



Investigations on genetic diversity and relationships among *Channa* species using AFLP-capillary electrophoresis

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ABSTRACT

The genetic diversity and relationships among eight species of *Channa* were investigated using amplified fragment length polymorphism (AFLP)-capillary electrophoresis. Ten *Eco*R I/*Mse* I primer pairs were selected out of thirty eight and were used for further AFLP analysis. The total amplified bands, consistency of bands and polymorphic bands ranged from 114 to 206, from 0 to 12 and from 114 to 196, respectively, with corresponding averages of 156.2; 4 and 152.2 respectively. The proportion of polymorphic bands ranged from 93.62 to 100%, with an average of 97.44%. The similarity coefficients among eight *Channa* species ranged from 0.5365 to 0.7875. The dendrogram generated after unweighted pair-group method with arithmetic means (UPGMA) clustering with NTSYS software indicated that the eight *Channa* species could be divided into four major clusters according to the genetic similarity coefficient of 0.58. *Channa striata* and *Channa micropeltes* were clustered into a separate branch that was far from the other six species and *Channa argus kimurai* (Shih) was closest to *Channa argus*. These results could be useful for conservation as well as for guiding research on captive breeding of *Channa* species.

Keywords: AFLP, Amplified fragment length polymorphism, Capillary electrophoresis, *Channa* species, Genetic diversity, Intergeneric relationships

Introduction

Snakehead (Perciformes: Channidae) are widely distributed in China (Cheng and Zheng, 1987; Courtenay and Williams, 2004), having high economic value and regarded as good food fish owing to their nutritional, medicinal and pharmaceutical properties (Liu *et al.*, 2000). The classification of some species of Channidae is still controversial. For example, *Channa argus kimurai* shares very similar morphology and overlapping distribution with *Channa argus*. It is named for the white colour of its entire body and has only been found in the Jialing River in Sichuan. According to the earliest documentation, *C. argus* has three subspecies: *C. argus argus* (Cantor, 1842); *O. argus warpachowskii* Berg, 1909; and *O. argus kimurai* Shih, 1936 (Shih, 1936; Nichols, 1943; Nikolski and Gao, 1960). Wang *et al.* (1992; 1993) suggested that *O. argus kimurai* should not be regarded as a subspecies of *C. argus*; rather, they concluded that it is an albino mutant of *C. argus*, based on their investigations on morphological characteristics, principal component analysis, lactate dehydrogenase and esterase isozymes. The study of 16S and 12S rRNA between two colour

morphs of northern snakehead also confirmed this point (Zhou, 2017a, b).

Amplified fragment length polymorphism (AFLP) markers are considered one of the best molecular markers for population analysis because they can be assessed in a short period of time and it is widely applied to analyse fish populations. AFLP is a DNA molecular marker based on restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) (Zabeau and Vos, 1993; Vos *et al.*, 1995). AFLP has been applied in germplasm identification, genetic diversity, genetic map construction, QTL location, animal genetics and breeding, disease detection and other areas (Li *et al.*, 2003; Zhang *et al.*, 2005; Liu *et al.*, 2005; Yang *et al.*, 2006; Krishnamurthy *et al.*, 2015). Chen *et al.* (2009), identified seven geographic groups of *Epinephelus akaara* using AFLP in the East China Sea and South China Sea and the UPGMA phylogenetic tree revealed that *E. akaara* mainly clustered into geographic groups, providing a scientific basis for germplasm resource protection of *E. akaara*. The late larval and adult fish of 11 *Lutjanus* species were analysed using AFLP electrophoretic pattern in the

Spratly Islands; the larvae were successfully identified and the phylogenetic and genetic relationships of *Lutjanus* were clearly explained, improving and complementing traditional morphological taxonomy findings (Zhang *et al.*, 2005). The proportion of AFLP polymorphs of *Cyprinus carpio* and *Ctenopharyngodon idella* were 6.7 and 58.9%, respectively, indicating a distant phylogenetic relationship between them. Notably, simple sequence repeat (SSR) markers showed the same results (David *et al.*, 2011). Capillary electrophoresis (CE), also known as high-performance CE (HPCE), is a new liquid phase separation technique that employs capillary action for the separation channel and high-voltage direct current electric field as the driving force. Electrophoresis results can be statistically analysed, effectively reducing the analysis time by eliminating complex steps such as gel destaining, immobilisation, washing, silver staining, developing and fixing while greatly improving resolution. Therefore, in the present study, AFLP-capillary electrophoresis was applied to evaluate the genetic diversity and relationships among different species under the genus *Channa*. The study also attempted to elucidate the molecular evidence for the classification between two colour morphs of northern snakehead.

Materials and methods

Sample collection and DNA extraction

Fin clippings and muscle tissue samples were collected and immediately soaked in 95% ethanol and then stored at -20°C. Total genomic DNA was extracted from the caudal fin using a standard extraction kit (DNeasy tissue kit, Baitaike Biotech Co. Ltd., China), DNA concentration was measured using Amersham Biosciences Ultrospec 2100 spectrophotometer and electrophoresed on 1% agarose gels and stored at -20°C. Basic information of sampling sites and size of *Channa* species used for the study are given in Table 1.

AFLP analysis

One hundred nanogram of DNA was digested with double restriction enzyme (*EcoR* I/*Mse* I) (New England Biolabs Co., Ltd., Beijing) and were ligated to two adapters (*EcoR* I- adapter/ *Mse* I- adapter) (Table 2) with T4 DNA ligase (New England Biolabs Co., Ltd., Beijing). Digestion and ligation reactions were carried out simultaneously. The reaction was carried out using a 20 µl reaction volume, containing 2 µl of 10×AFLP digest-ligation buffer, 1.8 µl AFLP digest-ligation enzyme mix, 1 µl (10 µM) *EcoR* I adaptor, 1 µl (10 µM) *Mse* I adaptor, 5 µl DNA template and up to 20 µl of double distilled water. The reaction conditions were 5 h at 25°C and electrophoresed on 1% agarose gel. One microliter of the ligated product was used for pre-amplification by PCR with adapter-homologous primers with one selective nucleotide. The PCR amplification profile was as follows: 3 min at 94°C, followed by 20 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C.

The pre-amplification product was diluted 20-folds and was subjected to selective amplification using primers with three selective nucleotides and ten pairs of selective AFLP primers (Table 2) were chosen. The selective amplification reaction was performed with touchdown PCR begun at 95°C for 5 min, followed by 12 cycles of 35 s at 95°C, 35 s at 65°C (decreasing 0.7°C each cycle) for 30 s, 1 min at 72°C. The last reaction was performed at 94°C for 30 s, 56°C for 30 s; 72°C for 1 min with 23 cycles and final extension of 72°C for 5 min.

Capillary electrophoresis

Formamide and molecular weight internal standards were mixed in the ratio of 100:1. Fifteen microliter was taken with 1 µl amplified product (diluted 10-folds) and was sent to Bio-ulab Biotech Co., Ltd. for high

Table 1. Basic information of sampling sites and size (n) of the eight *Channa* species

Species	Location	GPS coordinates	Altitude (m)	Year of collection
<i>C. argus kimurai</i>	Neijiang City, Sichuan Province, China	29°34'14.64"N 105°4'1.95"E	332	2014
<i>C. argus</i>	San Jiaozhen, Zhongshan City, Guangdong Province, China	22°39'14.31"N 113°25'43.95"E	1	2014
<i>C. maculata</i>				2015
Hybrid snakehead (<i>C. argus</i> ♂ x <i>C. maculata</i> ♀)	Shiban Cun, Qingyuan City, Guangdong Province, China	23°44'33.06"N 113°4'28.84"E	16	2015
<i>C. asiatica</i>	Guangzhou City, Guangdong Province, China	23°5'3.60"N 113°14'46.24"E	3	2015
<i>C. micropeltes</i>				2015
<i>C. lucius</i>	Hanoi City, Vietnam	21°1'41.45"N 105°51'48.94"E	164	2014
<i>C. striata</i>	Vientiane City, Laos	17°57'52.27"N 102°35'17.97"E	167	2014

Table 2. Adapters and primer sequences used in the analysis of AFLP-capillary electrophoresis

Adapters and primers	Sequence (5'-3')	Primers	Sequence (5'-3')
<i>Eco</i> R I adapter 1	CTCGTAGACTGCGTACC	<i>Mse</i> I adapter 1	GACGATGAGTCCTGAG
<i>Eco</i> R I adapter 2	AATTGGTACGCAGTCTAC	<i>Mse</i> I adapter 2	TACTCAGGACTCAT
Pre-Pe	GACTGCGTACCAATTC	Pre-Pm	GATGAGTCCTGAGTAA
E-AAC	GACTGCGTACCAATTCAAC	M-CCA	GATGAGTCCTGAGTAACCA
E-AAG	GACTGCGTACCAATTCAAG	M-CGA	GATGAGTCCTGAGTAACGA
E-ACT	GACTGCGTACCAATTCAC	M-CGC	GATGAGTCCTGAGTAACGC
E-AGC	GACTGCGTACCAATTCAGC	M-CTA	GATGAGTCCTGAGTAACTA
E-ATC	GACTGCGTACCAATTCATC	M-CTC	GATGAGTCCTGAGTAACTC
E-ATG	GACTGCGTACCAATTCATG	M-CTG	GATGAGTCCTGAGTAACTG
E-ATT	GACTGCGTACCAATTCATT	M-CTT	GATGAGTCCTGAGTAACTT
E-CAC	GACTGCGTACCAATTCCAC	M-GAC	GATGAGTCCTGAGTAAGAC
E-CAG	GACTGCGTACCAATTCCAG	M-GAG	GATGAGTCCTGAGTAAGAG
E-CAT	GACTGCGTACCAATTCCAT	M-GAT	GATGAGTCCTGAGTAAGAT

performance capillary electrophoresis employing ABI 3730XL.

Data analysis

For each individual, the DNA fingerprints were scored by apparatus inspection (ABI 3730) and the files were analysed with Genemarker V2.20. Polymorphic DNA bands ranging from 40–620 bp were scored as present (1) or absent (0). The genetic distance between each *Channa* species was calculated using the NTSYSpc 2.11 software package (Rohlf, 1997). The dendrogram for the eight *Channa* species were constructed using the unweighted pair-group method with arithmetic means (UPGMA) in MEGA 4.1 based on the genetic distance (Tamura *et al.*, 2007).

Results

Population genetic variation of *Channa* species

The proportions of polymorphic loci provide a good measure of genetic variations among the eight *Channa* species. A total of 1562 loci were detected by the ten AFLP primer pairs, of which 1522 (97.44%) were polymorphic (Table 3). The total loci scored per primer pair ranged from 114 to 206 loci with average of 156.2. The average number of polymorphic loci scored per primer was 152.2 loci and ranged from 114 to 196 loci (Table 3). The proportion of the polymorphic loci ranged from 65.96 to 81.97% with an average of 97.44% (Table 3). The number of amplification loci and the

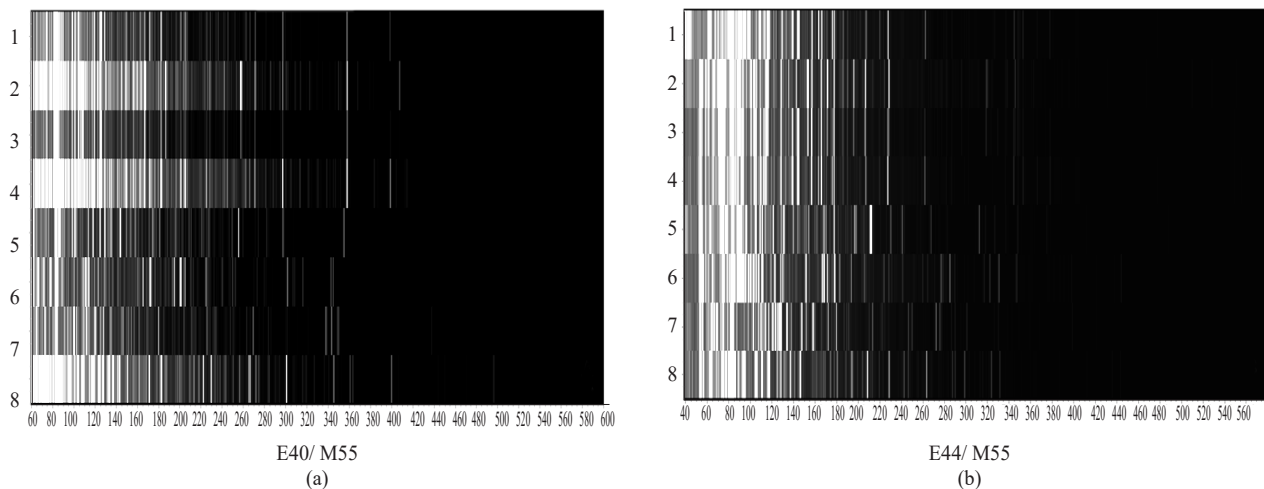


Fig. 1. AFLP high performance capillary electrophoresis gel patterns generated by (a) the lowest primer combination (E44/M59) and (b) the highest primer combination (E40/M55).

1. *C. argus*, 2. *C. maculata*, 3. Hybrid snakehead, 4. *C. argus kimurai*, 5. *C. asiatica*, 6. *C. striata*, 7. *C. micropeltes*, 8. *C. lucius*

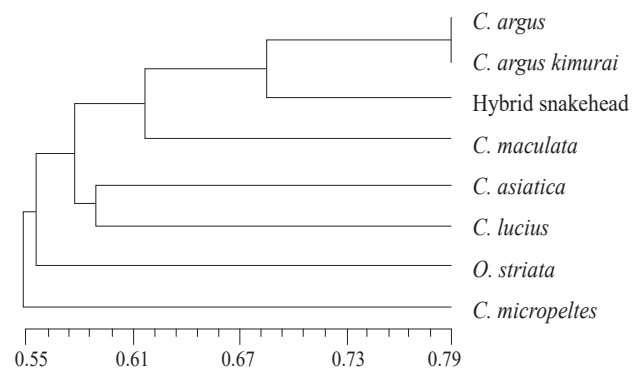
Table 3. The amplification results per AFLP primer pair of eight *Channa* species

Primer pair	Number of total loci	Number of consistent loci	Number of polymorphic loci	Proportion of polymorphic loci (%)
E32/M61	132	4	128	96.97
E33/M60	142	4	138	97.18
E38/M66	188	12	176	93.62
E40/M55	206	10	196	95.15
E44/M59	114	0	114	100.00
E45/M62	192	2	190	98.96
E46/M56	131	2	129	98.47
E48/M51	197	3	194	98.48
E49/M65	124	1	123	99.19
E50/M64	136	2	134	98.53
Total	1562	40	1522	
Mean	156.2	4	152.2	97.66

proportion of polymorphic loci were both large, which suggests that the AFLP marker provides abundant genetic information and it was a perfect genetic marker for population genetic analysis. AFLP-high performance capillary electrophoresis gel patterns generated by ten primers are depicted in Fig. 1. Only the highest (Fig. 1a) and lowest (Fig. 1b) number of primer combination are presented.

Population structure and differentiation among eight *Channa* species

A total of 10 pairs of AFLP primer combinations were used to detect genetic variation of pooled genomic DNA from eight *Channa* species (Table 4). The genetic distance and similarity among the eight *Channa* species ranged from 0.3425 to 1.0392 and from 0.5365 to 0.7875. Based on the genetic similarity coefficients, the UPGMA dendrogram displayed four major clusters. The genetic similarity coefficients between *C. argus* and *C. argus kimurai* was the greatest (0.7875) and then clustered with hybrid snakehead and *C. maculata*. *C. asiatica* and *C. lucius* formed a basal cluster, the relationship between *C. striata* and *C. micropeltes* was considerably far from each other and from other *Channa* species (Fig. 2).

Fig. 2. Phylogenetic tree constructed for eight *Channa* species using UPGMA method

Discussion

AFLP-capillary electrophoresis is a highly efficient molecular marker technique. It can amplify more than 200 sites with a pair of primers and detect site differences as small as 1 bp, which makes it easier to detect subtle differences among materials. Moreover, it reduces the error caused by manual operation, because it employs an efficient automation system that ensures standardised analysis of amplified samples and improves data reliability

Table 4. Genetic distance (above diagonal) and similarity coefficients (below diagonal) among eight *Channa* species based on AFLP data

Species	<i>Channa argus</i>	<i>C. maculata</i>	Hybrid snakehead	<i>C. argus kimurai</i>	<i>C. asiatica</i>	<i>C. striata</i>	<i>C. micropeltes</i>	<i>C. lucius</i>
<i>C. argus</i>	****	0.7712	0.6246	0.3425	0.9075	0.8690	0.9421	0.9541
<i>C. maculata</i>	0.5698	****	0.5126	0.7144	0.8638	0.8575	0.8217	0.9240
Hybrid snakehead	0.6620	0.6863	****	0.5229	0.8209	0.8762	0.9707	0.9487
<i>C. argus kimurai</i>	0.7875	0.5992	0.7106	****	0.9074	0.8834	0.9151	0.9411
<i>C. asiatica</i>	0.5768	0.5589	0.6152	0.5871	****	0.9143	0.9253	0.9939
<i>O. striata</i>	0.5621	0.5365	0.5723	0.5672	0.5717	****	0.8733	1.0209
<i>C. micropeltes</i>	0.5410	0.5499	0.5461	0.5589	0.5698	0.5589	****	1.0392
<i>C. lucius</i>	0.5762	0.5519	0.5915	0.5903	0.5922	0.5557	0.5525	****

(O'Hanlon and Peakall, 2000). In addition, high-quality DNA is required for establishing an AFLP reaction system and double digestion of genomic DNA and adapter binding are important factors in developing an AFLP system. Therefore, the key to success in the present study was the strict requirements for binding, pre-amplification and selective amplification.

In the selective amplification using 10 combinations of *EcoR* I and *Mse* I primers, each pair could generate 1562 clear sites (distributed between 60 and 600 bp). The average number of total loci scored per primer pair was 156.2. The primer combination E40/M55 amplified a maximum of 206 sites, while the lowest number was for the primer combination of E44/M59, which amplified 114. Each primer combination could generate highly polymorphic sites, the highest reached 100% and the average was 97.44%. Most teleost studies using AFLP focus on genetic diversity among different populations of the same species. A previous study of seven *E. akaara* groups found large differences in genetic diversity among different geographical groups (Chen *et al.*, 2009). An AFLP analysis of population genetic diversity of *Trachidermus fasciatus* revealed a low level of genetic diversity between two regional populations (Xu *et al.*, 2008). A comparative study of genetic diversity based on RAPD and AFLP markers reported a significantly higher polymorphism detection efficiency of AFLP markers compared to RAPD markers (Yang and Yu, 2006). Previous studies have successfully identified *Cyprinus carpio*, *Acceder oxyrinchus* and their hybrids, indicating that the technology can be extended to identify other similar organisms (Congiu *et al.*, 2011). Many researchers have employed AFLP to assess genetic relationships among plant species and successfully identified a high proportion of polymorphisms. AFLP can obtain more information for a given species and can effectively assess genetic diversity among species; therefore, it can play an important role in improving and complementing the traditional morphological taxonomy studies (Liu *et al.*, 2005; Zhang *et al.*, 2005).

C. argus kimurai shares very similar morphology and overlapping distribution with *C. argus*. There has been considerable controversy regarding its classification. According to the earliest documentation, they belong to two different subspecies. Some researchers have suggested that *C. argus kimurai* cannot be regarded as a subspecies of *C. argus*; rather, they consider it an albino mutant (Shih, 1936; Wang *et al.*, 1992; 1993; Zhou 2017a, b).

The phylogenetic tree constructed for the eight *Channa* species based on UPGMA cluster analysis shows that *C. argus kimurai* and *C. argus* have the highest genetic similarity coefficient (0.7875) (Fig. 2). This further

demonstrates that from the molecular biology perspective, *C. argus kimurai* may be an albino mutant rather than a subspecies of *C. argus*. These findings provide important molecular clues for correcting the classification of *C. argus kimurai* under the genus *Channa*.

The hybrid snakehead had a relatively small genetic distance from the parents, but the three species could be distinctly separated in the clustering tree (Fig. 2). This also demonstrates that AFLP-capillary electrophoresis is applicable to germplasm analysis for cross-breeding, especially when the parents and their offsprings have similar morphologies. Therefore this molecular marker could be effectively used for distinguishing the genetic effects of hybridisation between wild species, as well as for germplasm identification.

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