**Using Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) of *Barbodes* spp. in Thailand**

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**Abstract**

Genetic diversity was studied in this research. Randomly amplified polymorphic DNA analysis was used to determine levels of genetic diversity of three species; *Barbodes altus*, *B. gonionotus* and *Barbodes* sp. Two of twelve screened decanucleotide primers (OPA07 and UBC122), were selected for the genetic analysis of *Barbodes* genera. Thirty-five reproducible and polymorphic fragments (250-2150 bp in length) were generated across the investigated species. The percentages of polymorphic band were 7.69 %, 3.85 % and 6.25 % for *B. altus*, *B. gonionotus* and *Barbodes* sp., respectively. The UPGMA indicated a similarity coefficient between *B. gonionotus* and *Barbodes* sp. up to 90 %. Moreover, the candidate species-specific marker of UBC122 primer was found in only *Barbodes* sp. This approach indicates that *Barbodes* sp. may be a variant species of *B. gonionotus.*

Key words : Genetic diversity, *Barbodes*, DNA analysis, RAPD-PCR

**Introduction**

The diverse family Cyprinidae, the most species-rich family of all vertebrates, has been paid only limited attention in population genetics studies. In the few studies available, the primary focus has been on a few species that have shared the status of being either commercially important or popular game fish; exemplified by studies on common carp, *Cyprinus carpio* L. (Desvignes *et al*., 2001, Fuchs *et al*., 2000 and Zhou *et al*., 2003 ), gold fish,

*Carassius auratus* L. ( Zhou *et al*., 2001), European chub, *Leuciscus cephalus* L. (Larno *et al*., 2005) and the genetic model species zebrafish, *Danio rerio* Hamiltion (Shimoda *et al*., 1999). The family Cyprinidae with about 2,000 species seems to have originated in Southeast Asia but now is also distributed in fresh water lakes, rivers and streams of Europe, Asia, Africa and North America. About half the known species are native to Asia. Many important ornamental and aquarium fish belong to this family, ranging from barbs, danios, and rasboras to goldfish such as *Carassius auratus* and carp, *Cyprinus carpio* which have ornamental varieties. Because of the great ecological and economical importance of Cyprinidae as cultured food fish, many species have been distributed almost globally from their original range in Eurasia. In Thailand, the genus *Barbodes* has been cultivated because of the occurrence of high-backed and deep-bodied individuals with few scales and rapid growth (Bond, 1996). Moreover, it is highly efficient in food utilization. It is also well accepted as food. Rainboth (1996) identified the genus *Barbodes* using such external characteristics as serrated dorsal-fin spines, 8 branched pelvic-fin rays, skin of the lower lip separated from the lower jaw by a shallow groove, anal-fin base length that is 90% of head length and no tubercles on the snout. He identified 3 *Barbodes* species in the Klong river: *Barbodes gonionotus, B. altus* and *B. schwanenfeldii*. These species are also distributed in rivers in every region of Thailand, and as a result genetic markers are unavailable for the great majority of cyprinids. Since 2004, a fisherman from Surin province in northeastern Thailand, has been culturing *B. gonionotus* for several generations and has found that the body color of new generations changed in some individuals. Also offspring tend to increase in numbers when inbreeding occurs. This new fish strain looks like *B. gonionotus* but its body color is golden.

Classification of *Barbodes* has been based principally on external morphological characters. However, characteristics are apparently influenced by a variety of habitats and environmental conditions. Thus, two sympatric species may be morphologically similar but misidentified as a single species. On the other hand, allopatric populations inhabiting different habitats may show ecomorphological variation and have questionable species status. The basic information on numbers of species and/or population in a particular area is important for conservation programs (Carvalho and Hauser, 1994). In an earlier study, Holmen *et al*., 2005 established a platform for optimization of microsatellite markers for six different cyprinids based on cross-species amplification of markers that initially were developed for *D. rerio* and central stoneroller (*Campostoma anomalum* Rafinesque). Knowledge of genetic variation of *Barbodes* species in Thailand is, therefore, important for creating appropriate management schemes. However, information about genetic diversity of *Barbodes* species in Thailand is presently unavailable. Therefore, the objectives of this study were to study external morphology and genetic diversity using randomly amplified polymorphic DNA-polymerase (RAPD-PCR) of three *Barbodes* species.

RAPD reactions are PCR reactions, but they amplify segments of DNA which are essentially unknown to the scientist (random). Often, PCR is used to amplify a known sequence of DNA. Thus, the scientist chooses the sequence he or she wants to amplify, then designs and makes primers which will anneal to sequences flanking the sequence of interest. Thus, PCR leads to the amplification of a particular segment of DNA. However, in RAPD analysis, the target sequence(s) (to be amplified) is unknown. The scientist will design a primer with an arbitrary sequence. In other words, the scientist simply makes up a 10 base pair sequence (or may have a computer randomly generate a 10 bp sequence), then synthesizes the primer. The scientist then carries out a PCR reaction and runs an agarose gel to see if any DNA segments were amplified in the presence of the arbitrary primer. We chose RAPD analysis for this study because it is a simple and rapid method for determining genetic diversity in various organisms with the advantage that no prior knowledge of the genome under study is needed or the polymorphic function as genetic markers. DNA markers offer accurate identification (Williams *et al.*, 1990; Weising *et al*., 1995). This information is valuable for varietial (strain) classification and cross-breeding between different species for potential improvement in commercial production (Purdom, 1993).

## Material and methods

### External morphology study

*Barbodes* sp. samples were obtained from a Surin province fish farm in northeastern Thailand. Twenty specimens of unknown species were studied using external morphological characteristics as described by Rainboth (1996).

### Samples and DNA extraction

Thirty authenticated specimen samples from 3 species, *Barbodes gonionatus, B. altus* and *Barbodes* sp., were collected from a Surin province fish farm. The musculature portion was excised and frozen immediately in liquid nitrogen. It was then kept at -80 °C until DNA extraction. Total cellular DNA extraction was isolated from each sample using a phenol-chloroform-proteinase K method (Winnepenninckx *et al*., 1993). DNA concentrations were determined spectrophotometrically (Maniatis *et al.*, 1982), and extracted DNA were stored at 4 °C until required.

### RAPD-PCR analysis

Twelve decanucleotide primers were screened for amplification success in 3 representative individuals of each species. UBC122 (5’GTAGACGAGC3’) and OPA07 (5’GGTGACGCAG3’) primers were selected for RAPD

analysis. Amplification was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1% Triton-X 100, 2 mM $MgCl_2$, 100 mM each of dNTP, 0.4 µM of a primer, 1 unit of DyNAzyme TM II DNA Polymerase (Finnzymes) and 25 ng of DNA template. PCR was performed in a thermal cycler (Franklin,USA) for 40 cycles including denaturation at 94 °C for 10 seconds, annealing at 36°C for 30 seconds and extension at 72 °C for 90 seconds. The final extension was performed at 72°C for 5 minutes. After amplification, PCR products were electrophoretically analyzed through 1.5% agarose gels, in 0.5XTBE buffer (50 mM Tris ,50mM Boric Acid, 2.5 mM EDTA, pH 8.3). Gels were stained with ethidium bromide, and photographed under UV light. The sizes of the amplified products were determined by comparison with a 100 bp ladder. Bands were scored as a binary variable, (1) for presence, and (0) for absence of band. Only distinct well-resolved stable bands were considered for resolving.

**Data analysis**

Band-sharing analysis (Jaccard's similarity coefficient ) was conducted. Cluster analysis and dendrograms based on the single linkage method were generated to estimate relationships among *Barbodes* species using NTSYS-pc (Rohlf, 1992). The dendrogram was constructed based on data obtained from the two primers.

**Results**

External morphological studies found that *Barbodes* sp had serrated dorsal-fin spine, soft fins (6-7), 8 branched pelvic fin rays and no adipose fin. The anal fin had a gold color. The skin of the lower lip was separated from the lower jaw by a shallow groove. The anal fin base length was 90% of head length. Other characteristics noted were a flat body shape, small head, sharp premaxilla, 2 barbels and no tubercles on the snout. The origin of the dorsal fins was opposite the tenth scale of the lateral line which had 29-31 scales. The body from head to tail was yellow without any red color so the fish was classified as *Barbodes* species. Characteristics such as the shape and fin of *Barbodes* sp. are similar to *B. gonionatus*, but *Barbodes* sp. has a golden color whereas *B. gonionatus* is white. The color of *Barbodes* sp. is similar to *B. altus*, but has no red band on the distal fin.

For the result of RAPD analysis, a total of 35 scorable bands ranging from 250-2150 bp was observed from analysis of three species; *Barbodes altus*, *B. gonionotus* and *Barbodes* sp. using RAPD analysis of two oligonucleotide primers (OPA07 and UBC122) (Fig. 1, 2 and Table 1). The number of amplified bands of all investigated samples were 13 bands for primers OPA07 and 22 bands for UBC122. The percentage of polymorphic bands for each primer was moderate (61.53-86.36 %).

The average number of bands per primer were 12 for *B. gonionotus*, 8 for *Barbodes altus* and 13 for *Barbodes* sp. The percentage of polymorphic bands of the respective species were 3.9, 6.3 and 7.7% (Table 2) indicating low genetic diversity in each species. The percentage of monomorphic bands were 96.15, 93.75 and 92.39 %, respectively.

**Candidate species-specific RAPD markers**

Several RAPD fragments showed fixed frequencies in each of the particular species. These could be used as species-specific markers to distinguish *Barbodes* species in northeastern, Thailand. The 580 bp and 890 bp bands generated from UBC122 were fixed in *B. altus*, but 420 bp, 600 bp, 720 bp and 950 bp bands were found in *B. gonionotus* and *Barbodes* sp. Both 820 bp and 950 bp bands were found only in *B. altus* and 670 bp in *B. gonionotus* and *Barbodes* sp. from OPA07. Thus a distinction between *B. altus* and *B. gonionotus* and *Barbodes* sp. could be recognized by the existence of the 580 bp band from UBC122 in *B. altus* but which was absent in *Barbodes* sp. (Fig. 1, 2 and Table 3).

Cluster analysis of the genetic distance values (Jaccard's coefficient) from UBC122 was conducted to generate a dendrogram indicating relationships between the *Barbodes* species. The genetic distance was 97 % between *Barbodes* sp. and *B. gonionotus*, and 24% between *B. altus*, *Barbodes* sp. and *B. gonionotus.* OPA07 was 100 % between *Barbodes* sp. and *B. gonionotus*, and 38% between *B. altus* and *Barbodes* sp. and *B. gonionotus*, (Fig. 3) which indicates that the relationships between *Barbodes* sp. and *B. gonionotus* is closer than *B. altus*.

**Discussion and conclusion**

External morphological studies found that *Barbodes* sp. have characteristics described in Rainboth (1996) such as serrated dorsal-fin spines, soft fins (6-7) and 8 branched pelvic fin rays. Anal fins are gold. The skin of the lower lip is seperated from the lower jaw by a shallow groove. Anal fin base length is 90% of head length, there are no tubercles on the snout, it has a flat body shape, small head, sharp premaxilla and 2 barbels. The origin of the dorsal fin is opposite the tenth scale of the lateral line which has 29-31 scales. The body from head to tail is yellow and there is no red coloration on the body or fins. Therefore the new individual is classified as *Barbodes* sp. The characteristics of *Barbodes* sp. are similar to those of *B. gonionatus* and *B. altus*, although it has no red coloration on the body or fins.

Two of twelve screened decanucleotides were selected for genetic analysis of *Barbodes* genera. The results show the RAPD pattern of *B. gonionotus* and *Barbodes* sp. as identical main bands after performing with OPA07

and UBC122 primers. RAPD fragments using primer UBC122 found 400 bp, 460 bp, 600 bp, 700 bp, 800 bp and 920 bp, whereas *Barbodes altus* did not show 400 bp. When using primer OPA07 RAPD fragments show 650 bp and 1400 bp main bands in *B. gonionotus* and *Barbodes* sp. but which are absent in *B. altus.* These results are supported by the external morphological results of *Barbodes* sp. which differed from *B. altus* and *B. gonionotus*. *Barbodes* is more similar in external morphology to *B. gonionotus* than *B. altus*. Thirty-five reproducible and polymorphic fragments (250-2150 bp in length) were generated across the investigated species. The percentages of polymorphic band were 7.69 %, 3.85 % and 6.25 % for *Barbodes altus*, *B. gonionotus* and *Barbodes* sp., respectively. However, the polymorphic values that were performed with both primers were lower than mud crabs in eastern Thailand. Genetic diversity among samples from interspecies was higher than those from intraspecies. The UPGMA indicated that relationships between *Barbodes gonionotus* and *Barbodes* sp. were closer than *B. altus*. Moreover, a candidate species-specific marker of UBC122 primer was found in only *Barbodes* sp.

From the above considerations a clear differentiation of the *Barbodes* sp. from *B. gonionotus* was achieved. In conclusion, this is the first report showing genetic diversity of *Barbodes* sp in Thailand. However, an RAPD approach also requires a good quality DNA template for reliable and consistent results which may not be possible for field specimens. Significantly, this may cause false negative results from investigated samples which actually are *Barbodes* fishes. To eliminate this problem, candidate RAPD fragments should be converted to sequence-characterized amplified region (SCAR) markers for the next time.

Identification of *Barbodes* species and evaluation of heritability for the growth rate of each *Barbodes* species are necessary steps for broodstock selection and selective breeding programs in these taxa. Our results illustrate that RAPD analysis is a rapid and convenient technique to generate useful genetic markers in *Barbodes*. Species-diagnostic RAPD markers should be developed which can be utilized to determine from generation to generation a comparison of growth performance of each *Barbodes* species under commercial growing conditions.

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Table 1 : Sequences of oligonucleotide primers, sizes and number of scorable RAPD bands, and percentage of polymorphic bands resulting from RAPD analysis using primers OPA07 and UBC122

| Primer | sequence | size range (bp) (no.of scorable bands) | percentage of polymorphic bands (no. of polymorphic bands) |
|---|---|---|---|
| OPA07 | GGTGACGCAG | 300-1300 (13) | 61.53 (8) |
| UBC122 | GTAGACGAGC | 250-2150 (22) | 86.36 (17) |
| Average | | | 77.14 (12.5) |

Table 2 : Total number of bands, and percentage of polymorphic and monomorphic bands of each *Barbodes* species in this study based on RAPD analysis using primers OPA07 and UBC 122

| | *B . gonionotus* | | | *B. altus* | | | *Barbodes* sp | | |
|---|---|---|---|---|---|---|---|---|---|
| | OPA07 | UBC122 | Total | OPA07 | UBC122 | Total | OPA07 | UBC122 | Total |
| No. of bands | 10 | 16 | 24 | 8 | 8 | 16 | 10 | 16 | 26 |
| No. of polymorphic bands | 0 | 1 | 1(3.9%) | 0 | 1 | 1(6.3%) | 0 | 2 | 2 (7.7%) |
| No. of monomorphic bands | 10 | 15 | 25 (96.2%) | 8 | 7 | 15(93.8%) | 10 | 14 | 24 (92.4%) |

Table 3 : Candidate species-specific RAPD markers of *Barbodes* sp. in northeastern Thailand revealed by RAPD analysis

| Primer | RAPD marker | Species/Genus | Specificity in target species(%) |
|---|---|---|---|
| OPA07 | 820 and 950 | *B. altus* | 100% |
| UBC122 | 420,600,720 and 950 | *B. gonionotus* and *Barbodes* sp. | 100% |
| | 580 and 890 | *B. altus* | 100% |

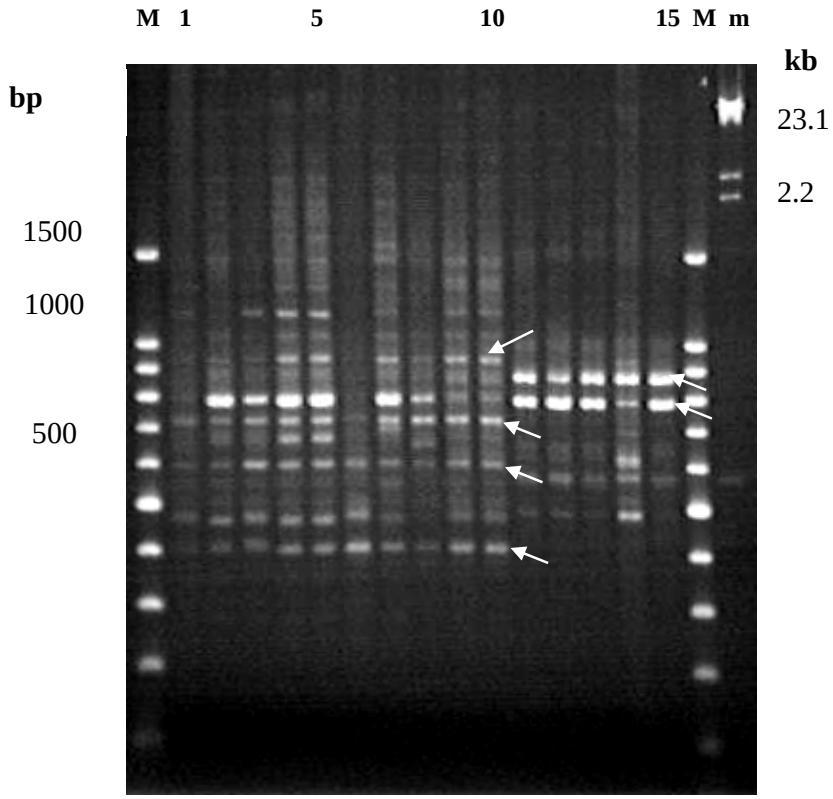


**Fig. 1** RAPD pattern of *Barbodes* sp. (lanes1-5) and *B. gonionotus* (lanes 6-10) and *B. altus* (lanes11-15) generated from RAPD-PCR using primer UBC122, a 100-bp ladder (lane M)and λ / *Hin*d III (lane m) were used as DNA markers. Arrowheads indicate species-diagnosetic RAPD bands described in Table 3.

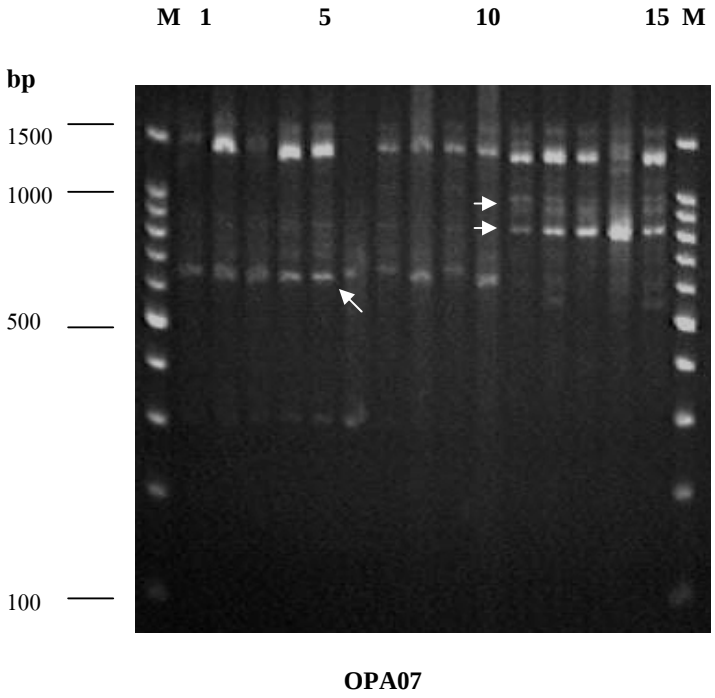


**Fig. 2** RAPD pattern of *Barbodes* sp. (lanes1-5) and *B. gonionotus* (lanes 6-10) and *B. altus* (lanes11-15) generated from RAPD-PCR using primer OPA07, a 100-bp ladder (lane M) was used as DNA markers. Arrowheads indicate species-diagnosetic RAPD bands described in Table 3.

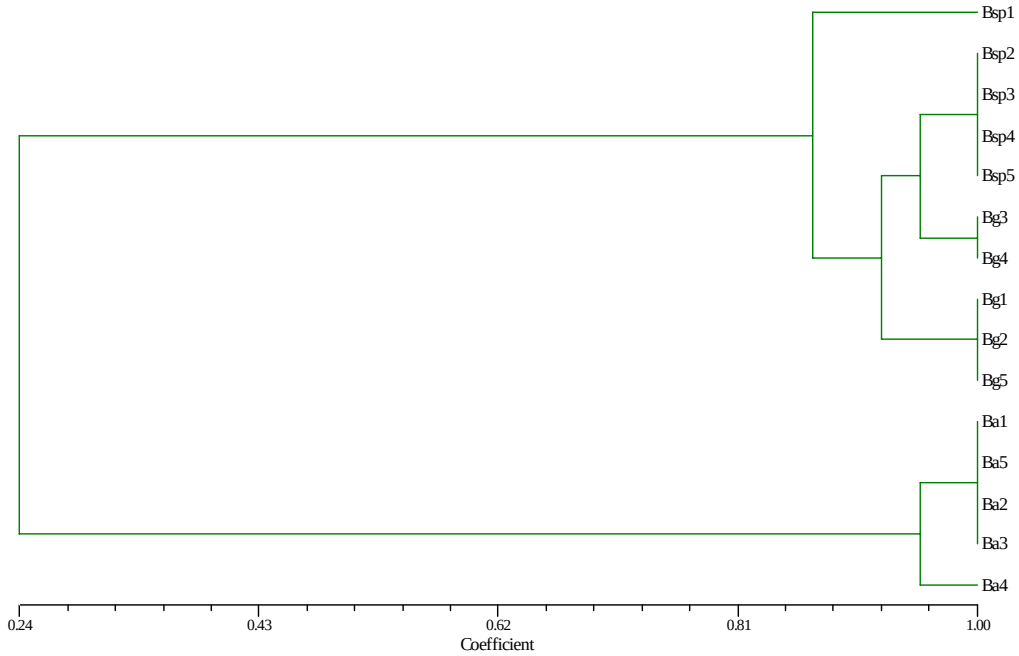


**Fig. 3** A hierarchical cluster analysis dendrogram of *Barbodes* sp.(Bsp), *B. gonionotus* (Bg) and *B. altus* (Ba) based on variation of RAPD pattern obtained with OPA07 and UBC122 primer