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Original Article

Species identification of non-hybrid and hybrid Pangasiid catfish using polymerase chain reaction-restriction fragment length polymorphism

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ABSTRACT

Species-diagnostic molecular markers are essential for the identification of species groups possessing overlapping morphological characters. They also play an important role in preventing the supply of incorrect species for the food industry and in verifying the species origins of various forms of products. Molecular genetic markers were developed for the identification of six Pangasiid species—*Pangasianodon gigas, Pangasianodon hypophthalmus, Pangasius bocourti, Pangasius conchophilus, Pangasius larnaudii* and *Pangasius sanitwongsei*—based on restriction analysis of mitochondrial *16S ribosomal* (*r*) *DNA* with *Tsp* 509I, *Hinc* II and *Mfe* I and nuclear *immunoglobulin M heavy chain constant region* (*IgM-H*) with *Taq* I and *Pst* I. Six non-overlapping composite restriction fragment length polymorphism (RFLP) patterns: AAB (AA) (AA), ACB(AA) (BB), BBB(BB) (BB), CCB(BB) (BB), DCA (BB) (CC) and DCB(−) (−) were observed across the respective species. Larvae of *P. gigas*, *P. hypophthalmus*, *P. bocourti*, *P. larnaudii* and *P. sanitwongsei* could be unambiguously differentiated using polymerase chain reaction (PCR)-RFLP analysis of *16S rDNA*. In addition, detection of *P. hypophthalmus* × *P. gigas*, *P. hypophthalmus* × *P. bocourti* and *P. hypophthalmus* × *P. larnaudii* hybrids could be carried out using PCR-RFLP analysis of *IgM-H*.

Introduction

The rapid increase in the global demand for *P. hypopthalmus* [production exceeding 1,100,000 t in 2014 (FAO, 2016) and an export value of approximately USD 1.6 billion (Globefish, 2016)] has triggered the expansion aquaculture involving of other Pangasiid catfish species in the lower Mekong basin. This includes basa (*Pangasius bocourti*) which is widely cultured in sections of the Mekong River basin in Thailand and Vietnam (Trong et al., 2002; Amornledpisan and Meng-umpan, 2010) and hybrids among member of the Pangasiid catfish group such as *P. hypophthalmus* × *P. gigas* (Meng-umpan, 2007). Apart from their economical importance, some Pangasiid catfish are important in terms of conservation. For example, the Mekong giant catfish (*Pangasianodon gigas*), which is endemic to the Mekong River, has been listed as a critically endangered species (IUCN, 2016) while *Pangasius sanitwongsei* is recognized as an endangered species in Thailand (Thailand Institute of Scientific and Technological Research, 1995; Na-Nakorn et al., 2009).

Identification of Pangasiid species based on morphology can be effectively applied in adults (Roberts and Vidthayanon, 1991), however, the morphological identification guidelines for larvae have not been well established for all Pangasiid species (Termvidchakorn and Hortle, 2013). In Thailand, inter-specific hybrids among Pangasiid species, such as *P. hypophthalmus* and *P. gigas*, (Mongkonpanya et al., 1996), *P. hypophthalmus* and *P. bocourti* (Annop Imsilp, personal communication), and *P. hypophthalmus* and *P. larnaudii* (Hanphongkittikul et al., 2010) have been produced and some have been adopted for aquaculture (Meng-umphan, 2007). Some of the hybrids were capable of reproduction (Panasey et al., 2013) and this increases the risk of genetic introgression among species as has been shown in some fish species (Na-Nakorn et al., 2004; Senanan et al., 2004; Prado et al., 2012). Moreover, the hybrids could not be unambiguously differentiated based on only morphological characters and this may lead to an admixture of parental species with hybrids, leading to genetic contamination of broodstock (Hashimoto

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et al., 2011; Prado et al., 2012). Furthermore, morphology-based species identification is not possible when the processed products of such fish are examined.

Molecular markers (such as polymerase chain reaction-restriction fragment length polymorphism; PCR-RFLP and single strand conformational polymorphism; SSCP) have been used for *P. hypophthalmus* product authentication (Rehbein, 2008; Wong et al., 2014). Hybrids of *P. hypophthalmus* × *P. gigas* could be discriminated using PCR-RFLP of the immunoglobulin M heavy chain (*IgM-H*) (Thongpan et al., 1997). Five Pangasiid species (*P. hypophthalmus, P. gigas, P. bocourti, P. larnaudii and Helicophagus leptorhynchus*) and their larvae were successfully identified using SSCP analysis. However, the patterns obtained from hybrids between *P. hypophthalmus* × *P. gigas* or *P. hypophthalmus* × *P. bocourti* could not differentiate the hybrids from the parental species (Sriphairoj et al., 2010). In the present study, PCR-RFLP was selected because of its advantages of simplicity and requiring less time (Teletchea, 2009) and the availability of the 16S rDNA sequence deposited in GenBank (Na-Nakorn et al., 2006).

This study focused on applying PCR-RFLP of mitochondrial *16S rDNA* and nuclear *IgM-H* gene segments for identification of six economically important Pangasiid catfishes in Thailand. Species origins of their larvae and *P. hypophthalmus* × *P. gigas*, or *P. hypophthalmus* × *P. bocourti* or *P. hypophthalmus* × *P. larnaudii* hybrids were assessed using either PCR-RFLP of the former or the latter gene segment. The developed markers are useful for species identification of wild and captive Pangasiid species as well as for the identification of hybrids among these species.

Materials and methods

Fish samples

Wild or captive adults of both of six Pangasiid species: *Pangasianodon gigas* (Pg, $N = 45$), *P. hypophthalmus* (Ph, $N = 37$), *Pangasiusbocourti* (Pb, $N = 38$), *P. conchophilus* (Pc, $N = 23$), *P. larnaudii* (Pl, $N = 49$) and *P. sanitwongsei* (Ps, $N = 51$), were collected from various locations in Thailand (Table 1). The species abbreviations in the parentheses above have been used in this study report. Post-hatch larvae of Pg ($N = 12$), Ph ($N = 6$), Pl ($N = 6$), Pb ($N = 11$) and Ps ($N = 41$) were also collected. In addition, hybrids of Ph (dam) × Pb (sire) ($N = 20$), were taken from the Sakon Nakorn Inland Fisheries Research and Development Centre, Department of Fisheries, Thailand. The presumed hybrids, Ph(dam) × Pg (sire) ($N = 10$) and Ph (dam) × Pl (sire) hybrids ($N = 27$) were collected from a private hatchery in Nakhonsawan province, Thailand and a fish market in Ubonratchatani province, Thailand, respectively. Fin clips of approximately 1 cm^2 were dissected out from adult fish and placed in absolute ethanol. The whole bodies of post-hatch larvae were collected and preserved in absolute ethanol. Specimens were transported back to the Fish Genetics Laboratory at Kasetsart University, Bangkok, Thailand and kept in a −20 °C freezer until needed.

DNA extraction

Genomic DNA was extracted from each specimen following the standard phenol-chloroform protocol (Taggart et al., 1992). Concentrations of extracted DNA were spectrophotometrically determined. Genomic DNA was kept at 4 °C until used.

Bioinformatic analysis

The genetic diversity and population structure of six Pangasiid species has been previously published based on polymorphism of *16S rDNA* (Na-Nakorn et al., 2006). Nucleotide sequences of Pg (4 haplotypes), Ph (8 haplotypes), Pb (10 haplotypes), Pc (3 haplotypes), Pl (11 haplotypes) and Ps (6 haplotypes) were retrieved from GenBank (http://ncbi.nlm.nih.gov) and multiply aligned using ClustalW (Thompson et al., 1994). Informative restriction endonucleases for differentiation of these species were predicted using NEBcutter V2.0 (Vincze et al., 2003).

Restriction analysis of mitochondrial16S rDNA

Initially, the partial *16S rDNA* gene segment of random samples of each species ($N = 3$) was amplified. PCR was carried out in a 30 μl reaction volume containing 50 ng genomic DNA, 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM each of dNTPS, 0.5 μM each of primers 16Sar (5′-CGCCTGTTTAACAAAAACAT-3′) and 16Sbr (5′–CCGGTC TGAACTCAGATCATG T-3′) (Palumbi et al., 1991) and 1 unit of *Taq* Polymerase (Promega; Madison, WI, USA). The thermal profile was initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 5 min. Then, 5 μL of each amplification reaction were electrophoresed through a 1.0% agarose gel to determine whether the resulting fragment was successfully amplified. The amplified product of each individual was purified using a PCR fragments extraction kit (RBC Bioscience Corp.; New Taipei City, Taiwan). Sequencing in both directions was conducted for confirmation of selected restriction endonucleases. Analysis of previously deposited *16S rDNA* sequences of six Pangasiid species (Na-Nakorn et al., 2006) revealed that *Tsp* 509I, *Hinc* II and *Mfe* I were informative for differentiation of the examined species and the preliminary study on restriction analysis of *16S rDNA* (approximately 610 bp) in limited samples ($N = 3$ for each species) confirmed the predicted results. These enzymes were further used for digestion of *16S rDNA* in non-hybrid Pangasiid adults ($N = 92–205$) and larvae ($N = 24–76$). Six microliters of the amplicon were singly digested with *Tsp* 509I, *Hinc* II and *Mfe* I in a 15 μl reaction volume using the conditions recommended by the manufacturer (Fermentas; Waltham, MA, USA). The digests were electrophoretically analyzed through 2.0% agarose gels and visualized in a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001). Subsequently, *16S rDNA* of adults and post-hatch larvae of each species was amplified (Table 2). PCR-RFLP was carried out as described above.

Restriction analysis of nuclear IgM-H

The nuclear *IgM-H* gene segment of *P. hypophthalmus*, *P. gigas*, and *P. bocourti* ($N = 2$ for each species) was amplified following the conditions described by Thongpan et al. (1997). The purified amplicons were sequenced and bioinformatically analyzed as described above. Two enzymes (*Pst* I and *Taq* I) were selected from sequence and restriction analyses of *IgM-H* (approximately 300 bp, $N = 2$). Subsequently, restriction analysis was carried out in parental species; Pg ($N = 19$), Ph ($N = 18$), Pb ($N = 18$), Pc ($N = 5$), Pl ($N = 18$) and in hybrids of Ph × Pb ($N = 20$), the presumed Ph × Pg ($N = 10$) and Ph × Pl ($N = 27$). *P. sanitwongsei* has not been used for hybrid production, and so the *IgM-H* gene of this species was not examined. Restriction patterns generated from each restriction endonuclease were given letter designations alphabetically according to their appearance. The composite PCR-RFLP patterns were constructed from combinations of restriction patterns of *16S rDNA* and *IgM-H*.

Ethics statement

All experimental procedures using the animal conformed to the guidelines established by the Animal Care Committee, Kasetsart

Table 1
Sample localities and sample sizes of six Pangasiid species used in this study.

Species/Locality	Sample size (*N*)	
	Adult	Larvae
Non-hybrid specimens		
Pangasianodon gigas (*N* = 45)		
Inland Fisheries Research Institute, Ayutthaya province	1	–
Chiangmai Inland Fisheries Research and Development Centre	14	12
Phayao Inland Fisheries Research and Development Centre	5	–
Pitsanulok Inland Fisheries Research and Development Centre	7	–
Kalasin Inland Fisheries Station	4	–
Tak Inland Fisheries Research and Development Centre	5	–
Songkla Inland Fisheries Research and Development Centre	5	–
Hatchery, Chaomudcha Farm, Supanburi province	3	–
Tonle Sap, Cambodia	1	–
Pangasianodon hypophthalmus (*N* = 37)		
Yasothon Inland Fisheries Research and Development Centre	–	6
Nongkhai Inland Fisheries Research and Development Centre	5	–
Sakaekrang River, Uthaitani province	6	–
Patumthani province	9	–
Chiangrai province	3	–
Tonle Sap, Cambodia	3	–
Hatchery, Narong Farm, Nakornsawan province	11	–
Pangasius bocourti (*N* = 38)		–
Nakornpanom province	32	–
Chiangrai province	4	–
Ubonratchatani province	2	–
SakonNakorn Inland Fisheries Research and Development Centre	–	11
Pangasiusconchophilus (*N* = 23)		
Mukdahan province	4	–
Nongkhai province	10	–
Nakornpanom province	3	–
SakonNakorn Inland Fisheries Research and Development Centre	6	–
Pangasiuslarnaudii (*N* = 49)		
Nakornpanom province	1	–
Khongjiem, Ubonratchatani province	7	–
Hatchery, Ubonratchatani province	2	–
Yasothon Inland Fisheries Research and Development Centre	6	6
Mekong River, Cambodia	5	–
Pitsanulok province	5	–
Pichit province	7	–
Uthaitani province	3	–
Chainat province	9	–
Patumtani province	4	–
Pangasiussanitwongsei (*N* = 51)		
Nongkhai Inland Fisheries Research and Development Centre	10	–
Nakornpanom province	14	–
Khongjiem, Ubonratchatani province	4	–
SakonNakorn Inland Fisheries Research and Development Centre	10	–
Chiangrai province	6	–
Chainat province	7	–
Phayao Inland Fisheries Research and Development Centre	–	41
Hybrid specimens		
P. hypophthalmus × *P. Gigas*[a]		
Private hatchery, Nakornsawan province	10	
P. hypophthalmus × *P. bocourti*		
SakonNakorn Inland Fisheries Research and Development Centre	20	
P. hypophthalmus × *P. larnaudii*[a]		
Fish market, Ubonratchatani province	19 (fin clip)	

[a] Specimens were collected by colleagues and external morphology was not available.

University, Thailand. This study was approved by the Animal Ethics Committee of Kasetsart University (Approval no. ACKU 60-ETC-002).

Results

PCR-RFLP analysis of 16S rDNA and IgM-H in adult Pangasiid catfishes

In total, four, three and two restriction patterns were generated from restriction analysis of *16S rDNA* with *Tsp* 509I, *Hinc* II, and *Mfe* I, respectively. Each of the restriction patterns was designated with an alphabetical code, for example, Pg and Ph shared pattern A of the *Tsp* 509I restricted *16S rDNA*; Pb, Pc showed pattern B, and C, respectively; and Pl and Ps shared pattern D (Fig. 1(A)). RFLP patterns A, B, C, and D of *Tsp* 509I RFLP revealed two (420 and 190 bp), three (325, 190 and 95 bp), three (325, 155 and 95) and three (309, 190 and 95) noticeable DNA bands on 2% agarose gel, respectively (Table 2). Likewise, restriction patterns of *Hinc* II and *Mfe* I were named accordingly (Table 2). RFLP patterns A, B and C of *Hinc* II showed two (365 and 245 bp), one (305 bp) and one (610 bp) DNA fragments on agarose gel, respectively (Table 2). Pg and Pb showed pattern A and B, respectively. Ph, Pc, Pl and Ps revealed pattern C (Fig. 1(B)). RFLP pattern A and B of *Mfe* I showed one DNA band of

Table 2
Restriction fragment patterns of mitochondrial DNA of Pangasiid catfish from restriction analysis of the 16S rDNA and *IgM-H* gene segments.

PCR-RFLP	Restriction pattern (fragment size)	Species	Adult (*N*)	Larvae (*N*)
16S rDNA				
Tsp 509I	A (420 and 190 bp)	*Pangasianodon gigas*	45	12
		Pangasianodon hypophthalmus	37	6
	B (325, 190 and 95 bp)	*Pangasius bocourti*	38	11
	C (325, 155 and 95 bp)	*Pangasius conchophilus*	23	–
	D (309, 190 and 95 bp)	*Pangasius larnaudii*	49	6
		Pangasius sanitwongsei	51	41
Hin cII	A (365 and 245)	*Pangasianodon gigas*	45	12
	B (305 and 305 bp)	*Pangasius bocourti*	2	–
	C (610 bp)	*Pangasianodon hypophthalmus*	37	6
		Pangasius conchophilus	1	–
		Pangasius larnaudii	2	6
		Pangasius sanitwongsei	5	–
Mfe I	A (305 and 305 bp)	*Pangasius larnaudii*	49	6
	B (610 bp)	*Pangasianodon gigas*	6	1
		Pangasianodon hypophthalmus	5	6
		Pangasius bocourti	3	1
		Pangasius conchophilus	5	–
		Pangasius sanitwongsei	51	7
IgM-H				
Pst I	AA (227, 52 and 31 bp)	*Pangasianodon gigas*	19	–
		Pangasianodon hypophthalmus	18	–
	BB (258 and 52 bp)	*Pangasius bocourti*	18	–
		Pangasius conchophilus	5	–
		Pangasius larnaudii	18	–
Taq I	AA (170 and 140 bp)	*Pangasianodon gigas*	19	–
	BB (170, 100 and 40)	*Pangasianodon hypophthalmus*	18	–
		Pangasius bocourti	18	–
		Pangasius conchophilus	5	–
	CC (272 and 38)	*Pangasius larnaudii*	18	–

305 and 610 bp, respectively (Table 2). Pl showed pattern A, whereas the remaining species revealed pattern B (Fig. 1(C)). PCR products of the five species of non-hybrid larvae (*P. gigas*, *P. hypophthalmus*, *P. bocourti*, *P. conchophilus*, *P. larnaudii* and *P. sanitwongsei*; $N = 24–76$, Table 2) were also examined using *Tsp* 509I, *Hinc* II and *Mfe* I. The restriction pattern of larvae of a given species was perfectly matched with that found in adults of the respective species.

In addition, the amplified *IgM-H* gene segments of all species except Ps were genotyped. Restriction analysis of *IgM-H* with *Pst* I and *Taq* I gave two and three digestion patterns, which were designated with alphabetical codes, respectively (Fig. 2 and Table 2). Two RFLP patterns were obtained from *Pst*I digestion. Three fragments (227, 52 and 31 bp) were generated for Pg and Ph (genotype AA) and two fragments (258 and 52 bp) were obtained for Pb, Pc and Pl (genotype BB). Three RFLP patterns were obtained from *Taq* I digestion. Two fragments of 170 and 140 bp were generated for Pg (genotype AA) and 272 and 38 bp for Pl (genotype CC). Three fragments of 170, 100 and 40 bp were generated for Ph, Pb and Pc (genotype BB) (Fig. 2 and Table 2). Three species (Pg, Ph and Pl) could be clearly differentiated using PCR-RFLP with both *Pst* I and *Taq* I. However, the Igm-H genotype of Pb and Pc was identical.

No intraspecific polymorphism was observed from each restriction enzyme. The six Pangasiid species in this study could not be differentiated by a single enzyme digestion of either gene. Composite restriction patterns from both gene segments of each specimen were generated and six composite patterns: AAB (AA) (AA), ACB(AA) (BB), BBB(BB) (BB), CCB(BB) (BB), DCA (BB) (CC) and DCB(−) (−), were observed across specimens (Table 3). Considering only those from *16S rDNA* or both gene segments, composite restriction enzyme digestion patterns did not overlap between species. In contrast, distributions of *IgM-H* composite restriction patterns alone overlapped between Pb and Pc where Ps was not genotyped for this gene (Table 3). Accordingly, species-diagnostic molecular markers were successfully developed in the examined species.

Identification of parental species of Pangasiid hybrids using IgM-H polymorphism

The species differentiation capacity of these markers was verified in the presumed hybrid specimens collected from farms and fish markets in northeast of Thailand. Among the samples analyzed, 20 individuals were unambiguously identified as hybrids between Ph × Pb by restriction patterns of *IgM-H*. In addition, 10 individuals presumed to be hybrids between Ph × Pg were revealed to comprise seven hybrids (genotypes AA and AB, respectively, to *Pst* I and *Taq* I and three individuals exhibiting genotypes unique to Ph (AA and BB for *Pst* I and *Taq* I, respectively; Fig. 2). Among the presumed Ph × Pl hybrid specimens collected from the fish market ($N = 27$), 19 individuals were Ph × Pl hybrids having a (AB) (BC) genotype (respective to *Pst* I and *Taq* I; Fig. 2), whereas the remaining individuals possessed an (AB) (BB) genotype indicating that they were Ph × Pb hybrids (data not shown).

Discussion

Species-specific PCR-RFLP markers were successfully developed for reliable species identification of six Pangasiid species consisting of Pg, Ph, Pb, Pc, Pl and Ps adults and larvae using restriction analysis of *16S rDNA* with a minimum of three restriction enzymes (*Tsp* 509I, *Hinc* II and *Mfe* I). Notably, the unavailability of Pc larvae prevented the inclusion of this species in the larval species identification. Previously, an AFLP-derived SCAR marker, *PL8*, was successfully developed for identification of species origins of Pg, Ph, Pb and Pl based on SSCP analysis; no intraspecific polymorphism of *PL8* was observed in these species (Sriphairoj et al., 2010). Nevertheless, the main disadvantage of PCR-SSCP is the reproducibility of the technique because SSCP patterns are strongly affected by temperature and the degree of gel cross-linking. Additionally, multi-allelic patterns of some nuclear DNA markers may make SSCP patterns too complicated for accurate determination of species

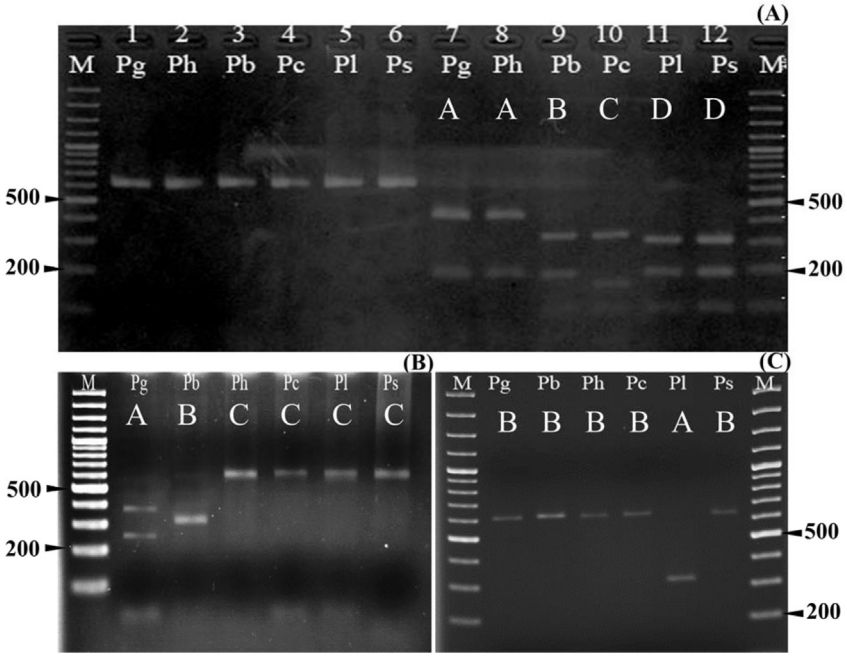

Fig. 1. Restriction analysis of the amplified *16S rDNA* gene segment of adult catfish with *Tsp*509I (A), *Hin* cII (B) and *Mfe* I (C). Four, three and two restriction patterns were observed from respective enzymes. The alphabetical codes under species name abbreviations indicate restriction patterns. Pg = *P. gigas* (Pattern AAB), Ph = *P. hypophthalmus* (Pattern ACB), Pb = *P. bocourti* (Pattern BBB), Pc = *P. conchophilus* (Pattern CCB), Pl = *P. larnaudii* (Pattern DCA) and Ps = *P. sanitwongsei* (Pattern DCB). Lanes 1–6 are 610 bp undigested polymerase chain reaction product of the six species. Lane M is the 100 bp DNA marker.

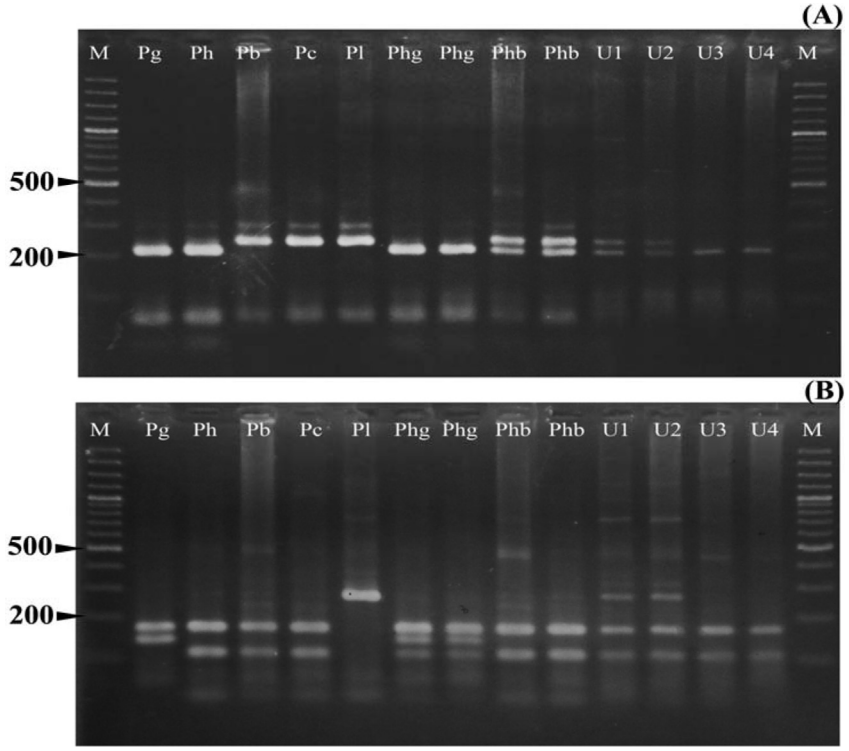

Fig. 2. Restriction analysis of the amplified *IgM-H* gene segment of Pangasiid catfish with *Pst* I (A) and *Taq* I (B). Two and three restriction patterns were observed from *Pst* I and *Taq* I, respectively. Pg = *P. gigas*, Ph = *P. hypophthalmus*, Pb = *P. bocourti*, Pc = *P. conchophilus*, Pl = *P. larnaudii*, Phg = hybrid offspring between *P. hypophthalmus* × *P. gigas*, Phb = hybrids offspring between *P. hypophthalmus* × *P. bocourti*, U1–U4 = unknown Pangasiid species. Polymerase chain reaction restriction fragment length polymorphism indicated that U1 and U2 are *P. hypophthalmus* × *P. larnaudii* hybrids while U3 and U4 are non-hybrid *P. hypophthalmus*. Lane M = the 100 bp DNA ladder.

origins of admixture samples (mixed species in a single sample). As a result, more reliable and simple species-diagnostic markers for identification of Pangasiid catfish still need to be developed.

Species-diagnostic molecular markers should be established from DNA segments exhibiting low genetic polymorphism within a particular species but showing high genetic divergence between

Table 3
Molecular diagnostic markers of Pangasiid species based on polymerase chain reaction restriction fragment length polymorphism (RFLP) analysis.

Composite RFLP pattern[a]	Species
AAB (AA) (AA)	*Pangasianodon gigas*
ACB(AA) (BB)	*Pangasianodon hypophthalmus*
BBB(BB) (BB)	*Pangasius bocourti*
CCB(BB) (BB)	*Pangasius conchophilus*
DCA(BB) (CC)	*Pangasius larnaudii*
DCB(−) (−)	*Pangasius sanitwongsei*

[a] Composite restriction patterns are arranged from single RFLP patterns of *16S rDNA* digeted with *Tsp*509I, *Hin* cII and *Mfe* I followed by those of *immunoglobulin M heavy chain* digested with *Pst* I and *Taq* I (in brackets), respectively. − = not determined.

different species. Overlapping patterns among different species or false positive and negative results or both should not exist (Thaewnon-ngiw et al., 2004). In addition, intraspecific genetic divergence within each species resulted in difficulties for the application of non-sequencing DNA techniques, for example, PCR-SSCP (Sriphairoj et al., 2010) and PCR-RFLP (the present study) in identifying these species. The levels of intraspecific variability should be carefully evaluated from many individuals covering the geographical range of each species (Sanjuan and Comesana, 2002; Civera, 2003) before non-sequencing species authentication approaches are implemented in practice. In the present study, specimens of each species were collected from different geographical locations and commercial farms. Therefore, the lack of intraspecific variability of restriction patterns observed herein suggested that they were conserved within species. This reflects the discriminating power of PCR-RFLP in the Pangasiid species in the present study.

Molecular genetic markers facilitate the correct species identification of adults and early development stages (egg, larvae) of fish which is useful for fishery biology studies. PCR-RFLP markers have been used to identify adult and egg/larvae of many marine species, the obtained information was applied for stock assessment and indicating catch rates (Li et al., 2006; Karaiskou et al., 2007; McKeown et al., 2015; Schmidt et al., 2015). Furthermore, the ability to discriminate early life history stages could be useful for the construction of spawning maps (Munk et al., 2009). The present results illustrated useful genetic markers in all examined species. Species-diagnostic PCR-RFLP patterns can be utilized to identify seed species and their distribution in natural resources.

In addition, due to increasing demand for Ph in global markets (Globefish, 2016), the authentication of Pangasiid catfish and catfish-derived products is important. PCR-RFLP markers have been developed and used for the identification of Ph and Pb (Rehbein, 2008; Wong et al., 2014) and their results demonstrated that the developed markers had more advantages than traditional DNA barcoding (Wong et al., 2014). The present study has reported for the first-time on molecular markers for six economically important Pangasiid catfishes. These markers would be useful for authentication of Pangasiid catfish and catfish-derived products in the food industry.

Interspecific hybrids may possess a serious threat to native species. The distinction between parental species and hybrids is a crucial step in developing conservation plans (Allendorf et al., 2001). Misidentification of parental species and hybrids may easily occur if the identification is based solely on morphology due to a phenotypic variation of hybrid (Prado et al., 2012). The application of molecular genetic markers greatly facilitates hybrid identification (Allendorf et al., 2001). PCR-RFLP has been used as an efficient tool for the detection of hybrids in aquaculture and of genetic introgression in wild stock (Hashimoto et al., 2010, 2011; Prado et al., 2012). Interspecific hybridization among Pangasiid species was documented as yielding reproductively fertile hybrids, for example, the hybrid of Ph × Pg (Panasey et al., 2013) and hence raised concerns on genetic introgression into wild populations (Epifanio and Philipp, 2001; Prado et al., 2012). As yet, evidence of gene introgression in natural populations of Pangasiid species has not been reported (Sriphairoj et al., 2010). This may be due partly to a lack of appropriate markers. In the present study, restriction analysis of nuclear *IgM-H* provided unambiguous identification of the hybrids in all cases. Therefore, the molecular markers developed herein would facilitate precise monitoring of genetic introgression in natural populations and hence may lead to development of conservation plans.

Although restriction analysis of *IgM-H* with *Pst*I and *Taq*I allows for identification of hybrids between Ph and the other three species, additional markers are still needed for authentication of those originating from complex hybridization circumstances (for example, subsequent generation of backcrosses or those generated from more than two parental species).

Conflict of interest

There is no conflict of interest in this research.

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