THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Zoology)
DEGREE

Zoology	Zoology
FIELD	**DEPARTMENT**

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

NAME: Mr. Surapop Sutthiwises

THIS THESIS HAS BEEN ACCEPTED BY

Pattanee Jantharotai
THESIS ADVISOR
(Associate Professor Pattanee Jantrarotai, Ph.D.)

COMMITTEE MEMBER
(Mr. Chavalit Vidthayanon, D.Sc.)

COMMITTEE MEMBER
(Mr. Wongpathom Kamonrat, Ph.D.)

Surin Peyachoknagul
COMMITTEE MEMBER
(Associate Professor Surin Peyachoknagul, Dr.Agr.)

Somphop Navephap
DEPARTMENT HEAD
(Associate Professor Somphop Navephap, M.S.)

APPROVED BY THE GRADUATE SCHOOL ON 13 Dec. 2006

Vinai Artkongharn
DEAN
(Associate Professor Vinai Artkongharn, M.A.)

THESIS

SPECIES IDENTIFICATION OF *Barbonymus gonionotus* AND 3 *Hypsibarbus* spp. (PISCES: CYPRINIDAE) USING PCR–RFLP OF *Cytochrome b* GENE

SURAPOP SUTTHIWISES

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Zoology)
Graduate School, Kasetsart University
2006

Surapop Sutthiwises 2006: Species Identification of *Barbonymus gonionotus* and 3 *Hypsibarbus* spp. (Pisces: Cyprinidae) Using PCR–RFLP of *Cytochrome b* Gene. Master of Science (Zoology), Major Field: Zoology, Department of Zoology. Thesis Advisor: Associate Professor Pattanee Jantrarotai, Ph.D. 62 pages.

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) was used to complement the morphological identification of 4 closely related cyprinid species; *Hypsibarbus wetmorei*, *H. vernayi*, *H. malcolmi* and *Barbonymus gonionotus*. In this study, the pair of primers was appropriately designed for amplification of mitochondrial *cytochrome b* gene (993 bp) in *B. gonionotus* and 3 *Hypsibarbus* spp. which showed single 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. One restriction enzyme; *Cfr*42I could be used to discriminate *B. gonionotus* from 3 *Hypsibarbus* spp. by generated 2 fragments (431 bp and 562 bp) in *B. gonionotus* and uncut fragment in 3 *Hypsibarbus* spp. 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 fragments of approximately 700 bp and 300 bp. Thus, PCR–RFLP technique could be used to complement the morphological identification of 4 closely related cyprinid species and the keys were developed to identify these species based on the external characters.

Student's signature

Thesis Advisor's signature

29 / Nov. / 06

ACKNOWLEDGEMENT

This thesis is the end of my long journey in obtaining Master degree in Zoology (6 years). There were many people who made this journey successful and it would be impossible without people who supported and believed in me.

First, I would like to express my deeply gratitude to **Associate Professor Dr. Pattanee Jantrarotai:** Department of Zoology, Kasetsart U., my thesis advisor for providing everything throughout my works, devoted time in supervision and continued to work with me to date including the writing of this thesis. She is not only a great scientist with deep vision but also most importantly a very nice and kind person. She inspired me in the most important moments of making right decisions, solve my numerous problems and I am very glad to be her advisee.

I am also very grateful to my thesis minor committee **Associate Professor Dr. Surin Peyachoknagul:** Department of Genetics, Kasetsart U. to my supervisor. She is a prudent teacher and gives me a profound knowledge in field of molecular genetics. In addition, she initiates me into the interesting in field of genetics after her lectures and I am very proud to be her student.

I would like to express my profound respect and gratitude to my 1st thesis major committee **Dr. Wongpathom Kamonrat:** Ornamental Fishes Research Institute, Department of Fisheries for giving me more than just a lab space but the understanding of what is "a goal of research". Within my thesis project, I learned a lot from him and I want to thanks him for take me on the process of learning and thinking.

Needless to say that, I am very grateful to **Dr. Chavalit Vidthayanon:** WWF International Thailand Programme, another thesis major committee who takes the viewpoint of my thesis topic and gives me a profound knowledge in fish systematics. I want to thanks him for help, kindness and discussion throughout the results.

I would like to express my gratitude to all of my thesis committees; "thank you doesn't seem sufficient but it is said with appreciation and respect".

My best regards, I want to acknowledge to **Dr. Piyama Tasanasuwan:** Department of Zoology, Kasetsart U, for devotes a lot of time to suggest and guidance in molecular techniques. I would like to give my special thanks to **Assistant Professor Dr. Jessada Denduangboripant:** Department of Biology, Chulalongkorn U., for suggest in sequence analysis and phylogenetic reconstruction.

In another aspect of life; I am also very indebted to **Mrs. Anong Swanyatipat:** Department of Zoology, Kasetsart U, for her provide and contact to scholarship source throughout my study, I wish to acknowledge kind assistance of her.

I warmly thanks to the native people in the provinces that I collected the specimens. Thank you for **Miss Anchana Boonma**; my lovely cousin for her contact

to the specimen collection, **Mr. Chaiwut** and **Mrs. Jarungjit Grudpan** for their valuable advice and friendly help.

This work was partially supported by The Thesis and Dissertation Support Fund, The Graduate School Kasetsart U. and I would like to acknowledge to Department of Zoology; Kasetsart U. and Ornamental Fishes Research Institute; Department of Fisheries for support me in lab instruments and space.

I would like to thanks all people; teachers, friends and Department of Zoology throughout 10 years in Kasetsart U.

Finally, my great appreciation is due to my family; dad, mom and 3 brothers for their supporting, continual beloving and taking care of me.

Thank you everybody and everything in my life.

Surapop Sutthiwises
December 2006

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INTRODUCTION

The genus *Hypsibarbus* Rainboth, 1996 (family Cyprinidae) consists of 12 species, generally distribute 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 (1996a) reported that there were 6 species in Thailand; *H. lagleri*, *H. malcolmi*, *H. salweenensis*, *H. suvattii*, *H. vernayi* and *H. wetmorei* base 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). However, the synonym of these fishes were ambiguous due to the most characters in each species were similar and the key characters in Rainboth's study had not been used by Sunairattanaporn's study.

From the different results, it was due to their morphological similarities between the species in the genus *Hypsibarbus*. In addition, the fishes in *Hypsibarbus* were also similar to some species of related genera such as *Barbonymus gonionotus* that commonly sympatric. Therefore, it caused the confusion in discrimination and identification of these fishes to species or genera levels.

Traditional identification of fishes species, are mostly base on morphological and anatomical features such as color, morphometric proportions, total gill raker, dorsal spine serration and the number of scales (Guzow–Krzeminska *et al*., 2001). Although morphological and anatomical features are sufficient for species identification, it is difficult to identify the species that have remarkably similar morphology such as *Hypsibarbus* spp. Furthermore, larval or juvenile stages within this genus or related genera are often morphologically similar and found in a large number. Hence it is difficult to accurately identification. Therefore, the identification of larval or juvenile *Hypsibarbus* spp. or related genera based on morphological characteristics alone is problematic. Moreover, most of the key characters are frequently refer to adult fishes (Mayr and Ashlock, 1991), whereas almost the characters of larval or juvenile fishes are highly variable from those of adult. From these identification problems; it consequently leads to the hurdle for effective aquaculture, stock management and species conservation.

In recent years; the molecular techniques have influenced all biological disciplines, including taxonomy. These techniques have the advantage over morphological method because there was no need to sacrifice the organisms. The techniques have facilitated the development of accurate species identification such as

the polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) technique has been used to discriminate morphologically similar species in hake species genus *Merluccius* (Quinteiro *et al*.,2001), hairtail species in family Trichiuridae (Chakraborty *et al*., 2005).

The purpose of this study is to enhance the reliable identification of 3 *Hypsibarbus* spp.; *H. wetmorei*, *H. vernayi*, *H. malcolmi* and *Barbonymus gonionotus* by using PCR–RFLP technique in complement of the traditional morphological identification. As these 4 species are closely related interm of taxonomic and morphological similarities.

The objectives of this study are:

1. To apply PCR–RFLP technique for discrimination of *Barbonymus gonionotus* from 3 *Hypsibarbus* spp.; *H. wetmorei*, *H. vernayi* and *H. malcolmi*.

2. To apply PCR–RFLP technique for identification of 3 *Hypsibarbus* spp.

3. To determine the reliable identification in 3 *Hypsibarbus* spp. through genetic analysis, for further development of simple key to identify these species based on external characters.

LITERATURE REVIEWS

1. Morphological Study

Fishes in genus *Hypsibarbus* Rainboth, 1996 belong to family Cyprinidae, order Cypriniformes. The cypriniform fishes comprise the most group of freshwater fish which contains approximately 2,700 species and classified to 5 families; Balitoridae, Catostomidae, Cobitidae, Gyrinocheilidae and Cyprinidae. The family Cyprinidae is one of the largest families in order Cypriniformes, found in a huge range in temperate and tropical waters of Europe, Africa, Asia, and North America (Nelson, 1994).

1.1 Family Cyprinidae

The family Cyprinidae (carps and minnows) is characterized by no jaw teeth, but present 1–3 rows of pharyngeal teeth (Figure 1), each row with a maximum of 8 teeth and the number of teeth is an important characteristic in distinguishing species (Nelson, 1994).

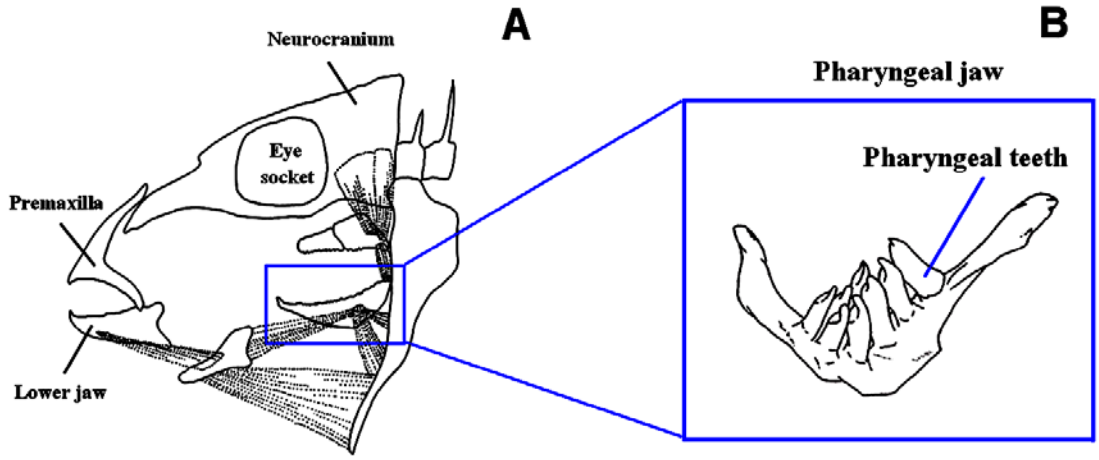

Figure 1 (A) The position of pharyngeal jaws within the cyprinid skull
(B) The pharyngeal teeth on pharyngeal jaw.
Source: (A) modified from Myers *et al*. (2006), (B) modified from Miranda and Escala (2005).

They are usually have large eyes and a body with conspicuous scales but no scales on the head, thin lips, papillae absent, mouth sometimes sucker–like. Some species have barbels but never more than 2 pairs (Figure 2), premaxilla usually borders, the upper jaw making the maxilla entirely or almost entirely excluded from the gape, usually protruding upper jaw (Nelson, 1994). They have only one dorsal and anal fin, dorsal fin with spine–like rays in some species, pectoral fins and pelvic fins on the abdomen; but never an adipose fin (Figure 3). Maximum length at least 2.5–3 m in *Catlocarpio siamensis*, many species less than 5 cm (Nelson, 1994).

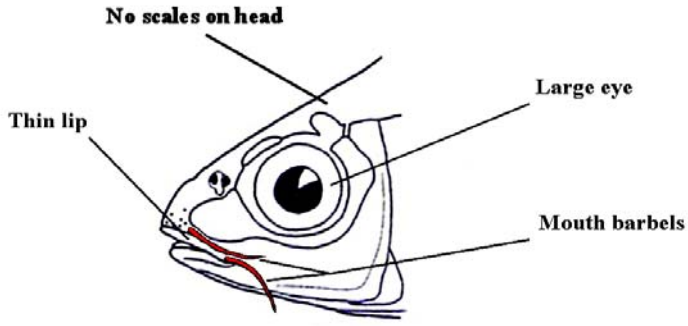

Figure 2 Characteristics of cyprinid fishes; large eye, thin lip, no scales on head and mouth barbels.
Source: modified from Rainboth (1996a).

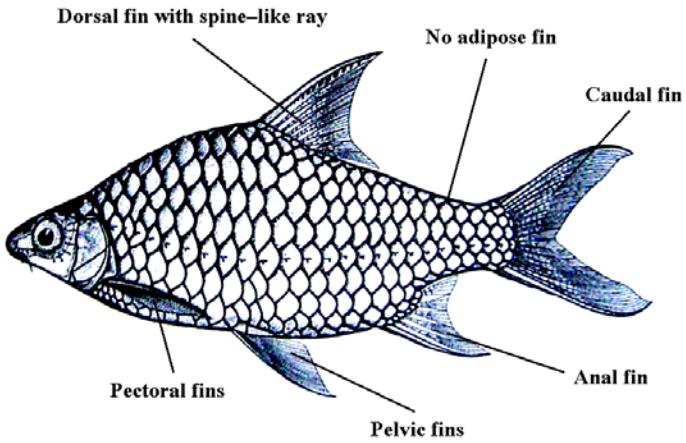

Figure 3 Characteristics and position of cyprinid fins.
Source: modified from Rainboth (1996a).

This huge family lives almost exclusively in freshwater, though some of its members stray into brackish water. Various sorts of carp are the best known, this family also includes minnows, barbins, daces, and bitterlings. They are very important in the aquaculture such as common carp (*Cyprinus carpio*), red tailed tinfoil (*Barbonymus altus*), Java barb (*B. gonionotus*) and gold foil barb (*B. schwanenfeldii*) and. In addition, numerous of the small beautifully colored cyprinids are popular and economically valuable in the aquarium fish industry (Hart and Reynolds, 2002).

In Thailand, there are many cyprinid genera such as *Puntius*, *Poropuntius*, *Barbodes*, *Scaphognathops*, *Hypsibarbus* and *etc.* The taxonomy of many individual cyprinid genera had been revised by Vidthayanon *et al.* (1997). However, the taxonomy of these fishes is necessary to be continuously revised (Vidthayanon *et al.*, 1997; Kottelat, 1999). Among the cyprinid genera, Sunairattanaporn (2001) reported that genus *Hypsibarbus* is closely related to genus *Barbodes* (*Barbodes* was revised to *Barbonymus* by Kottelat, 1999).

1.2 The Genus *Barbonymus* Kottelat, 1999

The genus *Barbonymus* (*Barbonymus*: *Barbus* = a generic name earlier applied to these fishes + anonymous = anonym or without name) was revised by Kottelat in 1999 and goldfoil barb (*Barbus schwanenfeldii* Bleeker, 1853) was used as the type species. Kottelat (1999) placed 3 *Barbodes* spp.; *B. altus*, *B. gonionotus* and *B. schwanenfeldii* that recorded by Rainboth (1996b) in this genus. However, Rainboth (1996b) and Sunairattanaporn (2001) still placed goldfoil barb in genus *Barbodes*.

For the diagnosis of genus *Barbonymus*; Kottelat (1999) refers to the diagnosis of genus *Barbodes* that commonly occurring from Thailand through Indonesia (Rainboth, 1996b).

They are characterized by serrated dorsal–fin spine, 8 branched pelvic–fin rays, skin of lower lip separated from lower jaw by a shallow groove, anal–fin base long 90% of head length and no tubercles on snout.

The members of *Barbonymus* in Thailand including 3 species; *B. gonionotus*, *B. altus* and *B. schwanenfeldii* that were sympatric species in main rivers such as Mekong, Chao Phraya and Meklong. These 3 fishes were the important species in aquaculture.

Barbonymus gonionotus; Java barb (Figure 4). This species was mostly morphological resemblance to *Hypsibarbus* spp. (Sunairattanaporn, 2001), was described as 6–7 branched anal fin rays; 26–32 lateral line scale; 9–11 predorsal scale; 4–6 upper transverse scale; the branched soft ray fin counts are: 6–8 dorsal, 11–16 pectoral and 6–9 pelvic; body is strongly compressed; head is small; the snout pointed with terminal mouth. The barbels are very minute or rudimentary especially the upper ones, which sometimes disappear entirely. Color when fresh is silvery white, sometimes with a golden tint. The dorsal and caudal fins are gray to gray–yellow; the anal and pelvic fins light orange, their tips reddish; the pectoral fins pale to light yellow. This fish was known from Thailand through Indonesia in Mekong, Chao Phraya basins, Malay Peninsula, Sumatra and Java (Sunairattanaporn, 2001).

Barbonymus altus; red tailed tinfoil (Figure 5). The species was characterized by 28–33 lateral line scale; 10–14 predorsal scale; 6–8 upper transverse scale; the branched soft ray fin counts are: 5–6 dorsal, 13–16 pectoral and 6–9 pelvic; caudal fin with red distal margin and grey base without darkened upper and lower margin; red pectoral, pelvic and anal fins. This fish was known only from the Mekong and Chao Phraya basins (Sunairattanaporn, 2001).

Barbonymus schwanenfeldii; goldfoil barb (Figure 6). This species is the type species of *Barbonymus*. They was described as 27–35 lateral line scale; 11–14 predorsal scale; 6–8 upper transverse scale; the branched soft ray fin counts are: 8 dorsal, 13–16 pectoral and 7–9 pelvic; red dorsal fin with a black blotch at the tip; red pectoral, pelvic and anal fins; red caudal fin with white margin and a black

submarginal stripe along each lobe. This species was found in Asia: Mekong and Chao Phraya basins, Malay Peninsula, Sumatra and Borneo (Sunairattanaporn, 2001).

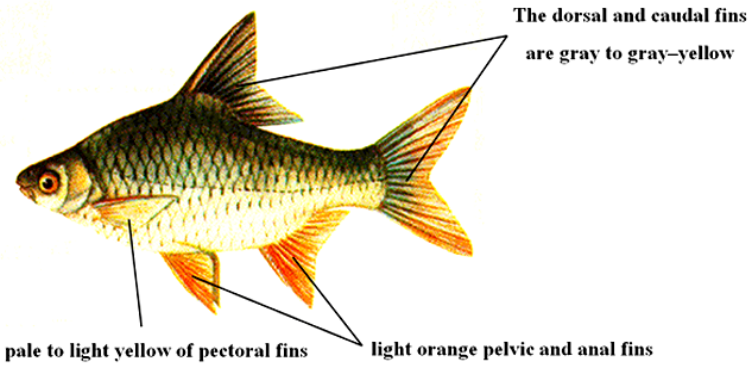

Figure 4 *B. gonionotus*, the mostly morphological resemblance to *Hypsibarbus* spp.
Source: modified from Bleeker (1977)

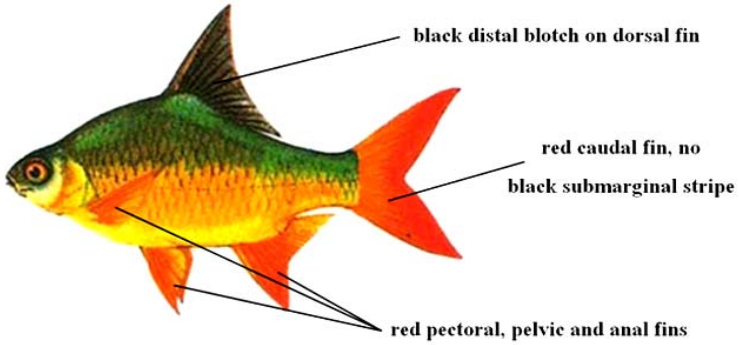

Figure 5 *B. altus*, one of the members of *Barbonymus* species in Thailand.
Source: modified from Bleeker (1977).

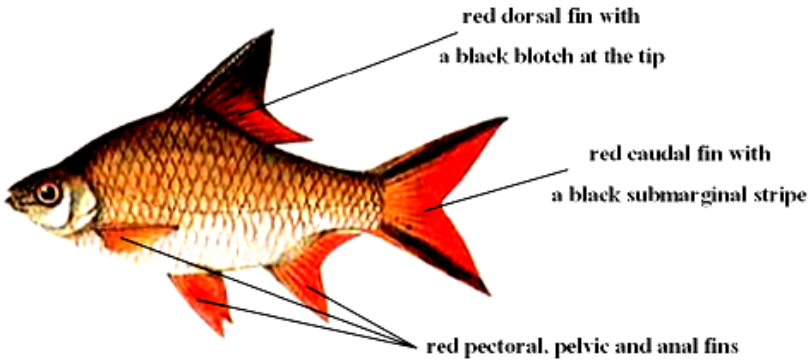

Figure 6 *B. schwanenfeldii*, the type species of genus *Barbonymus*.
Source: modified from Bleeker (1977)

1.3 The Genus *Hypsibarbus* Rainboth, 1996

The cyprinid fish genus *Hypsibarbus* (*Hypsibarbus*: hypsi = high + barbus = barbel) was described by Rainboth (1996a) as a new genus. The members of *Hypsibarbus* were included from 2 genera, *Puntius* (Hamilton, 1822) and *Acrossocheilus* (Oshima, 1919), using *A. malcolmi* as a type species. In addition the members of *Poropuntius* (Smith, 1931) were also included in these 2 genera. Therefore, the members of *Hypsibarbus* are most similar to those of genus *Poropuntius* and related genus *Barbonymus* (Kottelat, 1999). Rainboth (1996a) had given the description of fishes in *Hypsibarbus* based on morphological and meristic characters.

Fishes of this genus are deep bodies and strongly compressed. For the head characters; head length greater than height. Eye moderately large but its diameter less than the length of snout in adult, mouth sub–terminal with 2 pairs of barbels. There are well developed groove separating lips from both upper and lower jaws, the lip of the lower jaw is almost invisible when the mouth is closed.

Scales (Figure 7) large, with 23–32 lateral line scales, 20–24 circumferential scales, 14–16 circumpeduncular scales (most species have 14 scales), 7–12 predorsal scales row, 5–6 upper transverse scales row, 1–2 scales between vent and anal fin.

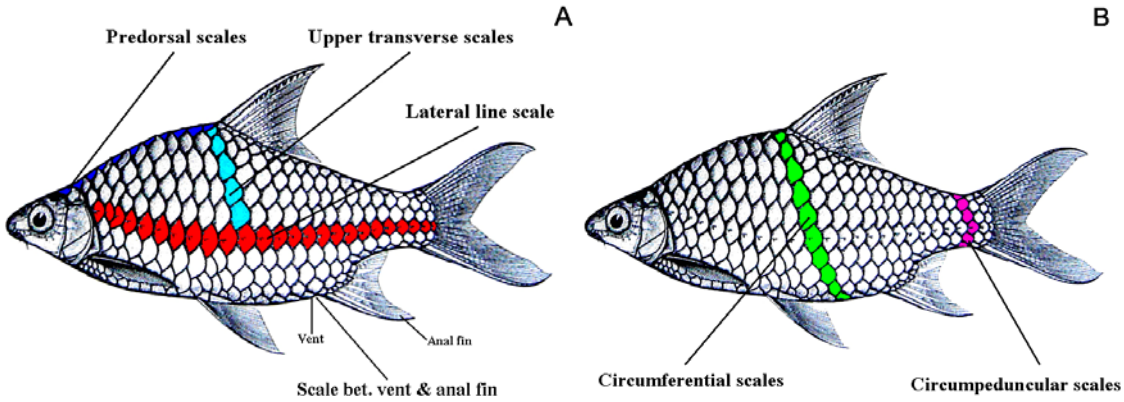

Figure 7 (A) The position of predorsal scales, upper transverse scales and lateral line scales. (B) Circumferential scales and circumpeduncular scales.
Source: modified from Rainboth (1996a)

The unbranched fin ray (Roman number) and the branched fin ray (Arabic number) counts are: iv–8 dorsal, i–13 to16 pectoral, i–8 pelvic and iii–5 anal.

Dorsal fin located at mid body. The top and edge of dorsal fin are bright colored while the lower parts of fins are usually white. The edge of caudal fin is gray, orange or red. The tip and mid of pelvic fin and anal fin are yellow to reddish orange; first basal of pelvic fin and anal fin may be milky.

1.4 Species Accounts of Genus *Hypsibarbus* in Thailand

Rainboth (1996a) reported that there were 6 species of *Hypsibarbus* in Thailand (Figure 8); *H. lagleri*, *H. malcolmi*, *H. salweenensis*, *H. suvattii*, *H. vernayi* and *H. wetmorei* base on 45 measurements per specimen, 23 counts (from 1,072 specimens) and geographic distribution.

***H. lagleri* (36 specimens, ranging from 42.4–177.8 mm of SL)**

Diagnosis: 6 upper transverse scales, usually 22 circumferential scales, highly compressed body with head width (HW) 51.6% of head length (HL), endemic in Mekong.

Description: 5 anal branched soft rays; 24–26 lateral line scales on body with 1–2 additional scales at base of caudal fin; 22 circumferential scales (86.11% of specimens); 14 circumpeduncular scales; 6 upper transverse scales (83% of specimens); 9–11 predorsal scales; 2 scales between vent and anal fin (97.22% of specimens); 10–17 dorsal spine serration; %HW from 46.6 to 58.3 of HL (mean = 51.7$\pm$2.4)

Distribution in Thailand: endemic to the middle Mekong basin of northern Thailand.

***H. malcolmi* (34 specimens, ranging from 58–405 mm SL)**

Diagnosis: 16 circumpeduncular scales, 7–8 gill rakers on 1st arch

Description: 5 anal branched soft rays; 24–28 lateral line scales on body with 1–2 additional scales at base of caudal fin; 20 circumferential scales (97.06% of specimens); 16 circumpeduncular scales; 5 upper transverse scales (97.06% of specimens); 8–10 predorsal scales; 2 scales between vent and anal fin; 12–20 dorsal spine serration; %HW from 50.4–63.5 of HL (mean = 56.6$\pm$2.9)

Distribution in Thailand: Tapi River**,** Chao Phraya, Mekong and Meklong.

***H. salweenensis* (19 specimens, largest specimen 200 mm SL)**

Diagnosis: 28–31 lateral line scales

Description: 5 anal branched soft rays; 28–31 lateral line scales on body with 1–2 additional scales at base of caudal fin; 22 circumferential scales (63.1% of specimens); 14 circumpeduncular scales (78.94% of specimens); 6 upper transverse scales (63.16% of specimens); 9–12 predorsal scales; 2 scales between vent and anal fin (94.74% of specimens); 14–20 dorsal spine serration; %HW from 49–60.4 of HL (mean = 54.9$\pm$2.9)

Distribution in Thailand: endemic in Salween basin.

***H. suvattii* (13 specimens, ranging from 63–218 mm SL)**

Diagnosis: usually 20 circumferential scales, 7–8 gill rakers on 1st arch, highly compressed body with HW 52.4% of HL.

Description: 5 anal branched soft rays; 23–24 lateral line scales on body with 1–2 additional scales at base of caudal fin; 20 circumferential scales (69.23% of specimens); 14 circumpeduncular scales; 5 upper transverse scales (69.23% of specimens); 7–9 predorsal scales; 2 scales between vent and anal fin (95.65% of specimens); %HW from 48.6–56 of HL (mean = 52.4±2.4)

Distribution in Thailand: Meklong basin, but does not occur in Mekong or Chao Phraya basin as far as known.

***H. vernayi* (46 specimens, ranging from 45–153 mm SL)**

Diagnosis: 1 row of scale between vent and anal fin, 3–5 gill rakers on 1st arch, 9–11 predorsal scales, 12–21 dorsal spine serrations and robust body not compressed.

Description: 5 anal branched soft rays; 26–29 lateral line scales on body with 1–2 additional scales at base of caudal fin; 20 circumferential scales (97.83% of specimens); 14 circumpeduncular scales; 5 upper transverse scales (95.65% of specimens); 9–11 predorsal scales; 1 row of scale between vent and anal fin (84.78% of specimens); 12–21 dorsal spine serration; %HW from 52.2–63.4 of HL (mean = 57.2±2.6)

Distribution in Thailand: Chao Phraya, Mekong and Meklong basins.

***H. wetmorei* (46 specimens, ranging from 45–153 mm SL)**

Diagnosis: 2 rows of scale between vent and anal fin, 3–5 gill rakers on 1st arch, 7–9 (mean 8.3) predorsal scales, 9–14 (mean 11.7) dorsal spine serrations, robust body not compressed.

Description: 5 anal branched soft ray; 24–27 lateral line scale on body with 1–2 additional scales at base of caudal fin; 20 circumferential scales (95.65% of specimens); 14 circumpeduncular scales; 5 upper transverse scales (95.65% of specimens); 7–9 predorsal scales; 2 rows of scale between vent and anal fin (95.65% of specimens); 9–14 dorsal spine serration; %HW from 52.6–72.3 of HL (mean = 58.3±4.1)

Distribution in Thailand: Chao Phraya, Mekong and Meklong basins.

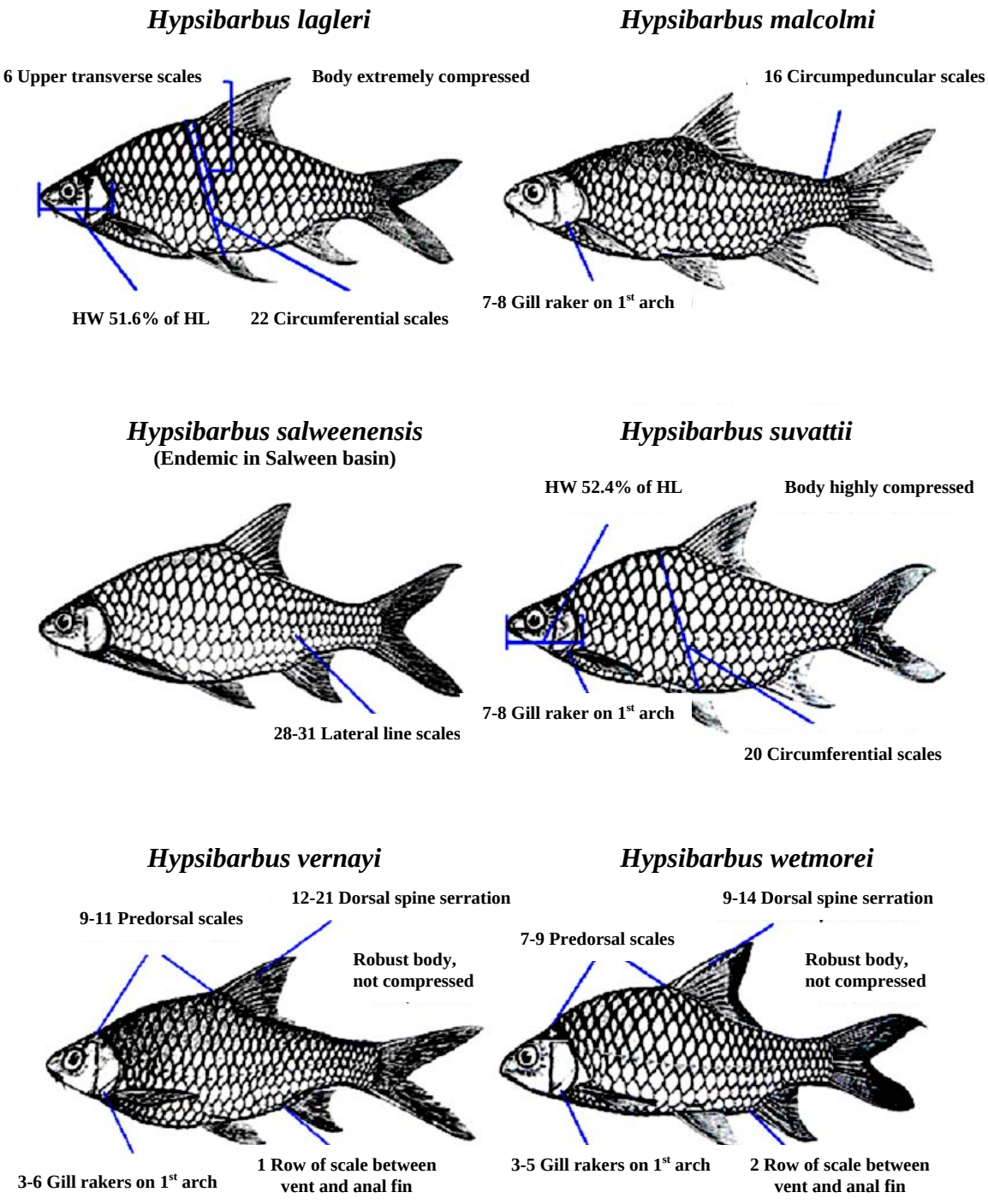

Figure 8 The diagnosis of 6 *Hypsibarbus* species reported by Rainboth (1996a); *H. lagleri*, *H. malcolmi*, *H. salweenensis*, *H. suvattii*, *H. vernayi* and *H. wetmorei*.

Source: modified from Rainboth (1996a)

However, Sunairattanaporn (2001) reported that there were 6 species; *H. lagleri, H. pierrei, H. salweenensis, H. tenasserimensis, H. vernayi* and *H. wetmorei* (Figure 9).

***H. lagleri* (34 specimens, ranging from 44.5–283.0 mm SL) (Described as synonym of *H. suvattii*)**

Diagnosis: colorless and highly compressed body, body depth at dorsal (BD) 40.92% of standard length (SL), head sharp and pointed more than other *Hypsibarbus* species.

Description: 22–26 lateral line scale on body; 14 circumpeduncular scales; 4–5 upper transverse scales; 8–11 predorsal scales; %HW from 55.26–66.67 of HL (mean = 60.61±2.57)

Distribution in Thailand: Mekong and Meklong basin.

***H. pierrei* (115 specimens, ranging from 49.0–428.5 mm SL) (Described as synonym of *H. malcolmi*)**

Diagnosis: mostly 16 circumpeduncular scales, body slender more than other *Hypsibarbus* species, BD 36 % of SL, pectoral and anal fins are orange–yellow color.

Description: 23–27 lateral line scale on body; mostly 16 circumpeduncular scales; 4–5 upper transverse scales; 8–10 predorsal scales; %HW from 54.76–69.23 of HL (mean = 60.71±3.28)

Distribution in Thailand: Tapi River**,** Mekong and Meklong basins.

***H. salweenensis* (40 specimens, ranging from 42.5–153.5 mm SL)**

Diagnosis: 27 or more lateral line scales, caudal fin with black margin, but the other fins with light orange colored.

Description: 27–31 lateral line scale on body; 14–16 circumpeduncular scales; 4–5 upper transverse scales; 9–12 predorsal scales; %HW from 56–72.22 of HL (mean = 63.33±3.68)

Distribution in Thailand: found only in Salween River.

***H. tenasserimensis* (53 specimens, ranging from 90–330 mm SL) (Described as a new species but it has been unpublished)**

Diagnosis: broadly upper and lower lips, brightly yellow colored fins, the inner of caudal fin with more dark color than the margin, 37 vertebra columns.

Description: 25–29 lateral line scale on body; 14 circumpeduncular scales; 4–5 upper transverse scales; 10–12 predorsal scales; %HW from 58.33–81.32 of HL (mean = 64.58±4.82)

Distribution in Thailand: Ta Nao Sri and Kra Buri basin

***H. vernayi* (126 specimens, ranging from 31.0–259.0 mm SL)**

Diagnosis: silver colored body; when up–folding the anal fin, its tip does not attach the base of caudal fin, black margin of caudal fin, the anal and caudal fins with orange colored.

Description: 23–27 lateral line scale on body; 14 circumpeduncular scales; 4–5 upper transverse scales; 9–11 predorsal scales; %HW from 52.63–81.25 of HL (mean = 65.22±5.25)

Distribution in Thailand: Chao Phraya, Mekong and Meklong basins.

***H. wetmorei* (80 specimens, ranging from 37.5–332.0 mm SL)**

Diagnosis: blunt lips, the tip of anal fin was sharp and pointed, the ventral part of body between pectoral and anal fins is yellow and the posterior margin of dorsal and caudal fins is pink.

Description: 22–27 lateral line scale on body; 14 circumpeduncular scales; 4–5 upper transverse scales; 7–10 predorsal scales; %HW from 58.62–96.39 of HL (mean = 66.67±6.31)

Distribution in Thailand: Chao Phraya, Mekong, Meklong and Pattani basins.

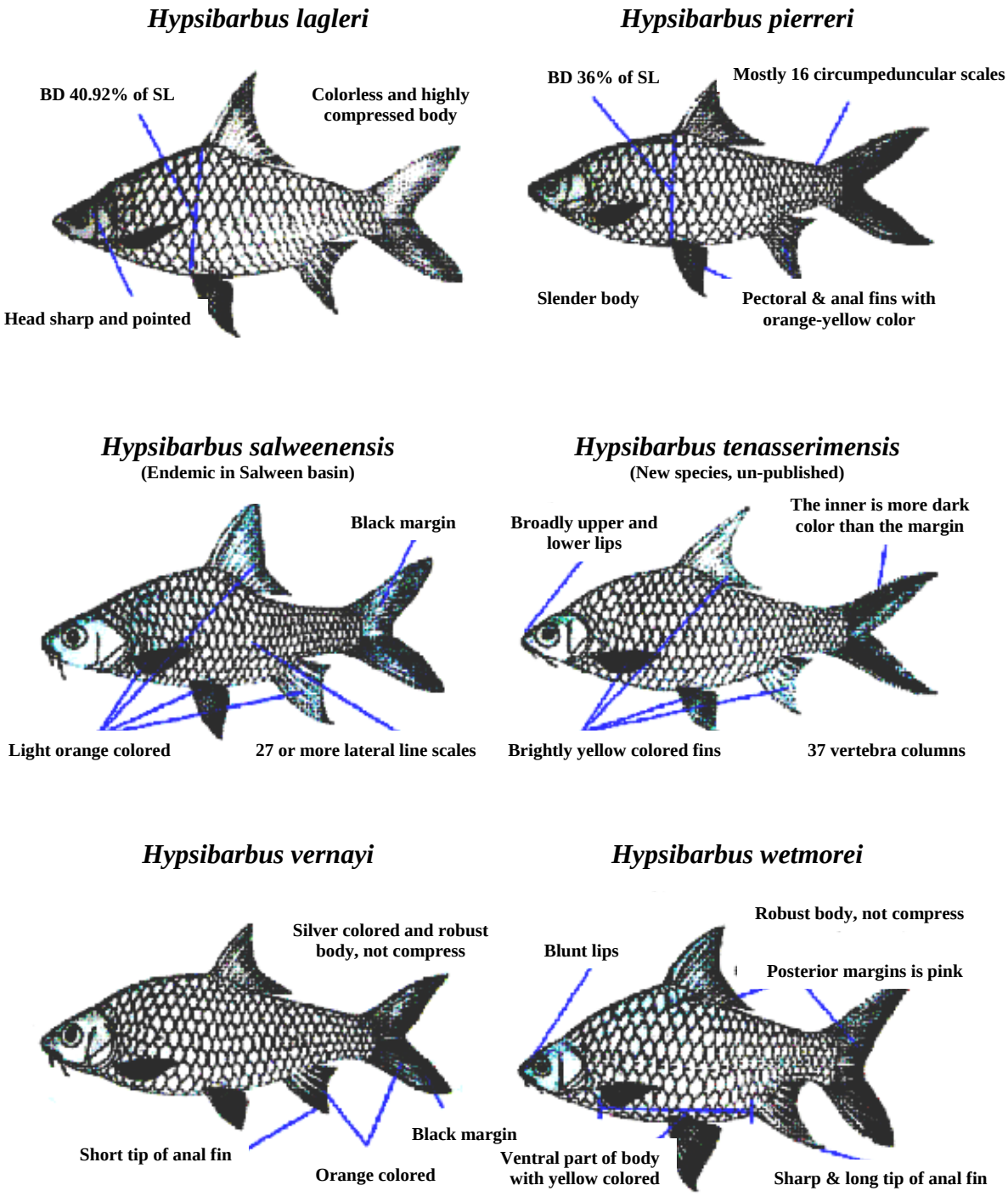

Figure 9 The diagnosis of 6 *Hypsibarbus* species reported by Sunairattanaporn (2001); *H. lagleri*, *H. pierreri*, *H. salweenensis*, *H. tenasserimensis*, *H. vernayi* and *H. wetmorei*.

Source: modified from Sunairattanaporn (2001)

The comparative of 6 *Hypsibarbus* species found in Thailand that reported by Rainboth (1996a.) and Sunairattanaporn (2001) were summarized in table 1. The external morphology of *H. malcolmi* and *H. suvattii* compared with *H. pierrei* and *H. lagleri* were shown in figure 10 and 11 respectively.

Table 1 The comparative of 6 *Hypsibarbus* spp. found in Thailand reported by Rainboth (1996a) and Sunairattanaporn (2001)

Species	Rainboth's report	Sunairattanaporn's report
H. lagleri	✓	✓
H. malcolmi	✓	—
		Described as synonym of *H. pierrei*
H. salweenensis	✓	✓
H. suvattii	✓	—
		Described as synonym of *H. lagleri*
H. vernayi	✓	✓
H. wetmorei	✓	✓
H. pierrei	No reported	✓
H. tenasserimensis	No reported	✓

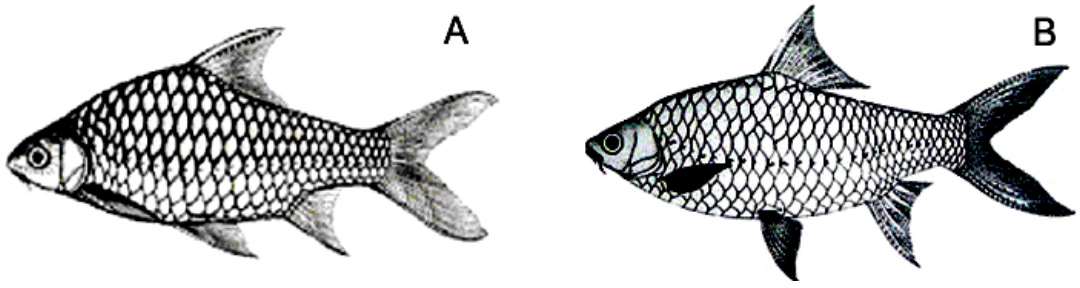

Figure 10 (A) *H. malcolmi* reported by Rainboth (1996a) compare with (B) *H. pierrei* reported by Sunairattanaporn (2001).
Source: (A) from Rainboth (1996a), (B) from Sunairattanaporn (2001)

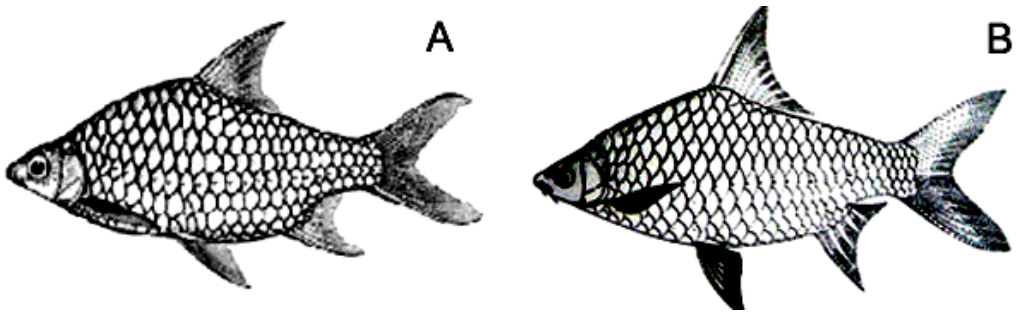

Figure 11 (A) *H. suvattii* reported by Rainboth (1996a) compare with (B) *H.lagleri* reported by Sunairattanaporn (2001).
Source: (A) from Rainboth (1996a), (B) from Sunairattanaporn (2001)

1.5 Problems in Morphological Identification

In all areas of biology, the accurate identification of species is crucial to the outcome of the work. Incorrect species identification can lead to misleading or incomplete conclusion because the species has been treated as a fundamental unit in biology (Hull, 1997; Wiens and Penkrot, 2002).

For species identification, the general morphological key mostly refers to adult individuals and do not address identification in larval, juvenile or fragmentary specimens. The key for identification are largely base on visible characters including patterning, color and morphometric proportion. However, these characters are varied such as patterning may be varied with age, color varied with food, insolation and humidity. Whereas, the morphometric proportions were changed in appearance during development due to the different part of the body may grow at different rates in each environmental condition (Ricker, 1979; Fuiman, 1983). In addition there are different in body shape between larvae and adults (Moser, 1981).

Thus, one of the essentials in good identification is to select the key characters that are relatively stable with a particular taxon (Simpson *et al*., 1960) such as the number of scales or the number of fin serration. These characters are widely use for fish identification. However; the counting of the fish scale and fin serration are time consuming especially in a large specimen number. In addition, some meristic counts may be varied due to the temperature gradation between areas during the seasons as described in juvenile Japanese flounder (*Paralichthys olivaceu*) in the Japan Sea (Kinoshita *et al*., 2000).

Furthermore, morphological variation may be appeared and within group of fish, dealing with distribution and geographic areas such as Coho salmon; *Oncorhynchus kisutch* (Hard *et al*., 2000), green sturgeon; *Acipenser medirostris* (North *et al*., 2002), lake charr; *Salvelinus namaycush* (Alfonso, 2004), Japanese charr; *Salvelinus leucomaenis* (Nakamura, 2003) and Galician three spine stickleback; *Gasterosteus aculeatus* (Hermida *et al*., 2005). Therefore, it may cause the problems when morphological characters are used for identification. However, this is not to say

that the morphological characters are always unsatisfactory in identification (Goto, 1982) as there are widely used in fish identification (Hermida *et al*., 2005).

Consequently, the alternative methods that have gained increasing importance to complement the traditional morphological identification are the molecular methods. These methods such as PCR–RFLP, AFLP and RAPD that have become widely used to assist species discrimination involving larval, juvenile, similar and fragmentary specimens.

2. Molecular Study

The molecular techniques have been developed over the last two decades and have allowed the development of authentic and reliable methods for species identification. Both nuclear and mitochondrial DNA have been targeted for species identification using PCR–RFLP technique.

2.1 Mitochondrial DNA (mtDNA)

The genetic materials of eukaryotic organisms are in three parts of cell; in the nucleus and two cytoplasmic organelles: plastids and mitochondria. Plants have genetic materials in both organelles while animals have only in mitochondria. Each of organellar genome is self–replicating, independent of that of the nuclear DNA, and has its own set of genes (Randi, 2000; Billington, 2003; Beebee and Rowe, 2004).

The structure of mtDNA is double–stranded circular DNA containing the heavy (H) strand (rich in G content) and the light (L) strand (rich in C content) (Hawkins, 1996; Randi, 2000; Billington, 2003). The genome of mitochondria has very high copy numbers per cell; it is usually 5–10 copies per mitochondria and hundreds to thousands of mitochondria in each cell. Mitochondrial DNA in each cell is haplotype (haploid molecule); it is homoplasmic (genetically identical) because it is mainly maternal inheritance (some exception of the paternal leak have been reported) (Randi, 2000; Billington, 2003). Therefore any tissue can be used as DNA source.

mtDNA is limited in size; so the genome is highly compact and efficiently organized. All mtDNAs have conserved gene coding for 22 tRNA; 2 rRNA and 13 enzymatic proteins involved in oxidative phosphorylation (including 7 NADH dehydrogenase subunits; 3 cytochrome oxidase subunits, ATPase6, ATPase8 and cytochrome b) (Randi, 2000; Billington, 2003) (Figure 12). Because of its compactness; the adjacent genes in mitochondria show some overlap; and all genes absence of intron. There are non–coding regions such as intergenic sequence (IGS) and control region. One specific site of control region called the D–loop (in vertebrates) or AT–rich region (in invertebrates) contains important sequence which regulate the replication and transcription of the entire mitochondrial genome (Randi, 2000). In addition, the codon usage of mtDNA gene uses slightly difference genetic code from that of nuclear DNA gene (Hawkins, 1996).

The size of the mtDNA is approximately 1% of cellular DNA; ranging from 13.8 kb in the nematode worm (*Caenorhabditis elegans*) up to 2,500 kb in musk melon (*Cucumis melo*) (Randi, 2000). In case of most animals; the length of mtDNA is rather conserved in 15–20 kb and approximately 16.7 kb remarkable for fish species (Sangthong, 2001).

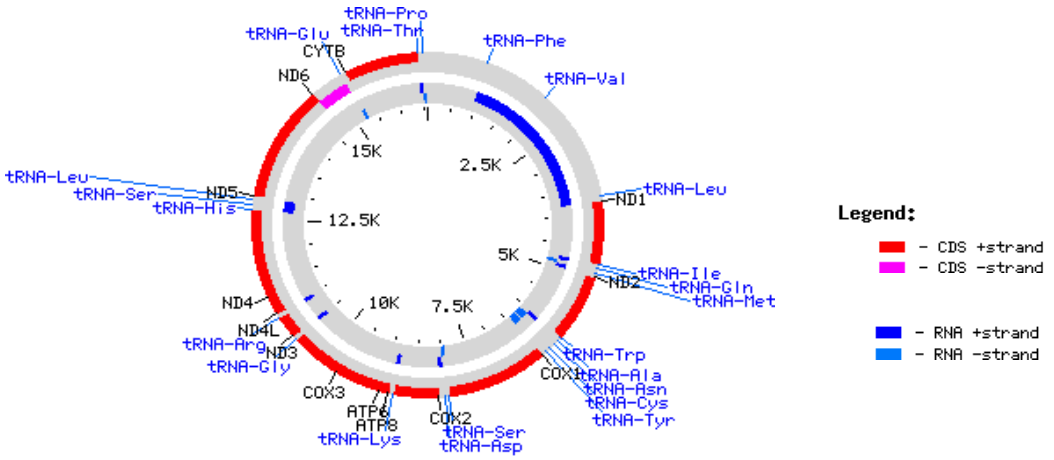

Figure 12 The genome organization of common carp (*Cyprinus carpio*); with 16575 bp length.
source: Huang (1993)

Among the mitochondrial genes, the *cytochrome b* gene, D–loop, 12S rRNA gene and 16S rRNA gene have been used for species identification (Girish *et al.*, 2004). The advantage of mitochondrial DNA as a molecular marker are:

1. Genome is small, compact gene packing and efficiently arrangement (Randi, 2000; Billington, 2003).

2. The different regions of the mitochondrial genome evolve at different rates, allowing suitable regions to be chosen for different study both inter and intraspecific variation (Randi, 2000; Saccone *et al.* 1991).

3. It is mainly maternal inheritance; the genetic variation is only due to mutation (Kondo *et al.*, 1990; Gyllestein *et al.*, 1991).

4. It lack of genetic recombination and therefore provides a set of completely linked, homologous markers that permit clear of maternal genealogies (Clayton, 1982, 1992; Hayashi *et al.*, 1985).

5. It present in multiple copies per cell, thus making it relatively easy to isolate and purify (Randi, 2000; Billington, 2003).

2.2 PCR–RFLP

PCR (Polymerase chain reaction) is the technique which allows an *in vitro* amplification of the specific interested DNA region. (Innis *et al*., 1995; Newton and Graham, 1997; Dale and Sanchantz, 2003). The PCR process was invented in 1985 by Kary Mullis, who received the Nobel Prize in Chemistry in 1993 (Newton and Graham, 1997; McPherson and Møller, 2000; Dale and Sanchantz, 2003). This technique does not require the purification of DNA template (Peyachoknagul, 2002).

PCR contains four important components; template DNA, oligonucleotide primers, deoxynucleoside triphosphates (dNTPs) and DNA polymerase (Innis *et al*., 1995; Newton and Graham, 1997; Dale and Sanchantz, 2003). The template DNA contains the target DNA sequence to be amplified. The oligonucleotide primers are the short single strands DNA molecules that bind by complementary base pairing to opposite strands of the amplified region of template DNA. The DNA polymerase is an enzyme that catalyzes the polymerization of DNA using dNTPs as the substrates (Winter *et al*., 1998).

The PCR processes are taken place in a thermal cycler and the number of PCR cycles usually between 30–35 cycles or 40 cycles at the maximum. The more cycles of PCR affects to non target DNA increase (Peyachoknagul, 2002). There are three major reactions; denaturation, annealing and extension or polymerization (Winter *et al*., 1998; McPherson and Møller, 2000).

1. Denaturation; using high temperature, usually 94 ºC to separate double stranded DNA into two–single strands. Each strand of DNA is used as a template to produce a complementary daughter strand (White, 1993; Harwood, 1996; McPherson and Møller, 2000; Peyachoknagul, 2002).

2. Annealing; the temperature for annealing is varied depending on the length and base sequence of primers (White, 1993 or McPherson and Møller, 2000), this step allow the primers to anneal (bind) their complementary sequences on the template strands (White, 1993; Harwood, 1996; McPherson and Møller, 2000).

3. Extension or Polymerization; to synthesize the new DNA strands, the temperature is adjusted to be optimal for DNA polymerase activity, approximately 72 ºC (White, 1993; Harwood, 1996; McPherson and Møller, 2000). The DNA polymerase initially synthesized new double stranded DNA molecules in the 5' to 3' direction from the 3' end of the primer (Peyachoknagul, 2002).

PCR is the powerful technique and uses extensively as a tool for molecular genetics research (Winter *et al*., 1998; McPherson and Møller, 2000). For example; DNA amplified by PCR has been used for DNA sequencing, to generate clone and uses as the molecular markers such as VNTR and microsatellites, SSCP, AFLP, RAPD, PCR–RFLP and others (Peyachoknagul, 2002; Dale and Sanchantz, 2003).

RFLP (Restriction Fragment Length Polymorphism) is the variation of DNA fragments banding patterns using restriction enzyme which cleave DNA at highly specific sites mutation; arising from their differing nucleotide sequence by which base substitution, addition, deletion and/or chromosome mutation (Peyachoknagul, 2000; Peyachoknagul, 2002). The difference in length of DNA fragments (Polymorphisms) indicated the genetically different individuals apart; so RFLP is used for identifying species including the study of phylogenetic relationship between individuals and among species using computer program (Peyachoknagul, 2000).

RFLP studies in chloroplast DNA (cpDNA) or mitochondrial DNA (mtDNA), the cpDNA or mtDNA of various samples were extracted, purified and cleaved with restriction enzyme into restriction fragments. The differences in numbers and sizes of DNA bands (RFLP) are separated by agarose gel electrophoresis and visualized by ethidium staining. RFLP studies of nuclear DNA (nDNA) was more difficult than cpDNA or mtDNA because of the largeness and complexity of nuclear genome; that led into the complicated detection because of its smear pattern result. So RFLP of nDNA can be detected by southern blotting hybridization with DNA probe (Peyachoknagul, 2000; Peyachoknagul, 2002).

For PCR–RFLP or CAPS (Cleaved Amplified Polymorphic Sequence) is the recent technique that allow the detection of point mutation in the PCR–products after cleaving into fragments with restriction enzyme (Peyachoknagul, 2002). The PCR–RFLP is more advantage than conventional RFLP technique (without PCR) because of its fastness, easiness and smaller amounts of DNA samples to be used. This technique plays an important role as a molecular marker for detecting polymorphisms at a particular locus; identify the closely related species or detecting the genetic variation within species.

For the species identification, the *cytochrome b* gene of mitochondrial DNA was selected for PCR–RFLP analysis, because of its low diversity and was widely used for species identification (Lin *et al*., 2005) such as freshwater eels (Lin *et al*., 2002), tuna fish (Pardo and Pérez–Villareal, 2003; Lin *et al*., 2005) and cod fish (Calo–Mata *et al*., 2003; Aranishi *et al*., 2005; Akasaki *et al*., 2006).

However; the other regions of mitochondrial DNA such as *cytochrome oxidase* subunit II gene (COII region), internal transcribed spacer 1 (ITS1) of the nuclear ribosomal RNA gene, 16S or 18S ribosomal RNA gene (rDNA) were selected for species identification in *Isotoma viridis* group (Burkhardt and Filser, 2004), five *Orius* spp. (Muraji *et al*., 2004), 10 dipteran species (Ratcliffe *et al*., 2003) and 4 closely related species of bivalves (Stepien *et al*., 2003), respectively.

In addition, the control region of the mitochondrial genome was selected for separation of the population in *Oligoryzomys* (González–Ittig *et al*., 2002), because of its higher diversity than other regions (Lin *et al*., 2005).

MATERIALS AND METHODS

1. Sample Collection

The total of 82 fish samples in the genus *Hypsibarbus* (*H. wetmorei*, *H. vernayi* and *H. malcolmi*) and *Barbonymus gonionotus* were collected or obtained during December 2002 to March 2006. The *Barbonymus gonionotus* samples were collected from Loei province. The genus *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; muscle tissue was dissected and preserved in 95% ethanol (ETOH) for DNA extraction. The samples were then stored on ice (in field) before preserved in 10% formalin, for further morphological investigation and identification checking.

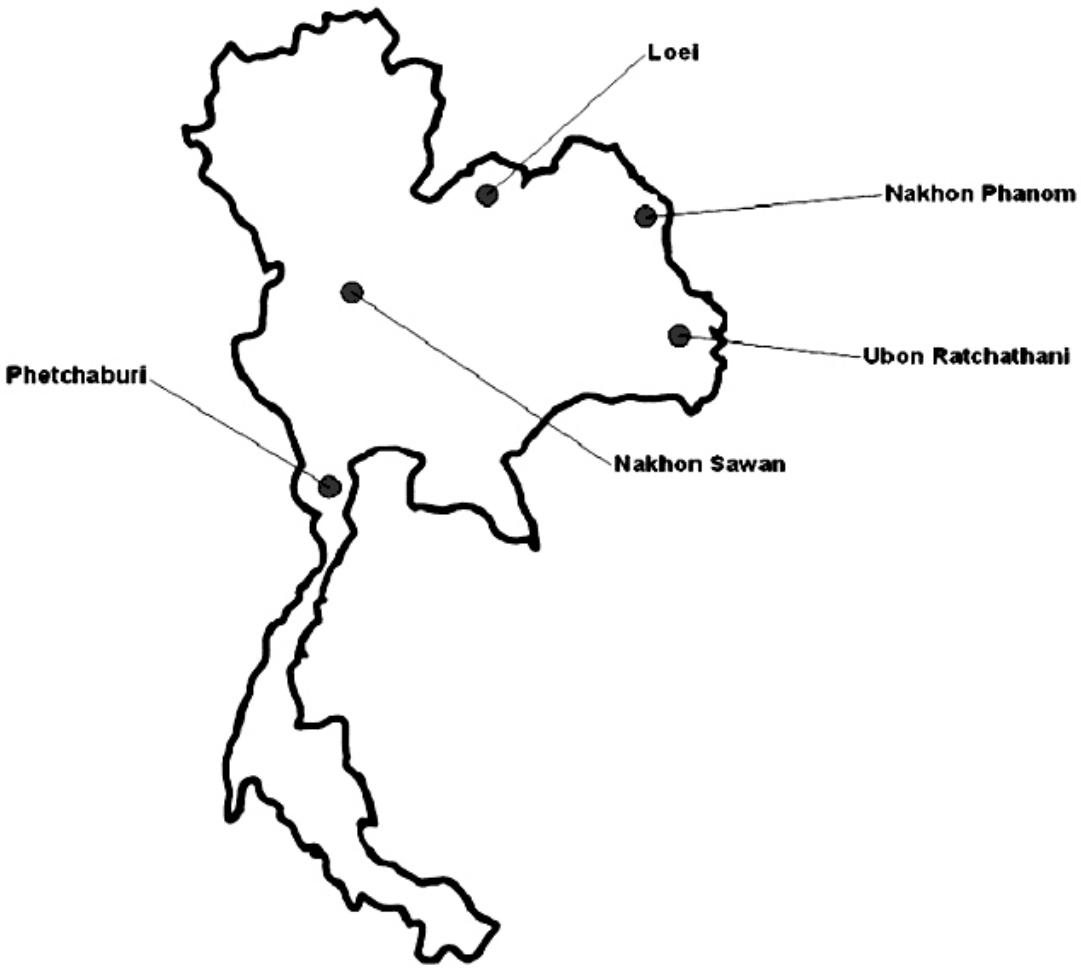

Figure 13 The locality of *B. gonionotus* and *Hypsibarbus* spp. that were collected or obtained.

2. Morphological Identification

2.1 Discrimination of *Barbonymus gonionotus* from *Hypsibarbus* spp.

Barbonymus gonionotus was a representative species of genus *Barbonymus* due to it was mostly morphological resemblance to *Hypsibarbus* species. The other species (*B. altus* and *B. schwanenfeldii*) were differing from *Hypsibarbus* spp. by having the difference in fin color pattern which was used in discrimination.

The key character for discrimination of *B. gonionotus* from *Hypsibarbus* spp. was the number of branched anal fin rays (Figure 14). In *B. gonionotus*, it presented 6–7 branched anal fin rays while those of *Hypsibarbus* spp., presented only 5 branched anal fin rays (Rainboth, 1996a).

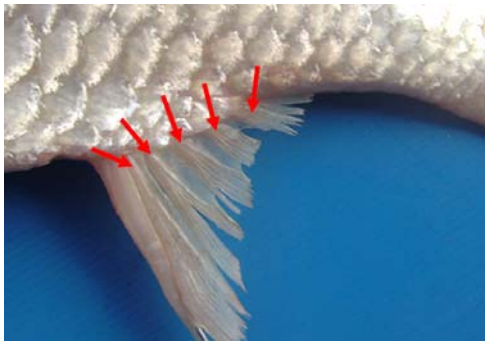

Figure 14 The five branched anal fin rays of *Hypsibarbus* spp.

2.2 Morphological Identification of *Hypsibarbus* spp.

The species identification of fish in the genus *Hypsibarbus* was basically followed almost diagnosis of Rainboth (1996a, 1996b) and Sunairatanaporn (2001). The species identification was focused into 4 main groups.

1. Morphometric characters: *i.e.* standard length, head length, head width and body depth at dorsal.

2. Meristic characters: *i.e.* predorsal scales, upper transverse scales and circumpeduncular scales.

3. The other external characters such as coloration of body and fins in live or fresh fishes.

4. The distribution of the fish.

A list of morphometric and meristic characters for species identification were given as below:

Standard length (SL); was measured from the anterior part of the snout to the mid lateral posterior edge of the hypural plate, expanded bones at the end of the backbone that supported the caudal fin. It could be determined by flexing the tail up while the caudal peduncle is held down (Figure 15).

Body depth at dorsal (BD); was measured perpendicularly from the anterior point of the dorsal fin to the ventral part of the body (Figure 15).

Head length (HL); the distance from the tip of the snout (or upper lip) to the posterior end of the operculum (Figure 15).

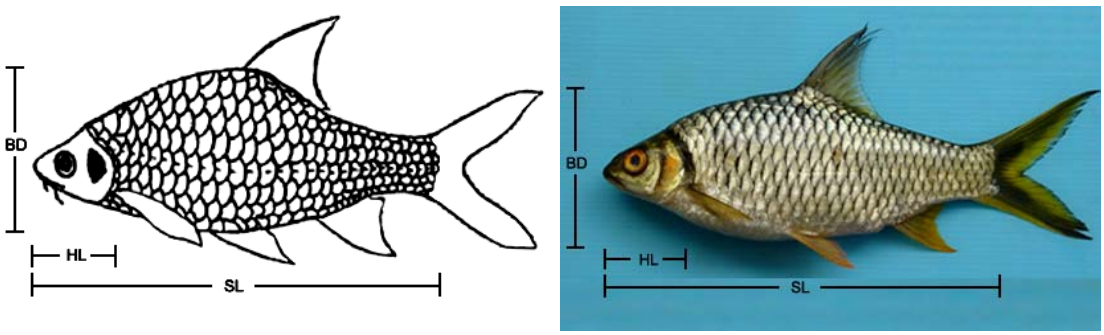

Figure 15 The measurement of standard length (SL), head length (HL) and body depth at dorsal (BD).

Head width (HW); was the transverse distance between margins at the widest area of the head (Figure 16).

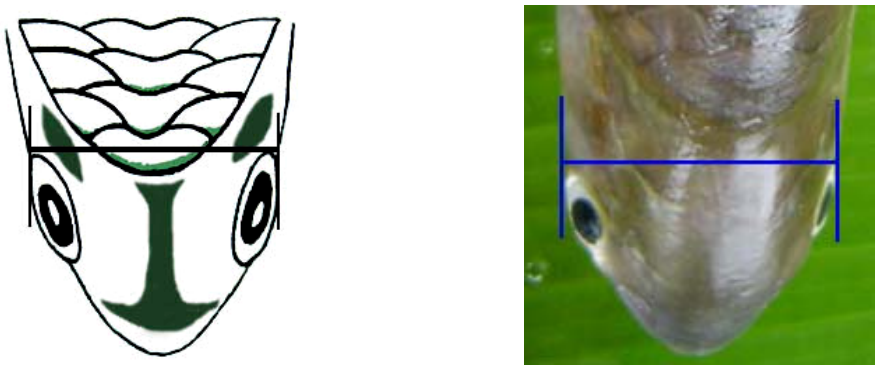

Figure 16 The measurement of head width (HW).

For the meristic characters of fish scale; the scale was always counted on the left side of each fish. If the scales on the left side were missing, the counts were made on the right. The abbreviation and the method of fish scale counts were given as below:

Predorsal scales (PDS); counted from the row of scales crossing the midline between the back of the skull to the anterior point of the dorsal fin (Figure 17).

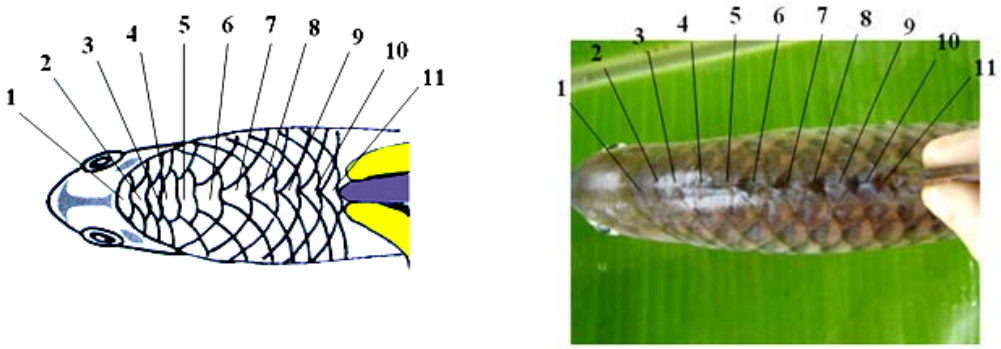

Figure 17 Predorsal scale counts.

Upper transverse scales (UTS); counted above the lateral line diagonally backwards and downwards across the sides of the body, starting at the scale at the anterior point of the dorsal fin to the lateral line (exclude the lateral line scale) (Figure 18).

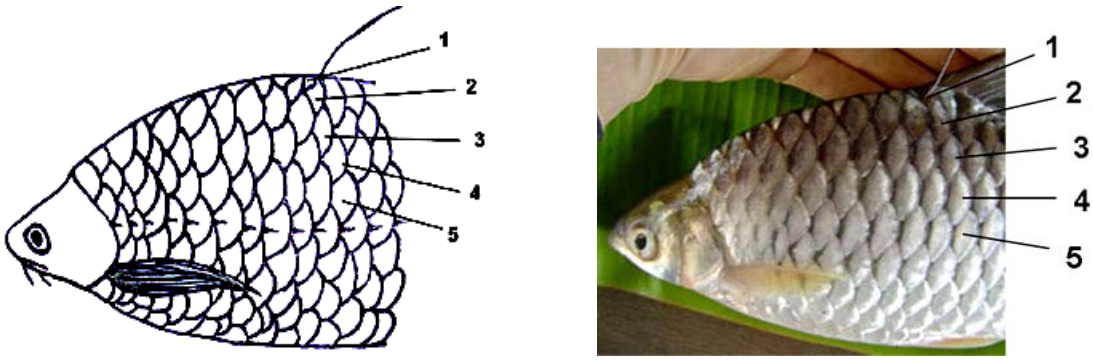

Figure 18 Upper transverse scale counts.

Circumpeduncular scales (CPS); was the number of scales around the narrowest portion of the caudal peduncle, counted diagonally from the first scale on the upper caudal peduncle downward to the lateral line and from the lateral line diagonally upward to the lower caudal peduncle. Repeat counts in another side of fish body and plus one scale of the dorsal midline and one scale of the ventral body (Figure 19).

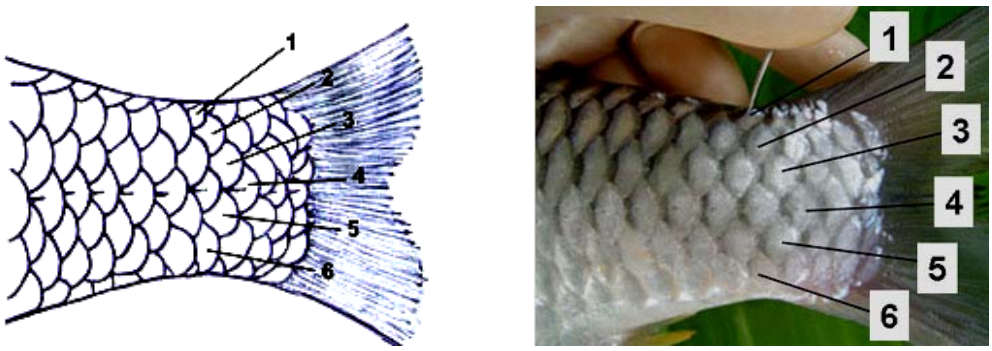

Figure 19 Circumpeduncular scale counts.

In this study; the characters in both diagnosis of Rainboth and Sunairattanaporn were used where applicable for species identification. In Rainboth's key some of characters such as total gill rakers, dorsal spine serration could not be used in identification. Since these characters are only appropriate for adult fish while specimens in this study were mostly juvenile stage. For Sunairattanaporn (2001), the characters such as the shape of mouth could not be followed for the same reason. Therefore, the identification of *Hypsibarbus* spp. in this study is referenced to applicable characters of either key as follows.

Keys I (derived from Rainboth, 1996a)

1a. 16 circumpeduncular scale rows……...….*H. malcolmi*
1b. 14 circumpeduncular scale rows……2

2a. 6 upper transverse scale rows; HW approximately 51.6% of HL…….*H. lagleri*
2b. 5 upper transverse scale rows; HW 57% to 58 % of HL……3

3a. usually 9 or fewer predorsal scales (95%)……..……..*H. wetmorei*
3b. usually 10 or more predorsal scales (75%)……..*H. vernayi*

Keys II (derived from Sunairattanaporn, 2001)

1a. When up–folding the anal fin; its tip does not reach the base of caudal fin…….…...*H. vernayi*
1b. When up–folding the anal fin; its tip reach to the base of caudal fin…….…...2

2a.The ventral part of body between pectoral and anal fins is yellow…..…*H. wetmorei*
2b.The ventral part of body between pectoral and anal fins is colorless…….3

3a.BD 40.92% (±2.84) of SL……*H. lagleri*
3b.BD 36.0% (±2.83) of SL……*H. pierrei*

3. Molecular Study

3.1 DNA Extraction

Prior to DNA extraction, the 95% ethanol (ETOH) preserved tissues were washed with distilled water to remove ethanol. The total DNA was extracted using standard phenol–chloroform method according to the protocol described by Peyachoknagul (2002).

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), briefly vortexed and followed by 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), inverted the tube twice, incubated for 5 minutes and centrifuged at 7,000 g for 5 minutes at room temperature (RT) to separate the phenol and aqueous phases. The aqueous phase with DNA was transfer 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 protocol in this DNA extraction were shown in Appendix table 1 and Appendix figure 1.

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}). The chemical reagents for DNA extraction and electrophoresis were shown in Appendix table 2.

3.2 Primer Design

One critical parameter for successful amplification in a PCR reaction is the correct design of the primers, because the primers also greatly affect the amount of the PCR–product yield. Therefore, the exactly designed primer for complementary base pairing to the template is crucial.

For the amplification the *cytochrome b* gene fragment in *Barbonymus gonionotus* and *Hypsibarbus* spp., 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* and which were accessed from GENBANK (http://www.ncbi.nlm.nih.gov/). The sequences were aligned (Appendix figure 2)

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 *Barbonymus gonionotus* and *Hypsibarbus* spp. were designed from the best matching areas: the 58^{th}–76^{th} region for the forward primer (FWD primer; L–strand); 5' GACCTACCAGCACCATCCA 3', and at the 1069^{th}–1089^{th} region for the reverse primer (REV primer; H–strand); 5' GAGGAATAGTGCGAAGTA TAG 3'.

3.3 PCR Amplification

The *cytochrome b* gene fragment (993 bp) of *Barbonymus gonionotus* and 3 *Hypsibarbus* spp. were amplified using standard PCR reaction described by Peyachoknagul (2002). The reaction was performed in total volumes of 100 µl containing the reaction components as shown in Table 2 and the condition were carried out as described in Table 3. The PCR–products were analyzed by 1.0% agarose gel electrophoresis.

Table 2 The reaction composition of 100 µl PCR reaction.

Reaction composition	Volume (µl)	Final concentration
1. 10X PCR Buffer	10	1 X
2. dNTPs (2 mM)	10	200 µM of each dNTPs
3. *Taq* DNA Pol (5Units/µl)	0.5	2.5 Units/Reaction
4. Primer		
4.1 FWD Primer (10µM)	2	0.2 µM
4.2 REV Primer(10µM)	2	0.2 µM
5. $MgCl_2$ (25mM)	12	3.0 mM
6. DNA template	1–3	100–300 ng
7. DDW	62.5	–
Total volume	100	–

Table 3 The thermal cycler conditions.

Step		Temperature (°C)	Time (minute)
1. Hold I	: Preliminary denaturation	92	3.00
2. PCR (35 cycles)	: Denaturation	92	1.00
	: Annealing	54	1.00
	: Extension	72	1.00
3. Hold II	: Final extension	72	7.00
4. Keep		4	∞

3.4 DNA Sequencing, Phylogenetic and Sequence analysis

The representative PCR–products (native specimens) of 3 *Hypsibarbus* spp. were sent to BSU (Bioservice unit) for DNA sequencing, the PCR–products were directly sequenced in both directions.

After the sequencing results were obtained, the sequences were edited with Chromas Lite 2.01 program, aligned with *Barbonymus gonionotus* sequence that provided in the GENBANK by CLUSTALW1.82 and constructed the phylogenetic tree using TreeTop program (http://www.genebee.msu.su/services/phtree_reduced.html)

To select the restriction enzyme for identification of *Barbonymus gonionotus* and 3 *Hypsibarbus* spp., the sequences were analyzed with the Webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html) to find out the different species–specific restriction sites. The informative restriction enzymes for RFLP analysis were originally chosen on the likelihood that they would produce specific patterns for species identification.

3.5 PCR–RFLP

The PCR–products were used as substrates for RFLP analysis. The reaction contained 200–500 ng of DNA (PCR–product) in the final volume of 10–20 μl. The reaction mixtures consisted of restriction enzyme (RE), buffer, DDW and PCR–product (Table 4). The reaction mixtures were incubated at the optimal temperature (usually 37 °C) for 1–2 hours and detected using 1.5% agarose gel electrophoresis.

Table 4 The reaction composition of 10 μl PCR–RFLP reaction.

Reagent	**Volume (μl)**	**Final concentration**
Restriction Buffer	1.0	1 X
DDW	2.0	–
RE	1.0	5 U
PCR–Product	6	200–500 ng
Total volume	10	–

RESULTS

1. Morphological Identification

In this study, the total of 82 specimens of fishes, *Barbonymus gonionotus* was successfully discriminated from 3 *Hypsibarbus* spp. (*Hypsibarbus* = 74 and *B. gonionotus* = 8) based on the number of branched anal fin rays. For the species identification of 74 *Hypsibarbus* specimens, they were identified using key I (derived from Rainboth, 1996a) and key II (derived from Sunairatanaporn, 2001). Species identification of *Hypsibarbus* specimens based on these 2 derived keys were shown in Table 5.

Table 5 The results of species identification of 74 *Hypsibarbus* specimens using the morphological key I and key II.

Specimens		Morphological Key I	Morphological Key II	Locality	SL (cm)
1.	L0001	Hw	Hv	L	11.7
2.	L002	Hw	Hv	L	9.1
3.	L0102	Hw	Hv	L	11.4
4.	L0201	Hm	Hv	L	11.6
5.	L0202	Hw	Hv	L	11.6
6.	L0701	Hw	Hw	L	26.4
7.	L0702	Hw	Hw	L	29.5
8.	L470301	Hv	Hw	L	14.2
9.	L470326	Hv	Hv	L	12.8
10.	L470327	Hv	Hv	L	13.1
11.	L480401	Hw	Hv	L	20.1
12.	L480402	Hw	Hp	L	19.9
13.	L480404	Hw	Hp	L	24.2
14.	L480406	Hw	Hp	L	22.5
15.	L480407	UI	Hp	L	16.8
16.	L480408	UI	Hl	L	26.4
17.	L480410	Hw	Hp	L	19.5
18.	L480412	Hw	Hp	L	18.6
19.	L480414	Hw	Hp	L	19.2
20.	L480415	Hv	Hp	L	16.9
21.	L480416	Hw	Hp	L	13.7
22.	L480419	Hw	Hw	L	30.1
23.	L480420	Hw	Hl	L	27.8
24.	L480421	Hw	Hp	L	25.3
25.	L480601	Hw	Hw	L	16.3
26.	L480602	Hw	Hl	L	27.2
27.	L490301	Hw	Hw	L	30.8
28.	L490302	UI	Hw	L	25.4
29.	L490303	Hw	Hw	L	20.1
30.	L490304	Hw	Hw	L	15.2

Table 5 (Continued)

Specimens		Morphological Key I	Morphological Key II	Locality	SL (cm)
31.	L490305	Hv	Hv	L	12.2
32.	L490306	Hv	Hv	L	9.4
33.	L490308	Hw	Hv	L	10.7
34.	L490310	Hw	Hw	L	30.5
35.	L490311	Hw	Hw	L	30.5
36.	L490312	Hw	Hw	L	10.3
37.	L490315	Hv	Hv	L	27.2
38.	L490316	Hv	Hv	L	25.5
39.	L490317	Hw	Hp	L	21.9
40.	L490318	Hw	Hp	L	20.5
41.	L490320	Hv	Hv	L	12.8
42.	L490321	Hw	Hp	L	22.3
43.	NP0802	Hw	Hw	NP	12.9
44.	NP0803	Hw	Hw	NP	9.1
45.	NP0805	Hw	Hw	NP	12.7
46.	NP0806	Hw	Hw	NP	12.8
47.	NP0808	Hw	Hw	NP	8.7
48.	NP0810	Hv	Hw	NP	8.1
49.	NP0811	Hw	Hw	NP	12.3
50.	NP0812	Hw	Hw	NP	10.5
51.	NP0813	Hv	Hw	NP	8.9
52.	NP0814	Hw	Hw	NP	8.6
53.	NP0815	Hw	Hw	NP	11.2
54.	NP0816	Hw	Hw	NP	8.4
55.	NP0817	Hw	Hw	NP	8.8
56.	NP0818	Hv	Hw	NP	12.3
57.	NS0803	Hw	Hw	NS	18.7
58.	NS0804	Hl	Hw	NS	20.1
59.	PB480301	Hw	Hv	PB	25.9
60.	PB480302	Hv	Hv	PB	23.8
61.	PB480303	Hv	Hv	PB	23.6
62.	PB480304	Hv	Hv	PB	23.2
63.	PB480305	Hl	Hv	PB	24.3
64.	PB480306	Hv	Hv	PB	24.6
65.	UB480701	Hw	Hv	UB	11.4
66.	UB480702	Hv	Hv	UB	47.5
67.	UB480703	Hv	Hw	UB	29.6
68.	UB480704	Hv	Hv	UB	13.2
69.	UB480705	UI	Hv	UB	15.5
70.	UB480706	UI	Hv	UB	11.8
71.	UB480707	Hm	Hv	UB	10.2
72.	UB480708	Hm	Hv	UB	10.3
73.	UB480709	Hv	Hv	UB	12.2
74.	UB480710	Hv	Hv	UB	13.2

Species; Hl = *Hypsibarbus lagleri*, Hm = *H. malcolmi*, Hp = *H. pierrei*, Hv = *H. vernayi*, Hw = *H. wetmorei* and UI = unable to identify

Locality; L = Loei, NP = Nakhon Phanom, NS = Nakhon Sawan, PB = Phetchaburi and UB = Ubon Ratchathani

Using morphological key I, the results showed that there were 43 of *H. wetmorei*, 21 of *H. vernayi*, 3 of *H. malcolmi*, 2 of *H. lagleri* and 5 specimens were unable to identify. Meanwhile the results from using morphological keys II, there were 28 of *H. wetmorei*, 30 of *H. vernayi*, 13 of *H. pierrei* (synonym to *H. malcolmi*) and 3 of *H. lagleri*.

2. Molecular Study

2.1 PCR Amplification

From this study, one pair of FWD and REV primers were used to amplify DNA fragments from 3 *Hypsibarbus* spp. (*H. wetmorei*, *H. vernayi* and *H. malcolmi*) and *Barbonymus gonionotus*. A single PCR–product of approximately 993 bp of *cytochrome b* gene was successfully amplified from 74 specimens of *Hypsibarbus* spp., and 8 specimens of *B. gonionotus*. The representative PCR–product from 3 *Hypsibarbus* spp. and *B. gonionotus* were shown in Figure 20.

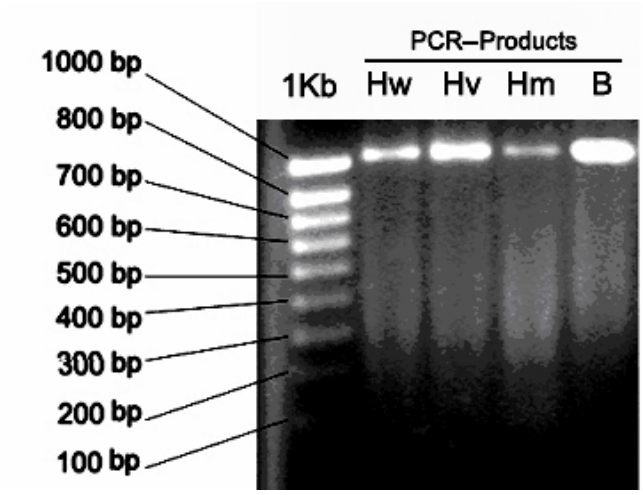

Figure 20 The PCR–products of approximately 993 bp *cytochrome b* gene amplified from *H. wetmorei* (Hw), *H. vernayi* (Hv), *H. malcolmi* (Hm) and *B. gonionotus* (B).

2.2 DNA Sequencing and Editing

The sequencing results of 3 *Hypsibarbus* samples (*H. wetmorei* = L490310, *H. vernayii* = L490316 and *H. malcolmi* = L0201) obtained from BSU and correctly edited with Chromas Lite version 2.01 were shown in Figure 21.

>*H. wetmorei* (859 bp)

```
GGCCTATTCCTAGCCATACACTACACTTCAGACATCTCAACTGCATTCTCATCAGTAACCCATATCTGCCGAGAC  75
GTAAACTACGGGTGACTAATTCGTAATATACACGCCAATGGGGCATCATTCTTCTTCATCTGTATTTACATACAC 150
ATCGCCCGAGGCCTATATTACGGGTCATACCTCTACAAAGAAACCTGAAATATCGGAGTAGTCCTCCTACTATTA 225
GTTATAATAACAGCCTTCGATGGCTACGTTCTCCCATGAGGACAAATGTCCTTCTGAGGCGCCACAGAAATTACA 300
AACCTCCAATCTGCCGTGCCATACATAGGGGACATATTAGTCCAATGAGTTTGAGGTGGGTTCTCGGTAGACAAC 375
GCAACRTTAACACGATTGTTTGCATTCCACTTCCTGCTACCATTCGTTATTGCTGCAGCAACCGTCCTACACCTA 450
CTATTCCTCCATGAAACAGGGTCAAATAACCCAATTGGCCTAAACTCAGATGCAGACAAAATCTCATTCCACCCA 525
TACTTCACGTACAAAGACCTCCTTGGATTCGTAATTATACTCGTAGGTCTTACACTACTAGCGCTATTCTCCCCT 600
AACCTATTAGGAGACCCAGAAAACTTCACCCCTGCCAACCCTCTAGTTACCCCACCACACATCAAACCAGAATGA 675
TATTTCCTATTTGCCTATGCCATTTTACGATCCATCCCAAATAAACTAGGAGGCGTCCTTGCACTACTATTCTCC 750
ATCCTAATTCTAATAGTAGTCCCCCTATTACATACCTCAAAGCAACGAGGACTAACATTCCGCCCAATTACCCAA 825
TTCCTATTCTGAACCCTAGTAGCAAGCATAATTA                                           859
```

>*H. vernayi* (770 bp)

```
GGCCTATTCCTAGCCATACACTACACCTCCGATATTTCSACCGCATTCTCATCAGTGACCCACATCTGTCGAGAC  75
GTAAACTACGGATGACTAATTCGTAATATACACGCTAACGGGGCATCATTCTTCTTCATCTGTATTTATATACAT 150
ATCGCCCGAGGCCTATATTACGGGTCATACCTCTACAAAGAAACCTGAAACATCGGAGTTGTCCTTCTACTACTG 225
GTCATAATAACAGCCTTCGTTGGTTACGTCCTCCCATGAGGACAAATGTCCTTCTGAGGTGCCACAGTAATCACA 300
AACCTCCTATCTGCCGTCCCATACATAGGAGACATGCTAGTCCAATGAATCTGAGGCGGATTCTCAGTAGACAAC 375
GCGACGCTGACGCGGTTCTTTGCATTCCACTTCCTACTACCATTTGTTATTGCCGCAGCAACAATTCTACACCTA 450
CTATTCCTCCACGAAACAGGATCAAACAACCCAATCGGACTAAACTCAGACGCAGATAAAATCTCATTCCACCCA 525
TACTTTACATACAAAGACCTCCTCGGATTCGTAATTATACTACTAGGCCTTACACTACTAGCACTATTCTCCCCC 600
AACCTGCTGGGAGAGCCAGAAAACTTCACCCCCGCCAACCCCCTAGTTACCCCACCACACATCAGGCCAGAATGA 675
TACTTCCTATTTGCCTACGCCATTCTACGATCCATTCCAAATAAACTAGGAGGCGTCCTCGCACTACTATTCTCC 750
ATCCTAGNCCTAATAGTAGT                                                         770
```

>*H. malcolmi* (946 bp)

```
GGCCTATTCCTAGCCATACACTACACTTCAGACATYTCACCCGCATTCTCATCAGTAACCCATATCTGCCGAGAC  75
GTAAACTACGGGTGACTAATTCGTAATATACACGCCAATGGGGCATCATTCTTCTTCATCTGTATTTACATACAC 150
ATCGCCCGAGGCCTATATTACGGGTCATACCTCTACAAAGAAACCTGAAATATCGGAGTAGTCCTCCTACTACTA 225
GTTATAATAACAGCCTTCGTTGGCTACGTTCTCCCATGAGGACAAATGTCCTTCTGAGGCGCCACAGTAATTACA 300
AACCTCCTATCTGCCGTCCCATACATAGGGGACATACTAGTCCAATGAATTTGAGGTGGGTTCTCGGTAGACAAC 375
GCAACATTAACACGATTCTTTGCATTCCACTTCCTGCTACCATTCGTTATTGCTGCAGCAACCGTCCTACACCTA 450
CTATTCCTCCATGAAACAGGGTCAAATAACCCAATTGGACTAAACTCAGATGCAGACAAAATCTCATTCCACCCA 525
TACTTCACGTACAAAGACCTCCTTGGATTCGTAATTATACTCCTAGGTCTTACACTACTAGCGCTATTCTCCCCC 600
AACCTATTAGGAGACCCAGAAAACTTCACCCCTGCCAACCCTCTAGTTACCCCACCACACATCAAACCAGAATGA 675
TATTTCCTATTTGCCTATGCCATTTTACGATCCATCCCAAATAAACTAGGAGGCGTCCTTGCACTACTATTCTCC 750
ATCCTAATTCTAATAGTAGTCCCCCTATTACATACCTCAAAGCAACGAGGACTAACATTCCGCCCAATTACCCAA 825
TTCCTATTCTGAACCCTAGTAGCAAACATAATTATTCTAACATGAATCGGAGGTATGCCAGTAGAACACCCATTC 900
ATTATCATCGGACAATTGCATCCATCTATACTTCGCACTATTCCTA                               946
```

Figure 21 The edited sequences of *cytochrome b* gene from *H. wetmorei*, *H. vernayi* and *H. malcolmi*.

2.3 Sequence Alignment

The results of sequences alignment from 3 *Hypsibarbus* spp. and *Barbonymus gonionotus* were shown in Figure 22. There were a few variation regions among 3 *Hypsibarbus* spp. and *B. gonionotus*.

```
                CLUSTAL W (1.83) multiple sequence alignment


H.wetmorei      GGCCTATTCCTAGCCATACACTACACTTCAGACATCTCAACTGCATTCTCATCAGTAACC 60
H.malcolmi      GGCCTATTCCTAGCCATACACTACACTTCAGACATYTCACCCGCATTCTCATCAGTAACC 60
H.vernayi       GGCCTATTCCTAGCCATACACTACACCTCCGATATTTCSACCGCATTCTCATCAGTGACC 60
B.gonionotus    GGCCTATTCCTAGCCATACACTACACTTCAGACATCTCACCCGCATTCTCATCAGTAACA 60
                ************************** ** ** ** **  * ************** **

H.wetmorei      CATATCTGCCGAGACGTAAACTACGGGTGACTAATTCGTAATATACACGCCAATGGGGCA 120
H.malcolmi      CATATCTGCCGAGACGTAAACTACGGGTGACTAATTCGTAATATACACGCCAATGGGGCA 120
H.vernayi       CACATCTGTCGAGACGTAAACTACGGATGACTAATTCGTAATATACACGCTAACGGGGCA 120
B.gonionotus    CACATTTGCCGAGACGTGAACTACGGATGACTGATCCGCAACATACACGCCAACGGAGCA 120
                ** ** ** ******** ******** ***** ** ** ** ******** ** ** ***

H.wetmorei      TCATTCTTCTTCATCTGTATTTACATACACATCGCCCGAGGCCTATATTACGGGTCATAC 180
H.malcolmi      TCATTCTTCTTCATCTGTATTTACATACACATCGCCCGAGGCCTATATTACGGGTCATAC 180
H.vernayi       TCATTCTTCTTCATCTGTATTTATATACATATCGCCCGAGGCCTATATTACGGGTCATAC 180
B.gonionotus    TCATTCTTCTTCATTTGTATTTATATACATATTGCTCGAGGCCTATACTATGGATCATAC 180
                ************** ******** ***** ** ** *********** ** ** ******

H.wetmorei      CTCTACAAAGAAACCTGAAATATCGGAGTAGTCCTCCTACTATTAGTTATAATAACAGCC 240
H.malcolmi      CTCTACAAAGAAACCTGAAATATCGGAGTAGTCCTCCTACTACTAGTTATAATAACAGCC 240
H.vernayi       CTCTACAAAGAAACCTGAAACATCGGAGTTGTCCTTCTACTACTGGTCATAATAACAGCC 240
B.gonionotus    CTTTACAAAGAAACCTGAAACATTGGAGTAATCCTTCTATTACTAGTTATGATAACAGCC 240
                ** ***************** ** *****  **** *** ** * ** ** *********

H.wetmorei      TTCGATGGCTACGTTCTCCCATGAGGACAAATGTCCTTCTGAGGCGCCACAGAAATTACA 300
H.malcolmi      TTCGTTGGCTACGTTCTCCCATGAGGACAAATGTCCTTCTGAGGCGCCACAGTAATTACA 300
H.vernayi       TTCGTTGGTTACGTCCTCCCATGAGGACAAATGTCCTTCTGAGGTGCCACAGTAATCACA 300
B.gonionotus    TTCGTCGGCTACGTCCTACCATGAGGACAAATATCCTTCTGAGGTGCCACAGTAATTACA 300
                ****  ** ***** ** ************** *********** ******* *** ***

H.wetmorei      AACCTCCAATCTGCCGTGCCATACATAGGGGACATATTAGTCCAATGAGTTTGAGGTGGG 360
H.malcolmi      AACCTCCTATCTGCCGTCCCATACATAGGGGACATACTAGTCCAATGAATTTGAGGTGGG 360
H.vernayi       AACCTCCTATCTGCCGTCCCATACATAGGAGACATGCTAGTCCAATGAATCTGAGGCGGA 360
B.gonionotus    AACCTCTTATCCGCCGTCCCTTACATGGGAGACATACTAGTCCAATGAATCTGAGGTGGA 360
                ******  *** ***** ** ***** ** *****  *********** * ***** **

H.wetmorei      TTCTCGGTAGACAACGCAACRTTAACACGATTGTTTGCATTCCACTTCCTGCTACCATTC 420
H.malcolmi      TTCTCGGTAGACAACGCAACATTAACACGATTCTTTGCATTCCACTTCCTGCTACCATTC 420
H.vernayi       TTCTCAGTAGACAACGCGACGCTGACGCGGTTCTTTGCATTCCACTTCCTACTACCATTT 420
B.gonionotus    TTCTCAGTAGACAACGCAACACTAACACGGTTCTTTGCATTCCACTTCCTACTACCATTT 420
                ***** *********** **  * ** ** ** ***************** ********

H.wetmorei      GTTATTGCTGCAGCAACCGTCCTACACCTACTATTCCTCCATGAAACAGGGTCAAATAAC 480
H.malcolmi      GTTATTGCTGCAGCAACCGTCCTACACCTACTATTCCTCCATGAAACAGGGTCAAATAAC 480
H.vernayi       GTTATTGCCGCAGCAACAATTCTACACCTACTATTCCTCCACGAAACAGGATCAAACAAC 480
B.gonionotus    ATTATTGCCGCGGCAACAATTCTACACCTATTATTCCTCCATGAAACTGGATCAAACAAC 480
                 ******* ** *****  * ********* ********** ***** ** ***** ***

H.wetmorei      CCAATTGGCCTAAACTCAGATGCAGACAAAATCTCATTCCACCCATACTTCACGTACAAA 540
H.malcolmi      CCAATTGGACTAAACTCAGATGCAGACAAAATCTCATTCCACCCATACTTCACGTACAAA 540
H.vernayi       CCAATCGGACTAAACTCAGACGCAGATAAAATCTCATTCCACCCATACTTTACATACAAA 540
B.gonionotus    CCAATTGGACTAAACTCAGACGCAGATAAAATCTCCTTCCACCCATACTTCACATACAAA 540
                ***** ** *********** ***** ******** ************** ** ******

H.wetmorei      GACCTCCTTGGATTCGTAATTATACTCGTAGGTCTTACACTACTAGCGCTATTCTCCCCT 600
H.malcolmi      GACCTCCTTGGATTCGTAATTATACTCCTAGGTCTTACACTACTAGCGCTATTCTCCCCC 600
H.vernayi       GACCTCCTCGGATTCGTAATTATACTACTAGGCCTTACACTACTAGCACTATTCTCCCCC 600
B.gonionotus    GACCTTCTTGGGTTCGTAGTTATACTTCTAGGGCTTACACTACTAGCACTATTCTCCCCC 600
                ***** ** ** ****** *******  **** ************** ***********
```

Figure 22 The multiple alignments of 3 *Hypsibarbus* spp. and *B. gonionotus*.

```
H.wetmorei      AACCTATTAGGAGACCCAGAAAACTTCACCCCTGCCAACCCTCTAGTTACCCCACCACAC 660
H.malcolmi      AACCTATTAGGAGACCCAGAAAACTTCACCCCTGCCAACCCTCTAGTTACCCCACCACAC 660
H.vernayi       AACCTGCTGGGAGAGCCAGAAAACTTCACCCCCGCCAACCCCCTAGTTACCCCACCACAC 660
B.gonionotus    AACCTGCTAGGAGATCCAGAGAACTTCACCCCTGCCAACCCCCTAGTCACCCCTCCACAC 660
                *****  * ***** ***** ********** ******** ***** ***** ******

H.wetmorei      ATCAAACCAGAATGATATTTCCTATTTGCCTATGCCATTTTACGATCCATCCCAAATAAA 720
H.malcolmi      ATCAAACCAGAATGATATTTCCTATTTGCCTATGCCATTTTACGATCCATCCCAAATAAA 720
H.vernayi       ATCAGGCCAGAATGATACTTCCTATTTGCCTACGCCATTCTACGATCCATTCCAAATAAA 720
B.gonionotus    ATTAAACCAGAATGATATTTCCTGTTCGCCTACGCTATTCTACGATCCATTCCAAATAAA 720
                ** *  ********** ***** ** ***** ** *** ********** *********

H.wetmorei      CTAGGAGGCGTCCTTGCACTACTATTCTCCATCCTAATTCTAATAGTAGTCCCCCTATTA 780
H.malcolmi      CTAGGAGGCGTCCTTGCACTACTATTCTCCATCCTAATTCTAATAGTAGTCCCCCTATTA 780
H.vernayi       CTAGGAGGCGTCCTCGCACTACTATTCTCCATCCTAGNCCTAATAGTAGT---------- 770
B.gonionotus    CTAGGAGGTGTCCTCGCACTACTATTTTCCATTCTAGTACTAATAGTAGTGCCCCTACTA 780
                ******** ***** ********** ***** ***   **********

H.wetmorei      CATACCTCAAAGCAACGAGGACTAACATTCCGCCCAATTACCCAATTCCTATTCTGAACC 840
H.malcolmi      CATACCTCAAAGCAACGAGGACTAACATTCCGCCCAATTACCCAATTCCTATTCTGAACC 840
H.vernayi       ------------------------------------------------------------
B.gonionotus    CACACCTCAAAACAACGAGGACTGACATTCCGCCCAATCACCCAATTCCTATTCTGAACC 840

H.wetmorei      CTAGTAGCAAGCATAATTA---------------------------------------- 859
H.malcolmi      CTAGTAGCAAACATAATTATTCTAACATGAATCGGAGGTATGCCAGTAGAACACCCATTC 900
H.vernayi       ------------------------------------------------------------
B.gonionotus    CTAGTAGCAGACATAATTATCCTAACCTGAATTGGAGGTATACCAGTAGAACATCCATTT 900

H.wetmorei      ------------------------------------------------------------
H.malcolmi      ATTATCATCGGACAATTGCATCCA--TCTATACTTCGCACTATTCCTA------------ 946
H.vernayi       ------------------------------------------------------------
B.gonionotus    ATCATTATTGGACAAATCGCATCAATCCTATACTTCGCACTGTTCCTAATCCTCATGCCA 960

H.wetmorei      ---------------------------------------
H.malcolmi      ---------------------------------------
H.vernayi       ---------------------------------------
B.gonionotus    CTAGCAGGATGACTAGAAAATAAAGCACTAGAATGAGCT 999
```

Figure 22 (Continued)

2.4 Phylogenetic Analysis

The sequence of 3 *Hypsibarbus* spp. were used for the construction of phylogenetic tree with the sequences of 13 cyprinid fishes in 6 genera that closely related to *Hypsibarbus*; *Barbonymus*, *Barbodes*, *Barbus*, *Acrossocheilus*, *Puntius* and *Cyprinus*. The phylogenetic analysis was constructed using TreeTop program. The tree of 16 cyprinid fish species based on the partial sequence of *cytochrome b* gene was shown in figure 23.

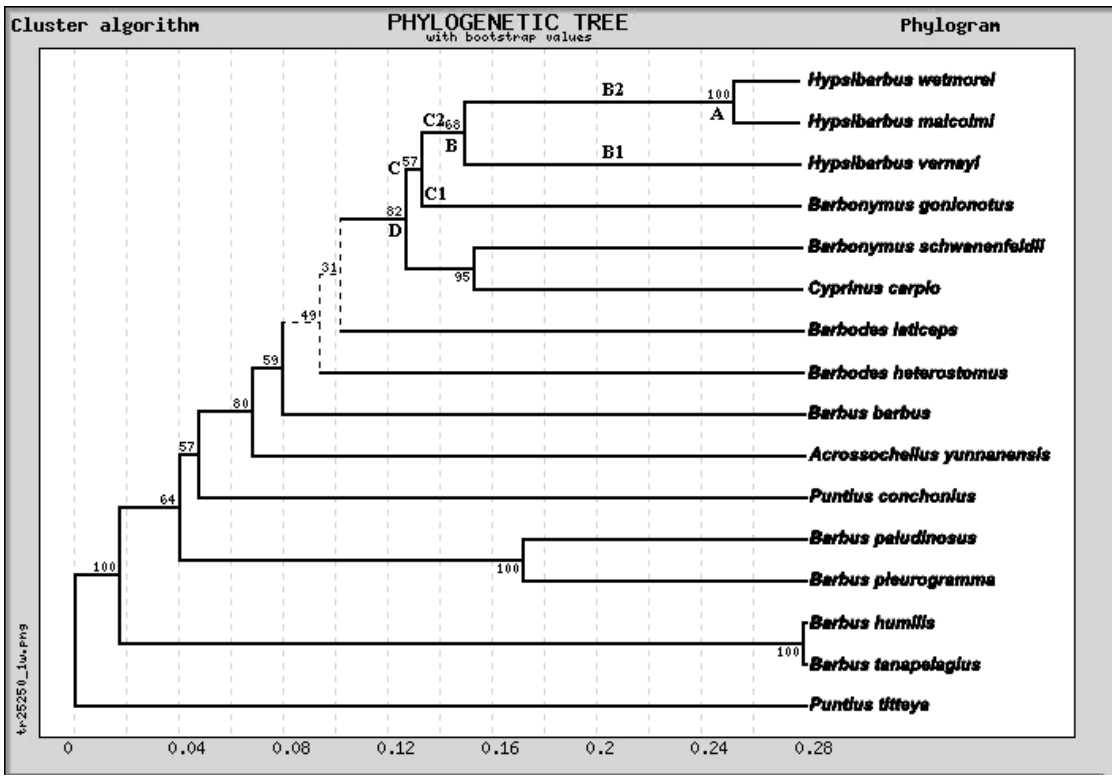

Figure 23 Phylogenetic tree with bootstrap of 16 cyprinid fishes species based on the partial sequence of *cytochrome b* gene

This phylogenetic tree showed that among 3 *Hypsibarbus* spp., the relationship between *H. wetmorei* and *H. malcolmi* was strong statistical support because the 2 species presented at the same node (A–node) with 100% bootstrap.

Among 3 genera; *Hypsibarbus*, *Barbonymus* and *Barbodes*, *Barbonymus* was the sister group to *Hypsibarbus* due to it presented at the same node (D–node) with 82% of bootstrap.

2.5 Sequence Analysis

After alignment the sequences of *Barbonymus gonionotus* and 3 *Hypsibarbus* spp., the sequence were analyzed with Webcutter 2.0, one restriction enzyme (*Cfr*42I) was selected to discriminate *B. gonionotus* from 3 *Hypsibarbus* spp. The expected restriction mapping and the diagram of expected RFLP patterns of *B. gonionotus* and 3 *Hypsibarbus* spp. were shown in figure 24.

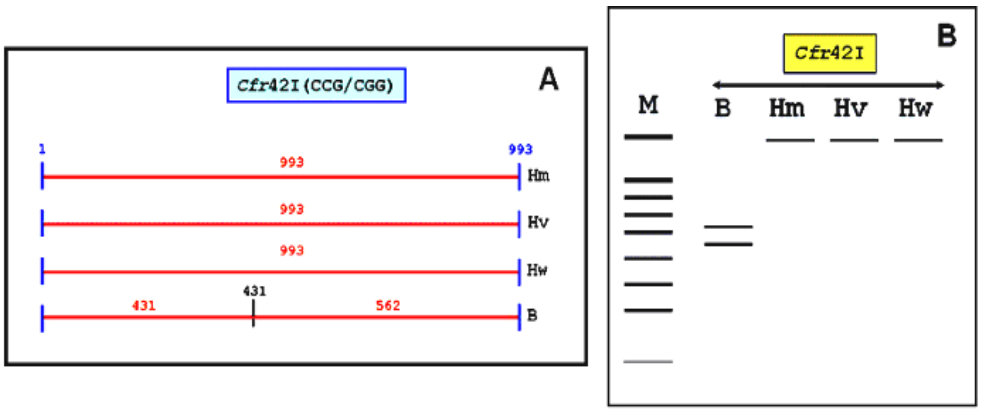

Figure 24 (A) The expected restriction mapping of 993 bp *cytochrome b* gene of *B. gonionotus* (B), *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with *Cfr*42I. (B) The diagram of expected RFLP patterns.

To discriminate 3 *Hypsibarbus* spp.; the combination of 2 restriction enzymes (*Bsp*143I and *Bcu*I) were selected. The expected restriction mapping and the diagram of expected RFLP patterns of 3 *Hypsibarbus* spp. were shown in figure 25.

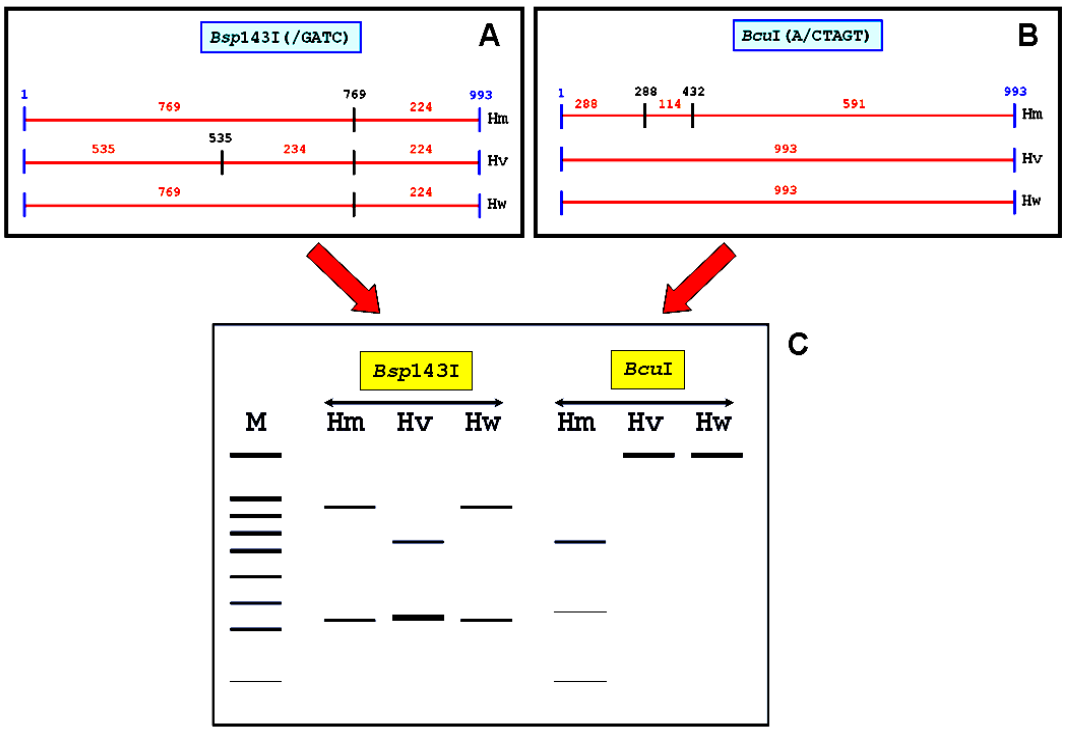

Figure 25 (A–B) The expected restriction mapping of 993 bp *cytochrome b* gene of *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with *Bsp*143I and *Bcu*I. (C) The diagram of expected RFLP patterns.

2.6 PCR–RFLP

In this study, the 993 bp PCR–products amplified from each sample were separately digested with the selected restriction enzymes resulted in specific restriction patterns.

In order to discriminate *Barbonymus gonionotus* from *Hypsibarbus* spp. in 84 specimens, one restriction enzyme (*Cfr*42I) could produce genus–specific restriction profiles. By using this enzyme, 2 fragments of approximately 431 bp and 562 bp were presented in 8 specimens of *B. gonionotus* whereas an uncut fragment was presented in 74 specimens of *Hypsibarbus* spp. (Figure 26). These patterns were corresponded to the expected patterns as described previously.

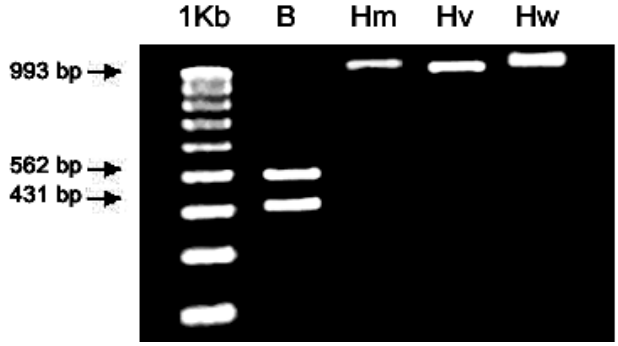

Figure 26 The RFLP patterns from PCR–products of 3 *Hypsibarbus* spp. and *B. gonionotus* when cleaved with *Cfr*42I.

The 3 *Hypsibarbus* spp. were discriminated using the combination of 2 restriction enzymes; *Bsp*143I and *Bcu*I, the first enzyme *Bsp*143I produced the same pattern for *H. malcolmi* and *H. wetmorei* by 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 27). Therefore, *Bsp*143I was useful to discriminate *H. vernayi* from *H. malcolmi* and *H. wetmorei*.

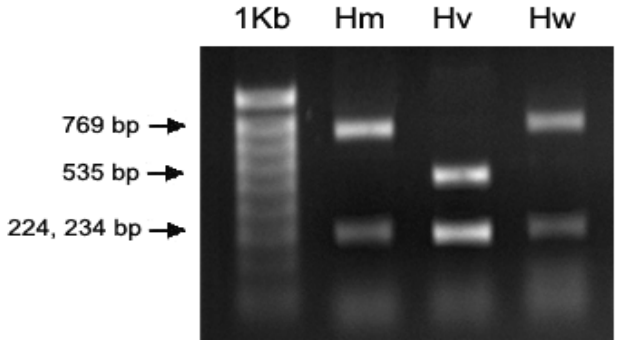

Figure 27 The RFLP patterns from PCR–products of *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with *Bsp*143I.

The remaining 2 species (*H. malcolmi* and *H. wetmorei*), were discriminated using the second enzyme *Bcu*I. 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 28).

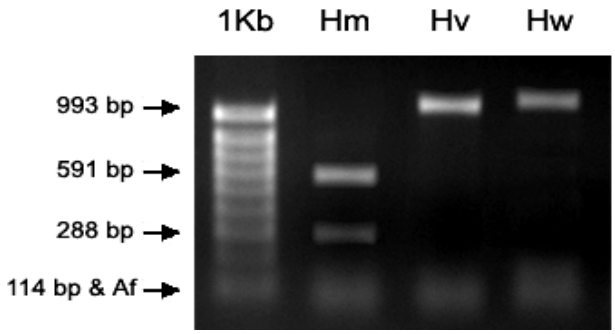

Figure 28 The RFLP patterns from PCR–products of *H. malcolmi* (Hm), *H. vernayi* (Hv) and *H. wetmorei* (Hw) when cleaved with *Bcu*I, Af was artifact from PCR reaction.

However, intraspecific restriction polymorphism was detected in *H. vernayi*. There were 2 patterns; one was an uncut fragment, another was 2 fragments (approximately 700 bp and 300). The RFLP polymorphic patterns of *H. vernayi* were shown in Figure 29.

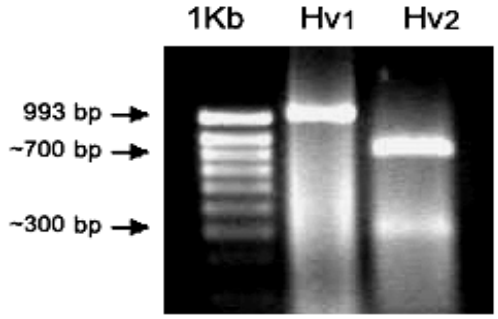

Figure 29 The RFLP polymorphic patterns (Hv1 and Hv2) in *H. vernayi* when cleaved with *Bcu*I.

According to the PCR–RFLP analysis of 74 *Hypsibarbus* specimens, 39 specimens were identified to be *H. wetmorei* patterns, 13 *H. malcolmi* and 22 *H. vernayi*.

The developed key for identification of *B. gonionotus* and 3 *Hypsibarbus* spp. using PCR–RFLP of 993 bp *cytochrome b* gene were shown below.

The molecular key for identification of *B. gonionotus* and 3 *Hypsibarbus* spp.

1a. Cleaved with *Cfr*42I, generated 431 and 562 bp fragments……...*B. gonionotus*
1b. Cleaved with *Cfr*42I, generated 993 bp (uncut fragment)…….2

2a. Cleaved with *Bsp*143I, generated 535 and single board band of 234 and 224 bp fragments…….…….....*H. vernayi*
2b. Cleaved with *Bsp*143I, generated 769 and 224 bp fragments……..…….3

3a. Cleaved with *Bcu*I, generated 591, 288 and 114 bp fragments.....*H. malcolmi*
3b. Cleaved with *Bcu*I, generated 993 bp (uncut fragment)……...…*H. wetmorei*

3. Morphology and Molecular Study

For the identification of 74 *Hypsibarbus* specimens using morphological method (key I and key II) and molecular method (PCR–RFLP technique), the results showed the matching of 72% and 72% species identified by morphological key I in comparison to PCR–RFLP and morphological key II in comparison to PCR–RFLP, respectively. Whereas, only 55.6% of the specimens were identified as the same species when using both key I and key II. The identification of 74 *Hypsibarbus* specimens using morphological key I, key II and PCR–RFLP technique were shown in Appendix table 3.

When using the molecular results for investigation the morphometric characters, the meristic characters and external morphology of 3 *Hypsibarbus* spp. The results showed that the range of morphometric characters such as HW/HL and BD/SL of each species were wide and therefore there were overlapped between species; HW/HL: *H. wetmorei* = 46.81–66.23, *H. vernayi* = 51.17–62.29 and *H. malcolmi* = 50.48–61.76; BD/SL: *H. wetmorei* = 38.18–57.63, *H. vernayi* = 31.45–48.90 and *H. pierrei*; (synonym to *H. malcolmi*) = 29.80–40.33.

The meristic characters; the number of predorsal scales in *H. wetmorei*, *H. vernayi* and *H. malcolmi* were 8–9, 9–11 and 8–11 repectively. However; 9 predorsal scales were presented in a large percentage of all species (Figure 30). In case of upper transverse scales; most of specimens were 5 scales (only 5 specimens were 6 scales). The circumpeduncular scales; there were 14 scales in *H. wetmorei* and *H. vernayi* whereas 14 or 16 scales were presented in *H. malcolmi* (Figure 31).

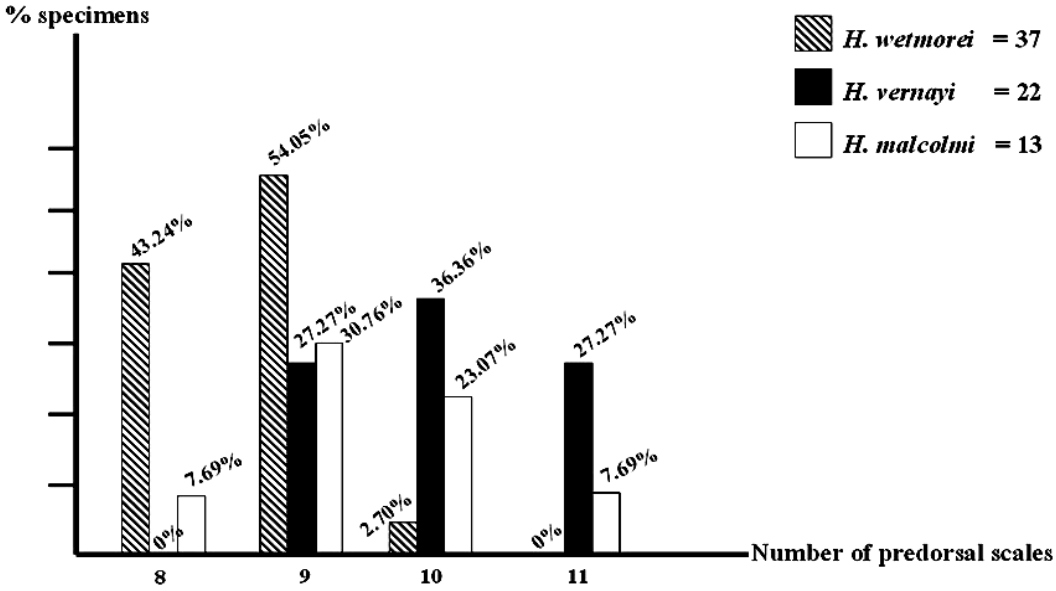

Figure 30 The histogram showed the number of predorsal scales in each species of *Hypsibarbus* specimens (N= 72; 2 specimens were fragmentary).

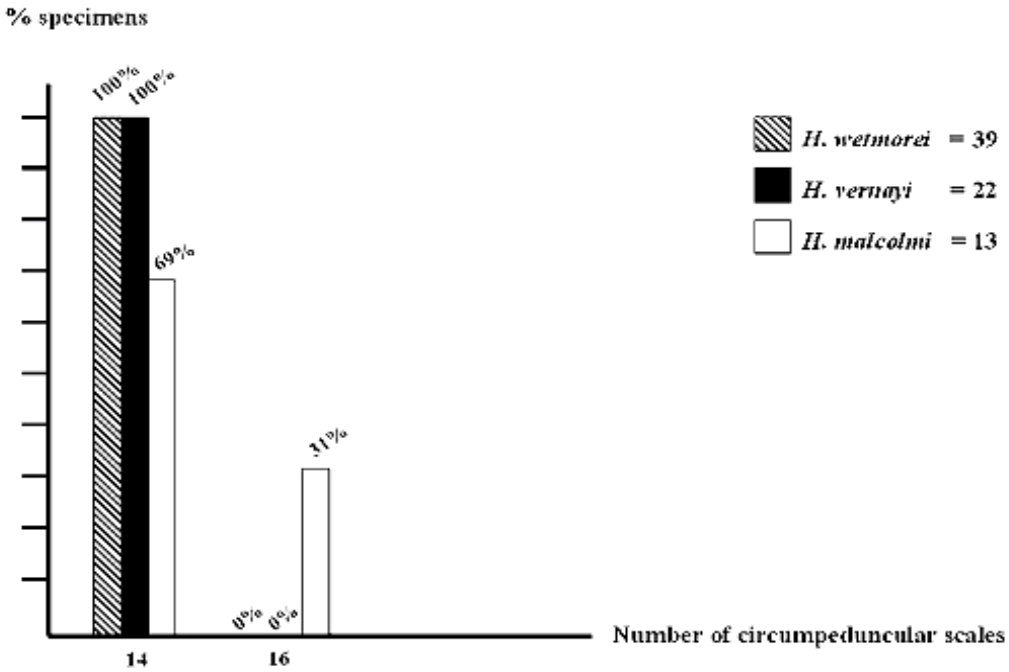

Figure 31 The histogram showed the number of circumpeduncular scales in each species of *Hypsibarbus* specimens (N= 74).

The external morphology in this study were color at the ventral part of body between pectoral and anal fin and anal fin up–folding. For the first character; most of *H. wetmorei* (76.92%) were yellow whereas most of *H. vernayi* (86.36%) and *H. pierrei* (84.61%) were colorless (Figure 32). The later character was identified by folding the tip of anal fin reached the base of caudal fin; the results showed that the tip of anal fin

in most of *H. wetmorei* (89.74%) reached the base of caudal fin whereas those of *H. vernayi* (90.90%) can not reached. For *H. pierrei*; this character was not so distinct and could not be used for species identification purpose because absent 53.84% of the samples can reach the base of caudal fin and about 46.51% can not (Figure 33).

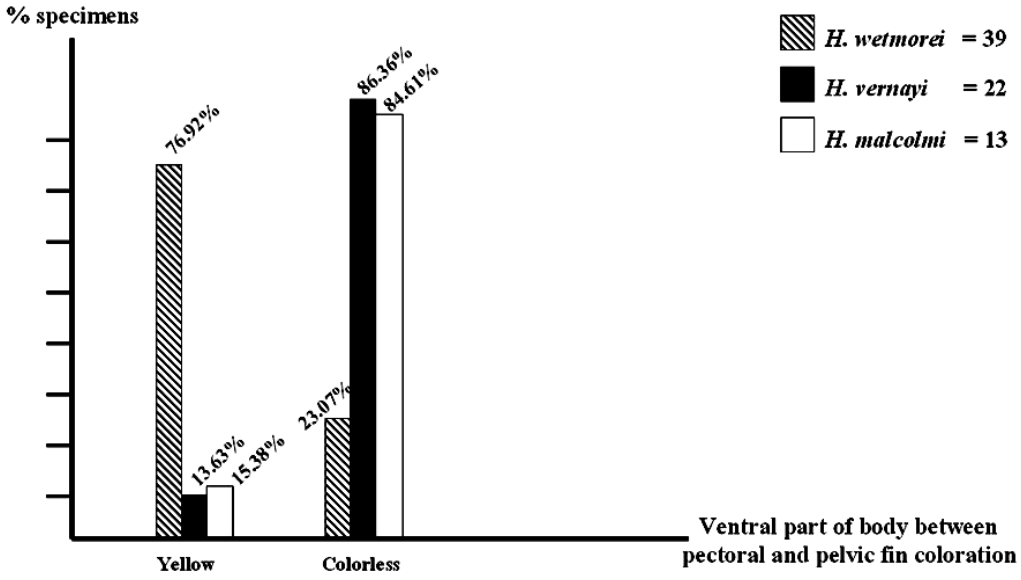

Figure 32 The histogram showed the coloration of ventral part of body between pectoral and anal fin in each species of *Hypsibarbus* specimens (N= 74).

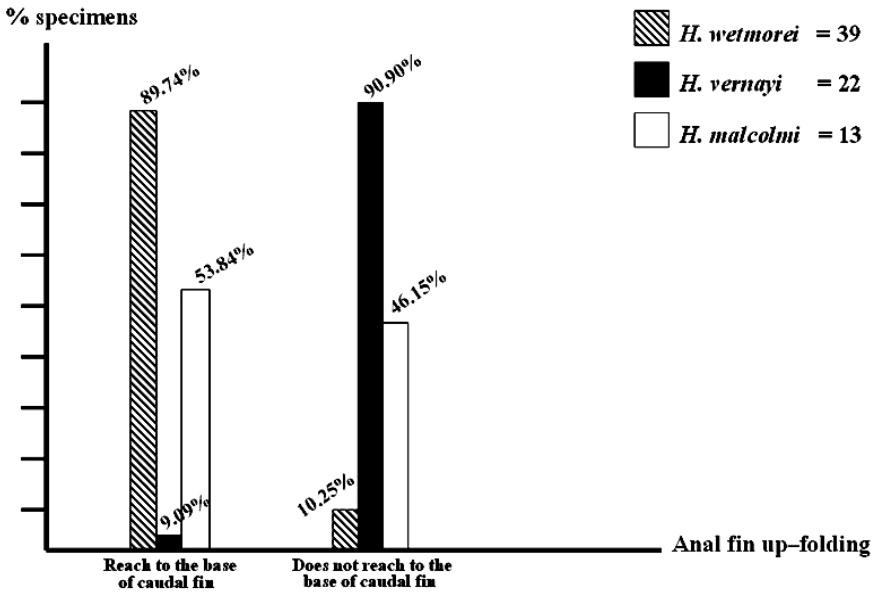

Figure33 The histogram showed the anal fin up–folding in each species of *Hypsibarbus* specimens (N= 74).

Therefore, some characters in Rainboth (1996a) and Sunairattanaporn (2001) were integrated for the reliable identification of *B. gonionotus* and 3 *Hypsibarbus* spp. Thus, the morphological keys were developed to identify *B. gonionotus* and 3 *Hypsibarbus* spp. as described below.

The developed morphological keys for identification of
B. gonionotus and 3 *Hypsibarbus* spp.

1a. 6–7 branched anal fin rays……. *B. gonionotus*
1b. 5 branched anal fin rays……..… 2

2a. Mostly 8–9 predorsal scales (97.3% of specimens), and the ventral part of body between pectoral and anal fins is yellow (76.92% of specimens)…….. *H. wetmorei*
2b. Mostly 10 or more predorsal scales, the ventral part of body between pectoral and anal fins is colorless……..... 3

3a. 37.26% (±2.48) of BD/SL ……..…….……...…… *H. vernayi*
3b. 34.47% (±1.53) of BD/SL ..... *H. malcolmi*

Using the developed morphological method key as described above; all of *Barbonymus gonionotus* were correspondingly identified to the molecular method (100%) whereas 78.38% of *Hypsibarbus* specimens (58 in 74 specimens) were correspondingly identified to the molecular method (PCR–RFLP technique).

DISCUSSION

In this study *Barbonymus gonionotus* was selected as a representative species of genus *Barbonymus* due to their mostly morphological resemblance to *Hypsibarbus* spp. (Kottelat, 1999; Sunairattanaporn, 2001). Using the meristic character; the number of branched anal fin rays is a key trait that used to rapidly distinguish *B. gonionotus* from *Hypsibarbus* spp. However; this character was appropriate for live or fresh, but not preserved or fragmentary specimens.

Identification of *Hypsibarbus wetmorei*, *H. vernayi* and *H. malcolmi* using morphological method (key I and key II) showed only 55.6% of the specimens were identified to be the same species. This was due to these methods were focused on morphometric characters, meristic characters and other external characters. These characters were widely used in fish identification but it caused some problems in this identification because the specimens were almost juvenile stage that was rather phenotypic variation within or among populations.

When using the molecular results for investigation the morphological characters; it showed that some characters such as the percentage of HW/HL that used to discriminate *H. lagleri* from *H. wetmorei* and *H. vernayi* (Rainboth, 1996a) and the percentage of BD/SL that was used to discriminate *H. lagleri* from *H. pierrei* (synonym to *H. malcolmi*) (Sunairattanaporn, 2001), these characters were variable characters. In this study *H. wetmorei* and *H. vernayi* had a wide range percentage of HW/HL from 46.81–66.23 and 51.17–62.29, respectively whereas the diagnosis key of both species were 57–58% (Rainboth, 1996a). Meanwhile the percentage of BD/SL that used to discriminate the percentage of BD/SL of *H. malcolmi* was 29.80–40.33 whereas the diagnosis key of this species was 36% ($\pm$2.83) (Sunairattanaporn, 2001).

Therefore the characters of the percentage of HL/HW and BD/SL were not the good characters for *Hypsibarbus* spp. identification and the results were similar to those of Coho salmon (Hard *et al*., 2000) and *Cynoscion* group (Aguirre and Shervette; 2005). Many authors suggest that the morphometric characters probably varied by the stage of maturation, sexual dimorphism, food or nutrition, geographic areas and environment (Ricker, 1979; Fuiman, 1983, Hard *et al*., 2000; Alfonso, 2003). However; these characters were easily measured interm of live, anaesthetized fish that free from size variation because it provided a rapid and simple way to classify fish.

Although the morphometric characters were not the good characters; the percentage of BD/SL was used to discriminate *H. vernayi* from *H. malcolmi* in this study due to the other external morphology of these 2 fishes were similar.

For the meristic characters; the number of predorsal scale, upper transverse scale and circumpeduncular scale were widely used in fish identification. However; the numbers in these characters were difficult to tell apart, partly due to within species variability and overlap in trait.

In this study, the number of predorsal scale was used to discriminate *H. wetmorei* (8–9 predorsal scale) from *H. vernayi* (9–11 predorsal scale). However; 9 predorsal scales were presented in both *H. wetmorei* and *H. vernayi* and found in a large number of specimens (54.05% and 30.76% respectively).

As well as the number of circumpeduncular scales; this character was used in key I to identify *H. malcolmi* (16 circumpeduncular scales) from *H. wetmorei* and *H. vernayi* (14 circumpeduncular scales). However, from the molecular's result; it showed that 14 circumpeduncular scales was also found in *H. malcolmi* (69%). Thus, the most confusion with the identification in 3 *Hypsibarbus* spp. occured when the specimens had 14 circumpeduncular scales; and if this character was used to distinguish *H. malcolmi* from *H. vernayi* because these 2 species shared many morphological traits.

Thus, the meristic counts in *Hypsibarbus* spp. were also the variable characters. This study was agree with the study in juvenile Japanese flounder; *Paralichthys olivaceu* (Kinoshita *et al*., 2000). However; the meristic characters could be effective in species identification when additional meristic characters were used (Hermida *et al*., 2005).

The external morphology for *Hypsibarbus* identification in key II; using the coloration of ventral part of body between pectoral and anal fin and anal fin up–folding. In this study, the ventral part of body between pectoral and anal fin in most *H. wetmorei* (76.92%) were yellow whereas those of *H. vernayi* and *H. pierrei* were colorless (86.36% and 84.61% respectively). Therefore, *H. wetmorei* could be discriminated from *H. vernayi* and *H. pierrei* when using this character,

For anal fin up–folding character; the results showed that the tip of anal fin in most *H. wetmorei* (89.74%) can reached the base of caudal fin whereas most of *H. vernayi* (90.90%) can not. For *H. pierrei*; this character could not be used to identify this species because the percentage of specimens that the tip of anal fin reached and could not reached were 53.84% and 46.15% respectively.

In this study; using morphological method, 5 specimens (L480408, L480420, L480602, NS0804 and PB480305) were identified to *H. lagleri*. The 2 specimens (NS0804 and PB480305) were identified by using 6 upper transverse scales, these results were not correspond to PCR–RFLP's result due to *H. lagleri* was endemic species in Mekong basin (Rainboth, 1996a; Sunairattanaporn, 2001) so their distribution should occur only in this area, not in Chao Phraya basin or Phetchaburi basin. The other specimens (L480408, L480420 and L480602) were identified by using the percentage of BD/SL (40.92$\pm$2.84). However, this key character was ambiguous due to this range was also fall into those of 3 *Hypsibarbus* spp. (*H. wetmorei* = 40.00$\pm$24.69, *H. vernayi* = 37.76$\pm$3.18 and *H. pierrei* = 36.00$\pm$2.83).

In general; the molecular method is benefit for fish identification in case of morphological ambiguity such as with larval stage (Olson *et al.*, 1991). In this study the *cytochrome b* gene could be used to identify *B. gonionotus* and 3 *Hypsibarbus*

spp. in all specimens due to 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).

In this finding; the specific PCR–products of partial mitochondrial *cytochrome b* gene (993 bp) in 3 *Hypsibarbus* spp. and *B. gonionotus* 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 the PCR–products of 3 *Hypsibarbus* spp. were sequenced and analyzed, it showed that there were very low interspecific variations. This result was similar to those of 4 *Thunnus* spp. and therefore the mitochondrial *cytochrome b* gene was useful for species identification (Lin *et al.*, 2005).

In this study, the species identification was consistent in all specimens when using partial mitochondrial *cytochrome b* gene (993 bp), indicated that this DNA region (Lin *et al.*, 2005) was useful for identification of *B. gonionotus* and 3 *Hypsibarbus* spp. This study was confirm with the study of Lin *et al.*, 2005 on the attempt to identify 4 tuna species; *Thunnus* spp.

For the phylogenetic tree of 3 *Hypsibarbus* spp. and the other 13 cyprinid fishes, the tree showed that among 3 *Hypsibarbus* spp.; *H. wetmorei* presented at the same node with *H. malcolmi* (A–node) with 100% bootstrap, indicated that *H. wetmorei* is more closely to *H. malcolmi* than *H. vernayi*. These closely relationship are represented by the number of bootstrap which is the statistic parameter to estimate confidence levels. The higher bootstrap is the more confident of the relationship of species within the node.

For *Barbonymus*; it was more closely related to *Hypsibarbus* than the other 12 cyprinid species because it presented at the same node (C–node, 57% bootstrap). This result is corresponded to the recent fish systematics (Kottelat, 1999; Sunairattanaporn, 2001). In addition; the 2 species of *Barbonymus* (*B. gonionotus* and *B. schwanenfeldii*) are more closely related to *Hypsibarbus* spp. than 2 species of *Barbodes*; *B. laticeps* and *B. heterostomus*. This result suggested that it is more accurate to classify *Barbonymus gonionotus* and *B. schwanenfeldii*, which previously belong to *Barbodes* into a new genus *Barbonymus*.

Although, the directly DNA sequencing could be used for species identification, but it was time consuming, expensive and technically demanding (Pardo and Pérez–Villareal, 2003). At present, PCR–RFLP was the technique of choice for species identification and it had been used in several fish such as freshwater eels (Lin *et al*., 2002), tuna fish (Pardo and Pérez–Villareal, 2003; Lin *et al*., 2005) and cod fish (Calo–Mata *et al*., 2003; Aranishi *et al*., 2005; Akasaki *et al*., 2006). Therefore the PCR–RFLP technique allowed species identification in this study due to the smaller amounts of DNA samples in use and no purity required (Peyachoknagul, 2002). This technique was found to unambiguously identify *B. gonionotus* and 3 *Hypsibarbus* spp.

As a result, *B. gonionotus* could be discriminated from 3 *Hypsibarbus* spp. by using 1 restriction enzyme (*Cfr*42I). This enzyme could easily and effectively in discrimination, because it showed different haplotypes between *B. gonionotus* and 3 *Hypsibarbus* spp.

When identifying the 3 *Hypsibarbus* spp., the combination of 2 restriction enzymes; *Bsp*143I and *Bcu*I, were used. The first enzyme *Bsp*143I could easily discriminate *H. vernayi* from *H. wetmorei* and *H. malcolmi* by producing the same RFLP pattern in all *H. vernayi* specimens and this pattern was differ from those of *H. wetmorei* and *H. malcolmi*. The second enzyme *Bcu*I was found to unambiguously identify *H. wetmorei* from *H. malcolmi*.

For *Bcu*I, this enzyme produced 2 haplotypes in *H. vernayi*; indicated that it may be the intraspecific variation in *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, including the developed PCR–RFLP method for the reliable this species identification (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 haplotyes of *H. vernayi* were differ from those of *H. wetmorei* and *H. malcolmi* haplotypes.

Finally, the results of the present study showed the established PCR–RFLP could identify *B. gonionotus* and 3 *Hypsibarbus* spp. especially in juvenile stage and fragmentary specimens.

The assemblage of the results from molecular study and morphological study (both key I and key II), the developed morphological key for identification of *B. gonionotus* and 3 *Hypsibarbus* spp.; was characterized by 6–7 branched anal fin rays while those of 3 *Hypsibarbus* spp had 5 branched anal fin rays. Among 3 *Hypsibarbus* spp.; *H. wetmorei* was characterized by the ventral part of body between pectoral and anal fin is yellow, usually 8–9 scales. For *H. vernayi* was characterized by the ventral part of body between pectoral and anal fin is colorless, 37.26% of BD/SL and when up–folding the anal fin; its tip usually does not reach to the base of caudal fin. And the *H. malcolmi* was characterized by the ventral part of body between pectoral and anal fin is colorless, 34.47% of BD/SL and when up–folding the anal fin; its tip may reach or does not reach to the base of caudal fin.

CONCLUSION

The specific PCR–product of partial mitochondrial *cytochrome b* gene (993 bp) in *Barbonymus gonionotus* and 3 *Hypsibarbus* spp. (*H. wetmorei*, *H. vernayi* and *H. malcolmi*) was useful in species identification. There was very low interspecific variation of partial *cytochrome b* gene among these species. However; it could be used to discriminate by RFLP analysis.

To discriminate *B. gonionotus* from 3 *Hypsibarbus* spp., one restriction enzyme (*Cfr*42I) could produce genus–specific restriction profile by generated 2 fragments (431 bp and 562 bp) in *B. gonionotus* and uncut fragment in 3 *Hypsibarbus* spp.

For the identification of 3 *Hypsibarbus* spp., the combination of 2 restriction enzymes; *Bsp*143I and *Bcu*I were used. The first enzyme *Bsp*143I could discriminate *H. vernayi* from *H. wetmorei* and *H. malcolmi*; by generated 2 fragments of 769 bp and 224 bp in *H. wetmorei* and *H. malcolmi*, whereas 3 fragments of 535 bp, 234 bp and 224 bp in *H. vernayi*. The later enzyme *Bcu*I was used to discriminate *H. wetmorei* from *H. malcolmi* by generated 3 fragments of 591 bp, 288 bp and 114 bp in *H. malcolmi* and uncut fragment in *H. wetmorei*. However, this enzyme produced 2 haplotypes in *H. vernayi* by generated 2 patterns; uncut fragment or 2 fragments of approximately 700 bp and 300 bp. Therefore; to establish PCR–RFLP technique for identification of 3 *Hypsibarbus* spp., it could be used to complement the morphological identification by generating the diagnosis characters of *H. wetmorei*, *H. vernayi* and *H. malcolmi*.

SUGGESTION

1. The morphological identification was the basic methodology in species identification; so that, to develop the easy and certain external key characters that were not or less varied due to the environment were importance and led to effective species identification.

2. For DNA extraction; according to most DNA extraction protocols in the incubating step, liquid N_2 were recommended, but in this study it was not necessary as well as the tissue were small pieces. DNA extracts from ethanol–preserved tissue were less protein contamination and more concentration of DNA than fresh or frozen tissues. However the unpurity of DNA extracts (contaminated with RNA or proteins) were less effect in PCR reaction due to the small amounts of DNA in use.

3. For PCR amplification; the optimal concentration of primers (0.1–0.3µM) and Mg^{2+} (to be adjusted) were increasing the yield of PCR product. The general annealing temperature in thermal cycler was 55–60 ºC. However; out of this range could be found, to find the optimal annealing temperature, using the thermal gradient PCR machine was recommended. All PCR supplements (tip, microcentrifuge tube, micropipette, lots of reagents) were effective in the stable of PCR reaction; it should be using the same lot, pack or brand for the more consistent amplification in all specimens.

4. For DNA sequencing; the PCR–product should be proved by sequenced in both strands for the accurate interpretation, and the sequence editing by computer program should be required.

5. The DNA sequencing of the specimens from difference locality were required for detecting the intraspecific variation.

6. In RFLP incubation; according to many protocols, the incubating time over 1.00 hours were not necessary in RFLP analysis whereas the optimal percentage of agarose gel and the staining time with ethidium bromide were effective in the clearly results.

7. To develop the reliable species identification by PCR–RFLP method; the large numbers of specimens, the standard species sampling from various locations were required for detecting the intraspecific variation.

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APPENDIX

Appendix Table 1 The protocol of DNA extraction using standard phenol–chloroform method as described by Peyachoknakul (2002).

Step	Methods
1.	Cut 0.10–0.15 g of tissue into small pieces and transfer to 1.5 ml microcentrifuge tube. Add 500 µl of STE Buffer, 30 µl of 20% SDS and 30 µl of proteinase K (10 mg/ml of STE buffer) and briefly vortex, for homogenizing and digestion.
2.	Incubated the samples at 55°C for 2 hours or until the digestion was completed, temporary shaking during incubation.
3.	The resulting homogeneous solution was extracted with 500 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1), then inverted the tube twice and incubate for 5 minutes at room temperature.
4.	Centrifuged at 7,000 g for 5 minutes at room temperature. The phenol phase and aqueous phase were separated.
5.	Recovered the aqueous phase into the new microcentrifuge tube and repeated the extraction with 500 µl of chloroform: isoamyl alcohol (24: 1).
6.	Centrifuged at 7,000 g for 3 minutes at room temperature.
7.	Pipetted the aqueous phase into the new microcentrifuge tube. (be careful the lower phase of chloroform leak into the tip of micropipette)
8.	Precipitated the DNA with 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.
9.	Centrifuged at 14,000g 4°C for 3 minutes.
10.	Discarded the supernatant and cleaned the precipitate with 500 µl of 70% ETOH, centrifuge finally at 14,000g 4°C for 2 minutes.
11.	Discarded the supernatant, dried the pellet and finally dissolved it in 100–200 µl of TE buffer (maybe overnight at 4°C for completely dissolved); kept DNA solution at –20°C for long term using or 4°C for short term using.

Appendix Table 2 The chemical reagents in phenol–chloroform DNA extraction

1 M Sodium chloride (NaCl: Stock solution)		
Ingredients	**Weight**	**Volume**
1. NaCl	2.922 g	–
2. DDW	–	adjust to 50 ml
Total volume	–	50 ml

1 M Tris–HCl pH 8.0 (Stock solution)		
Ingredients	**Weight**	**Volume**
1. Tris–HCl	12.114 g	–
2. pH buffer	–	adjust
3. DDW	–	adjust to 100 ml
Total volume	–	100 ml

0.5 M EDTA pH 8.0 (Stock solution)		
Ingredients	**Weight**	**Volume**
1. EDTA	18.612 g	–
2. pH buffer	–	adjust
3. DDW	–	adjust to 100 ml
Total volume	–	100 ml

STE buffer		
Ingredients	**Volume**	**Final concentration**
1. 1M NaCl (stock)	10 ml	100 mM
2. 1M Tris–HCl, pH 8.0 (stock)	5 ml	50 mM
3. 0.5 M EDTA, pH 8.0 (stock)	0.2 ml (200 µl)	1 mM
4. DDW	84.8 ml	–
Total volume	100 ml	–

TE buffer		
Ingredients	**Volume**	**Final concentration**
1. 1M Tris–HCl, pH 8.0 (stock)	1 ml	10 mM
2. 0.5 M EDTA, pH 8.0 (stock)	0.2 ml	1 mM
3. DDW	98.8 ml	–
Total volume	100 ml	–

20% SDS		
Ingredients	**Weight**	**Volume**
1. Sodium dodecyl sulfate (SDS)	20 g	–
2. DDW	–	adjust to 100 ml
Total volume	–	100 ml

Proteinase K (10mg/ml STE)		
Ingredients	**Weight**	**Volume**
1. Proteinasr K	0.005 g	–
2. STE buffer	–	adjust to 500 µl
Total volume	–	500 µl

Phenol–Chloroform–Isoamyl alcohol (25:24:1)	
Ingredients	**Volume**
1. Phenol	50 ml
2. Chloroform	48 ml
3. Isoamyl alcohol	2 ml
Total volume	100 ml

Chloroform–Isoamyl alcohol (24:1)	
Ingredients	**Volume**
1. Chloroform	96 ml
2. Isoamyl alcohol	4 ml
Total volume	100 ml

3M Sodium acetate (CH_3COONa) pH 5.2		
Ingredients	**Weight**	**Volume**
1. CH_3COONa	8.16 g	–
2. DDW	–	adjust to 20 ml
Total volume	–	20 ml

50X TAE buffer pH 8.0		
Ingredients	**Weight**	**Volume**
1. Tris–base	242 g	–
2. glacial acetic acid	–	57.1 ml
3. 0.5 M EDTA, pH 8.0 (stock)	–	100 ml
4. DDW	–	adjust to 1,000 ml
Total volume		1,000 ml

DNA loading dye (6X)		
Ingredients	**Volume**	**Final concentration**
1. Bromophenol blue	25 mg	0.25%
2. Xylene Xyanol	25 mg	0.25%
3. Sucrose	4 g	40%
4. DDW	adjust to 10 ml	–
Total volume	10 ml	–

Ethidium bromide (stock: 10mg/ml)		
Ingredients	**Weight**	**Volume**
1. Ethidium bromide	100 mg	–
2. DDW	–	10 ml
Total volume	–	10 ml

Appendix Table 3 The comparative results of *Hypsibarbus* identified using Morphological methods (key I and key II) and molecular method (PCR–RFLP technique).

Specimens		**Morphological Key I**	**Morphological Key II**	**PCR–RFLP**	**Locality**
1.	L0001	Hw	Hv	Hv	L
2.	L002	Hw	Hv	Hv	L
3.	L0102	Hw	Hv	Hw	L
4.	L0201	Hm	Hv	Hm	L
5.	L0202	Hw	Hv	Hw	L
6.	L0701	Hw	Hw	Hw	L
7.	L0702	Hw	Hw	Hw	L
8.	L470301	Hv	Hw	Hw	L
9.	L470326	Hv	Hv	Hm	L
10.	L470327	Hv	Hv	Hm	L
11.	L480401	Hw	Hv	Hw	L
12.	L480402	Hw	Hp	Hm	L
13.	L480404	Hw	Hp	Hw	L
14.	L480406	Hw	Hp	Hv	L
15.	L480407	UI	Hp	Hm	L
16.	L480408	UI	Hl	Hw	L
17.	L480410	Hw	Hp	Hw	L
18.	L480412	Hw	Hp	Hm	L
19.	L480414	Hw	Hp	Hw	L
20.	L480415	Hv	Hp	Hm	L
21.	L480416	Hw	Hp	Hw	L
22.	L480419	Hw	Hw	Hw	L
23.	L480420	Hw	Hl	Hw	L
24.	L480421	Hw	Hp	Hw	L
25.	L480601	Hw	Hw	Hw	L
26.	L480602	Hw	Hl	Hw	L
27.	L490301	Hw	Hw	Hw	L
28.	L490302	UI	Hw	Hw	L
29.	L490303	Hw	Hw	Hw	L
30.	L490304	Hw	Hw	Hw	L
31.	L490305	Hv	Hv	Hv	L
32.	L490306	Hv	Hv	Hv	L
33.	L490308	Hw	Hv	Hv	L
34.	L490310	Hw	Hw	Hw	L
35.	L490311	Hw	Hw	Hw	L

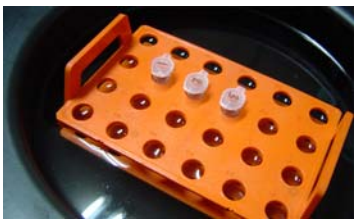
Appendix Table 3 (Continued)

Specimens		Morphological Key I	Morphological Key II	PCR–RFLP	Locality
36.	L490312	Hw	Hw	Hm	L
37.	L490315	Hv	Hv	Hv	L
38.	L490316	Hv	Hv	Hv	L
39.	L490317	Hw	Hp	Hm	L
40.	L490318	Hw	Hp	Hm	L
41.	L490320	Hv	Hv	Hm	L
42.	L490321	Hw	Hp	Hw	L
43.	NP0802	Hw	Hw	Hw	NP
44.	NP0803	Hw	Hw	Hw	NP
45.	NP0805	Hw	Hw	Hw	NP
46.	NP0806	Hw	Hw	Hw	NP
47.	NP0808	Hw	Hw	Hw	NP
48.	NP0810	Hv	Hw	Hw	NP
49.	NP0811	Hw	Hw	Hw	NP
50.	NP0812	Hw	Hw	Hw	NP
51.	NP0813	Hv	Hw	Hw	NP
52.	NP0814	Hw	Hw	Hw	NP
53.	NP0815	Hw	Hw	Hw	NP
54.	NP0816	Hw	Hw	Hw	NP
55.	NP0817	Hw	Hw	Hw	NP
56.	NP0818	Hv	Hw	Hw	NP
57.	NS0803	Hw	Hw	Hw	NS
58.	NS0804	Hl	Hw	Hw	NS
59.	PB480301	Hw	Hv	Hv	PB
60.	PB480302	Hv	Hv	Hv	PB
61.	PB480303	Hv	Hv	Hv	PB
62.	PB480304	Hv	Hv	Hv	PB
63.	PB480305	Hl	Hv	Hv	PB
64.	PB480306	Hv	Hv	Hv	PB
65.	UB480701	Hw	Hv	Hv	UB
66.	UB480702	Hv	Hv	Hv	UB
67.	UB480703	Hv	Hw	Hv	UB
68.	UB480704	Hv	Hv	Hv	UB
69.	UB480705	UI	Hv	Hv	UB
70.	UB480706	UI	Hv	Hv	UB
71.	UB480707	Hm	Hv	Hm	UB
72.	UB480708	Hm	Hv	Hm	UB
73.	UB480709	Hv	Hv	Hv	UB
74.	UB480710	Hv	Hv	Hv	UB

Species; Hl = *Hypsibarbus lagleri*, Hm = *H. malcolmi*, Hp = *H. pierrei*, Hv = *H. vernayi*, Hw = *H. wetmorei* and UI = unable to identify

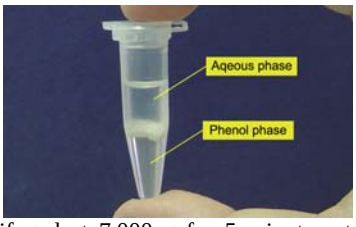
Locality; L = Loei, NP = Nakhon Phanom, NS = Nakhon Sawan, PB = Phetchaburi and UB = Ubon Ratchathani

1. The small pieces of tissue sample with STE buffer, 20% SDS and proteinase K.



2. Incubated at 55°C for 2 hours.

3. Added phenol: chloroform: isoamyl alcohol (25:24:1) , inverted the tube and incubated for 5 minutes at RT.

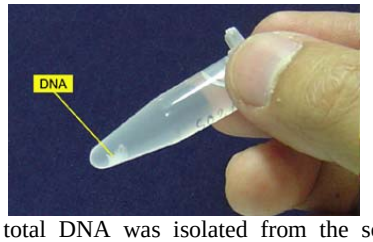


4. Centrifuged at 7,000 g for 5 minutes at RT, the phenol phase and aqueous phase were separated.

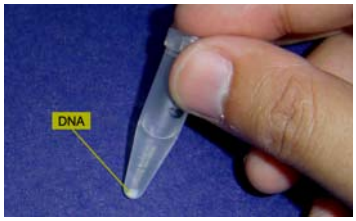
5. Recovered the aqueous phase into the new tube.

6. Added chloroform: isoamyl alcohol (24:1) and centrifuged at 7,000 g for 3 minutes at RT.

7. Recovered the aqueous phase into the new tube, added 3M CH_3COONa (pH 5.2) and absolute ETOH.



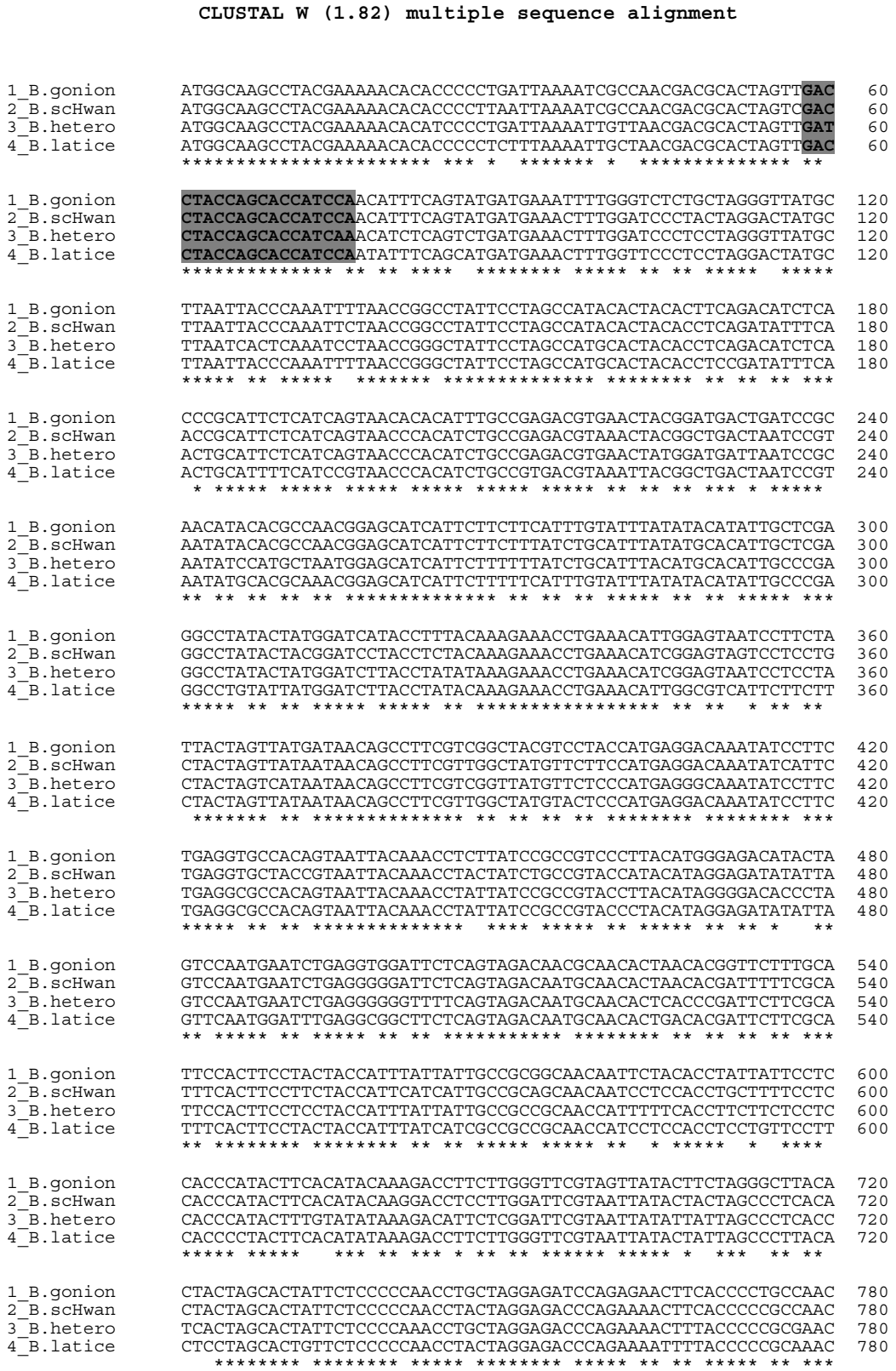
8. The total DNA was isolated from the solution. Centrifuged at 14,000g 4°C for 3 minutes.



9. The pellet DNA was precipitated, discarded the supernatant and cleaned the DNA pellet with ETOH.

10. Discarded ETOH, dried the pellet and then dissolved in 100–200 µl of TE buffer.

Appendix Figure 1 The steps of DNA extraction using standard phenol–$CHCl_3$ method.



```
                    CLUSTAL W (1.82) multiple sequence alignment

1_B.gonion      ATGGCAAGCCTACGAAAAACACACCCCCTGATTAAAATCGCCAACGACGCACTAGTTGAC   60
2_B.scHwan      ATGGCAAGCCTACGAAAAACACACCCCTTAATTAAAATCGCCAACGACGCACTAGTCGAC   60
3_B.hetero      ATGGCAAGCCTACGAAAAACACATCCCCTGATTAAAATTGTTAACGACGCACTAGTTGAT   60
4_B.latice      ATGGCAAGCCTACGAAAAACACACCCCCTCTTTAAAATTGCTAACGACGCACTAGTTGAC   60
                ********************** *** *  ******* *  ************** **

1_B.gonion      CTACCAGCACCATCCAACATTTCAGTATGATGAAATTTTGGGTCTCTGCTAGGGTTATGC  120
2_B.scHwan      CTACCAGCACCATCCAACATTTCAGTATGATGAAACTTTGGATCCCTACTAGGACTATGC  120
3_B.hetero      CTACCAGCACCATCAAACATCTCAGTCTGATGAAACTTTGGATCCCTCCTAGGGTTATGC  120
4_B.latice      CTACCAGCACCATCCAATATTTCAGCATGATGAAACTTTGGTTCCCTCCTAGGACTATGC  120
                ************** ** ** ****  ******** ***** ** ** *****  *****

1_B.gonion      TTAATTACCCAAATTTTAACCGGCCTATTCCTAGCCATACACTACACTTCAGACATCTCA  180
2_B.scHwan      TTAATTACCCAAATTCTAACCGGCCTATTCCTAGCCATACACTACACCTCAGATATTTCA  180
3_B.hetero      TTAATCACTCAAATCCTAACCGGGCTATTCCTAGCCATGCACTACACCTCAGACATCTCA  180
4_B.latice      TTAATTACCCAAATTTTAACCGGGCTATTCCTAGCCATGCACTACACCTCCGATATTTCA  180
                ***** ** *****  ******* ************** ******** ** ** ** ***

1_B.gonion      CCCGCATTCTCATCAGTAACACACATTTGCCGAGACGTGAACTACGGATGACTGATCCGC  240
2_B.scHwan      ACCGCATTCTCATCAGTAACCCACATCTGCCGAGACGTAAACTACGGCTGACTAATCCGT  240
3_B.hetero      ACTGCATTCTCATCAGTAACCCACATCTGCCGAGACGTGAACTATGGATGATTAATCCGC  240
4_B.latice      ACTGCATTTTCATCCGTAACCCACATCTGCCGTGACGTAAATTACGGCTGACTAATCCGT  240
                 * ***** ***** ***** ***** ***** ***** ** ** ** *** * *****

1_B.gonion      AACATACACGCCAACGGAGCATCATTCTTCTTCATTTGTATTTATATACATATTGCTCGA  300
2_B.scHwan      AATATACACGCCAACGGAGCATCATTCTTCTTTATCTGCATTTATATGCACATTGCTCGA  300
3_B.hetero      AATATCCATGCTAATGGAGCATCATTCTTTTTTATCTGCATTTACATGCACATTGCCCGA  300
4_B.latice      AATATGCACGCAAACGGAGCATCATTCTTTTTCATTTGTATTTATATACATATTGCCCGA  300
                ** ** ** ** ** ************** ** ** ** ***** ** ** ***** ***

1_B.gonion      GGCCTATACTATGGATCATACCTTTACAAAGAAACCTGAAACATTGGAGTAATCCTTCTA  360
2_B.scHwan      GGCCTATACTACGGATCCTACCTCTACAAAGAAACCTGAAACATCGGAGTAGTCCTCCTG  360
3_B.hetero      GGCCTATACTATGGATCTTACCTATATAAAGAAACCTGAAACATCGGAGTAATCCTCCTA  360
4_B.latice      GGCCTGTATTATGGATCTTACCTATACAAAGAAACCTGAAACATTGGCGTCATTCTTCTT  360
                ***** ** ** ***** ***** ** ***************** ** **  * ** **

1_B.gonion      TTACTAGTTATGATAACAGCCTTCGTCGGCTACGTCCTACCATGAGGACAAATATCCTTC  420
2_B.scHwan      CTACTAGTTATAATAACAGCCTTCGTTGGCTATGTTCTTCCATGAGGACAAATATCATTC  420
3_B.hetero      CTACTAGTCATAATAACAGCCTTCGTCGGTTATGTTCTCCCATGAGGGCAAATATCCTTC  420
4_B.latice      CTACTAGTTATAATAACAGCCTTCGTTGGCTATGTACTCCCATGAGGACAAATATCCTTC  420
                 ******* ** ************** ** ** ** ** ******** ******** ***

1_B.gonion      TGAGGTGCCACAGTAATTACAAACCTCTTATCCGCCGTCCCTTACATGGGAGACATACTA  480
2_B.scHwan      TGAGGTGCTACCGTAATTACAAACCTACTATCTGCCGTACCATACATAGGAGATATATTA  480
3_B.hetero      TGAGGCGCCACAGTAATTACAAACCTATTATCCGCCGTACCTTACATAGGGGACACCCTA  480
4_B.latice      TGAGGCGCCACAGTAATTACAAACCTATTATCCGCCGTACCCTACATAGGAGATATATTA  480
                ***** ** ** **************  **** ***** ** ***** ** ** *   **

1_B.gonion      GTCCAATGAATCTGAGGTGGATTCTCAGTAGACAACGCAACACTAACACGGTTCTTTGCA  540
2_B.scHwan      GTCCAATGAATCTGAGGGGGATTCTCAGTAGACAATGCAACACTAACACGATTTTTCGCA  540
3_B.hetero      GTCCAATGAATCTGAGGGGGGTTTTCAGTAGACAATGCAACACTCACCCGATTCTTCGCA  540
4_B.latice      GTTCAATGGATTTGAGGCGGCTTCTCAGTAGACAATGCAACACTGACACGATTCTTCGCA  540
                ** ***** ** ***** ** ** *********** ******** ** ** ** ** ***

1_B.gonion      TTCCACTTCCTACTACCATTTATTATTGCCGCGGCAACAATTCTACACCTATTATTCCTC  600
2_B.scHwan      TTTCACTTCCTTCTACCATTCATCATTGCCGCAGCAACAATCCTCCACCTGCTTTTCCTC  600
3_B.hetero      TTCCACTTCCTCCTACCATTTATTATTGCCGCCGCAACCATTTTTCACCTTCTTCTCCTC  600
4_B.latice      TTTCACTTCCTACTACCATTTATCATCGCCGCCGCAACCATCCTCCACCTCCTGTTCCTT  600
                ** ******** ******** ** ** ***** ***** **  * *****  *  ****

1_B.gonion      CACCCATACTTCACATACAAAGACCTTCTTGGGTTCGTAGTTATACTTCTAGGGCTTACA  720
2_B.scHwan      CACCCATACTTCACATACAAGGACCTCCTTGGATTCGTAATTATACTACTAGCCCTCACA  720
3_B.hetero      CACCCATACTTTGTATATAAAGACATTCTCGGATTCGTAATTATATTATTAGCCCTCACC  720
4_B.latice      CACCCCTACTTCACATATAAAGACCTTCTTGGGTTCGTAATTATACTATTAGCCCTTACA  720
                ***** *****   *** ** *** * ** ** ****** ***** *  ***  ** **

1_B.gonion      CTACTAGCACTATTCTCCCCCAACCTGCTAGGAGATCCAGAGAACTTCACCCCTGCCAAC  780
2_B.scHwan      CTACTAGCACTATTCTCCCCCAACCTACTAGGAGACCCAGAAAACTTCACCCCCGCCAAC  780
3_B.hetero      TCACTAGCACTATTCTCCCCAAACCTGCTAGGAGACCCAGAAAACTTTACCCCCGCGAAC  780
4_B.latice      CTCCTAGCACTGTTCTCCCCCAACCTACTAGGAGACCCAGAAAATTTTACCCCCGCAAAC  780
                   ******** ******** ***** ******** ***** ** ** ***** ** ***
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Appendix Figure 2 The multiple alignment of 4 *Barbodes* species; the highlight bases were the conserve region that selected for primer design.

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1_B.gonion      CCCCTAGTCACCCCTCCACACATTAAACCAGAATGATATTTCCTGTTCGCCTACGCTATT  840
2_B.scHwan      CCCCTAGTCACTCCTCCCCACATCAAACCAGAATGATATTTCCTATTTGCATATGCCATC  840
3_B.hetero      CCTCTAGTTACTCCACCCCACATTAAGCCAGAATGATACTTTCTATTTGCATACGCCATC  840
4_B.latice      CCACTAGTCACTCCCCCACATATCCAACCAGAATGATACTTCTTATTCGCCTACGCCATC  840
                ** ***** ** ** ** ** **  * ********** **  * ** ** ** ** **

1_B.gonion      CTACGATCCATTCCAAATAAACTAGGAGGTGTCCTCGCACTACTATTTTCCATTCTAGTA  900
2_B.scHwan      CTACGATCTATTCCAAACAAACTCGGAGGAGTCCTTGCATTACTATTCTCCATCCTAGTA  900
3_B.hetero      CTACGATCAATTCCAAACAAACTCGGAGGTGTTCTCGCATTACTATTTTCCATCCTAGTA  900
4_B.latice      CTACGATCAATCCCAAACAAACTAGGAGGTGTTCTCGCCCTACTTTTTTCAATCCTCGTA  900
                ******** ** ***** ***** ***** ** ** **  **** ** ** ** ** ***

1_B.gonion      CTAATAGTAGTGCCCCTACTACACACCTCAAAACAACGAGGACTGACATTCCGCCCAATC  960
2_B.scHwan      CTAATGGTAGTTCCCCTACTACACACCTCAAAACAACGAGGCCTAACATTCCGCCCAATT  960
3_B.hetero      TTAATAGTGGTACCACTATTACACACCTCAAAACAACGAGGATTAACATTCCGCCCAATC  960
4_B.latice      CTAATAGTGGTACCACTACTACACACCTCAAAGCAGCGAGGACTAACATTCCGCCCCCTC  960
                 **** ** ** ** *** ************* ** *****  * ***********  *

1_B.gonion      ACCCAATTCCTATTCTGAACCCTAGTAGCAGACATAATTATCCTAACCTGAATTGGAGGT 1020
2_B.scHwan      ACTCAATTCCTTTTCTGAACCCTAGTAGCAGACATAATCATCCTAACATGAATCGGAGGT 1020
3_B.hetero      ACCCAATTTCTCTTCTGAACCCTAGTAGCAGACATAGTTATTTTAACATGAATTGGAGGC 1020
4_B.latice      ACCCAATTCCTATTCTGAGCCCTAGTGGCAGATATGATTATCTTAACATGAATTGGGGGT 1020
                ** ***** ** ****** ******* ***** **  * **  **** ***** ** **

1_B.gonion      ATACCAGTAGAACATCCATTTATCATTATTGGACAAATCGCATCAATCCTATACTTCGCA 1080
2_B.scHwan      ATACCAGTAGAACACCCATTCATCATTATTGGACAAATCGCATCCGTTCTATACTTCGCA 1080
3_B.hetero      ATACCAGTAGAACACCCATTTATCATTATTGGACAAATCGCATCCATCTTATACTTCGCA 1080
4_B.latice      ATACCAGTAGAACACCCATTTATTATTATTGGACAAATTGCATCTGTATTATACTTCGCA 1080
                ************** ***** ** ************** *****  *  ************

1_B.gonion      CTGTTCCTAATCCTCATGCCACTAGCAGGATGACTAGAAAATAAAGCACTAGAATGAGCT 1140
2_B.scHwan      CTATTCCTCGTCCTCATACCATTAGCAGGATGATTAGAAAATAAAGCACTAGAATGAGCT 1140
3_B.hetero      CTATTCCTCATCTTCATTCCACTAGCAGGATGACTGGAAAATAAAGCACTAGAATGAGCC 1140
4_B.latice      CTATTCCTCATTTTCATCCCCCTGGCAGGATGATTAGAAAATAAAGCGCTAGAATGAGCT 1140
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Appendix Figure 2 (Continued)

Barbonymus gonionotus , Nature
Juvenile; SL= 16.5 cm

Hypsibarbus malcolmi, Nature
Juvenile; SL= 11.6 cm (L0201)

Hypsibarbus vernayi, Nature
Juvenile; SL= 13.2 cm (UB480704)

Hypsibarbus wetmorei, Nature
Juvenile; SL= 12.8 cm (NP0806)

Hypsibarbus vernayi, Nature
Adult; SL= 47.5 cm (UB480702)

Hypsibarbus wetmorei, Nature
Adult; SL= 30.8 cm (L490301)

Hypsibarbus vernayi, Fishery station
Subadult; SL= 25.9 cm (PB480301)

Hypsibarbus wetmorei, Fishery station
Subadult; SL= 21.9 cm (L490317)

Appendix Figure 3 The photograph of *B. gonionotus* and 3 *Hypsibarbus* spp.