การวิเคราะห์ พีซีอาร์ อาร์เอฟแอลพี ของไมโตคอนเดรียดีเอ็นเอ เพื่อบ่งชี้ความแตกต่างของประชากรปลาหมอ (*Anabas testudineus*) ในประเทศไทย

PCR-RFLP Analysis of Mitochondrial DNA to Differentiate Populations of Climbing Perch (*Anabas testudineus*) in Thailand

Syarif Hidayat[1, 2] and Wansuk Senanan[1]*

[1]Department of Aquatic Science, Faculty of Science, Burapha University.

[2]Department of Aquaculture, Faculty of Fisheries, Brawijaya University, Malang-Indonesia.

บทคัดย่อ

การเพาะเลี้ยงปลาหมอ (*Anabas testudineus*) ได้รับความสนใจในหลายๆ ประเทศ เนื่องจากเป็นปลาที่มีการบริโภคอย่างแพร่หลาย เป็นแหล่งโปรตีนที่มีราคาถูก และเพิ่มความสำคัญทางเศรษฐกิจในระดับนานาชาติ กรมประมงของประเทศไทยจึงมีความสนใจที่จะคัดเลือกปลาชนิดนี้ให้มีลักษณะที่ดีขึ้นสำหรับการเพาะเลี้ยงสัตว์น้ำ อย่างไรก็ตามยังขาดข้อมูลเบื้องต้นถึงระดับความหลากหลายทางพันธุกรรม และความแตกต่างทางพันธุกรรมของแหล่งปลาที่ใช้ในการปรับปรุงพันธุ์ การศึกษานี้จึงใช้เทคนิคพีซีอาร์อาร์เอฟแอลพี (polymerase chain reaction restriction fragment length polymorphism; PCR-RFLP) ของบริเวณดีลูปของไมโตคอนเดรีย ในการบ่งชี้ความแตกต่างระหว่างประชากรธรรมชาติ (6 ประชากร) และประชากรโรงเพาะฟัก (2 ประชากร) ของปลาหมอ (*Anabas testudineus*) (n = 21-29) การวิเคราะห์ความแปรปรวนแสดงความหลากหลายทางพันธุกรรมของตัวอย่างจากโรงเพาะฟักที่น้อยกว่าตัวอย่างจากธรรมชาติอย่างมีนัยสำคัญ (P = 0.01; ค่าเฉลี่ยความหลากหลายแฮพโพลไทป์ = 0.52 และ 0.10 ในตัวอย่างจากธรรมชาติ และจากโรงเพาะฟัก ตามลำดับ) อย่างไรก็ตาม ไม่พบความสัมพันธ์ระหว่างการปรากฏรูปแบบของแฮพโพลไทป์ กับแหล่งของตัวอย่าง การศึกษานี้ยังพบความแตกต่างกลุ่มตัวอย่างในเกือบทุกกลุ่ม โดยการวิเคราะห์ความแปรปรวนทางพันธุกรรม (Analysis of Molecular Variance; AMOVA) พบ 57.83% ของความแปรปรวนเกิดจากความต่างระหว่างประชากร นอกจากนี้ค่า F_{ST} ระหว่างคู่ตัวอย่าง ยังบ่งชี้ความแตกต่างระหว่าง เกือบทุกคู่การทดสอบ ยกเว้นสองคู่ (ระหว่างตัวอย่างจากโรงเพาะฟัก 2 ตัวอย่าง และ ระหว่าง ชลบุรี, CH และ ราชบุรี, RT) แผนภูมิแสดงความสัมพันธ์ทางวิวัฒนาการ (Neighbor-Joining dendrogram) ที่สร้างจากค่าระยะห่างทางพันธุกรรม Nei's genetic distance ยังบ่งชี้ความแตกต่างระหว่างตัวอย่างจากจังหวัดสกลนคร (SN) กับตัวอย่างอื่นๆ แต่ไม่ได้แสดงความสัมพันธ์อย่างชัดเจนระหว่างตัวอย่างที่เหลือ (นครปฐม (NP), RT, CH และนครศรีธรรมราช (NS)) ซึ่งเป็นตัวแทนของแม่น้ำสายหลักในประเทศไทย ดังนั้นเทคนิคพีซีอาร์อาร์เอฟแอลพีของบริเวณดีลูป จึงเป็นเทคนิคที่มีประสิทธิภาพในการประเมินความหลากหลายทางพันธุกรรมภายในและระหว่างประชากรปลาหมอได้ อย่างไรก็ตาม ระดับความหลากหลายทางพันธุกรรมดังกล่าวอาจยังไม่เพียงพอในการแสดงความสัมพันธ์ทางพันธุกรรมระหว่างกลุ่มตัวอย่างบางกลุ่มได้ ข้อมูลจากการศึกษานี้สามารถใช้ประกอบการตัดสินใจในการจัดการระดับความหลากหลายทางพันธุกรรมที่มีอยู่ในปัจจุบันของประชากรโรงเพาะฟัก และช่วยในการออกแบบประชากรตั้งต้นในการคัดเลือกปรับปรุงพันธุ์ของปลาชนิดนี้ได้

คำสำคัญ : พีซีอาร์อาร์เอฟแอลพี บริเวณดีลูป ไมโตคอนเดรียดีเอ็นเอ Anabas testudineus ประเทศไทย

Corresponding author. E-mail: wansuk@buu.ac.th

Abstract

Climbing perch (*Anabas testudineus*) is a common tropical fresh- and brackish water fish species in Asia. Its aquaculture has received attention in many Asian countries both as an emerging economic species and affordable protein source. The Thai Department of Fisheries, therefore, has been interested in a selective breeding program for this species. However, the lack of genetic data prevents such systematic breeding program. We performed polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of mitochondrial D-loop region to differentiate six wild and two hatchery populations of *Anabas testudineus* in Thailand (n = 21 - 29). Analysis of variance indicated that hatchery populations had lower genetic diversity than wild populations (P = 0.01; average haplotype diversity = 0.52 and 0.10 in the wild and hatchery samples, respectively). However, there was no clear relationship between the presence of haplotypes and geographic locations. Almost all populations were genetically distinct. Analysis of Molecular Variance (AMOVA) indicated 57.83% among population genetic variation. Pairwise F_{ST} values showed significant divergence in almost all population pairs, except for two pairs (two hatchery samples; and Chonburi, CH and Ratchaburi, RT). Neighbor-Joining dendrogram based on Nei's genetic distance suggested high divergence between Sakhon Nakhon, SN and the remaining samples, but it did not resolve the relationships among Nakhon Pathom (NP), RT, CH and Nakhon Si Thammarat (NS) representing major river systems in Thailand. The PCR-RFLP of the mitochondrial D-loop region was an effective technique to evaluate genetic diversity within and among populations. However, the level of polymorphism may not be adequate to resolve the relationships among some populations. The results can aid the management of existing genetic variation within hatcheries and the development of a base population for selective breeding.

Keywords : PCR-RFLP, D-loop region, mitochondrial DNA, Anabas testudineus, Thailand

Introduction

Climbing perch (*Anabas testudineus*) is a common tropical fresh- and brackish water fish species in Asia. Its aquaculture has received attention in many Asian countries both as an emerging economic species and affordable protein source. Production of *A. testudineus* in Thailand increased from 7,700 tons in 2001 to 16,200 tons in 2005 (Thailand Department of Fisheries, 2006). With growing number of farms (550 farms (recorded in 2005 and 1,024 farms recorded in 2007) in Thailand, there are interests in developing domesticated broodstock of this species (Dr. Sanga Leesanga, Chumphon Fisheries Test and Research Center, Personal communication).

An initial step for a breeding program is to develop a base population. A critical requirement for an effective breeding program is to know whether the species exists as a single genetic unit (homogeneous) or as series of relatively genetically distinct groups (heterogeneous) (Beaumont & Hoare, 2003). Ferguson (1994) suggested that a commercial breeding program should start from a base population with a wide-range genetic base. This will ensure for long-term genetic responses (Hayes *et al*., 2006). To develop such base population, genetic data of the source populations will be critical. The genetic data will help us determine whether potential sources for the base population are genetically distinct. In addition, these data can aid the decisions to conserve original gene pool for future application (Tinni *et al*., 2007).

Mitochondrial DNA (mtDNA) genetic marker has been proven successful in differentiating populations of several fish species such as swordfish (Chow *et al*., 1997), sockeye salmon (Brykov *et al*., 2003), Dolly varden (Oleinik *et al*., 2004), and freshwater goby (Takahashi & Ohara, 2003). MtDNA can be more sensitive than allozyme or nuclear DNA to detect genetic differences between populations that are recently diverged (Parker *et al*., 1998). It has smaller effective population size (Liu & Cordess, 2004) and higher rate of nucleotide substitutions than allozymes or nuclear DNA (Brown *et al*., 1979). In addition, mtDNA is appropriate for population genetic study because mitochondrial genome is small, maternally inherited, and only small amount of sample needed for analysis. The D-loop or control region (non-coding region) of mtDNA is generally more variable than the coding region due to its lower functional constrains and lack of selection pressure (Liu & Cordess, 2004). In this study, we evaluated genetic variation of *A. testudineus* from different geographic areas using PCR-RFLP of mtDNA D-loop region. The information obtained from the study can help design management strategies to maximize genetic variation in hatchery populations as well as to help identify populations with unique genetic identity for conservation.

Materials and methods

We collected fin clips of adult *A. testudineus* from six wild and two hatchery populations (Table 1). Samples were collected between years 2007 to 2008. The 2007 samples were collected by the staff of the Chumphon Fisheries Test and Research Center. Wild samples came from Nakhon Si Tammarat (NS), Uttaradit (U), Sakhon Nakhon (SN), Chonburi (CH), Ratchaburi (RT), and Nakhon Pathom (NP), while hatchery samples were from Chumphon Fisheries Test and Research Center (CU1) and a private farm in Chumphon (CU2). Samples were preserved in 95% ethanol.

DNA was extracted following a protocol modified from Aljanabi and Martinez (1997). PCR amplification was performed using primer MTA-01 (5'-AAG CCA GGA TTC TAA ATT AAA-3') and MTA-02 (5'-TCT TCA GTG TTA TGC TTT GA-3') designed from available sequence of mitochondrial D-loop region of *A. testudineus* in the online database, Genbank (accession number EF179144). A 10 µl PCR reaction contains 1X PCR buffer, 0.4mM $MgCl_2$, 0.2mM of dNTP, 0.2µM of each primers, 10-100 ng of DNA template, and 0.2 unit of

Table 1. Descriptions of *A. testudineus* samples used in this study.

No	Location	Sample code	Sample number	River system	Geographic region	Collection year
1	Uttaradit	U	21	Chao Phraya	Northern	2007
2	Nakhon Pathom	NP	23	Chao Phraya	Central	2008
3	Sakhon Nakhon	SN	22	Mekong	Northeast	2007
4	Ratchaburi	RT	26	Maeklong-Petchaburi	Central	2007
5	Chonburi	CH	29	Eastern	Eastern	2008
6	Nakhon Si Tammarat	NS	21	Peninsular	Southern	2007
7	Chumphon Fisheries Test and Research Center	CU1	28	-	-	2007
8	Private farm in Chumphon	CU2	21	-	-	2007

Taq polymerase (Vivantis). PCR was performed in a thermal cycler (TGradient) with a temperature profile consisting of 40 cycles of denaturation at 93°C for 1 minute, annealing at 49°C for 1 minute 30 seconds and extension at 72°C for 1 minute. The cycle ended with 1 cycle of 72°C for 10 minutes. PCR amplified about 903 basepairs (bp) fragment of the D-loop region.

We analyzed restriction patterns of seven restriction enzymes; *Apo*I, *Ava*II, *Eco*RII, *Hae*II, *Hind*II, *Afl*II, and *Bcc*I (Table 2). We analyzed the potential restriction sites using a complete D-loop sequence from GenBank (EF179144) and six individuals from NS, CH and NP. Digestion reaction was performed using 1 µg of PCR product for 1 Unit of restriction enzyme. Digestion was done in 10 µl of reaction volume and incubated for at least six hour at the specified incubation temperature. DNA fragments cleaved by the restriction enzyme were electrophorezed on 1.5% agarose gel and stained with

Table 2. Specificity of restriction enzymes used in this study and the potential fragment size after restriction (generated using a complete sequence of *A. testudineus* D-loop region from Genbank, EF179144, and a partial sequence obtained in our study).

Enzyme	Recognition sites	Incubation temperature (°C)	Potential restriction cuts (basepairs)
*Apo*I	RAATTY	50	71, 217, 620
*Ava*II	GGWCC	37	409, 499
*Eco*RII	CCWGG	37	8, 143, 160, 220, 377
*Hae*II	RGCGCY	37	392, 516
*Hind*II	GTYRAC	37	81, 167, 660
*Afl*II	CTTAAG	37	655, 114*
*Bcc*I	$CCATC(N)_4$	37	86, 95, 239, 488

* Using result from the DNA sequencing result in the preliminary study (769 bp)

A = Adenosine; G = Guanine; T = Thymidine; C = Cytosine; R = G or A; Y = T or C; W = A or T; N = Any base.

ethidium bromide. The electrophoretic migration of restricted DNA fragments (haplotype) on a gel were compared against 50 bp DNA ladder. The DNA fragments were visualized under UV light (VILBER LOURMAT ETX-40M). Each distinct haplotype pattern was assigned with a letter. Each individual fish was assigned by multiple letters representing its composite haplotype.

We evaluated two levels of genetic variation (within and among populations). For within population genetic variation, we estimated haplotype diversity (h) and nucleotide diversity (π) using DA implemented in REAP (McElroy *et al*., 1992). Homogeneity test was performed using MONTE implemented in REAP to determine whether the haplotype frequencies differ significantly among populations. To test whether within-population genetic variation differs among populations, we performed analysis of variance (ANOVA) of haplotype number and haplotype diversity using SPSS 11 (SPSS Inc.). To examine whether the presence of particular haplotypes correlate with geographic locations of the samples, we constructed a Neighbor-Joining dendrogram based on Nei's genetic distance of composite haplotypes using PHYLIP version 3.68 (Felsenstein, 1989).

To evaluate population genetic divergence, two approaches were used: (1) analysis of molecular variance (AMOVA), and (2) genetic distance and cluster analysis. AMOVA test was calculated using ARLEQUIN version 3.11 (Excoffier *et al*., 2005). A pairwise comparison among populations (F_{ST}) and Exact tests were also performed using ARLEQUIN. A Nei's genetic distance matrix (a hundred bootstrap replicates) was obtained using MTDIS (Danzmann, 1998). The distance matrix then used to construct a Neighbor-Joining dendrogram using PHYLIP version 3.68 (Felsenstein, 1989).

Results and discussions

Within population genetic variation

Restriction analysis yielded fifteen composite haplotypes with the number of haplotype in each sample ranging from one (CU2) to six (NP and SN) (Table 3). SN had a very unique set of composite haplotypes to other populations. This observation may indicate that SN sample is genetically distinct from the others, although small number of samples could also explain why there were no shared haplotypes between SN and other populations. Among all haplotypes, *h2* was the most common haplotype which present in almost all samples except NS and SN. CH and NP shared the highest number of haplotype with three haplotypes present in both samples. Monte Carlo simulation test (1000 simulations) for haplotype frequency heterogeneity among populations showed significant differences in haplotype distribution among all populations (χ^2 = 98.53, $P < 0.001$). Cluster analysis based on the level divergence of mtDNA haplotypes did not reveal strong geographical association (Hidayat, 2010).

Both haplotype diversity and nucleotide diversity suggested a similar genetic diversity pattern with NP having highest haplotype diversity (0.78) and nucleotide diversity (0.028) and CU2 having lowest haplotype diversity (Table 3). Nucleotide diversity values were much lower than haplotype diversity in every sample. Most samples had relatively low nucleotide diversity (0.000-0.028). The level of MtDNA diversity of the examined *A. testudineus* populations was similar to other freshwater fishes. The haplotype diversity values (h) observed in the *A. testudineus* wild populations (h = 0.39-0.78; average = 0.52) (Table 3) are within the ranges observed in other freshwater species, such as an Amazonia species, *Brycon opalinus*, wild populations (h = 0.00-0.89; average = 0.60; Hilsdorf *et al*., 2002); and Goldfish, *Carassius auratus gibelio*, (h = 0.00-0.84; average = 0.55; Brykov *et al*., 2002). The values observed in this study are higher than those observed in a migratory fish species such as brown trout, *Salmo trutta*, (h = 0.00-0.54; average = 0.30; Weiss *et al*., 2000).

Although the average value of haplotype diversity found in this study was lower than those observed in

Table 3. Composite haplotypes, haplotype frequencies, haplotype diversity (h±SE), and nucleotide diversity (π) of the studied *A. testudineus* populations.

Composite haplotypes		Sampling locations							
		CH (n = 29)	**NP** (n = 23)	**NS** (n = 21)	**SN** (n = 22)	**U** (n = 21)	**CU1** (n = 28)	**CU2** (n = 21)	**RT** (n = 26)
h1	*BBBBBBB*	21 (0.72)	4 (0.17)	-	-	-	-	-	13 (0.50)
h2	*BBBBBBC*	7 (0.24)	8 (0.34)	-	-	12 (0.57)	25 (0.89)	21 (1.00)	13 (0.50)
h3	*BBBBBBA*	-	-	-	-	8 (0.38)	3 (0.11)	-	-
h4	*BBBBABC*	-	-	-	-	1 (0.04)	-	-	-
h5	*BBBBAAB*	-	-	-	1 (0.04)	-	-	-	-
h6	*CBBBBBB*	-	1 (0.04)	16 (0.76)	-	-	-	-	-
h7	*CBBBBBC*	1 (0.03)	3 (0.13)	4 (0.19)	-	-	-	-	-
h8	*CBBBBAB*	-	-	-	2 (0.09)	-	-	-	-
h9	*CBBBBAC*	-	-	-	16 (0.72)	-	-	-	-
h10	*CBBBABB*	-	-	1 (0.04)	-	-	-	-	-
h11	*CBBAAAB*	-	-	-	1 (0.04)	-	-	-	-
h12	*CBBAAAC*	-	-	-	1 (0.04)	-	-	-	-
h13	*CABBBAC*	-	-	-	1 (0.04)	-	-	-	-
h14	*DBBBBBB*	-	1 (0.04)	-	-	-	-	-	-
h15	*DBBBBBC*	-	6 (0.26)	-	-	-	-	-	-
Haplotype diversity (h±SE)		0.42±0.06	0.78±0.03	0.39±0.08	0.47±0.09	0.54±0.04	0.20±0.07	0.00±0.00	0.51±0.01
Nucleotide diversity (π)		0.004	0.028	0.000	0.000	0.001	0.000	0.000	0.000

several marine fishes, some populations had reasonably high level of genetic diversity. Both samples from the Chao Phraya river system (U and NP) showed higher genetic diversity than the other river systems (average h = 0.66 and 0.45 for samples from the Chao Phraya and other river systems, respectively). This result is similar to the observation in another species with similar life history to *A. testudineus*. Na-Nakorn *et al.* (2004) detected higher level of genetic diversity of *Clarias macrocephalus* in the Chao Phraya river system compared to the Mekong, South-east, and Peninsular river systems; the authors suggested that the Chao Phraya river system might provide a good breeding ground with little geographical barriers. Thus, these fish species were able to maintain a large population size encountering the effects of genetic drift.

The ability of *A. testudineus* to 'walk' and survive on land for an extended period of time might increase the chance for gene flow between neighboring populations. Genetic contribution from distinct genetic sources will increase genetic variation of the receiving populations. Sokheng *et al.* (1999) reported the history of lateral migration of this species from the Mekong mainstream or other permanent water bodies to flooded areas during the flood season.

ANOVA suggested that wild populations had higher genetic variation compared to hatchery populations (average h = 0.52 and 0.10, respectively; P = 0.01). The findings are typical for several species. Sekino *et al.* (2002) found that wild populations of Japanese Flounder *Paralichthys olivaceus* had higher mtDNA variation (average h = 0.99) than hatchery populations (h = 0.69-0.79; average = 0.76). Study of *Hemigrammocypris rasborella* in Japan by Watanabe *et al.* (2009) also showed higher mtDNA variation in the wild populations (h = 0.27-0.79; average = 0.51) compared to the hatchery populations (h = 0.00-0.79; average = 0.39). Possible reasons for the reduction in genetic variation in these hatchery populations include bottleneck effects and inbreeding.

In *A. testudineus* hatchery populations, some aquaculture practices, such as selective breeding, using unequal numbers of male and female breeders, and using small number of founders may lead to the reduction of genetic variation. The individuals from both hatchery populations have undergone at least four generations of domestication and some selection for body size and growth (Dr. Sanga Leesanga, Chumphon Fisheries Test and Research Center, personal communication). A breeding scheme used at the Chumphon Fisheries Test and Research Center (CU1 sample) is a group spawning of 100-200 individuals with a 1:1 male to female ratio (Dr. Sanga Leesanga, Chumphon Fisheries Test and Research Center, personal communication). However, mass spawning might allow for differential broodstock contribution which will reduce genetic variation (Frost *et al.*, 2006). Even though the broodstock at the Chumphon Fisheries Test and Research Center was derived from the broodstock at the private farm, the level of genetic diversity in CU1 sample was slightly higher than that observed in the CU2 sample. This observation may reflect the difference in practices between these two hatcheries (e.g., the intensity of selection, sex ratio, and number of breeders).

Low level of genetic diversity in hatchery populations might also reflect a consequence of domestication selection. This type of selection usually favors individuals (or genotypes) with the ability to survive in captivity. It may inadvertently reduce genetic variation in the successive generations.

Among Population Genetic Variation

All analyses indicated genetic differences among populations. AMOVA indicated that 57.83% of the observed genetic variation was a result of among population genetic variation. Pairwise F_{ST} values ranged from 0.06 (CU1-CU2) to 0.87 (NS-CU2). Pairwise F_{ST} also indicated significant genetic differences in almost all

Table 4. Pairwise F_{ST} comparisons among populations. * and ** indicated significant levels at $P < 0.05$ and 0.002 after Bonferroni correction, respectively.

	CH	NP	NS	SN	U	CU1	CU2	RT
CH								
NP	0.33**							
NS	0.56**	0.48**						
SN	0.72**	0.53**	0.65**					
U	0.48**	0.23**	0.75**	0.70**				
CU1	0.61**	0.27**	0.84**	0.76**	0.15*			
CU2	0.66**	0.29**	0.87**	0.77**	0.31**	0.06		
RT	0.06	0.19**	0.60**	0.68**	0.27**	0.39**	0.45**	

sample pairs (Table 4), except for the CH-RT, CU1-CU2, and U-CU1 (*0.002<P<0.05*). Neighbor-Joining dendrogram based on Nei's genetic distance also suggested genetic divergence among samples, except for CU1 and CU2 (Figure 1). However, it could not resolve the relationships among NP, RT, CH and NS.

The examined *A. testudineus* populations exhibited a pattern of genetic variation typical to many freshwater fish species, such as *Opsariichthys bidens* and *Xiphophorus cortezi* (among population genetic variation = 92.21% and 95.03%, respectively) (Perdices *et al*., 2005; Rodríguez *et al*., 2007). However, a study on *Hypseleotris compressa* populations in Australia did not show this pattern of genetic diversity (among population genetic variation = 1.4%); the authors suspected that this observation may be due to historical connectivity between the populations (McGlashan & Hughes, 2001).

RFLP of mtDNA used in this study revealed a higher level of population differentiation compared to nuclear DNA. The RAPD study of *A. testudineus* detected 17% among populations genetic variation (Unpublished data; an identical set of populations except for CH and NP). This difference may be due to higher variability and sensitivity to genetic drift of D-loop mtDNA compared to nuclear DNA.

Most populations examined were genetically distinct. Among wild population pairs, only the CH-RT pair did not show significant F_{ST} value (F_{ST} = 0.06; P > 0.05) (Table 4). Among the genetically distinct populations, highest F_{ST} value in wild population pairs was between NS and U (0.75; $P < 0.002$) while the lowest value was found between NP and RT (0.19; $P < 0.002$). Geographical distance probably explained the pattern of this genetic differentiation. The insignificant F_{ST} value between CH and RT might reflect historical connectivity

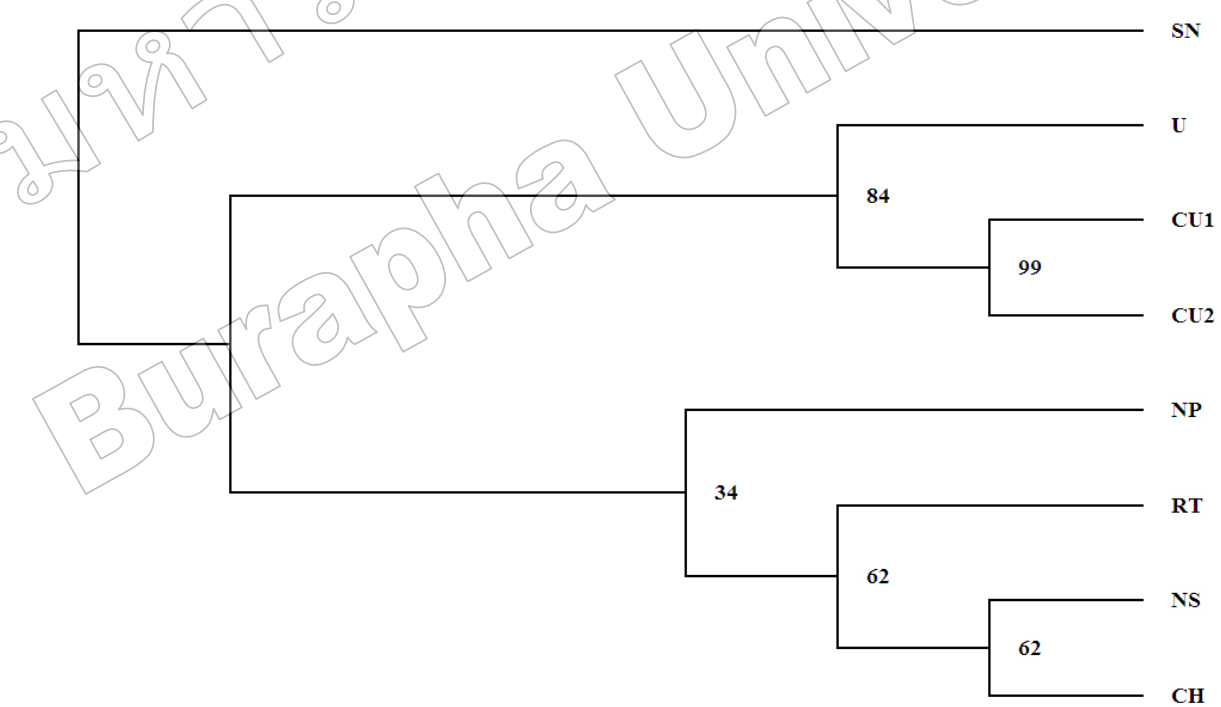

Figure 1. A consensus genetic dendrogram based on Nei's genetic distance. Numbers on each node represent numbers of the same node generated over 100 bootstrap replicates.

of the rivers (Maeklong-Petchaburi and Eastern river systems) or it might be coincident. There has been no record of the first speculation. Furthermore, NP which is a population located between the two populations was genetically distinct from both populations. The second speculation is possible as genetic drift as a random process that can lead to a similar genetic outcome in independent populations, especially if these populations are small.

The low F_{ST} value between the CU1 and CU2 samples (F_{ST} = 0.06, P > 0.05) reflected the historical exchange between these two populations. The CU1 sample was derived from the CU2 which had been domesticated for some generations (Dr. Sanga Leesanga, Director of Chumphon Fisheries Test and Research Center; Personal communication). From the haplotype found in this study (Table 3) and the genetic dendrogram (Figure 1), some founders for the CU2 sample may have came from the central part of Thailand.

Genetic dendrogram generated in this study suggested three major clusters (Figure 1). SN was highly diverged from other samples. This finding coincides with the presence of unique haplotypes found in SN. The dendrogram also differentiated U from the remaining wild samples (bootstrap value = 84%). However, the dendrogram did not resolve the relationships among some populations located in different river systems (NP, RT, NS, and CH representing Chao Phraya, Maeklong-Petchaburi, Peninsular and eastern river systems respectively). An extensive stocking program all over the country using breeders from the Choa Phraya River basin may also explain this observation (Dr. Sanga Leesanga, Chumphon Fisheries Test and Research Center, Personal communication). In contrast to the strong population structure of this species detected by allozymes (Sekino & Hara, 2000), the level of polymorphism of RFLP of mtDNA D-loop may not be adequate to resolve these relationships. Also, small sample sizes used in the study may not allow the detection of all available polymorphism in each population.

Recommendations for broodstock management

After only a few generations of domestication, the level of genetic variation in hatchery populations was quite low. The hatcheries may need to consider changing some aquaculture practices to increase genetic variation and prolong the loss of genetic variation. These practices may include using a 1:1 male to female ratio, and avoid using a group spawning for broodstock development.

Breeding scheme used at the Chumphon Fisheries Test and Research Center is already 1:1 male to female ratio. However, in a group spawning, the contribution of parents is likely unknown. Group spawning also tends to skew family representation in the next generations (Frost *et al*., 2006). It would be interesting to asses the contribution of the parents in each spawning using parentage assignment of hyper variable markers such as microsatellite DNA.

Declining in genetic variation in hatchery populations could also due to low number of individuals in a founding population. Kapuscinski & Miller (2007) recommended using 60 to 500 individuals. Although the Chumphon Fisheries Test and Research Center have been using 100-200 individuals to start a population, using domesticated stock as a source population may have restricted the available genetic diversity. To increase genetic variation, these hatcheries may need to introduce new genetic material from the wild or other genetically distinct hatchery stocks. Study comparing natural and hatchery populations of *Betta splendens* in Thailand by Meejui *et al.* (2005) found that observed heterozygosity in hatchery populations was higher than the wild (0.081-0.125 for the hatchery populations and 0.065 in natural population) as a result of routine stock exchange between hatcheries.

Conclusions

We detected high haplotype diversity in the samples that located in the Chao Phraya river

system compared to other river systems. Both hatchery samples had lower haplotype diversity compared to the wild samples. Significant differences found in almost all population pairs of *A. testudineus* examined with 57.83% of variation was due to variation among population. However, cluster analysis did not showed strong population structuring based on the geographic locations of the rivers.

RFLP of mtDNA is an effective tool for evaluating genetic variation of populations. From this study, despite the low number of samples, mtDNA marker showed high value of genetic variation in NP and U. It successfully revealed genetic differences among almost all population pairs, and it even successfully differentiated CH from NP although they were located close to each other. However, restriction mapping in this part of DNA might under represent the available level of genetic variation, especially when genetic changes occur in nucleotides not specific to restriction enzyme. This restricted level of variation may have limited our ability to provide strong population structure among samples. Genetic markers with higher resolution such as sequencing of mtDNA D-loop region may provide additional insights on genetic structure of these populations.

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