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Genetic variation of five species of *Yasuhikotakia* in Thailand using AFLP

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The genetic variation of *Yasuhikotakia* was assessed using amplified fragment length polymorphism (AFLP) of 5 species, including *Y. sidthimunki, Y. nigrolineata*, *Y. morleti*, *Y. modesta* and *Y. lecontei*. Four primer-pairs were screened, from which ten primer-pairs (E-AAG/M-CCG, E-AGC/M-GAC, E-ATG/M-GCG and E-CAG/M-CCG) were used. A total of 228 AFLP fragments were detected, polymorphic was 74.79%, and average heterozygosity was 0.19. Nei's unbiased genetic distance among populations ranged from 0.0409 - 0.4378. An unweigthed pair-group method of arithmetic average (UPGMA) constructed dendrogram based on Nei's unbiased genetic distances indicated two clusters. The first cluster included *Y. sidthimunki* and *Y. nigrolineata*, with more closely related. In the second cluster was composed of *Y. morleti*, *Y. modesta* and *Y. lecontei*.

Key words: genetic variation, *Yasuhikotakia* , AFLP

Introduction

Yasuhikotakia (pronounced yah-soo-high-ko-tay-kee-ah) includes a number of common aquarium botias including *Y. eos, Y. caudopunctata, Y. lecontei, Y. longidorsalis, Y. modesta, Y. morleti, Y. nigrolineata, Y. splendida* and *Y. sidthimunki* (Ahlander, 2004). They are in Laos, Thailand, Cambodia, Vietnam and China. *Yasuhikotakia* belongs to the subfamily Botiinae, family Cobitidae, Loaches, order Cypriniformes, carp like fish. Later all of them were *Botia*. Until 2004 when the Swiss ichthyologist Maurice Kottelat made the genus *Botia* that divided into four genus, besides *Chromobotia* (C*lown loaches) Yasuhikotakia* (Mekong loaches), *Syncrossus* (Tiger loaches) and *Botia* (Indian loaches). (Ahlander, 2004; Clarke, 2004; Kottelat, 2004) *Yasuhikotakia*

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distinguished from the other genera within the subfamily botiinae by the following combination of characters: mental lobe not developed in a barbel, with a pair of fleshy papillae at its anterior edge. Colour pattern: most species with pale body and more or less distinct blotch at end of caudal peduncle: a few species with stripes along flank or back; most species with ontogenic changes in colour pattern, usually a vertical pattern in juveniles which disappear with growth (Kottelat, 2004). However, number of the catch has rapidly declined in the recent years. This definitely reflects the decline of these genus in natural habitats. *In Thailand Red Data: Fish: Y. sidthimunki* is Critically Endangered, *Y. nigrolineata* is endangered, *Y. eos, Y. longidorsalis* and *Y. splendid* are vulnerable (Vidthayanon, 2005).

The conservation of genetic diversity is important for the long-term interest of any species (Song *et al*., 2006). Molecular markers are useful tools in the assessment of genetic diversity (Powell *et al*., 1996). Amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) depends on the reliability of RFLP and the high efficiency of PCR to amplify the digested genome DNA segment selectively, and is highly reliable for the assessment of genetic variation among and within populations (Keiper and McConchie, 2000). AFLP has provided genetic markers for DNA fingerprinting, genetic mapping, and studies of genetic relationships among fish (Gwo *et al*., 2008; Congiu *et al*., 2002; Han and Ely, 2002; Ezaz *et al*., 2004; Chong *et al*., 2000; Dorenbosch, 2006; McMillan *et al*., 2006). The aim of the present study was to reconstruct the phylogenetic relationships among 5 species of the genus *Yasuhikotakia* using AFLP.

Materials and methods

The present study comprises 64 individuals of 5 species. Geographic locations and sample size are given in Fig. 1 and Table 1. Muscle samples from each individual were cut and stored frozen at -70^{o}C until analysis. Genomic DNA of the samples in each individual was extracted from muscle tissue by using with DNATrap II (DNATEC). DNA concentration was measured with and UV spectrophotometer .The quality of extracted DNA was assessed by 1.0% agarose gel electrophoresis.

The genomic DNA samples were used for AFLP analysis as described by Vos *et al*. (1995). Fingerprint patterns were visualized on a 4.5% denaturing polyacrylamide gel using silver staining method. DNA template for AFLP reactions were generated by restriction of *EcoRI* and *MseI* in A buffer (Borhinger Mannheim: Roche) at 37^{o}C for 3 h. To generate DNA templat for subsequent PCR amplification, the digested DNA fragments were ligated with 7.5 pmol *EcoRI*-adapter and 75 pmoy *MseI*-adapter in reaction mixture

containing 1.2 mM BSA, 1X ligase buffer, 1.2 mM ATP and 1.2 U T4DNase at at 37°C for 3 h. Preamplification PCR reaction was conducted using GeneAmp^R PCR System 9700 with p pair of primers containing a single selective nucleotide, Amplification was performed at an annealing temperature of 56°C for 1min. The PCR product mixture was diluted 10-fold with distilled aster and used as templates for the subsequent selective PCR amplification. The selective amplification was performed using three pairs of primers, EcoRI + MseI (AAA-CCg, AGC-GAC, ATG-GCG, CAG-CCG). Fragment data are collected using TFPGA package (Miller, 1997)

Table 1. Sample collection sited of 5 species of *Yasuhikotakia.*

species	Sample size	Collected places (provinces)
Y. sidthimunki	6	Bangkok (market)
Y. nigrolineata	17	Nan
Y. morleti	10	Nan
Y. modesta	16	Ubonrachatani
Y. lecontei	15	Nakhonpanom
Total	64	-

Results and discussion

Four informative AFLP primer combinations generated a total of 228 reproducible amplification fragments across five species of *Yasuhikotakia*, among which 227 bands were polymorphic. The number of amplified AFLP bands per primer pair varied from 46 to 76 with and average of 57 bands.

Table 2. Average heterozygosity and percentage polymorphic loci for 5 species of *Yasuhikotakia* across 228 AFLP loci.

species	n	Percentage polymorphic loci	Average heterozygosity
Y. sidthimunki	6	17.1053	0.0597
Y. nigrolineata	17	25.9649	0.1038
Y. morleti	10	15.7895	0.0476
Y. modesta	16	9.2105	0.0257
Y. lecontei	15	9.2105	0.0282
	64	98.6842	0.1877

A single species produced 50 (in *Y. modesta*) to 101 (in *Y. nigrolineata*) bands (Table 3). Species differed in the levels of genetic diversity among

individuals, with 37.74% (in *Y. lecontei*) to 77.23% (in *Y. nigrolineata*) of the bands scored as polymorphic. The proportion of species-specific markers identified to the total number of bands scored for each species ranged from 0.99% (in *Y. nigrolineata*) to 35.85% (in *Y. lecontei*).

Table 3. Number of amplified bands and polymorphic bands scored from AFLP analysis in five *Yasuhikotakia* species.

	Y. sidthimunki	***Y. nigrolineata***	***Y. morleti***	***Y. modesta***	***Y. lecontei***
Sample size	6	17	10	16	15
Total no. of bands amplified	79	101	71	50	53
No. of polymorphic bands	40	78	36	21	20
Percentage of polymorphic bands	50.63	77.23	50.70	42.00	37.74
No. of species-specific bands	1	1	15	15	19
Percentage of species-specific bands	1.27	0.99	21.13	30.00	35.85

Genetic distances ranged from 0.0409 (between *Y. sidthimunki* and *Y. nigrolineata*) to 0.4378 (between *Y. sidthimunki* and *Y. morleti*) (Table 4) Cluster analysis using UPGMA (unweighted pair group method with arithmetic mean) was performed to examine genetic relationships among five species of *Yasuhikotakia*. Five species of *Yasuhikotakia* were divided into two major clusters. The first cluster consisted of *Y. sidthimunki* and *Y. nigrolineata*, with more closely related. The other cluster comprised *Y. morleti*, *Y. modesta* and *Y. lecontei*. This finding is similar to the work of Vidthayanon (2005).

Table 4. Nei's unbiased (1978) distance between five *Yasuhikotakia* species.

species	***Y. sidthimunki***	***Y. nigrolineata***	***Y. morleti***	***Y. modesta***	***Y. lecontei***
Y. sidthimunki	*****				
Y. nigrolineata	0.0409	*****			
Y. morleti	0.4378	0.3583	*****		
Y. modesta	0.3609	0.3162	0.2889	*****	
Y. lecontei	0.4020	0.3381	0.3030	0.3346	*****

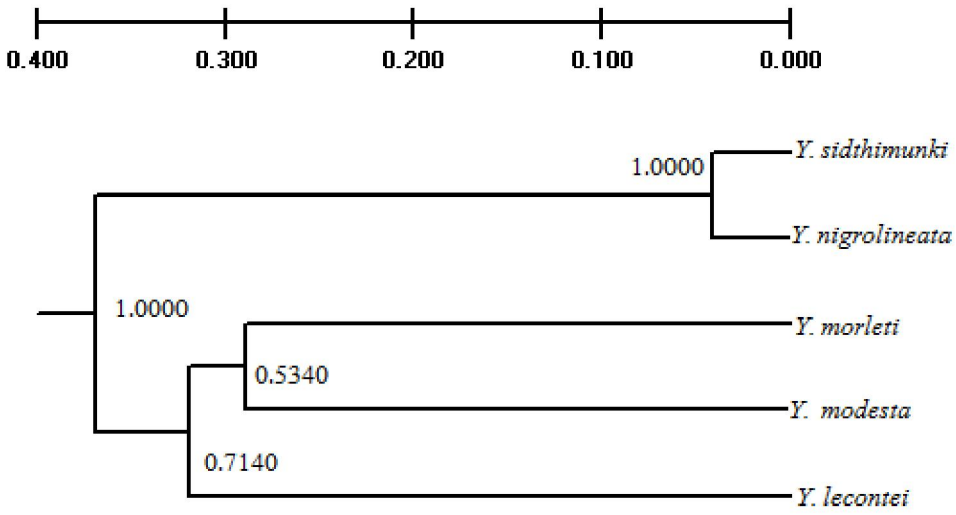

Fig. 1. UPGMA Cluster of five *Yasuhikotakia* species using Nei's (1978) unbiased distance, number above the branches indicate bootstrap value. (1,000 permutations conducted)**.**

In the present study, AFLP analysis using four sets of primers generated more than 50 fragments for each of the five *Yasuhikotakia* species. Within a single species, 37-77% of the fragments are polymorphic (Table 1). Such high level of polymorphism would make AFLP a powerful technique in elucidating genetic differentiation in *Yasuhikotakia* species. The species-specific AFLP marker would be useful in genetic identification of larvae which are hard to be identified to the species level using morphological characters as stated by Kottelat (2004). UPGMA Cluster, *Y. sidthimunki* and *Y. nigrolineata*, with more closely related. The results comfirmed that the *Y. sidthimunki* is very similar in morphology to *Y. nigrolineata*. It is suggested that AFLP is a useful tool to study genetic variation of *Yasuhikotakia* as stated by Vos *et al*. (1995) and Keiper and McConchie (2000).

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