

Full Paper

Identification of *Pagasianodon gigas*, *P. hypophthalmus* and their hybrids using amplified fragment length polymorphism markers

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Received: 18 August 2013 / Accepted: 29 September 2014 / Published: 9 October 2014

Abstract: Freshwater Pangasiid catfish species and hybrids are difficult to identify on the basis of morphological characteristics, especially for larvae and fingerlings. In this study, we identified genetic markers for Pangasiid catfish breeds, viz. *Pagasianodon gigas*, *Pagasianodon hypophthalmus* and their F₁ and F₂ hybrids. Twenty primer combinations were used to screen for specific amplified fragment length polymorphism (AFLP) markers. A total of 486 DNA bands were identified and 335 polymorphic bands were found to be segregated among these catfish types. The level of polymorphism detected using 20 different primer combinations ranged between 65.38-100%. Eleven distinct markers were found to be significantly associated with *P. gigas* (two markers), *P. hypophthalmus* (three markers), their F₁ hybrid (three markers) and F₂ hybrid (three markers). Using these markers, 100% of the individuals were accurately assigned to their correct species. These results demonstrate that AFLP markers can be used to genetically identify Pangasiid catfish species and hybrids.

Keywords: *Pagasianodon gigas*, *P. hypophthalmus*, Pangasiid catfish, AFLP markers, species identification, F₁ hybrid, F₂ hybrid

INTRODUCTION

Freshwater catfish from the Pangasiidae family represent an economically important protein source for local consumers in South-east Asia, including *Pangasius bocourti* in Vietnam [1], *P. djambal* in Indonesia [2] and *Pagasianodon hypophthalmus* in Thailand [3]. Adult Pangasiid catfish can be identified on the basis of morphology [4]; however, morphological characteristics are not enough to differentiate larvae and fingerlings from different species [5]. Most fish farmers also have

difficulties distinguishing between pure line and hybrid individuals. DNA-based techniques have been developed and proved to be analytically accurate in identifying species [6, 7]. These methods can help to identify and authenticate fish and seafood species. They include techniques such as restriction fragment length polymorphism (RFLP), single strand conformation polymorphism (SSCP), forensically informative nucleotide sequencing (FINS) and amplified fragment length polymorphism (AFLP). These methods have been used to identify of numerous fish species and other aquatic organisms [8] including gadoids [9, 10], flatfish [11, 12], salmonids [13, 14], scombroids [15, 16], sardines and anchovies [17-19], and mollusks [20, 21]. Mitochondrial DNA and nuclear ribosomal DNA fragments have recently been proposed as additional methods for species identification [22, 23].

AFLP was first described by Vos et al. [24]. It is essentially a fingerprinting technique that draws upon aspects of randomly amplified polymorphic DNA (RAPD) and RFLP techniques [25]. AFLP is the tool of choice when it comes to unravelling genetic information of complex traits, as it is a fast and inexpensive tool particularly suitable for screening the genome [25-27]. AFLP, like the RAPD technique, presents the advantages of being capable of differentiating among species even in the absence of prior knowledge of the DNA sequence. In addition, AFLP shows greater levels of polymorphism and reproducibility than does RAPD [28, 29]. However, many studies using AFLP have used the data to construct genetic linkage maps rather than to differentiate among species. In this study we investigate the potential of AFLP markers to identify and classify two Pangasiid catfish species and their two hybrids.

MATERIALS AND METHODS

Animal Samples

A total 16 individual catfish comprising *Pagasianodon gigas* (n = 4), *P. hypophthalmus* (n = 4), their F₁ hybrid (n = 4) and F₂ hybrid (n = 4) were cultured and collected as samples. The specimens were obtained from the hatcheries of the Plabuk Integrated Utilisation Project at the Faculty of Fisheries and Aquatic Resources, Maejo University.

DNA Extraction

Total genomic DNA was extracted from fish fin samples using phenol-chloroform extraction procedure adapted from that of Sambrook and Russel [30]. The samples were cut in small pieces and suspended in 700 µL of TNES-urea buffer (1mM Tris-HCL pH 8.0, 6 M NaCl, 0.5M EDTA pH 8.0, 10% sodium dodecyl sulphate, and 4M urea) with 4 µL of proteinase K solution (20 µg/mL) (Invitrogen, USA). After adding the proteinase K, the samples were incubated at 37°C overnight. An equal volume of phenol-chloroform (1:1 v/v) was added and the two phases were mixed until an emulsion formed. The two phases were separated by centrifugation at 10,000 rpm for 10 min. The aqueous supernatant solution was collected in fresh tubes. The phenol-chloroform extraction procedure was repeated twice. Sodium acetate (3.0M, pH 5.2) in 1/10 volume and equal volume of isopropanol were added to the collected supernatant and the samples were shaken gently until DNA precipitated. The precipitated DNA was washed twice with 70% ethanol and dried. Finally, the DNA was resuspended in Tris-EDTA buffer and kept at 4° until further use. The final DNA concentration from each sample was tested using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc.) and measured on 1% agarose-gel electrophoresis.

AFLP Marker Methodology

The AFLP assay was performed using the protocols described by Wimmers et al. [31]. The DNA of the 16 individual fish was analysed using the adapter and primer sequences shown in Table 1. In brief, 100 ng of genomic DNA was digested with two enzymes: FastDigest[®] *EcoRI* and *TaqI* restriction endonuclease (Fermentas, USA) following the manufacturer's instructions. The digested products were ligated to 50 μmols of *EcoRI*-Adapters and 50 μmols of *TaqI*-Adapters (Fermentas, USA) in 30 μL of a solution containing 6 U T4 DNA ligase (Fermentas, USA) and 46mM ATP (Fermentas, USA) and incubated overnight at 4° to ensure ligation. The concentration of the ligated DNA templates was adjusted to 10 ng/μL and diluted fivefold with distilled water for pre-selective amplification.

Table 1. AFLP primer combinations used to generate polymorphism markers between *P. gigas*, *P. hypophthalmus*, F₁ hybrid and F₂ hybrid (The combinations were randomly designed from *TagI* and *EcoRI* nucleotide adapters.)

Combination	Primer- <i>EcoRI</i>	Primer- <i>TaqI</i>
1	5-GACTGCGTACCAATTC ACC -3	5-GATGAGTCCTGACCGA CAC -3
2	5-GACTGCGTACCAATTC ACC -3	5-GATGAGTCCTGACCGA CAT -3
3	5-GACTGCGTACCAATTC ACC -3	5-GATGAGTCCTGACCGA CGA -3
4	5-GACTGCGTACCAATTC ACC -3	5-GATGAGTCCTGACCGA CTG -3
5	5-GACTGCGTACCAATTC ACC -3	5-GATGAGTCCTGACCGA CGT -3
6	5-GACTGCGTACCAATTC ACG -3	5-GATGAGTCCTGACCGA CAC -3
7	5-GACTGCGTACCAATTC ACG -3	5-GATGAGTCCTGACCGA CAT -3
8	5-GACTGCGTACCAATTC ACG -3	5-GATGAGTCCTGACCGA CGA -3
9	5-GACTGCGTACCAATTC ACG -3	5-GATGAGTCCTGACCGA CTG -3
10	5-GACTGCGTACCAATTC ACG -3	5-GATGAGTCCTGACCGA CGT -3
11	5-GACTGCGTACCAATTC AAG -3	5-GATGAGTCCTGACCGA CAC -3
12	5-GACTGCGTACCAATTC AAG -3	5-GATGAGTCCTGACCGA CAT -3
13	5-GACTGCGTACCAATTC AAG -3	5-GATGAGTCCTGACCGA CGA -3
14	5-GACTGCGTACCAATTC AAG -3	5-GATGAGTCCTGACCGA CTG -3
15	5-GACTGCGTACCAATTC AAG -3	5-GATGAGTCCTGACCGA CGT -3
16	5-GACTGCGTACCAATTC AAC -3	5-GATGAGTCCTGACCGA CAC -3
17	5-GACTGCGTACCAATTC AAC -3	5-GATGAGTCCTGACCGA CAT -3
18	5-GACTGCGTACCAATTC AAC -3	5-GATGAGTCCTGACCGA CGA -3
19	5-GACTGCGTACCAATTC AAC -3	5-GATGAGTCCTGACCGA CTG -3
20	5-GACTGCGTACCAATTC AAC -3	5-GATGAGTCCTGACCGA CGT -3

The conditions for the pre-selectively amplified polymerase chain reaction (PCR) were as follows: 10 ng of genomic DNA, 1X *Taq* buffer [20 mM Tris-HCl, pH 8.4 and 50mM (NH₄)₂SO₄] (Fermentas, USA), 3.0mM MgCl₂, 0.25mM each of the four deoxynucleotide triphosphates (dNTPs) (Fermentas, USA), 0.25U *Taq* polymeerase (Fermentas, USA), 4 pmols of pre-selectively amplified *EcoRI* primer (E-A: 5'-GAC TGC GTA CCA ATT CA-3') (Pacific Science, USA) and 4 pmols of pre-selectively amplified *TaqI* primer (T-C: 5'-GAT GAG TCC TGA CCG AC-3') (Pacific Science,

USA) in a final volume of 25 μL . The PCR programme was: 3 min. at 94°, 20 cycles of 30 sec. each at 94°, 1 min. at 56° and 30 sec. at 72°, followed by 5 min. at 72°, and a final step at 4°. PCR products were analysed using 6% polyacrylamide-gel electrophoresis (120 V; 400 mA), stained with ethidium bromide. The pre-amplified products were diluted twentyfold with distilled water and then used as selectively amplified DNA templates.

For the selective amplification, 20 primer combinations were used in the following PCR reaction mix: 2.5 μL of diluted pre-selectively amplified products, 1X *Taq* buffer, 3.0mM MgCl_2 , 0.25mM each of the four dNTPs, 0.25U *Taq* polymerase, 2 pmols of selectively amplified *EcoRI* primer (Pacific Science, USA) and 2 pmols of selectively amplified *TaqI* primer (Pacific Science, USA) in a total volume of 12.5 μL . A touchdown thermal protocol was used for the selective PCR amplification. The selectively amplified products were resolved on a 6% denatured polyacrylamide-gel electrophoresis with constant power (50W) for 3 hr. The AFLP fingerprints were visualised using silver staining.

Statistical Analysis

The individual samples were scored for the presence or absence of each AFLP band. Only polymorphic AFLP bands were analysed using the logistic regression model as follows:

$$\text{logit(LP)} = \ln(P_i/1-P_i) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_n X_n + e \quad (1^*)$$

where LP = logistic probability of a catfish to be predicted as *P. gigas* or non - *P. gigas* (*P. hypophthalmus*, F₁ hybrid and F₂ hybrid)

β_0 = value of y-intercept

$\beta_1, \beta_2, \dots, \beta_n$ = regression coefficient of molecular markers: X_1, X_2, \dots, X_n

X_1, X_2, \dots, X_n = molecular markers: X_1, X_2, \dots, X_n

e_{ij} = residual error term

The best-fit model was selected using a backward elimination regression, which tends to be the preferred method for performing exploratory analyses. The predicted probability to be assigned to *P. gigas* was used as follows:

$$P_i^* = e^{LP^*} / 1 + e^{LP^*}$$

where P_i^* = predicted probability test of *P. gigas* species: if probability ≥ 0.5 , it is *P. gigas* species; if probability < 0.5 it is non - *P. gigas* species.

e = value of exponential constant

LP^* = $\ln(P_i/1-P_i)$ from equation (1*)

The accuracy test of these markers was described by Wassertheil-Smoller [32]: accuracy = no. of correct assessments / no. of all assessments. Separate logistic regressions were done for other species (*P. hypophthalmus*, F₁ hybrid and F₂ hybrid) using equations similar to the above.

RESULTS AND DISCUSSION

The 20 primer combinations generated a total of 487 AFLP fragments from the 16 Pangasiid catfish individuals (Figure 1). The results showed 9-45 bands per primer combination and 335 markers covering a range of 65.38-100.0% (Table 2). A total of 335 polymorphic bands were used to analyse the association between markers and species assignment using logistic regression. Two markers were found to be significantly associated with *P. gigas*, whereas three markers helped

distinguish *P. hypophthalmus*, F₁ hybrid and F₂ hybrid (Table 3). The identification of *P. gigas*, *P. hypophthalmus*, F₁ hybrid and F₂ hybrid was 100% accurate using these markers (Table 4).

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M

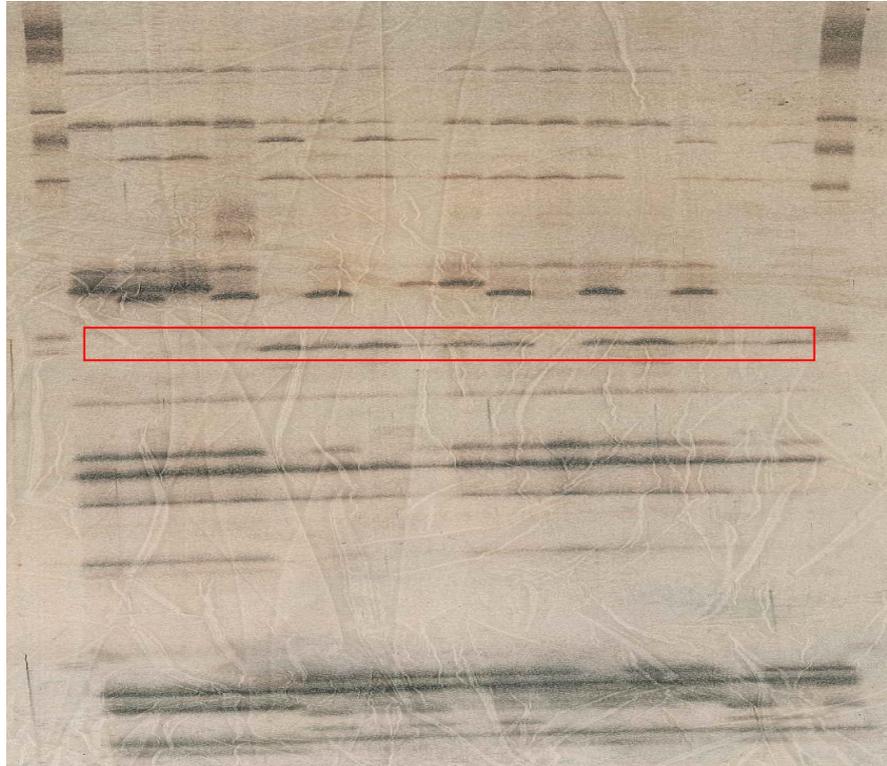


Figure 1. AFLP fingerprints of *P. gigas* (1-4), *P. hypophthalmus* (5-8), F₁ hybrids (9-12) and F₂ hybrids (13-16). DNA bands were amplified by primer E-ACC/ T-CTG. Red box = specific bands present in *P. hypophthalmus*, F₁ hybrids and F₂ hybrids but absent in *P. gigas*. M = DNA ladder 100 bp.

In a previous study, Sriphairoj et al. [33] identified species-specific markers for four economically important Pangasiid catfish, i.e. *P. gigas*, *P. hypophthalmus*, *Pangasius bocourti* and *Pangasius larnaudii*, using seven primers developed by means of AFLP. They used SSCP to screen for potential species-specific markers and only one marker (*PL8*) was shown to be conserved within species, and thus this could be useful for distinguishing among different species. However, these markers failed to separate hybrids of *P. gigas*, *P. hypophthalmus* and *Pangasius bocourti* from their parents. Thus, the present study extends these results by including additional markers. Our results have shown that these markers are also useful for distinguishing hybrids from their parental species.

AFLP markers can be assigned to single-locus markers such as sequence-characterised amplified regions (SCARs) as described by Xu et al. [34]. Kai et al. [35] used AFLP to distinguish three colour morphotypes, in which diagnostic AFLP loci were identified as well as loci with significant frequency differences. In the same way, Chong et al. [36] used AFLP to analyse Malaysian river catfish (*Mystus nemurus*) population in five geographical regions and found that AFLP was more efficient for the differentiation of sub-populations and for the identification of genotypes within the population than RAPD, although similar clusters of the population were

concluded with either analysis. Moreover, Zhang and Cai [14] used AFLP to develop a species-specific marker for rainbow trout. The marker was distinctly present in all the rainbow trout samples tested and was absent from all other Atlantic salmon samples. The species-specific SCAR could only be detected in the admixture containing 10-50% rainbow trout DNA in Atlantic salmon DNA for processed product in 10 replicates. Our results suggest that AFLP makers can be used to identify Pagasiid catfish and their F₁ and F₂ hybrids; however, further testing using more samples per species will be required to test the efficacy of this method. For further study, we plan to identify species-specific DNA fragments using sequencing and to develop DNA or SCAR markers for testing in a larger sample size.

Table 2. Estimation of average number of DNA bands in each primer, number of polymorphic bands and per cent polymorphic

Primer combination	No. of DNA bands in each primer	No. of polymorphic bands	% Polymorphic
1	15	14	93.33
2	21	17	80.95
3	18	16	88.88
4	24	22	91.66
5	14	12	85.71
6	9	7	77.77
7	26	25	96.15
8	12	12	100.00
9	23	23	100.00
10	20	20	100.00
11	25	19	76.00
12	34	26	76.47
13	15	15	100.00
14	45	32	71.11
15	35	33	94.28
16	26	17	65.38
17	37	27	72.97
18	21	20	95.23
19	35	23	65.71
20	32	26	81.25
Total	487	406	1,712.85
Average	24.35	20.30	85.64

Table 3. Estimated values of logistic regression, P-value and odds ratio for the identification of Pangasiid catfish

Species	Variant	Estimated logistic regression	P-value ($Pr > X^2$)	Odds ratio
<i>P. gigas</i>	Intercept	9.4032±55.065	0.3134	-
	2(E3-T3)	-17.5301±80.116	0.0253	<0.001
	26(E3-T5)	-18.7468±64.614	0.0006	<0.001
	% concordant = 100.0; % discordant 0.0; % tied = 0.0			
<i>P. hypophthalmus</i>	Intercept	-20.5324±44.481	0.0004	-
	2(E3-T3)	-15.6238±49.607	0.0472	<0.001
	132(E7-T20)	28.7925±62.553	0.0088	<0.001
	270(E15-T22)	13.9173±35.076	0.0455	<0.001
% concordant = 100.0; % discordant 0.0; % tied = 0.0				
F ₁ hybrid	Intercept	-40.2265±87.031	0.0004	-
	310(E15-T23)	16.2689±50.459	0.0285	<0.001
	326(E15-T23)	16.7919±44.716	0.0209	<0.001
	386(E16-T5)	15.3156±46.502	0.0254	<0.001
% concordant = 91.7; % discordant 0.0; % tied = 8.3				
F ₂ hybrid	Intercept	-7.6711±40.081	0.0004	-
	32(E3-T5)	15.8337±52.644	0.0455	<0.001
	77(E3-T22)	-16.7655±50.935	0.0077	<0.001
	98(E7-T3)	16.4146±46.167	0.0158	<0.001
% concordant = 100.0; % discordant 0.0; % tied = 0.0				

Table 4. Accuracy test of molecular markers for *P. gigas*, *P. hypophthalmus*, F₁ hybrid and F₂ hybrid

Molecular markers for <i>P. gigas</i>		Observed		% Correct
	<i>P. gigas</i>	<i>P. gigas</i>	Non - <i>P. gigas</i>	
<i>P. gigas</i>	4	0	100%	
Non - <i>P. gigas</i>	0	12	100%	
Molecular markers for <i>P. hypophthalmus</i>		Observed		% Correct
	<i>P. hypophthalmus</i>	Non - <i>P. hypophthalmus</i>		
<i>P. hypophthalmus</i>	4	0	100%	
Non - <i>P. hypophthalmus</i>	0	12	100%	
Molecular markers for F ₁ hybrid		Observed		% Correct
	F ₁ hybrid	Non - F ₁ hybrid		
F ₁ hybrid	4	0	100%	
Non - F ₁ hybrid	0	12	100%	
Molecular markers for F ₂ hybrid		Observed		% Correct
	F ₂ hybrid	Non - F ₂ hybrid		
F ₂ hybrid	4	0	100%	
Non - F ₂ hybrid	0	12	100%	

CONCLUSIONS

This study shows that AFLP markers can be used to differentiate Pangasiid catfish (*Pagasianodon gigas* and *P. hypophthalmus*) and their F₁ and F₂ hybrids from their parents. Consequently, this technique may help differentiate taxonomic units and identify genetic relationships between the Pangasiae species. The technique may also provide valuable information for breeding and conservation programmes as well as for ecological, evolutionary and traceability studies

ACKNOWLEDGEMENTS

We are grateful to Thailand Research Fund (TRF) for financial support through the Royal Golden Jubilee Ph.D. Program (PHD/ 0149/ 2554). We are grateful to the Plabuk Integration Knowledge Base, Faculty of Fisheries Technology and Aquatic Resources, Maejo University for providing fish samples. We wish to express our gratitude to the Centre of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Commission on Higher Education (AG-BIO/PERDO-CHE), Ministry of Education, as well as the Project of the Service and Technology Transfer of Agricultural Biotechnology Centre, Faculty of Agriculture, Chiang Mai University, for providing research facilities.

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