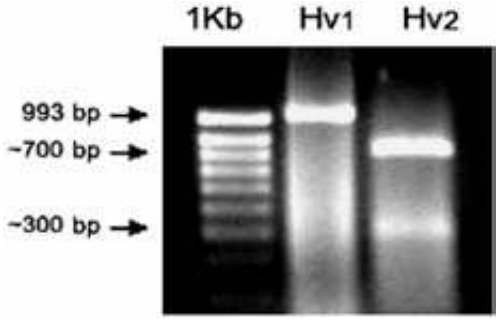

Figure 5 The RFLP polymorphic patterns (Hv1 and Hv2) in *H. vernayi* when cleaved with *Bcu*I.

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; *Bsp*143I and *Bcu*I 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 *Bsp*143I could discriminate *H. vernayi* from *H. wetmorei* and *H. malcolmi*. The second enzyme *Bcu*I was used to discriminate *H. malcolmi* from *H. wetmorei*.

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