MORPHOLOGICAL DIFFERENCES AND GENETIC DIVERSITY OF RICE FIELD FROGS, *Hoplobatrachus rugulosus* (Wiegmann, 1835), FROM NATURAL HABITATS IN THAILAND

Mr. Anusorn Pansook

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biological Sciences
Faculty of Science
Chulalongkorn University
Academic Year 2010

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ความแตกต่างทางสัณฐานวิทยาและความหลากหลายทางพันธุกรรมของกบนา
Hoplobatrachus rugulosus (Wiegmann, 1835) จากแหล่งอาศัยธรรมชาติในประเทศไทย

นายอนุสรณ์ ปานสุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

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Thesis Title	MORPHOLOGICAL DIFFERENCES AND GENETIC DIVERSITY OF RICE FIELD FROGS, *Hoplobatrachus rugulosus* (Wiegmann, 1835), FROM NATURAL HABITATS IN THAILAND
By	Mr. Anusorn Pansook
Field of Study	Biological Sciences
Thesis Advisor	Associate Professor Putsatee Pariyanonth
Thesis Co-advisor	Assistant Professor Sanit Piyapattanakorn, Ph.D.

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อนุสรณ์ ปานสุข: ความแตกต่างทางสัณฐานวิทยาและความหลากหลายทางพันธุกรรมของกบนา *Hoplobatrachus rugulosus* (Wiegmann, 1835) จากแหล่งอาศัยธรรมชาติในประเทศไทย. (MORPHOLOGICAL DIFFERENCES AND GENETIC DIVERSITY OF RICE FIELD FROGS, *Hoplobatrachus rugulosus* (Wiegmann, 1835), FROM NATURAL HABITATS IN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ผุสตี ปริยานนท์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ศานิต ปิยพัฒนากร, 120 หน้า.

การศึกษาความแตกต่างทางสัณฐานวิทยาของกบนา *Hoplobatrachus rugulosus* โดยวิธี Principal canonical analysis (PCA) และ Cluster analysis และการศึกษาความหลากหลายทางพันธุกรรม โดยวิธี Inter-simple sequence repeat (ISSR) และการหาลำดับเบสของไมโทคอนเดรีย ใน 18 พื้นที่ (6 ภูมิภาค) ของประเทศไทย

จากการศึกษาลักษณะทางสัณฐานวิทยาของกบนาโตเต็มวัย เพศผู้จำนวน 109 ตัว และเพศเมียจำนวน 91 ตัว โดยวิธี PCA พบว่า ขนาดลำตัวระหว่างเพศผู้และเพศเมีย มีความแตกต่างกัน จากแผนภูมิการจัดกลุ่มของลักษณะทางสัณฐานวิทยา โดยวิธี Cluster analysis สามารถแบ่งกบนาเพศผู้และเพศเมียออกเป็น 6 และ 7 กลุ่มตามลำดับ แต่ความแตกต่างทางสัณฐานวิทยาของกลุ่มกบนาเหล่านี้ไม่สอดคล้องกับข้อมูลการกระจายพันธุ์ทางภูมิศาสตร์ของตัวอย่างกบนาที่ใช้ในการศึกษาในครั้งนี้

การศึกษาความหลากหลายทางพันธุกรรมของกบนา โดยวิธี ISSR จากประชากรกบนาจำนวน 230 ตัว จาก 18 พื้นที่ ใน 6 ภูมิภาค พบว่าประชากรกบนาในเขตภาคตะวันออกมีความหลากหลายทางพันธุกรรมสูงสุด ส่วนประชากรกบนาในเขตภาคเหนือความหลากหลายทางพันธุกรรมต่ำสุด โดยพบว่าระยะห่างทางพันธุกรรมกับระยะห่างทางภูมิศาสตร์ไม่มีความสัมพันธ์กัน สำหรับการศึกษาความสัมพันธ์ทางสายวิวัฒนาการด้วยลำดับเบสของไมโทคอนเดรียดีเอ็นเอ จากลำดับเบสของยีน cyt-*b* ความยาว 564 คู่เบส และลำดับเบสของยีน 12S rRNA และยีน 16S rRNA ความยาว 813 คู่เบส สามารถแบ่งกลุ่มประชากรได้เป็น 2 สายวิวัฒนาการอย่างชัดเจน และความแตกต่างของลำดับเบสระหว่าง 2 สายวิวัฒนาการมีค่าสูงมากจนอาจจะทำให้สามารถจำแนกกบนาในประเทศไทยออกเป็น 2 ชนิดหรือชนิดย่อยได้

จากผลการศึกษาที่พบว่าลักษณะทางสัณฐานวิทยาของกบนาที่ได้กับข้อมูลทางโมเลกุลไม่สัมพันธ์กัน โดยพบว่าลักษณะทางสัณฐานวิทยาระหว่างกลุ่มประชากรจาก 6 ภูมิภาค มีความแตกต่างกันน้อย ในขณะที่ข้อมูลทางโมเลกุลมีความแตกต่างกันมากจนอาจสามารถจำแนกกบนาในประเทศไทยได้เป็น 2 ชนิด อย่างไรก็ตามเพื่อความชัดเจนจึงควรจะต้องมีการศึกษาข้อมูลของกบนาเพิ่มเติม เช่น เสียงร้อง, สรีรวิทยา, นิเวศวิทยา และพฤติกรรม เพื่อที่จะทำให้สามารถยืนยันได้ว่ากบนาในประเทศไทยสามารถจำแนกออกเป็น 2 ชนิดได้หรือไม่

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: *Hoplobatrachus rugulosus* / MORPHOMETRIC / GENETIC DIVERSITY / PRINCIPAL CANONICAL ANALYSIS (PCA) / CLUSTER ANALYSIS / INTER-SIMPLE SEQUENCES REPEAT (ISSR) / MITOCHONDRIAL DNA

ANUSORN PANSOOK: MORPHOLOGICAL DIFFERENCES AND GENETIC DIVERSITY OF RICE FIELD FROGS, *Hoplobatrachus rugulosus* (Wiegmann, 1835), FROM NATURAL HABITATS IN THAILAND. ADVISOR: ASSOC. PROF. PUTSATEE PARIYANONTH, CO-ADVISOR: ASST. PROF. SANIT PIYAPATTANAKORN, Ph.D., 120 pp.

Morphological differences in rice field frogs, *Hoplobatrachus rugulosus*, were examined using Principal Canonical Analysis (PCA) and cluster analysis while the genetic diversity was investigated using inter-simple sequence repeat (ISSR) and mitochondrial DNA sequence analysis from 18 localities (6 regions) in Thailand.

Morphometric investigation in this species of 109 male and 91 female adult frogs showed a clear size sexual dimorphism. The dendrograms from the cluster analysis for separate male and female adult frogs grouped samples into six and seven groups for adult male and female, respectively. Nevertheless, these groups in both male and female did not correspond to any geographic region in Thailand.

The genetic diversity based on ISSR to investigate population genetic structure of *H. rugulosus* from 18 localities in 6 regions of Thailand found that the highest genetic variability was found in the eastern region, whereas the lowest genetic variability was in the northern region. Moreover, the genetic distances and the geographic distances among populations from 6 regions were not correlated. The phylogenetic relationship using two mitochondrial DNA sequences, a 564 bp fragment of the cyt-*b* gene and a 813 bp combined fragment of the 12S and 16S rRNA genes, clearly revealed *H. rugulosus* into two distinct clades. The high sequence divergences between the two major clades suggest that *H. rugulosus* as currently recognized may contains two distinct species in Thailand.

According to these results, the morphological data did not provide resolution sufficient to reveal any difference in morphological characters among 6 regions. On the other hand, the molecular data yielded better resolution and revealed at least one "hidden" entity within *H. rugulosus*. However, additional information including, but not limiting to, bioacoustic, physiological, ecological and behavioral characters, will be needed to further elucidate the species status of taxonomic of *H. rugulosus*.

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Student's Signature Anusorn Pansook
Advisor's Signature P. Pariyanonth
Co-advisor's Signature Sanit Piyapattanakorn.

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Finally, I would like to dedicate all the best of my dissertation to my glorious parents for their love, encouragement, help and understanding during my study.

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CHAPTER I

GENERAL INTRODUCTION

Amphibians (caecilians, frogs and salamanders) are a conspicuous component of the world's vertebrate fauna. They currently include about 6,000 recognized species with representatives found in virtually all terrestrial and freshwater habitats. The number of recognized species of amphibians has grown enormously, with a ~ 48.2% increase since 1985 (Frost et al., 2006), but many species of amphibians have and still are disappearing because of habitat loss or fragmentation, and in addition due to unsustainable levels of hunting for food and the international pet-trade (Alford and Richards, 1999; Blaustein et al., 2003).

Chan-ard (2003) reported that 141 species of amphibians, from the three orders of Gymnophiona, Caudata and Anura, were found in Thailand. Most of these species (134) belong to the order Anura, with six and one species from the orders Gymnophiona and Caudata, respectively. The genus *Hoplobatrachus* is one of the genera within the order Anura with representative species in Africa and Asia (Frost, 2010). This genus is presently recognized to consist of four species, *H. crassus*, *H. occipitalis*, *H. tigerinus* and *H. rugulosus*.

H. rugulosus, also known as the rice field frog (Figure 1.1), is the only species of this genus that is widely distributed in Thailand (Chan-ard, 2003; Inthara et al., 2004; Taksintum et al., 2009). This species is widespread from East Asia to Myanmar through Thailand, Laos, Viet Nam and Cambodia (Figure 1.2). Its natural habitats are floodplain wetlands, forest pools and the like, but it has adapted to man in as much as it is now found in paddy fields, irrigation infrastructure, fishponds, ditches and so on (Frost, 2010).

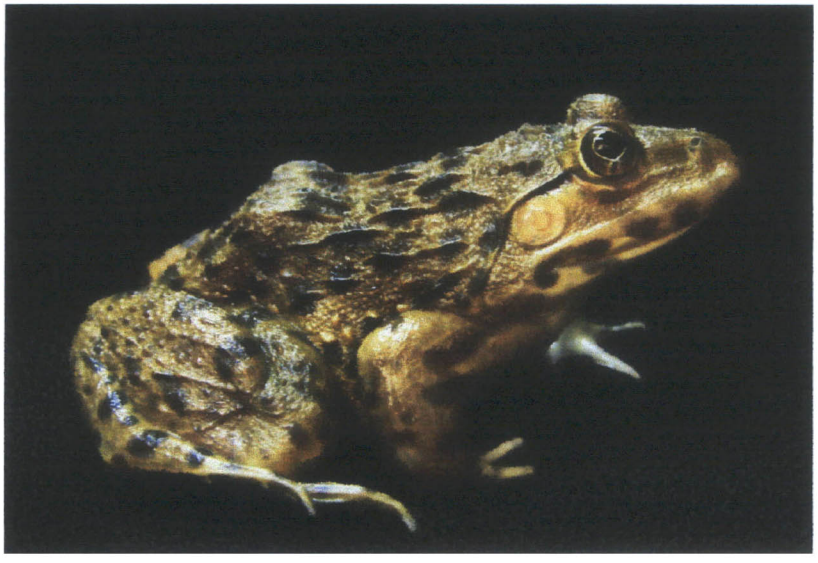

Figure 1.1 *Hoplobatrachus rugulosus*

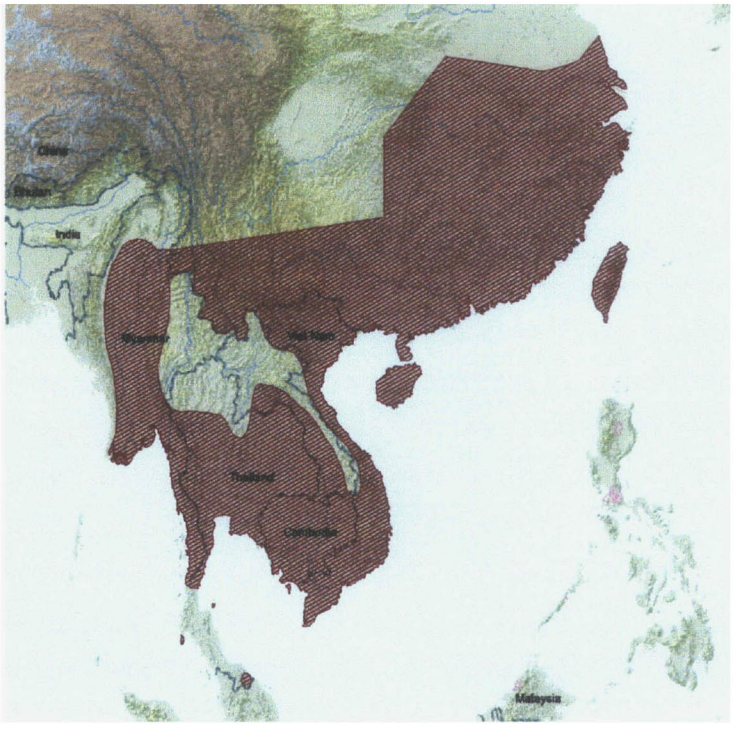

Figure 1.2 The known distribution of *H. rugulosus* (modified from http: //www. iucnredlist.org/apps/redlist/details/58300/0/rangemap)

Currently, this species has decreased in numbers in both its natural and adapted anthropological habitats in Thailand, principally because of habitat loss and

toxic contaminants in the environment (Jiwyam et al., 2006). The other reason for this decline is non-sustainable levels of hunting because this species is an edible and an economic animal. Lau et al. (1999) reported that more than six million specimens of this species were imported into Hong Kong from Thailand in 1999. Whilst some of these frogs were from commercial farms, many were wild caught. Indeed, although as an economically important species the rice field frog has been domesticated for more than 30 years, it is important to note that even here the brooder stocks for such commercial farms are taken from the natural environment leading to their further decline (Pariyanonth and Daorerk, 1995).

While the natural population of this species has decreased in Thailand, the rice field frog's morphology and genetic diversity within and between geographical populations, all remain uncertain. Thus, whether human agriculture and environment adapted frogs represent the complete natural genetic diversity or only a small subset following either, for example, habitat-dependent adaptive selection or foundress-bottlenecks, is unknown. Likewise, the effect the natural habitat destruction is having on the genetic makeup of the frog populations that remain in these habitats, and on all sympatric populations regardless of any natural / agricultural habitat divisions, is unknown. To address this, this study specifically studied the degree of variation between these populations using; 1) morphometric analysis to determine if there are any morphological differences within and between geographical populations, 2) genetic diversity and population structure, as evaluated with inter-simple sequence repeat (ISSR) markers, and 3) the genetic diversity and phylogeographic relationships as evaluated by mitochondrial DNA (mtDNA) sequence analysis. The knowledge gained from this study can be used in the conservation of genetic diversity, future strain improvement and stock identification of the rice field frogs in natural habitats.

CHAPTER II

MORPHOMETRIC DIFFERENCES IN RICE FIELD FROGS, (*Hoplobatrachus rugulosus*), FROM NATURAL HABITATS IN THAILAND

Introduction

The rice field frog, *Hoplobatrachus rugulosus,* has a widespread distribution from central, southern and south-western China, including Taiwan, Hong Kong and Macau, to Myanmar, Thailand, Viet Nam and Cambodia and south to the Thai-Malay peninsula. *H. rugulosus* is the only species in the genus *Hoplobatrachus* that is widely distributed in Thailand. (Chan-ard, 2003; Inthara et al., 2004; Taksintum et al., 2009).

Based on morphological data, the classification of rice field frog in Thailand was first reported by Malcolm A. Smith (1917). Two distinct forms of tadpole collected from Bangkok were reported, one with a long snout and elongated body and another with a shorter snout and more rounded body. He remarked one of these different forms was similar to the morphological characters of *Rana tigrina* tadpoles. This was later supported by Taylor (1962), who reported the morphological characters of *H. rugulosus* (as *R. rugulosa*) with slightly similar to *R. tigrina* (as *R. tigerina pantherina*), but that the body, arm and leg lengths of *H. rugulosus* were shorter than *R. tigrina,* and that *H. rugulosus* can be found in northern and northeastern regions of Thailand whilst *R. tigrina* was found in all the parts of Thailand (Taylor, 1962; Nuttaphan, 2001). However, *R. tigrina* is now recognized as a synonym of *H. rugulosus* (Dubois, 1992) leading Chan-ard (2003) to propose only one species, *H. rugulosus,* which can be found in all parts of Thailand. A study based upon

mitochondrial DNA sequence analysis revealed highly divergent sequences between *H. rugulosus* populations (Alam et al., 2008), potentially separating *H. rugulosus* into more than one species, except that in this study the samples were collected from only three populations rather than throughout Thailand. Thus, it is still unclear what the morphological diversity of rice field frogs is in Thailand, and if this relates to variation within populations of the same species or to different sub-species.

Morphometrics is the measurement of organisms or of their parts to study the variation and change in the size and shape within and between species. There are several methods for extracting data from shapes, each with their own benefits and weaknesses. These include the measurement of lengths and angles, landmark analysis and outline analysis. Whichever measurement(s) is / are taken, their analysis typically begins with a PCA, which highlights any trends and makes it easy to spot any correlation with other features. Morphometric studies aim to describe the size or shape of organisms in the simplest possible fashion, removing extraneous information and thereby facilitating comparison between different organisms. As such, morphometrics is the prevalent technique to study the morphological variation in allopatric population groups of many organisms, including plants (Nybom et al., 1997), termites (Koshikawa et al., 2002), moths (Miles, 1983), fish (Hard et al., 2000; Silva, 2003), skinks (Faizi and Rastegar-Pouyani, 2006), lizards (Zug et al., 2006) and geckos (McMahan and Zug, 2007). In the case of amphibians, morphological variations have been reported in many species of amphibians, such as moor frogs (*R. arvalis*) (Babik and Rafiński, 2000), golden-striped salamanders (*Chioglossa lustianica*) (Alexandrino et al., 2005), Japanese salamanders (*Hynobius naevius*) (Tominaga et al., 2005), yellow-bellied toads (*Bombina variegata*) (Vukov et al.,

2006) and Tunisian green frogs (*R. saharica*) (Amor et al., 2009) amongst others, and morphometrics can be applied to clarify the morphological variation of these species.

Morphological variation in *H. rugulosus* was first reported by Schmalz and Zug (2002), who analyzed the morphological variation of this species on a larger geographical scale, that is among four different countries (Myanmar, Thailand, Hong Kong and Taiwan), with only one population sampled from Thailand. Moreover, there are no previous morphological differences reported for *H. rugulosus* found in Thailand.

In this chapter, the morphological characters are examined for any differences in *H. rugulosus* populations in each region of Thailand. The results can be applied to clarify the morphological variations of *H. rugulosus* in Thailand.

Materials and methods

Sample collection

In the majority of reported studies on variation in amphibians, the main analyses have been made on adults to standardize for variation in different developmental stages (Wilson and Larsen, 1999; Breder et al., 2000; Vukov et al., 2006; Amor et al., 2009; Chuaynkern et al., 2010). Thus, all adult samples of *H. rugulosus* were collected from natural habitats in six biogeographic regions (16 localities) of Thailand, namely the North, Northeast, Central, West, East and South (Nabhitabhata and Chan-ard, 2005) (Figure 2.1). Adult males were identified by the presence of the vocal sac. On the other hand, adult females are not easily distinguishable by their external features, and so discrimination was made by the results of subsequent dissection to reveal the internal sexual organs. The samples sizes collected and analyzed ranged from 17 to 62 (Table 2.1).

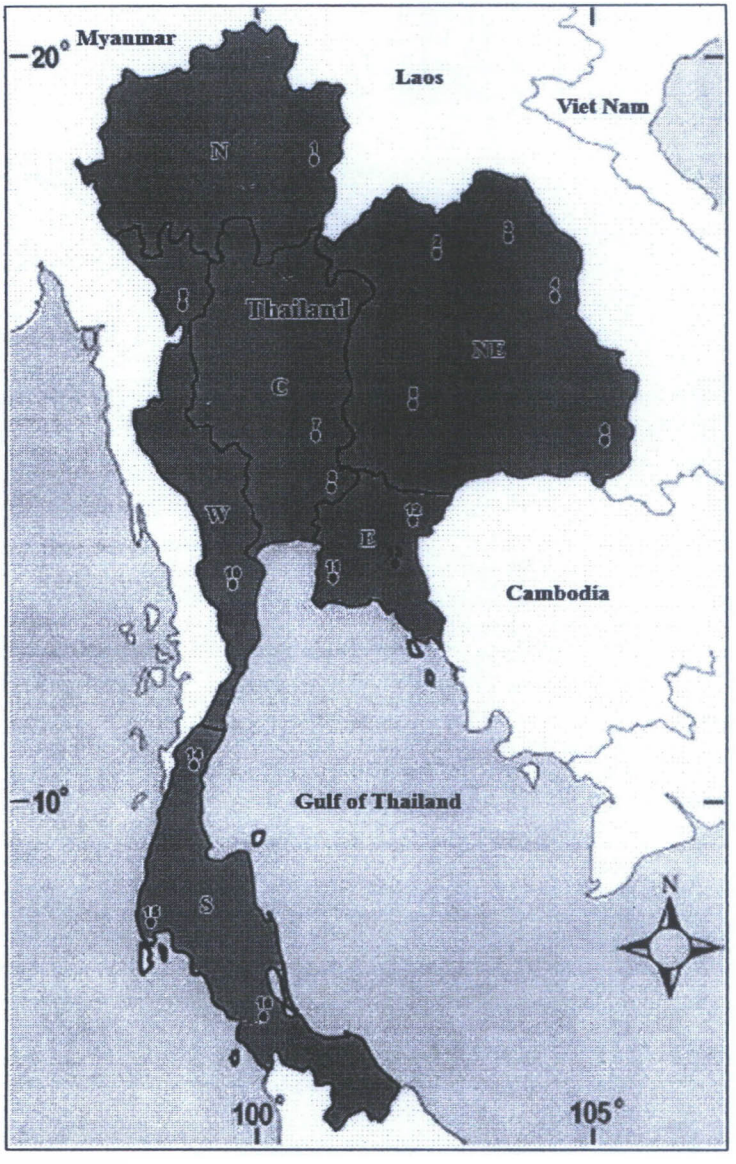

Figure 2.1 A map of Thailand showing the collection localities of *H. rugulosus* samples in each region. For the locality numbers, refer to Table 2.1. Geographical regions indicated on the map are; N = North, NE = Northeast, C = Central, W = West, E = East and S = South.

Table 2.1 The localities and number (*n*) of *H. rugulosus* samples collected and analyzed in each region

Region	Locality		Sample sizes (n)	
	Map[1]	Name (Code)	Male	Female
North	1	Nan	14	11
Northeast	2	Udon Thani (UDN)	7	6
	3	Sakon Nakhon (SKN)	11	5
	4	Mukdahan (MDH)	1	3
	5	Nakhon Ratchasima (NKR)	10	10
	6	Ubon Ratchathani (UBR)	9	-
Central	7	Lopburi (LOP)	1	1
	8	Nakhon Nayok (NKN)	7	12
West	9	Tak (TAK)	2	2
	10	Phetchaburi (PCB)	16	14
East	11	Chonburi (CBR)	5	5
	12	Sa-Kaeo (SKO)	18	9
	13	Chanthaburi (CTR)	2	2
South	14	Chumphon (CHP)	1	2
	15	Phang-nga (PNA)	4	8
	16	Songkhla (SKL)	1	1

[1]*Numbers refer to the indicated locality on the map of Figure 2.1*

Samples were measured after they were euthanized in a glass jar of a saturated solution of 1,1,1-trichloro-2-methyl-2-propanol hemihydrate (Chloretone).

Sample measurement

All samples were measured with digital vernier caliper with an accuracy of 0.01 mm. Twenty character dimensions were measured for each sample, as shown in Figure 2.2. Descriptions of character dimensions are presented in Appendix A (Matsui, 1984). These include most of the lengths hitherto measured in the taxonomy of anurans. Usually, paired structures were measured on the left side of the body

unless there was a defect or anomaly on that side. Direct line distance was measured for each dimension, unless otherwise noted.

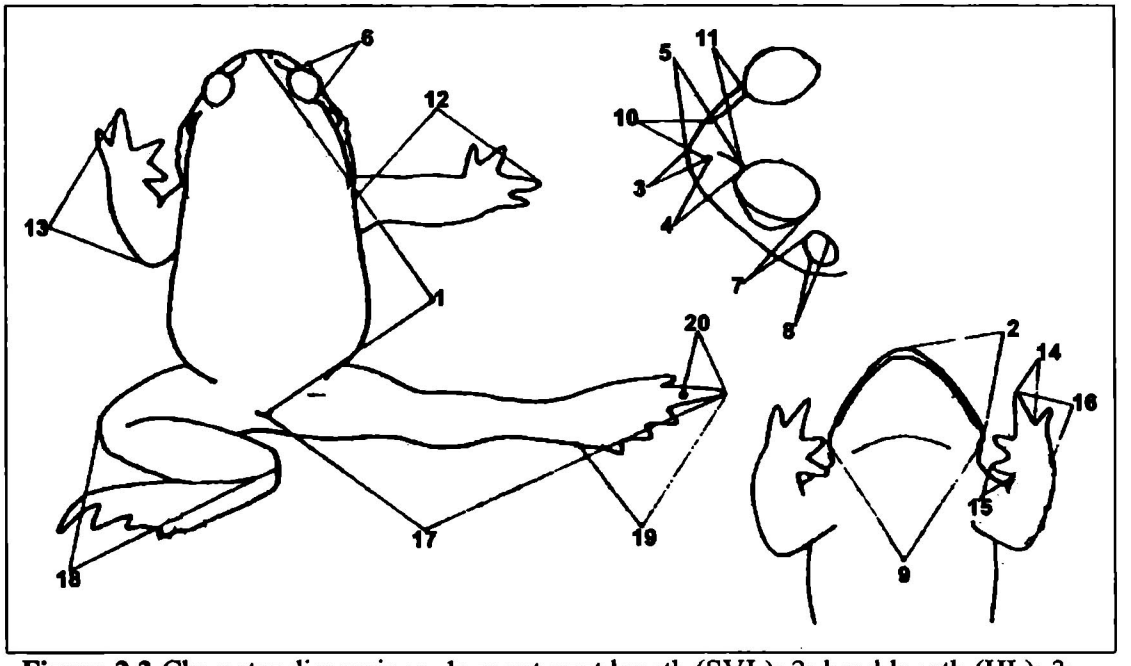

Figure 2.2 Character dimensions. 1: snout-vent length (SVL); 2: head length (HL); 3: snout-nostril length (S-NL); 4: nostril-eye length (N-EL); 5: snout length (SL); 6: eye length (EL); 7: tympanum-eye length (T-EL); 8: tympanum diameter (TD); 9: head width (HW); 10: internarial distance (IND); 11: intercanthal distance (ICD); 12: forelimb length (FLL); 13: lower arm length (LAL); 14: third finger length (TFL); 15: first finger length (FFL); 16: hand length (HAL); 17: hindlimb length (HLL); 18: tibia length (TL); 19: foot length (FL); 20: fourth toe length (FTL) (modified from Matsui, 1984).

Data analysis

The data was log-transformed in order to meet the assumption of normality more closely and to remove any allometric effects on the body size (Hayek et al., 2001). PC-ORD version 4.0 software was used for all statistical analysis, principal

component analysis (PCA) with correlation matrix and no rotation, and cluster analysis with Euclidean distance measurement. The PCA was performed to explore the morphometric variability independent of the regional group assignment. The data from adult males and females were analyzed separately because size sexual dimorphism in *H. rugulosus* has been reported (Schmalz and Zug, 2002).

Results

Based on 200 samples of *H. rugulosus* (109 males and 91 females) from the six different regions of Thailand, the average values, standard deviation (S.D.), coefficient of variation (*CV*) and ranges for all the character dimensions measured of rice field frog *H. rugulosus* samples are represented in Tables 2.2 - 2.7. The average of the average SVL for adult male and female *H. rugulosus* from the six regions of Thailand were 99.4 ± 7.7 mm and 110.2 ± 11.8 mm, respectively. *H. rugulosus* populations from the central region exhibited the highest average SVL values of male (111.1 mm) and female (132.2 mm) frogs, whilst the lowest average SVL values of male (90.1 mm) and female (99.4 mm) frogs were exhibited in the populations from the eastern and northern regions, respectively. For a comparison of variability in the SVL values between males and females, sexual dimorphism was evident ($t = 6.315$, df = 198, $p < 0.05$), and adult females were generally larger in the SVL than adult males. The coefficient of variation (*CV*) for all the character dimensions range from 13.7% to 31.0% and from 13.0% to 24.0% for adult male and female *H. rugulosus*, respectively. The highest *CV* were 31.0% (ICD) and 24.0% (IND) for adult males and females, respectively.

Table 2.2 The average values, standard deviation (S.D.), coefficient of variation (*CV*) and ranges for all the measured character dimensions of adult male *H. rugulosus* from the northern and northeastern regions of Thailand. Character abbreviations are as defined in Figure 2.2. Sample sizes are represented in parentheses. The average values, S.D. and ranges are represented in mm.

Region		SVL	HL	HW	S-NL	N-EL	SL	EL	T-EL	TD	IND	ICD	FLL	LAL	TFL	FFL	HAL	HLL	TL	FL	FTL
North (14)	Average	101.99	30.58	30.53	6.88	6.74	13.61	8.97	4.79	6.77	3.42	7.89	53.52	37.47	11.26	11.84	19.46	141.17	43.32	66.74	14.41
	S.D.	6.78	1.59	2.60	0.50	0.58	0.99	0.77	0.77	0.62	0.82	1.34	4.08	2.22	0.93	0.76	0.82	9.50	2.93	3.78	1.01
	CV	6.65	5.20	8.52	7.22	8.56	7.24	8.63	16.06	9.09	24.10	16.98	7.63	5.93	8.26	6.41	4.22	6.73	6.77	5.66	7.01
	Minimum	93.41	28.27	26.96	6.04	6.11	12.47	8.03	3.40	5.89	2.41	5.72	46.08	34.28	10.03	10.71	18.46	128.88	39.38	61.75	13.01
	Maximum	116.75	33.41	37.29	7.76	8.20	15.96	10.22	6.31	8.15	5.57	11.08	60.33	41.63	12.70	13.30	21.64	160.30	48.65	74.34	16.55
Northeast (38)	Average	93.06	28.72	29.64	6.92	6.14	13.06	8.85	4.40	6.64	3.75	6.24	48.17	33.73	10.49	9.79	18.35	137.30	39.73	61.59	12.94
	S.D.	14.84	3.98	4.27	1.08	1.10	2.03	0.99	0.92	1.03	1.05	2.32	8.46	5.85	1.96	2.32	2.88	20.32	8.09	9.78	2.17
	CV	15.95	13.85	14.40	15.59	17.85	15.53	11.21	21.01	15.56	27.92	37.25	17.56	17.36	18.67	23.68	15.70	14.80	20.36	15.87	16.76
	Minimum	68.90	21.30	21.86	4.85	3.80	8.65	7.20	2.71	4.83	2.04	3.02	33.18	22.22	7.55	3.53	13.11	106.75	26.49	43.48	8.43
	Maximum	116.92	35.97	38.19	8.65	8.34	16.92	10.80	6.19	8.49	5.78	11.59	63.08	47.10	14.87	13.82	23.04	174.74	52.41	74.70	16.43

Table 2.3 The average values, standard deviation (S.D.), coefficient of variation (*CV*) and ranges for all the measured character dimensions of adult male *H. rugulosus* from the central and western regions of Thailand. Character abbreviations are as defined in Figure 2.2. Sample sizes are represented in parentheses. The average values, S.D. and ranges are represented in mm.

Region		SVL	HL	HW	S-NL	N-EL	SL	EL	T-EL	TD	IND	ICD	FLL	LAL	TFL	FFL	HAL	HLL	TL	FL	FTL
Central (8)	Average	111.13	36.24	35.73	8.19	6.65	14.84	9.91	5.94	7.86	4.80	6.31	61.04	43.64	13.95	12.36	23.87	154.82	45.85	73.86	15.33
	S.D.	13.08	5.24	5.04	1.16	0.85	1.86	1.05	0.78	0.84	0.57	0.69	8.73	6.53	2.05	1.44	2.82	18.93	5.54	8.97	1.79
	CV	11.77	14.46	14.11	14.17	12.77	12.50	10.56	13.10	10.69	11.93	10.93	14.30	14.96	14.69	11.68	11.81	12.23	12.09	12.14	11.67
	Minimum	82.05	24.02	24.76	5.76	5.08	10.84	7.56	4.17	6.41	3.71	5.30	43.37	29.92	10.07	9.60	18.80	109.39	33.50	53.55	11.80
	Maximum	124.90	41.44	41.41	9.65	7.92	16.76	10.80	6.63	9.34	5.48	7.04	72.69	50.89	16.58	14.18	27.66	168.90	50.13	82.15	17.38
West (18)	Average	96.37	28.97	29.97	7.17	6.49	13.65	9.19	5.37	6.81	3.45	8.29	49.35	36.01	11.47	10.46	18.94	129.76	40.78	61.85	13.80
	S.D.	13.52	3.73	4.06	0.80	0.97	1.62	1.02	1.14	1.03	0.69	1.47	6.81	4.99	1.51	1.77	3.36	17.59	6.07	8.47	2.02
	CV	14.03	12.88	13.55	11.20	14.91	11.88	11.05	21.19	15.08	19.91	17.76	13.79	13.85	13.19	16.97	17.74	13.56	14.90	13.69	14.66
	Minimum	63.70	20.50	20.00	5.60	4.91	10.60	7.11	3.50	4.48	2.41	5.34	36.16	24.35	7.83	6.18	11.65	88.51	27.05	42.80	9.66
	Maximum	113.61	33.77	34.65	8.62	8.33	16.07	11.51	7.89	8.90	4.88	10.87	61.95	42.99	13.90	13.01	22.55	150.76	48.28	72.33	16.66

Table 2.4 The average values, standard deviation (S.D.), coefficient of variation (*CV*) and ranges for all the measured character dimensions of adult male *H. rugulosus* from the eastern and southern regions of Thailand. Character abbreviations are as defined in Figure 2.2. Sample sizes are represented in parentheses. The average values, S.D. and ranges are represented in mm.

Region		SVL	HL	HW	S-NL	N-EL	SL	EL	T-EL	TD	IND	ICD	FLL	LAL	TFL	FFL	HAL	HLL	TL	FL	FTL
East (25)	Average	90.12	28.62	28.12	6.52	6.48	13.09	8.20	4.08	6.38	3.66	5.08	45.51	14.16	9.41	8.28	17.18	124.73	38.23	55.67	12.36
	S.D.	15.17	4.37	4.33	1.14	1.16	2.01	1.49	1.06	0.97	0.74	1.13	11.17	7.37	2.19	3.08	3.63	21.46	6.39	13.55	2.65
	CV	16.83	15.25	15.40	17.52	17.93	15.36	18.12	26.06	15.14	20.34	22.30	24.54	22.68	23.23	37.14	21.15	17.20	16.70	24.33	21.47
	Minimum	69.60	20.70	20.60	4.52	4.52	9.78	4.91	2.24	4.72	2.29	3.31	31.10	23.50	6.99	3.51	12.90	96.90	29.30	31.30	9.20
	Maximum	121.10	37.13	37.13	8.95	9.34	16.88	11.08	6.43	8.48	5.96	8.19	67.70	46.47	14.17	14.60	23.79	166.90	50.92	80.67	17.74
South (6)	Average	103.74	32.86	31.96	7.64	6.56	14.20	9.40	5.36	6.99	3.84	6.43	52.88	37.51	11.21	11.25	19.88	141.62	43.27	66.78	14.60
	S.D.	7.79	3.34	3.13	0.30	0.65	0.92	0.80	0.83	0.64	0.54	1.50	5.13	3.28	1.55	1.53	1.54	8.23	3.37	3.76	1.48
	CV	7.51	10.16	9.80	3.90	9.97	6.47	8.51	15.55	9.21	14.20	23.35	9.71	8.74	13.85	13.56	7.75	5.81	7.78	5.63	10.11
	Minimum	97.10	30.13	28.80	7.17	5.93	13.10	8.23	4.09	6.00	3.35	5.36	45.38	34.34	9.55	8.90	17.25	131.10	39.73	61.88	12.49
	Maximum	117.66	39.12	36.97	7.98	7.74	15.72	10.63	6.46	7.67	4.83	9.36	59.27	43.46	13.43	12.77	22.07	150.30	47.82	70.80	16.76

Table 2.5 The average values, standard deviation (S.D.), coefficient of variation (*CV*) and ranges for all the measured character dimensions of adult female *H. rugulosus* from the northern and northeastern regions of Thailand. Character abbreviations are as defined in Figure 2.2. Sample sizes are represented in parentheses. The average values, S.D. and ranges are represented in mm.

Region		SVL	HL	HW	S-NL	N-EL	SL	EL	T-EL	TD	IND	ICD	FLL	LAL	TFL	FFL	HAL	HLL	TL	FL	FTL
North (11)	Average	99.41	30.67	31.54	7.35	7.17	14.48	8.86	5.03	6.74	3.46	8.01	53.15	37.02	11.23	11.76	19.46	140.33	42.43	66.17	14.10
	S.D.	10.52	2.75	3.17	0.73	0.76	1.43	0.88	0.93	0.98	0.63	1.08	6.15	4.08	1.54	1.49	2.40	15.86	4.65	7.35	1.42
	CV	10.59	8.97	10.05	9.95	10.58	9.86	9.90	18.52	14.54	18.24	13.48	11.58	11.02	13.74	12.68	12.31	11.30	10.97	11.11	10.08
	Minimum	76.49	24.83	24.77	5.78	5.88	11.66	7.26	3.53	4.33	2.34	6.31	39.08	27.07	7.67	8.99	15.50	103.11	31.56	48.90	11.59
	Maximum	114.83	33.86	36.43	8.39	8.17	15.84	9.83	6.68	7.70	4.41	10.08	60.16	41.63	13.14	13.45	23.37	157.76	48.79	75.61	16.14
Northeast (24)	Average	111.44	33.67	36.74	8.20	7.82	16.02	9.73	5.46	7.74	4.82	8.02	57.38	40.21	12.63	12.36	21.76	157.38	47.33	72.58	15.11
	S.D.	18.38	3.71	4.99	1.14	0.99	1.99	1.19	1.52	1.20	1.15	2.36	9.92	6.66	1.88	2.39	3.01	21.18	8.61	10.13	1.54
	CV	16.50	11.03	13.57	13.87	12.62	12.40	12.19	27.87	15.46	23.89	29.41	17.29	16.55	14.86	19.32	13.82	13.46	18.18	13.96	10.17
	Minimum	89.50	27.45	29.50	6.38	5.94	12.50	7.49	3.40	5.70	3.22	4.87	43.80	32.30	10.39	9.12	17.70	129.30	32.80	60.30	12.70
	Maximum	152.08	43.01	47.34	10.80	9.61	19.87	11.61	8.85	10.04	8.55	11.87	78.86	56.98	17.16	17.46	29.03	195.17	64.42	95.54	18.46

Table 2.6 The average values, standard deviation (S.D.), coefficient of variation (*CV*) and ranges for all the measured character dimensions of adult female *H. rugulosus* from the central and western regions of Thailand. Character abbreviations are as defined in Figure 2.2. Sample sizes are represented in parentheses. The average values, S.D. and ranges are represented in mm.

Region		SVL	HL	HW	S-NL	N-EL	SL	EL	T-EL	TD	IND	ICD	FLL	LAL	TFL	FFL	HAL	HLL	TL	FL	FTL
Central (13)	Average	132.21	41.10	41.77	9.21	8.21	17.41	11.13	7.66	8.77	5.57	6.64	71.12	48.49	15.11	14.75	25.74	177.18	52.94	84.28	17.71
	S.D.	14.09	4.36	5.06	1.01	1.04	1.83	1.01	1.18	0.66	0.93	1.01	8.67	6.03	1.75	2.30	2.79	16.83	5.50	8.28	2.60
	CV	10.66	10.60	12.12	10.96	12.67	10.52	9.11	15.44	7.52	16.71	15.15	12.19	12.43	11.55	15.58	10.83	9.50	10.38	9.82	14.68
	Minimum	100.17	32.80	32.07	7.03	6.67	13.70	8.84	5.38	7.94	3.91	4.37	53.96	37.61	10.49	12.05	19.96	136.16	42.90	64.90	12.86
	Maximum	152.40	46.86	49.34	10.66	10.66	20.84	12.78	9.26	9.90	6.96	8.09	84.15	57.15	17.70	19.46	30.20	197.50	61.45	94.69	22.02
West (16)	Average	102.25	30.25	32.06	7.42	6.92	14.34	9.39	5.73	7.31	3.67	8.98	53.94	36.97	11.78	11.04	20.09	134.60	42.48	64.51	14.05
	S.D.	11.30	2.98	4.23	1.03	0.83	1.75	0.78	1.01	0.69	0.68	0.83	5.76	4.28	1.22	1.31	2.23	15.67	4.91	7.15	1.56
	CV	11.05	9.84	13.20	13.87	11.95	12.21	8.26	17.62	9.49	18.61	9.30	10.67	11.57	10.37	11.83	11.10	11.64	11.56	11.09	11.07
	Minimum	71.02	22.38	22.73	5.09	5.35	11.24	7.67	3.42	5.63	2.61	6.68	43.28	27.56	9.46	9.04	15.12	97.55	30.25	48.14	10.84
	Maximum	119.96	33.98	39.72	9.49	8.80	17.53	10.33	7.34	8.24	5.11	10.19	65.59	43.15	13.39	13.83	23.28	156.37	49.01	75.08	16.63

Table 2.7 The average values, standard deviation (S.D.), coefficient of variation (*CV*) and ranges for all the measured character dimensions of adult female *H. rugulosus* from the eastern and southern regions of Thailand. Character abbreviations are as defined in Figure 2.2. Sample sizes are represented in parentheses. The average values, S.D. and ranges are represented in mm.

Region		SVL	HL	HW	S-NL	N-EL	SL	EL	T-EL	TD	IND	ICD	FLL	LAL	TFL	FFL	HAL	HLL	TL	FL	FTL
East (16)	Average	104.88	34.32	33.77	7.42	7.24	14.85	9.36	5.30	7.05	4.05	5.75	54.81	37.90	11.26	10.70	20.58	143.81	42.78	66.56	14.61
	S.D.	15.81	4.43	4.51	1.28	1.20	2.05	1.48	1.29	0.94	0.78	1.06	12.72	7.30	2.20	2.91	3.37	20.21	5.92	12.70	2.44
	CV	15.08	12.91	13.36	17.29	16.57	13.81	15.77	24.28	13.30	19.15	18.48	23.20	19.26	19.51	27.22	16.36	14.05	13.85	19.07	16.70
	Minimum	84.50	28.80	27.50	5.17	5.17	11.48	6.59	3.76	5.82	3.08	4.37	37.20	27.80	8.00	7.11	14.10	115.50	35.50	48.30	10.30
	Maximum	138.40	44.42	44.63	10.91	9.90	18.85	11.75	8.70	8.74	5.34	8.09	77.31	52.03	15.73	16.07	27.51	183.70	55.75	90.98	19.37
South (11)	Average	110.95	34.81	35.68	8.38	7.78	16.16	10.00	6.01	7.15	4.32	6.88	57.71	40.21	12.38	11.81	21.93	154.77	45.27	73.38	15.71
	S.D.	6.13	2.76	2.54	0.82	0.86	1.43	1.10	0.66	0.95	0.58	1.17	3.44	2.72	1.10	1.09	1.34	10.12	2.85	3.97	0.90
	CV	5.52	7.94	7.12	9.74	11.10	8.84	10.99	10.95	13.29	13.49	16.98	5.97	6.76	8.92	9.21	6.12	6.54	6.30	5.41	5.71
	Minimum	101.90	31.56	31.94	7.25	6.48	14.29	8.63	4.64	5.87	3.31	5.30	51.37	35.49	9.91	10.03	20.29	132.60	40.63	64.54	13.88
	Maximum	119.44	39.28	39.75	9.92	9.14	18.39	12.79	6.83	8.56	5.14	9.48	62.82	43.67	14.01	13.77	23.95	167.30	49.81	78.00	17.09

For evaluation of the morphological differences in *H. rugulosus* populations within and between regions and sexes, PCA analysis was used to examine the morphological differences of *H. rugulosus* among the six regions in Thailand. In adult males, the first principal component (PC 1) explained 78.0% of the total variance, whilst 92.5% of the total variance could be explained by as many as six principal components and PCs 2 - 6 each explained a similar amount of variance. The first two PCs were the most important in explaining the morphological differences of adult male *H. rugulosus* [eigenvalue 1 (λ_1) = 15.59 and λ_2 = 1.00), explaining 83.0% of the total variance (Table 2.8). The SVL and the IND represented the highest negative loadings in PC1 (-0.2462) and PC 2 (-0.5479), respectively. The PC comparison of adult males from 16 localities (six regions) shows a cluster for only three localities, at Nakhon Ratchasima (NKR) and Ubon Ratchathani (UBR) from the northeastern region and Sa-Kaeo (SKO) from the eastern region (Figure 2.3).

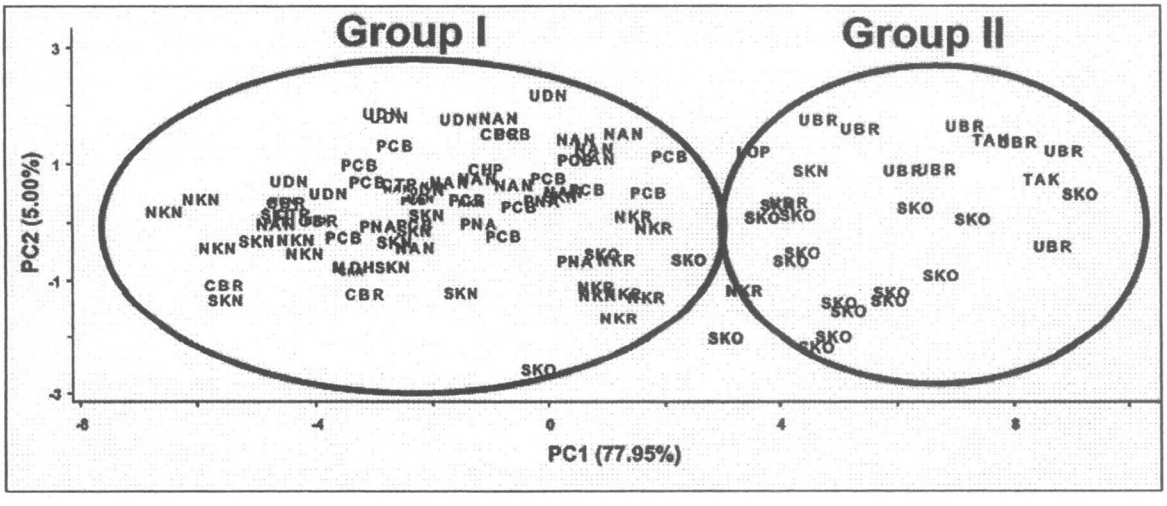

Figure 2.3 Scatter-plot of the principal component scores (PC1 and PC2) of adult male *H. rugulosus* from 16 localities in the six regions of Thailand. Locality abbreviations are as defined in Table 2.1

The result of PCA of adult females was broadly similar to that for adult males. PC 1 explained 77.4% of the total variance for adult females and 92.9% of the total variance was explained by six principal components, with PCs 2 - 6 explaining similar amounts of variance. When comparing the eigenvalues, those of the first two PCs of adult females are not different to that for the adult males (λ_1 = 15.49 and λ_2 = 1.13), and they explained 83.1% of the total variance (Table 2.9). The SVL and the ICD in adult females represented the highest negative (-0.2463) and positive (+0.8189) loadings in PC 1 and PC 2, respectively. The PC comparison revealed two major groups but only three localities, the populations from Nakhon Ratchasima (NKR) in the northeastern region, Nakhon Nayok (NKN) in the central region and Sa-Kaeo (SKO) population in the eastern region were clustered for adult females (Figure 2.4).

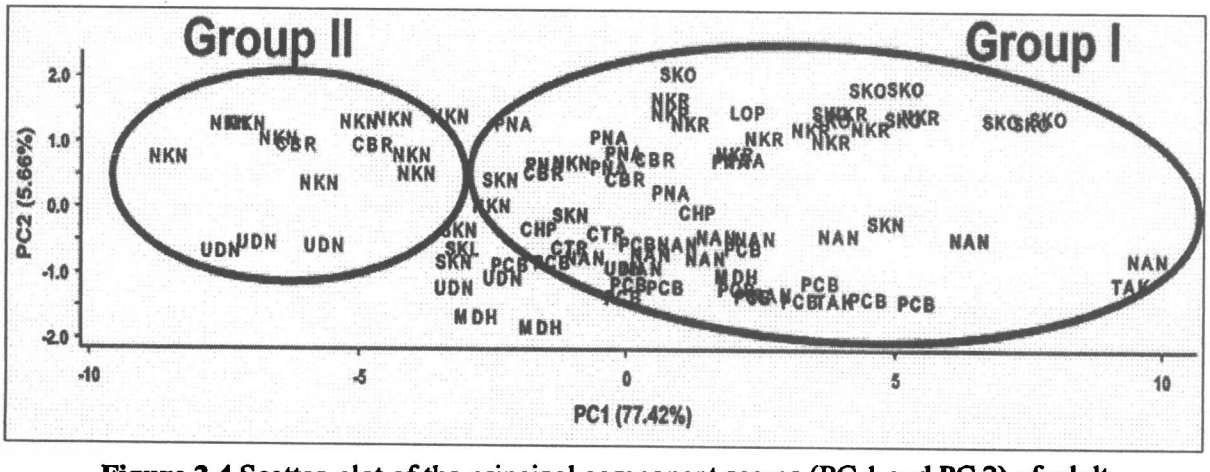

Figure 2.4 Scatter-plot of the principal component scores (PC 1 and PC 2) of adult female *H. rugulosus* from 16 localities in six regions Locality abbreviations are as defined in Table 2.1.

Table 2.8 Principal component loadings (PC 1 to PC 6) of adult male *H. rugulosus*.

Character	1	2	3	4	5	6
SVL	-0.2462	0.0167	0.0225	0.0697	0.0455	-0.0677
HL	-0.2333	-0.1669	0.1300	0.2021	0.0956	0.0050
HW	-0.2370	-0.1227	0.1550	0.0791	0.0149	-0.0724
S-NL	-0.2246	-0.1606	-0.0328	0.0816	0.0369	0.5391
N-EL	-0.1933	-0.4128	-0.5298	0.1090	0.1369	-0.2005
SL	-0.2273	-0.3218	-0.2966	0.1051	0.0859	0.1784
EL	-0.1909	0.0278	0.2003	-0.5593	0.7173	0.0174
T-EL	-0.2124	0.1140	-0.2351	-0.2131	-0.0987	0.6076
TD	-0.2257	-0.0321	0.1396	-0.0999	0.0837	-0.2123
IND	-0.1574	-0.5479	0.4838	-0.3033	-0.4883	0.0284
ICD	-0.1925	0.1459	-0.4022	-0.5955	-0.3465	-0.2520
FLL	-0.2433	0.1298	0.0672	0.0823	0.0165	-0.0857
LAL	-0.2403	0.0944	0.1311	0.0775	-0.0518	-0.0297
TFL	-0.2231	0.3201	0.1410	0.0455	-0.0236	0.1337
FFL	-0.2121	0.4003	-0.0145	0.0446	-0.2386	0.0544
HAL	-0.2337	0.1257	0.1177	0.0810	-0.0499	0.0370
HLL	-0.2394	0.0065	0.0670	0.1520	0.0551	-0.1618
TL	-0.2389	0.0185	-0.0914	-0.0147	-0.0392	-0.2622
FL	-0.2403	0.0967	0.0392	0.1738	0.0146	-0.1147
FTL	-0.2377	0.0823	-0.0899	0.1632	-0.0590	-0.1002
Eigenvalue	15.59	1.00	0.61	0.54	0.42	0.33
% of variance	77.95	5.00	3.07	2.70	2.12	1.63
Cumulative %	77.95	82.95	86.02	88.72	90.84	92.47

**Character abbreviations are defined in Figure 2.2*

Table 2.9 Principal component loadings (PC 1 to PC 6) of adult female *H. rugulosus*.

Character	1	2	3	4	5	6
SVL	-0.2463	0.0287	-0.1452	0.0740	0.0747	0.1560
HL	-0.2278	0.3154	-0.0752	-0.0141	0.0769	0.2035
HW	-0.2418	0.1233	-0.0031	0.0414	0.1507	0.1047
S-NL	-0.2216	0.0316	0.3053	-0.0399	-0.2803	0.3030
N-EL	-0.2022	0.0088	0.5665	-0.3418	0.0105	-0.1123
SL	-0.2287	0.0487	0.4506	-0.1699	-0.1147	0.1073
EL	-0.2192	-0.1025	0.0113	0.3276	-0.3310	-0.2808
T-EL	-0.2122	-0.1415	-0.1967	0.3444	-0.4632	0.3591
TD	-0.2190	-0.0804	0.0217	0.1232	0.6128	0.0572
IND	-0.1847	0.3015	0.2887	0.6387	0.0959	-0.3749
ICD	-0.1009	-0.8189	0.2012	0.1824	0.1366	0.0131
FLL	-0.2354	-0.0609	-0.2237	-0.1077	-0.0703	-0.0047
LAL	-0.2439	-0.0184	-0.1666	-0.1150	0.0157	0.0759
TFL	-0.2263	-0.0355	-0.2470	-0.0841	0.1094	-0.4098
FFL	-0.2212	-0.2203	-0.1332	-0.3049	-0.1426	-0.3721
HAL	-0.2406	0.0693	-0.1292	-0.0714	0.0121	-0.1097
HLL	-0.2439	0.0709	-0.0375	-0.0203	0.0991	0.1075
TL	-0.2383	-0.0599	-0.0681	0.0229	0.2542	0.2872
FL	-0.2460	-0.0148	-0.1110	-0.1359	-0.0310	-0.0220
FTL	-0.2271	0.1230	-0.0363	-0.1166	-0.1829	-0.1918
Eigenvalue	15.49	1.13	0.74	0.54	0.37	0.32
% of variance	77.42	5.66	3.69	2.69	1.83	1.59
Cumulative %	77.42	83.08	86.77	89.46	91.29	92.88

**Character abbreviations are as defined in Figure 2.2*

For the cluster analysis, the dendrograms revealed six and seven major groups (75% information remaining) for adult males and females, respectively. The morphological variations of *H. rugulosus* among the six regions were a little different so the samples from different regions were mixed and clustered into all group except the samples from the Sa-Kaeo (SKO) population were divided within Group V (Figure 2.5). In case of adult females, the results were broadly similar to adult males in that most samples among six regions were mixed and clustered into all group except for from the Nakhon Ratchasima (NKR), Nakhon Nayok (NKN) and Sa-Kaeo (SKO) populations. Almost of the samples from the Nakhon Ratchasima (NKR) and Sa-Kaeo (SKO) populations were divided within Group V whilst the samples from the Nakhon Nayok (NKN) population were divided within Group VII (Figure 2.6).

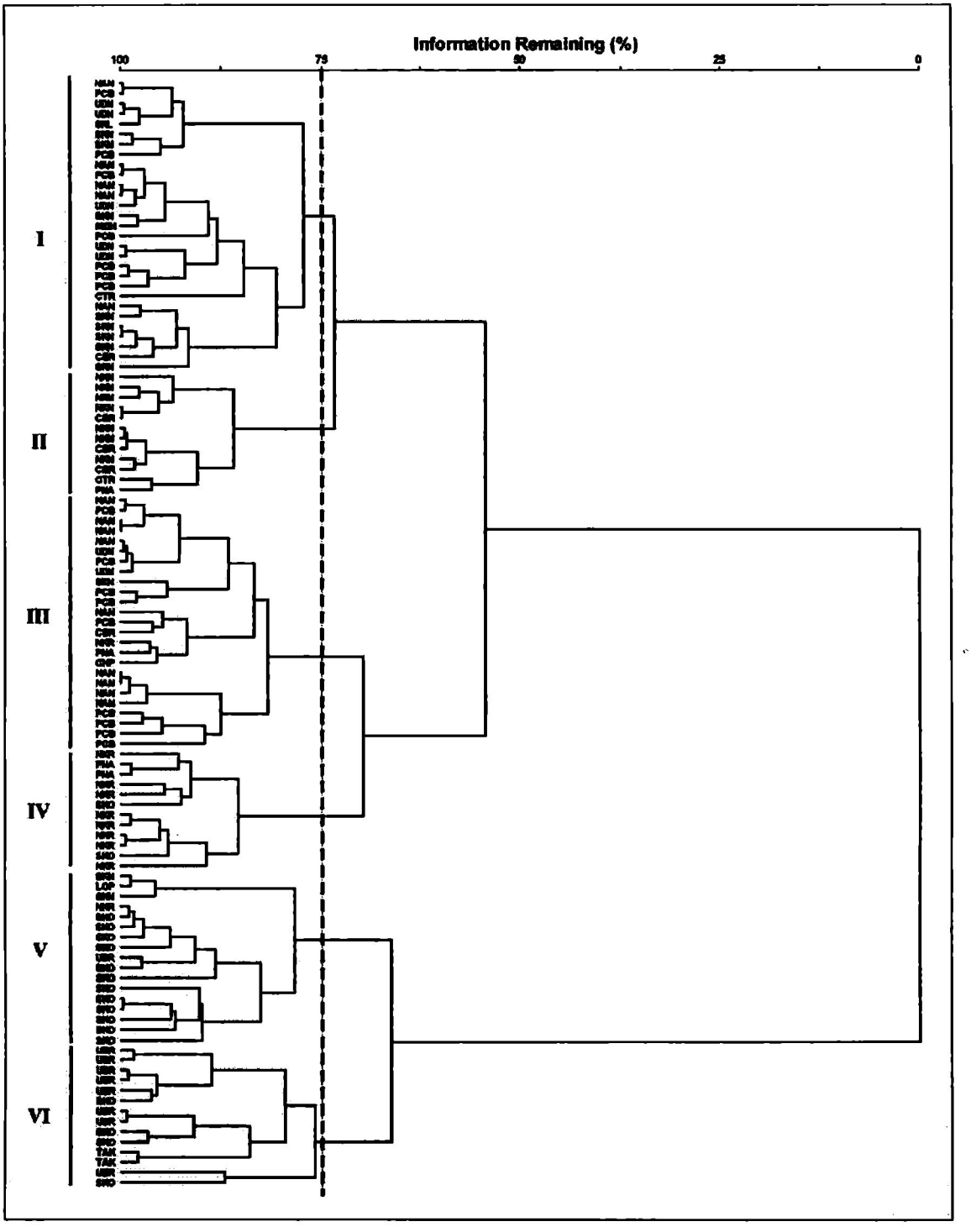

Figure 2.5 Dendrogram representing the morphometrical similarities between adult male *H. rugulosus*. Locality abbreviations are as defined in Table 2.1.

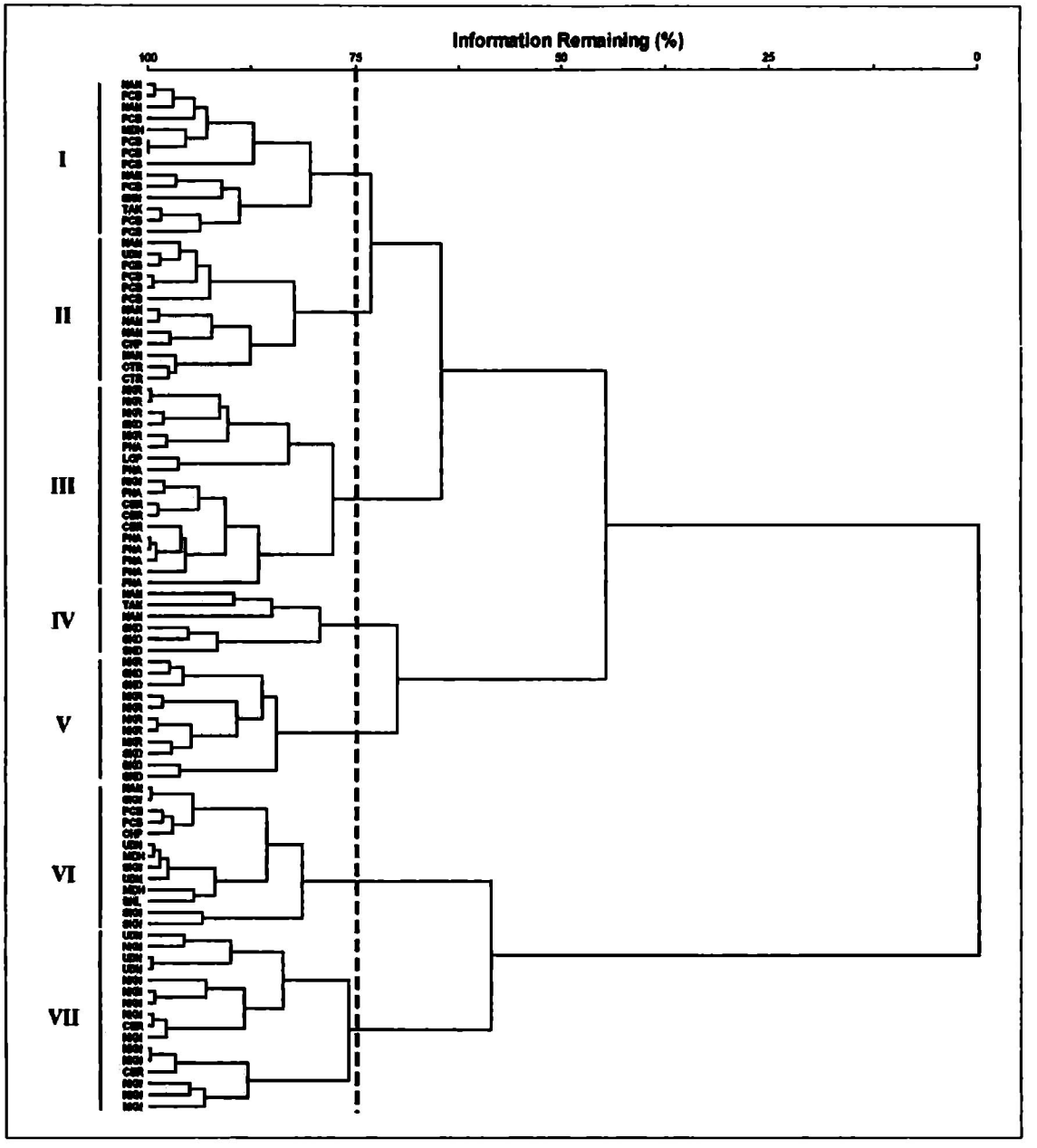

Figure 2.6 Dendrogram representing the morphometrical similarities between adult female *H. rugulosus*. Locality abbreviations are as defined in Table 2.1.

Discussion

Sexual dimorphism in sizes is a common aspect of anuran morphology (Zug, 1993). In this study, the average SVL of adult females was larger than that of adult males by about 15 mm. The size sexual dimorphism of *H. rugulosus,* as revealed by the analysis of morphological variation, confirmed the previous data (Schmalz and Zug, 2002), except that the CV of all character dimensions in this study was higher than their data. Hayek et al. (2001) recommended twenty repeated measurements on each character dimension of the same sample to reduce the CV derived from inter-observer variation. However, we measured the character dimensions of each sample only once, while Schmalz and Zug (2002) applied the repeated measurement for same samples. This then likely accounts for at least part if not all of the higher CV seen for all the character dimensions of this report compared to those reported by Schmalz and Zug (2002).

The PCA of the morphological variables revealed a degree of differentiation within the *H. rugulosus* populations. The PCA results segregated individuals on the basis of size on the first component for adult males and females. *H. rugulosus* from the central region had, on average, a relatively longer SVL than individuals from the other regions for PC 1 in adult males and females, respectively. However, the difference in the SVL seen in this study did not correlate with the previously reported east to west trend across the geographic region (Schmalz and Zug, 2002). Morphological differences in amphibian species are usually related to the geographic variation (environmental factors), such as the relative altitude (Sotiropoulos et al., 2008), temperature (Castellano and Giacoma, 1998) and humidity (Alexandrino et al., 2005). Because the morphological differences observed here did not appear to correlate with the geographic regions, then the observed morphological differences in

H. rugulosus might be affected more by ecological (*e.g.*, effects of coexisting species) than by physical factors.

H. rugulosus is an economically important species because it is a favourite food dishes among many Thai people and it is also a pet and experimental subject. Thus, *H. rugulosus* from various parts of Thailand have been caught and transported to cities for human utilization. Artificially introduced amphibians often establish stable colonies and steady widen the distribution range, such as *Eleutherodactylus johnstonei* (Pough et al., 1977), *R. catesbeiana* (Adams, 1999), *Bufo marinus* (Crossland, 2000) and *Ambystoma tigrinum* (Riley et al., 2003). Doubtlessly a portion of the *H. rugulosus* artificially transported for commercial use will either escape or be released and then may establish a new population if the environmental conditions are suitable. Indeed, *H. rugulosus* has been artificially transferred much more than is usually expected. This then may explain why no significant morphological differences in *H. rugulosus* between regions could be found and that samples from different parts were mixed.

The previous morphological data of *H. rugulosus* classified it as only one species (Chan-ard, 2003). In accord, our morphological analysis here, which reveals very little regional morphological variations in *H. rugulosus* across the six regions of Thailand, is consistent with there being only one species of *H. rugulosus* in Thailand. However, these morphological differences were based on mensural character dimensions only. Meristic characters (*e.g.* number of glandular fold, anterior body folds, posterior body folds) and body color pattern should be also applied to analyze the morphological variation of *H. rugulosus* to further test this notion in the future.

CHAPTER III

GENETIC DIVERSITY OF RICE FIELD FROGS, (*Hoplobatrachus rugulosus*), FROM NATURAL HABITATS IN THAILAND, AS EVALUATED WITH INTER SIMPLE SEQUENCE REPEAT (ISSR) ANALYSIS

Introduction

Recently, molecular genetic methods have been using to study the genetic diversity of many and diverse organisms, especially using those methods based upon the polymerase chain reaction (PCR). A number of PCR-based methods have been developed and proven useful for assessing genetic variation, biodiversity and genetic studies of populations (Zhang and Hewitt, 2003). Two types of markers can be generated: co-dominant, single and known markers, such as microsatellites (SSR) and minisatellites (VNTR), and dominant multilocus markers, such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP). The dominant multilocus markers have been popular in genetic diversity studies, due to the simplicity of some of the methods. ISSR markers are a targeted PCR method based on SSR-like primers (i.e. containing a repeated one to 4 base pair motif) that are then anchored at either the 5' or the 3' end by one, two or three other nucleotides, and so allow the amplification of the DNA sequences between two-inverted SSR (Figure 3.1). It thus permits the detection of polymorphism in microsatellites and inter-microsatellite loci without prior knowledge of the DNA sequence (Zietkiewicz et al., 1994). A series of studies have indicated that ISSR can reproducibly produce large numbers of polymorphic fragments at a low

cost (Han et al., 2007; Chen et al., 2008; Hu et al., 2010). The amplification and data scoring methods used for ISSR markers are similar to RAPD markers, but ISSR markers have the advantages of avoiding some of the limitations of RAPD markers in that the annealing temperature for amplification is usually higher, resulting in a higher degree of stringency for the amplified fragments (Wolfe and Liston, 1998), and the cost of the analysis is relatively lower than that of some other markers, such as RFLP, SSR and AFLPS.

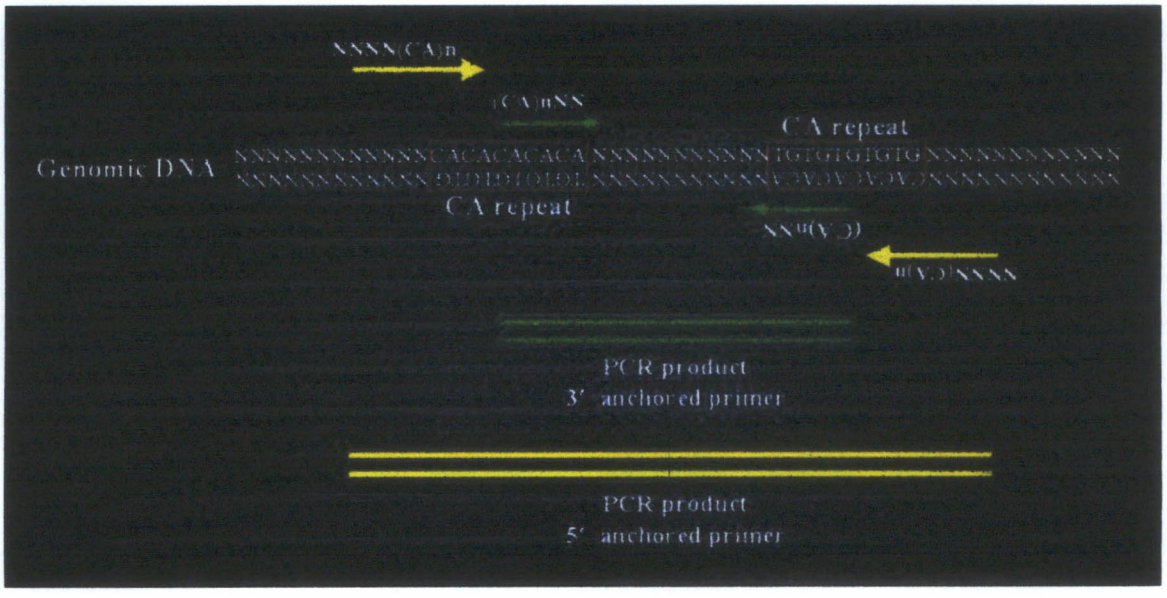

Figure 3.1 Amplification of ISSR segments using 5' and 3' anchored primers for $(CA)_n$ repeats. Arrows indicate primers and double lines indicate the amplicons (http://sunserver.cdfd.org.in:9999/PHP/SILKSAT/index.php?f=protocol_issr)

ISSR markers have been widely used for population and conservation genetics (Cully and Wolfe, 2001), and investigations in natural populations (Crawford et al., 2001). It has also demonstrated the hypervariable nature of the markers and its potential power for examining the genetic relationships within and among species and population studies (Culley and Wolfe, 2001). Moreover, based on the published, unpublished and in-progress studies that have recommended using ISSR markers, it is

clear that ISSR markers have a great potential for use in genetic studies of natural populations (Wolfe et al., 1998).

Population genetic theory posits a direct, positive relationship between genetic variation and population viability (Dolan, 1994). Genetic variation provides the resources on which a population can draw for short-term adaptation to environmental change and for long-term evolutionary change (Frankham et al., 2002). Estimating the level and structure of genetic variation within and among populations of threatened species is necessary for species conservation (Fritsch and Riseberg, 1996). ISSR is a relatively popular method for studying genetic variation within and among populations. It was introduced and used in the genetic study on cultivated plants (Zietkiewicz et al., 1994), fungi (Vandenkoornhuyse et al., 2001) and animals (Chatterjee and Mohandas, 2003). Although, ISSR has been popularly used by plant biologists for a variety of applications (Bornet and Branchard, 2001; Chen et al., 2005; Shen et al., 2006; Dong et al., 2007; Terzopoulos and Bebeli, 2008; Hasan et al., 2010), it has only rarely been used in animals (Kostia et al., 2000; Abbot, 2001; Glazko, 2003; Ursenbancher et al., 2008).

For amphibians, there are few reports about this genetic diversity determined by ISSR markers. In this chapter, the genetic diversity within and among regions of the rice field frog (*H. rugulosus*) sampled from 18 locations across the six regions of Thailand was investigated using ISSR markers. The main aims of this study were to 1) assess the level of genetic diversity of natural populations and 2) to reveal the partitioning of the genetic variations within and among populations within the six geographical regions of Thailand.

Materials and methods

Tissue sampling

A total of 230 individual adult rice field frogs (*H. rugulosus*) were collected using the Visual Encounter Survey (VES) technique from 18 geographically separate localities (populations) in each of the six biogeographic regions of Thailand, namely the North, Northeast, Central, West, East and South (Nabhitabhata and Chan-ard, 2005) (Figure 3.2, Table 3.1). All tissue samples (toe clip or liver) were immediately placed into absolute ethanol and were stored at -20 °C until required.

DNA extraction

Total DNA was extracted from the dissected tissue sample of each sampled adult animal using standard protocols of proteinase K digestion followed by phenol/chloroform extraction (Hillis et al., 1996). A small piece of tissue was dissected from each sample, placed in a 1.5 ml eppendorf tube containing 335 µl of TEN (0.1 M NaCl, 10 mM Tris and 1 mM EDTA, pH 8.0) + 1% (w/v) SDS buffer and mixed. Then, 15 µl of proteinase K solution (7 mg/ml; 30 U/ mg) was added and incubated at 55 °C for three hours or until the tissue was completely dissolved. An equal volume (350 µL) of phenol: chloroform (1:1 (v/v)) solution was then added to each tube, shaken vigorously for 1 min and then centrifuged at 14,000 rpm (16,000x g) for 8 min for complete phase separation and precipitation of denatured proteins. The upper aqueous phase was removed carefully and transferred to a new 1.5 ml eppendorf tube, and the phenol-chloroform extraction repeated as above until no more precipitated material was visible at the phase interface after centrifugation. Then, 700 µl of absolute ethanol was added, inverted gently to mix and then stored at -20 °C overnight.

Table 3.1 Details of the sampling sites and numbers of specimens of *H. rugulosus* used in this study.

Region	Locality[1]	Locality code	No. of Samples
North	Nan (1)	NAN	25
Northeast	Udon Thani (2)	UDN	13
	Sakon Nakhon (3)	SKN	16
	Mukdahan (4)	MDH	4
	Nakhon Ratchasima (5)	NKR	20
	Wang Nam Khiao (6)	WNK	25
	Ubon Ratchathani (7)	UBR	9
Central	Lopburi (8)	LOP	2
	Nakhon Nayok (9)	NKN	19
West	Tak (10)	TAK	17
	Phetchaburi (11)	PCB	25
East	Chonburi (12)	CBR	10
	Sa-Kaeo (13)	SKO	23
	Chanthaburi (14)	CTR	4
	Trad (15)	TRA	1
South	Chumphon (16)	CHP	3
	Phang-nga (17)	PNA	12
	Songkhla (18)	SKL	2
TOTAL	18		230

[1]*Numbers refer to the indicated locality on the map of Figure 3.2*

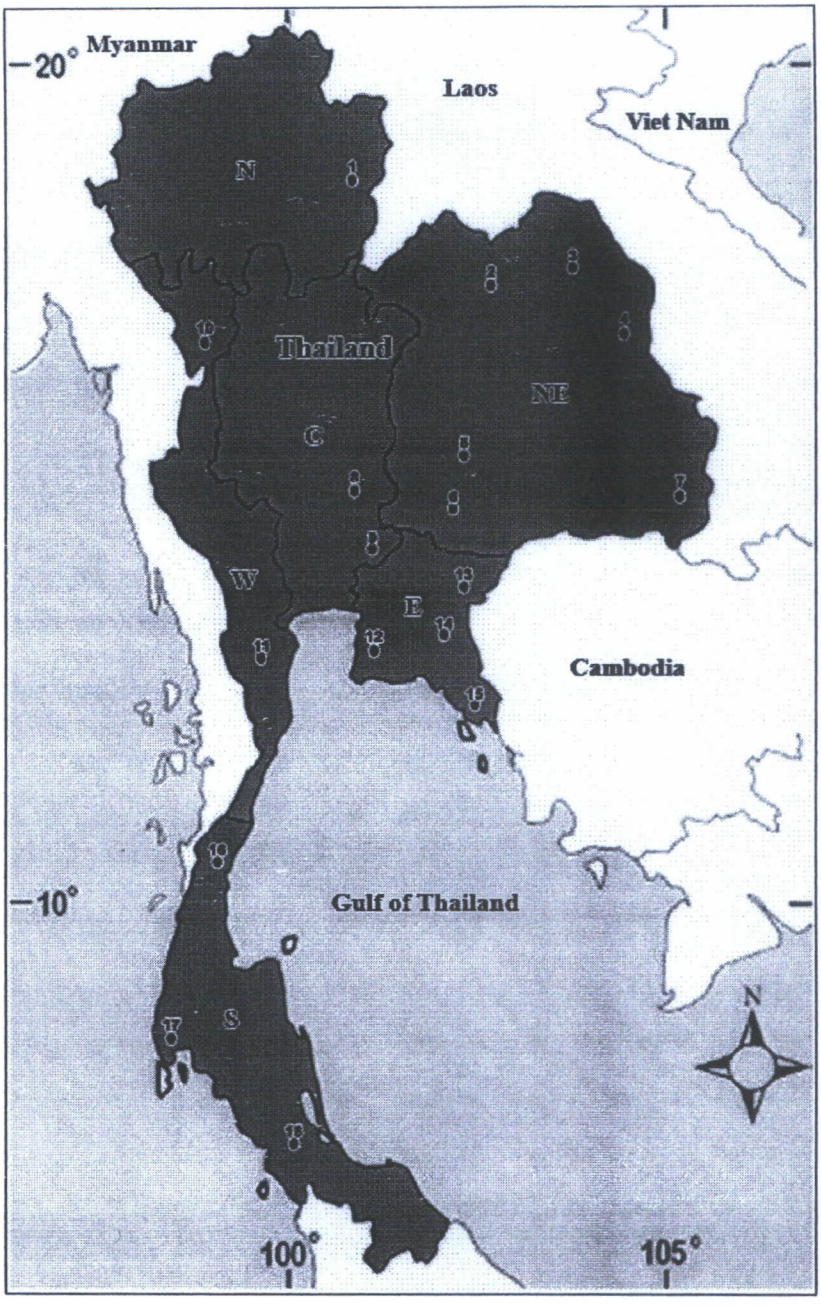

Figure 3.2 A map of Thailand showing the collection localities of *H. rugulosus* samples. For the locality numbers, refer to Table 3.1. Geographical regions indicated on the map are; N = North, NE = Northeast, C = Central, W = West, E = East and S = South.

The precipitated DNA was recovered by centrifugation at 14,000 rpm (16,000x g) for 15 min, the supernatant removed and the pellet washed in 700 µl of 70% (v/v) ethanol. The DNA pellet was then air-dried at room temperature and

dissolved in 25 µl TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and kept at –20 °C for further analysis.

Genomic DNA analysis

The quality and quantity of the extracted DNA was evaluated using agarose-TBE gel electrophoresis. 2 µl of the resolvated extracted DNA in TE was mixed with 2 µl of loading dye (0.15% (w/v) orange G, 0.05% (w/v) xylene cyanol FF and 60% (v/v) glycerol) and 7 µl of distilled water. The mixed DNA was then loaded onto 0.8% (w/v) agarose 0.5x TBE gel (0.89 M Tris-base, 0.89 M boric acid and 0.02 M EDTA) containing 0.6 µg/ml of ethidium bromide with λ/*Hin*d III (Fermentas™) DNA maker to allow the determination of the molecular weight and estimation of size of the extracted DNA, as per the manufactures instructions. After loading, electrophoresis was carried out at 80 volts for approximately 45 min. Finally, DNA band(s) were visualized under UV transillumination and photographed using a gel document system (Bio-Rad). Subsequentially, the extracted DNA solution in TE was adjusted to approximately 20 - 30 ng/µl prior to use in the PCR amplification.

Primer Screening and ISSR-PCR amplification

Sixty ISSR primers, designed by the University of British Columbia (UBC; UBC 801 - UBC 860) (Table 3.2) were screened with the DNA samples of each population. PCR reactions were performed in a final volume of 20 ↘l containing 20 - 30 ng total DNA, 250 µM of each dNTPs, 0.5 µM of each primer, 1.5 mM of $MgCl_2$, 1x PCR buffer and 1 unit *Taq* DNA polymerase (Fermentas™).

Table 3.2 ISSR primers used for screening to amplify of DNA samples of *H. rugulosus*

ISSR primers	Sequence 5'-3'	ISSR primers	Sequence 5'-3'
UBC 801	$(AT)_8T$	UBC 831	$(AT)_8Y^*A$
UBC 802	$(AT)_8G$	UBC 832	$(AT)_8Y^*C$
UBC 803	$(AT)_8C$	UBC 833	$(AT)_8Y^*G$
UBC 804	$(TA)_8A$	UBC 834	$(AG)_8Y^*T$
UBC 805	$(TA)_8C$	UBC 835	$(AG)_8Y^*C$
UBC 806	$(TA)_8G$	UBC 836	$(AG)_8Y^*A$
UBC 807	$(AG)_8T$	UBC 837	$(TA)_8R^*T$
UBC 808	$(AG)_8C$	UBC 838	$(TA)_8R^*C$
UBC 809	$(AG)_8G$	UBC 839	$(TA)_8R^*G$
UBC 810	$(GA)_8T$	UBC 840	$(GA)_8Y^*T$
UBC 811	$(GA)_8C$	UBC 841	$(GA)_8Y^*C$
UBC 812	$(GA)_8A$	UBC 842	$(GA)_8Y^*G$
UBC 813	$(CT)_8T$	UBC 843	$(CT)_8R^*A$
UBC 814	$(CT)_8A$	UBC 844	$(CT)_8R^*C$
UBC 815	$(CT)_8G$	UBC 845	$(CT)_8R^*G$
UBC 816	$(CA)_8T$	UBC 846	$(CA)_8R^*T$
UBC 817	$(CA)_8A$	UBC 847	$(CA)_8R^*C$
UBC 818	$(CA)_8G$	UBC 848	$(CA)_8R^*G$
UBC 819	$(GT)_8A$	UBC 849	$(GT)_8Y^*A$
UBC 820	$(GT)_8C$	UBC 850	$(GT)_8Y^*C$
UBC 821	$(GT)_8T$	UBC 851	$(GT)_8Y^*G$
UBC 822	$(TC)_8A$	UBC 852	$(TC)_8R^*A$
UBC 823	$(TC)_8C$	UBC 853	$(TC)_8R^*T$
UBC 824	$(TC)_8G$	UBC 854	$(TC)_8R^*G$
UBC 825	$(AC)_8T$	UBC 855	$(AC)_8Y^*T$
UBC 826	$(AC)_8C$	UBC 856	$(AC)_8Y^*A$
UBC 827	$(AC)_8G$	UBC 857	$(AC)_8Y^*G$
UBC 828	$(TG)_8A$	UBC 858	$(TG)_8R^*T$
UBC 829	$(TG)_8C$	UBC 859	$(TG)_8R^*C$
UBC 830	$(TG)_8G$	UBC 860	$(TG)_8R^*A$

**Single letter abbreviations for mixed base positions: Y = C or T; R = A or G*

PCR conditions started with an initial denaturation at 95 °C for 5 min followed by 40 cycles of 94 °C for 45 s, 54 °C for 45 s and 72 °C for 2 min, plus a

final 72 °C for 10 min. The negative control was run by replacing the template DNA with ddH_2O to test for the possibility of contamination. PCR products were coresolved with a DNA ladder (100-3,000 bp; Fermentas™) by electrophoresis through a 2.0% (w/v) agarose-TBE gel containing 0.6 μg/ ml of ethidium bromide at 100 volts for 210 minutes and then photographed under UV transillumination using a gel document system (Bio-Rad). ISSR primers that showed easily discernable and reproducible bands with inter-population polymorphisms were then selected for use in the analysis of inter-ISSR genetic diversity of all the samples.

Data analysis

Assuming two alleles per locus, ISSR profiles were scored for each individual as discrete dominant characters based on the presence (1) or absence (0) of amplified bands. Smeared and weak bands were excluded from the analysis. The resulting presence/absence data matrix of the ISSR phenotypes was analyzed including the percentage of polymorphic loci (PPB), Nei's genetic diversity (*He*) (Nei, 1973), and Shannon indices of diversity (*I*) (Shannon and Weaver, 1949). These parameters were used to investigate genetic diversity for each region.

Nei's unbiased genetic distances separating populations (Nei, 1978) were determined. The differentiation of *H. rugulosus* within each region was analyzed for polymorphism among regions by G_{st}. Corresponding estimates of gene flow (*Nm*) were estimated from $Nm = 0.5\ (1\text{-}G_{st})/G_{st}$ (McDermott and McDonald, 1993). All calculations were performed using POPGENE 1.3.2 software (Yeh et al., 1999) and assumed that populations are in Hardy-Weinberg equilibrium. Analyses of molecular variance (AMOVA) were conducted to partition the total phenotypic variance into that within a given region and that amongst regions (North, Northeast, Central, West,

East and South) (Excoffier et al., 1992). Unlike Nei's analysis using POPGENE, AMOVA is not based on the assumption of Hardy-Weinberg equilibrium. Rather, AMOVA assumes that deviations from Hardy-Weinberg equilibrium and from linkage equilibrium are similar at different sites. The fixation index (F_{st}) was also estimated. These analyses were conducted using the program ARLEQUIN 2.001 (Excoffier and Schneider, 2005). Significance tests were made after 1,000 permutations.

The dendrogram construction was performed using the unweighted pair group method with an arithmetic average (UPGMA) by PHYLIP version 3.67 (Felsenstein, 2007) based on Nei's unbiased genetic distances, and the dendrogram was drawn using the Treeview (Win32) 1.6.6 program.

Results

DNA extraction

Genomic DNA was extracted from the tissue samples (toe clip or liver) of *H. rugulosus* samples using a standard proteinase K and phenol/chloroform extraction procedure. The quality and quantity of extracted genomic DNA was determined by comparison with a coresolved λ/*Hin*d III DNA ladder (Figure 3.2). The extracted DNA was adjusted to approximately 20 - 30 ng/μl for use in PCR amplification

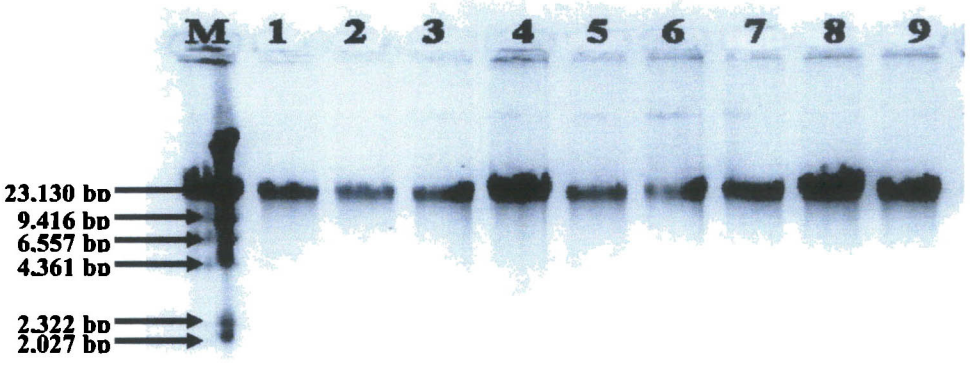

Figure 3.3 Genomic DNA was carried out on 0.8% (w/v) agarose-TBE gel and stained with 0.6 μg / ml ethidium bromide. Lane M: λ/*Hin*d III DNA ladder and Lanes 1 – 9: representative samples of *H. rugulosus* DNA extractions.

Primer Screening and ISSR-PCR amplification

The 60 3'-anchored ISSR primers (UBC 801 - UBC 860) were screened and nine out of them (UBC 807, UBC 825, UBC 826, UBC 827, UBC 829, UBC 835, UBC 840, UBC 841 and UBC 856) were selected based on the amplification of discrete, reproducible and strong bands with a degree of inter-population polymorphism.

A total of 230 individual adult rice field frogs (*H. rugulosus*) from 18 natural populations across the six geographical regions of Thailand were screened with the nine selected primers. The primers generated 155 reproducible bands (assumed loci), an average of 17.22 bands per primer, of which 150 (96.8%) were polymorphic among at least some of the populations. The amplicon sizes ranged from 200 to 2,000 bp (Table 3.3) and representative banding patterns of the resolved PCR amplicons are shown in Figures 3.3 - 3.5.

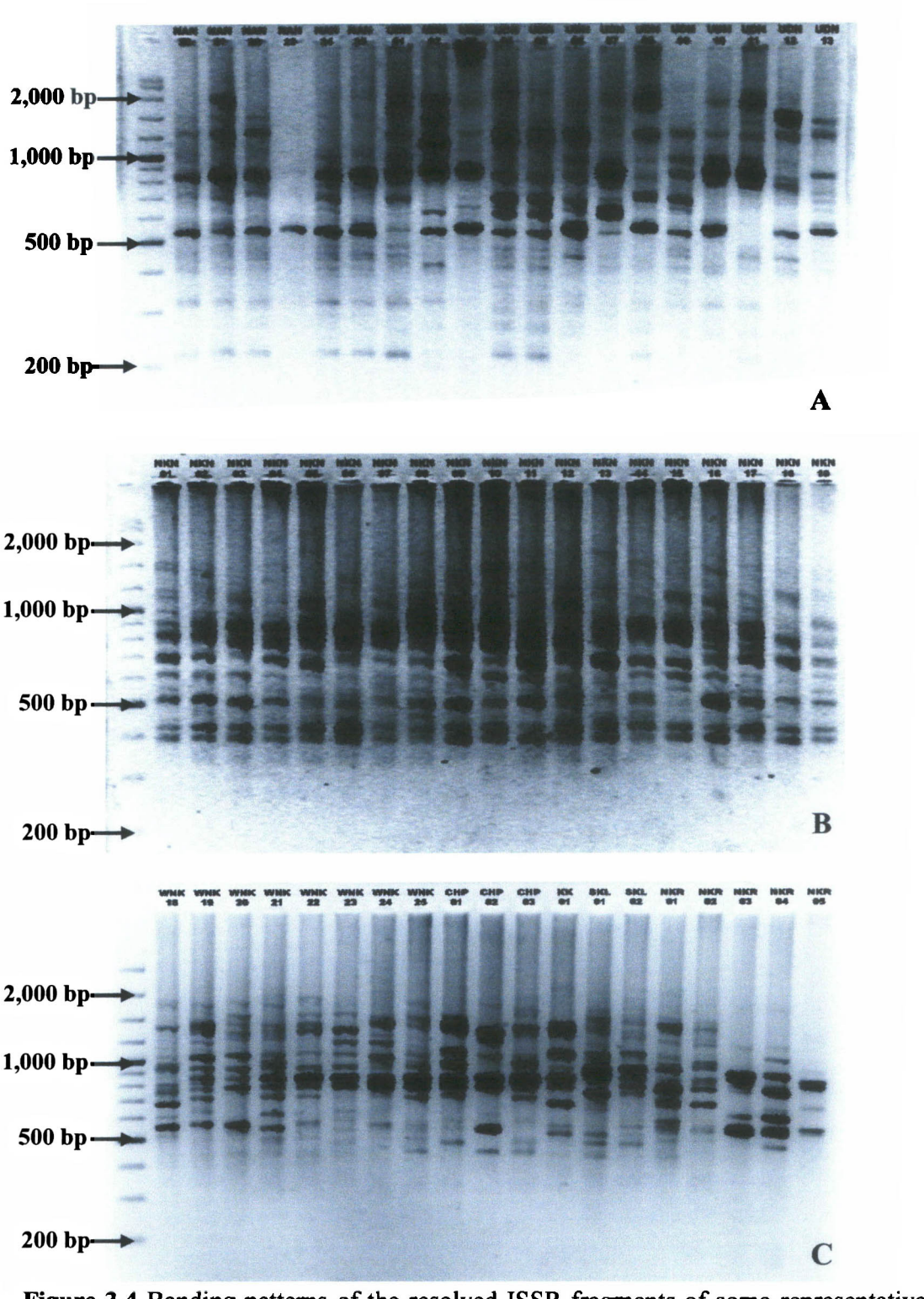

Figure 3.4 Banding patterns of the resolved ISSR fragments of some representative *H. rugulosus* samples after PCR amplification with primers (A) UBC 807, (B) UBC 825 and (C) UBC 826. Lane M, DNA ladder (Fermentas™); other lane abbreviations refer to locality codes in Table 3.1

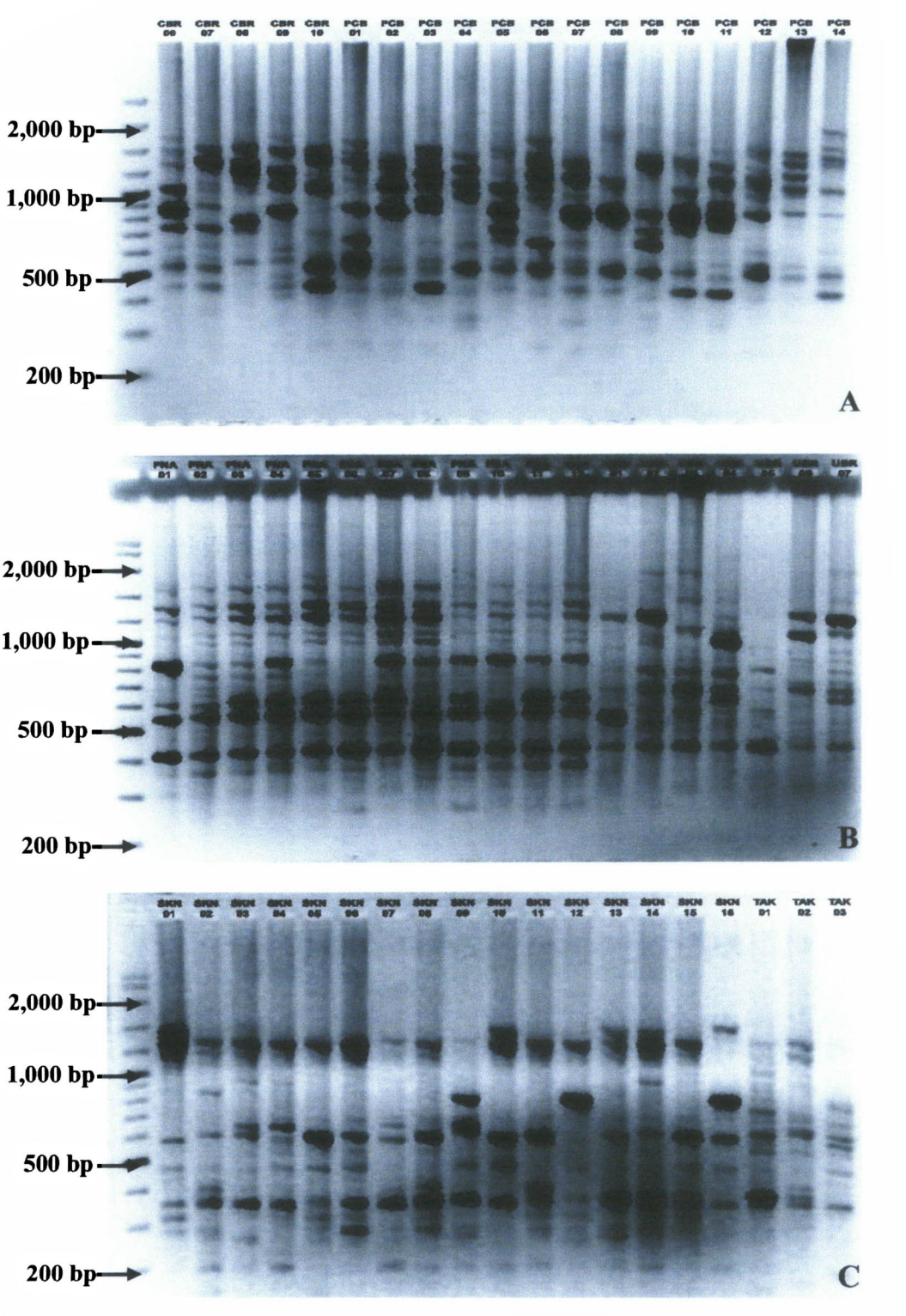

Figure 3.5 Banding patterns of the resolved ISSR fragments of some representative *H. rugulosus* samples after PCR amplification with primers (A) UBC 827, (B) UBC 829 and (C) UBC 835. Lane M, DNA ladder (Fermentas™); other lane abbreviations refer to locality codes in Table 3.1

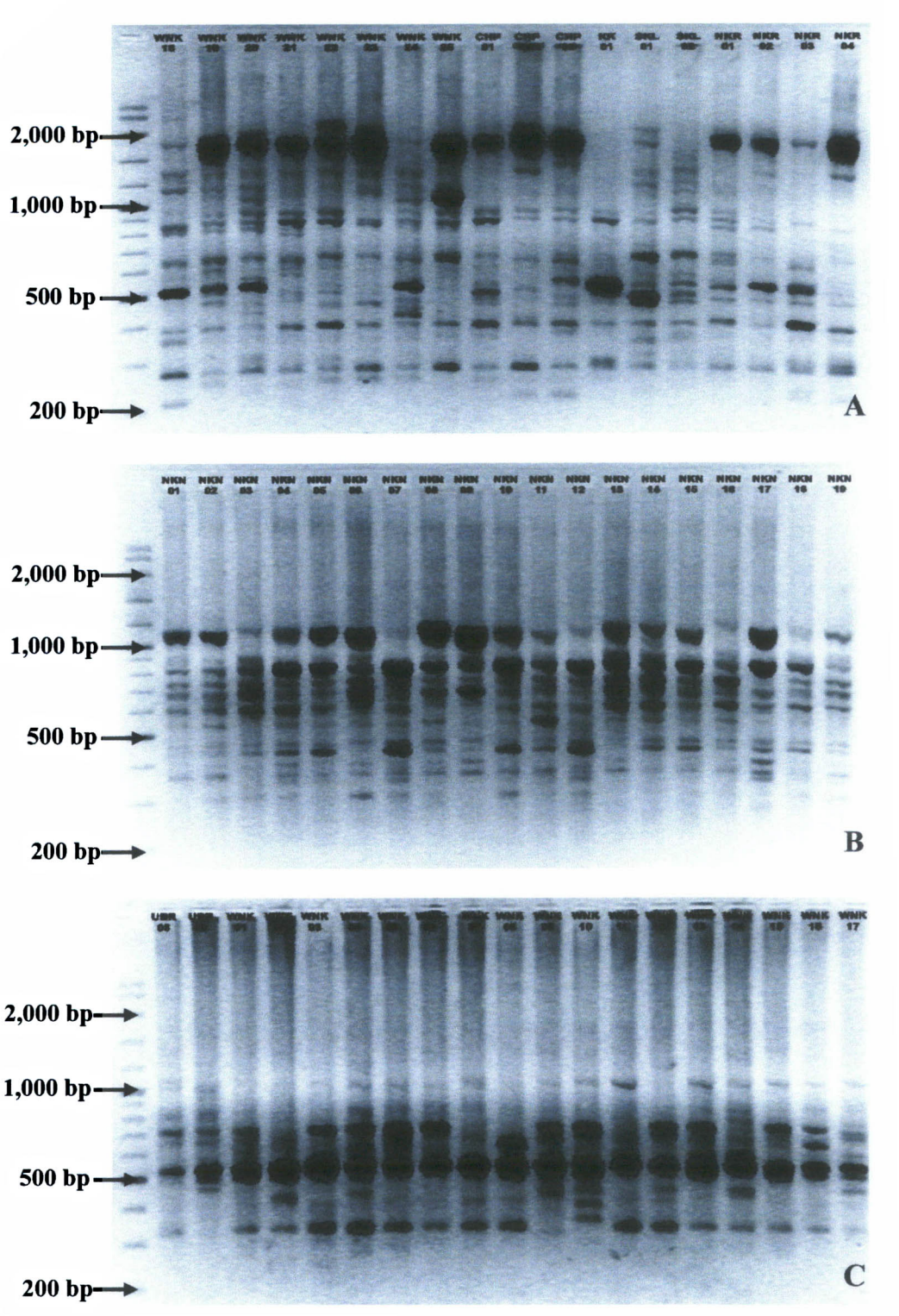

Figure 3.6 Banding patterns of the resolved ISSR fragments of some representative *H. rugulosus* samples after PCR amplification with primers (A) UBC 840, (B) UBC 841 and (C) UBC 856. Lane M, DNA ladder (Fermentas™); other lane abbreviations refer to locality codes in Table 3.1

Table 3.3 Total number of discernable bands, their size range, number of polymorphic bands and percentage of polymorphic bands (PPB) of each ISSR primer

ISSR primers	No. of bands	No of polymorphic bands	PPB[1] (%)	Size range (bp)
UBC 807	19	19	100	300 - 2,000
UBC 825	10	10	100	400 - 800
UBC 826	17	17	100	400 - 1,500
UBC 827	21	21	100	400 - 1,500
UBC 829	19	19	100	300 - 1,500
UBC 835	19	18	94.74	200 - 1,500
UBC 840	22	21	95.45	300 - 2,000
UBC 841	14	12	85.71	300 - 1,000
UBC 856	14	13	92.86	300 - 1,000
Total	155	150	96.77	200-2,000

[1]*PBB = Percentage of polymorphic bands, which for simplicity is assumed to be the percentage of polymorphic loci.*

Data analysis

Genetic diversity of *H. rugulosus* populations across the six geographical regions of Thailand

At the region level, the PPB ranged from 49.03% to 90.97%, with an average of 72.47%. Nei's genetic diversity (*He*) varied between 0.1642 and 0.3040, with an average of 0.2304, and Shannon's information index (*I*) ranged from 0.2465 to 0.4573, with an average of 0.3504. When calculated at the species level, the *He* and *I* values equaled 0.3184 and 0.4817 respectively, demonstrating a relatively high level of genetic diversity. Among the six regions of Thailand, the highest genetic variability was found in the eastern region (PPB = 90.97%; *He* = 0.3040; *I* = 0.4573), whereas

the lowest genetic variability was found in the northern region (PPB = 49.03%; *He* = 0.1643; *I* = 0.3689), as shown in Table 3.4

Table 3.4 Genetic variability of *H. rugulosus* in each region as determined by ISSR analyses

Region	No. of samples	No. of polymorphic bands	PPB (%)	*He*	*I*
North	25	76	49.03	0.1642 (0.1966)	0.2465 (0.2826)
Northeast	87	139	89.68	0.2676 (0.1702)	0.4107 (0.2290)
Central	21	100	64.52	0.2016 (0.1970)	0.3119 (0.2783)
West	42	117	75.48	0.2313 (0.1829)	0.3538 (0.2585)
East	38	141	90.97	0.3040 (0.1684)	0.4573 (0.2234)
South	17	101	65.16	0.2139 (0.2008)	0.3220 (0.2812)
Mean		112.33	72.47	0.2304 (0.0453)	0.3504 (0.0685)
Species level		150	96.77	0.3184 (0.1505)	0.4817 (0.1912)

Numbers in parenthesis are standard deviations; PPB, percentage of polymorphic loci; He = genetic diversity; I = Shannon's information index.

Genetic structure of *H. rugulosus* populations and relationships

According to Nei's genetic diversity, the coefficient of genetic differentiation between regions (G_{st}) was 0.2844. The level of gene flow (*Nm*) was 1.2584 individuals per generation between regions. Nei's unbiased genetic distances between seventeen populations of *H. rugulosus* ranged from 0.0600 (between the northeastern and eastern regions) to 0.2387 (between the northern and central regions) (Table 3.5).

Table 3.5 Nei's unbiased measures of identity (above diagonal) and genetic distance (below diagonal) among regions of *H. rugulosus* populations

Region	North	Northeast	Central	West	East	South
North	-	0.8597	0.7876	0.8292	0.8163	0.8168
Northeast	0.1511	-	0.8306	0.9066	0.9418	0.9082
Central	0.2387	0.1856	-	0.8891	0.8429	0.8314
West	0.1873	0.0980	0.1175	-	0.9122	0.8927
East	0.2030	0.0600	0.1709	0.0919	-	0.8803
South	0.2024	0.0963	0.1846	0.1135	0.1275	-

Populations of *H. rugulosus* were recognized in the six biogeographic regions of Thailand, namely the North, Northeast, Central, West, East and South (Nabhitabhata and Chan-ard, 2005). According to the analysis of molecular variance (AMOVA) of the populations from within each of the six biogeographic regions, only 28.8% of the total variation could be accounted for among the biogeographic regions, with 71.2% of the variation occurring within biogeographic regions. The fixation index (F_{st}) was 0.28791. In addition, the AMOVA showed a highly significant partitioning of the genetic differentiation between these six biogeographic regions ($P < 0.001$) (Table 3.6).

In order to represent the relationship among regions, an UPGMA dendrogram was produced using the Nei's unbiased genetic distances between regions (Figure 3.6). The dendrogram indicated three clusters; the northern region as cluster I, the central and western regions as cluster II, and the northeastern, eastern and southern regions as cluster III (Figure 3.7).

Table 3.6 Analysis of molecular variance (AMOVA) within / among biogeographic regions of *H. rugulosus*

Source of variance	d.f.	SSD	Variance component	Total variance (%)	Fixation index	*P*-value[1]
Among regions	5	1559.541	8.22460	28.79	$F_{st} = 0.28791$	< 0.001
Within regions	224	4556.694	20.34238	71.21		
Total	229	6116.235	28.56698			

d.f.: degrees of freedom; SSD: sum of squares. [1] *Significance tests after 1,000 permutations.*

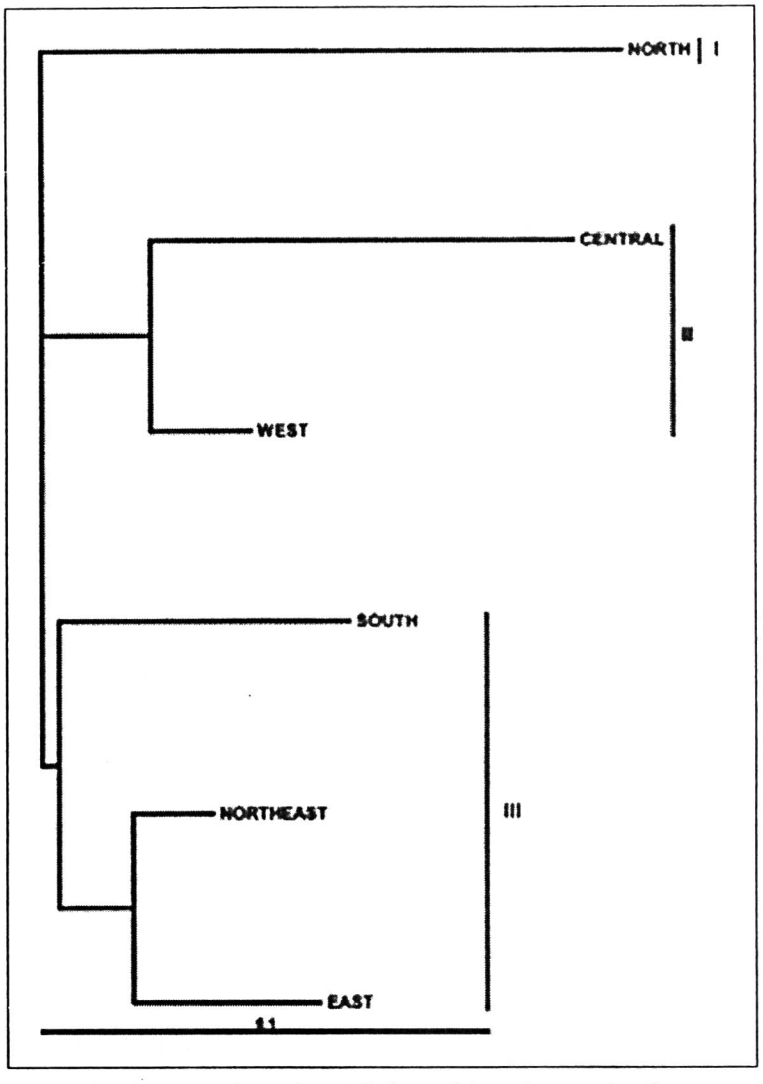

Figure 3.7 UPGMA dendrogram based on Nei's unbiased genetic distances

DISCUSSION

Genetic diversity of *H. rugulosus* in Thailand

ISSR analysis has a high potential power for the detection of polymorphism and as such is one of the powerful anonymous approaches for the assessment of genetic variation among populations, especially for species like *H. rugulosus* in which no molecular genetic information is previously available. Compared to the limited available data on other amphibian species, a relative higher level of genetic diversity within (PPB varies from 49.0% to 91.0%; mean value of 72.5%), and among (PPB = 96.8%) populations of *H. rugulosus* was detected in this study. For example, based on allozyme analysis, Hitchings and Beebee (1997) reported a lower genetic diversity among populations (44.3%) of *R. temporaria*. Grobler et al. (2003), also using allozyme data, reported a very low genetic diversity within populations and at the population level of *Heleophry natalensis* in South Africa (PPB values were <8% and 32%, respectively).

However, the levels of genetic diversity reported here for *H. rugulosus* were in agreement to that for some other amphibians when screened with more sensitive systems, such as the high level of genetic diversity such by RAPD analysis in 18 populations (PPB value was 97%) of *Physalaemus cuvieri* in Central Brazil (Telles et al., 2006). In addition, *H. rugulosus* is widely distributed in Thailand (Chan-ard, 2003) and, in general, widespread species tend to have a higher level of genetic variability than narrowly distributed ones (Hamrick and Godt, 1996). This may also, then, contribute to the high level of genetic diversity that was observed in this study for *H. rugulosus* across Thailand. Comparison of the genetic diversity of *H. rugulosus* in different habitats within Thailand suggested that the level of genetic diversity of *H.*

rugulosus from natural habitats is higher than in those populations of *H. rugulosus* that were cultured in farms (PPB varied from 26.0% to 47.5%) (Jiwyam et al., 2006).

Population genetic structure of *H. rugulosus* in Thailand

In this study, the genetic diversity (*He*) of *H. rugulosus* within the six geographical regions across Thailand, as determined by ISSR analysis, varied from 0.1642 to 0.3040, with the lowest genetic diversity in the northern region. However, the samples were collected from only one population in the northern region. This may reflect limited sampling sizes or a disproportionate sampling of genetically related frogs, rather than sampling across the whole regional population. Certainly, the number of samples assayed in a population, as well as the level of coverage of sampling across the sympatric range, are likely to affect the level of genetic variability. Moreover, the samples from the northern region were collected from the rice field at the valley. They may be restricted to the other populations so the genetic diversity within this population from the northern region was the lowest. For this reason, the apparent low genetic diversity observed in the *H. rugulosus* population in the northern region should be treated with care. On the other hand, the eastern region had the highest genetic diversity. The distance between populations within the eastern region is shorter than the distance between populations within each of the other regions so, excluding human transportation, migration between populations may be easier in the eastern region than in the other regions and may reflect to the high level of gene flow. When gene flow is high, the genetic diversity within population will be increased (Beebee and Rowe, 2008).

Generally, as the geographic distance between populations increases, there is a higher degree of genetic differentiation between them. But the genetic differentiation did not related to the geographic distance in this study. The distance

between the northern and southern regions is higher than the other regions in Thailand but in contrast the genetic distance between the northern and central regions was the highest. Here it is of note that the main natural habitat of *H. rugulosus* is rice fields or paddy fields in all parts of Thailand. In the central region, the farmers typically cultivate rice twice (or maybe thrice) in one year while the farmers in the other regions cultivate rice only once a year (or rarely twice). In addition, farmers in the central region use more agrochemicals in their crops, especially chemical fertilizer, than those in the other regions in Thailand (Office of Agricultural Economics, 2010). How this more disturbed habitat in the central region will impact upon the gene pool in the *H. rugulosus* populations is unknown.

From the UPGMA dendrogram, the populations from the southern region were found to be closely related to the populations from the eastern and the northeastern regions, even though the southern region is more than 500 km direct distance from the eastern and northeastern regions and it is separated by the Gulf of Thailand (< 100 m deep). These regions may have been connected by land bridges through the continent in the past (Hall, 1998; Voris, 2000; Sathiamurthy and Voris, 2006) so the gene flow between these populations could have occurred though a route over the present Gulf of Thailand in the past. This notion is not refuted by the level of the genetic diversity seen within *R. nigrovittata* in Thailand, where the southern population of *R. nigrovittata* was reported to be genetically closely related to the eastern populations, at least as based on allozyme analysis (Matsui et al., 2001). Gene flow between these regions in the past may have occurred between populations in the southern, eastern and the northeastern regions. However, alternative (and not mutually exclusive) possibilities could also explain this trend, including the movement of frogs between the regions by humans. Perhaps the use of more rapidly evolving

polymorphic markers, like nuclear microsatellite (SSR) or single nucleotide polymorphism (SNP), could resolve this issue in the future.

By using ISSR molecular markers in this study, AMOVA analysis revealed that there is a significant population structure for *H. rugulosus* in Thailand ($p < 0.001$; 1,000 permutation), as found for many other species of anurans worldwide (Driscoll, 1998; Telles et al., 2006; Silva et al., 2007; Arens et al., 2007). AMOVA analysis further revealed a relative low level of inter-population genetic differentiation (28.8%) in *H. rugulosus* across the six regions of Thailand. The F_{st} value (0.28791) was large compared with those reported for other species, such as 0.23 in *R. temporaria* (Palo et al., 2004), 0.14 in *R. arvalis* (Knopp and Merilã, 2009), 0.0878 in *R. chensinensis* (Zhan et al., 2009) and 0.215 in *R. kukunoris* (Zhao et al., 2009). Indeed, a F_{st} value above 0.25 is largely accepted as indicating a high genetic variation within regions (Wright, 1978). In addition, Nei's differentiation coefficient (G_{st}) was 0.2844, supporting that the genetic variation was mainly found within regions. The high genetic variation within regions was further confirmed from the high level of inter-population gene flow (Nm = 1.2584). This value is negatively related to the F_{st} value. Moreover, if $Nm < 1$, then local populations tends to differentiate, but if $Nm \geq 1$ then there will be little differentiation among populations and migration is more important than genetic drift (Wright, 1951). The results further indicated extensive inter-population gene flow among regions of *H. rugulosus* in Thailand so the genetic variation of *H. rugulosus* within regions was high.

CHAPTER IV

PHYLOGENETIC RELATIONSHIP OF RICE FIELD FROGS, (*Hoplobatrachus rugulosus*), FROM NATURAL HABITATS IN THAILAND, AS INFERRED FROM MITOCHONDRIAL DNA SEQUENCE ANALYSIS

Introduction

The new molecular genetic methods that have been implemented over the last three decades, particularly PCR amplification and DNA sequencing, have been extensively applied to the study of population genetics and phylogenetics of living organisms. Mitochondrial DNA (mtDNA) is a small extra-nuclear part of the genome found as multiple copies in mitochondria, organelles that occur in the cytoplasm of most eukaryotic cells (Beebee and Rowe, 2008). Animal mtDNAs is a circular double-stranded molecule and contains 13 protein-coding genes, 22 transfer RNAs and two ribosomal RNAs. There is also a control region that contains sites for replication and transcription initiation. Most of the sequences are unique, i.e. they are non-repetitive, and there is little evidence of either spacer sequences between genes or intervening sequences within transcribed genes. Although some rearrangement of mitochondrial genes has been found in different animal species, the overall structure, size and arrangement of genes are relatively conserved (Freeland, 2005) (Figure 4.1). In most animals mitochondrial DNA is inherited maternally, meaning that it is passed down from mothers to their offspring.

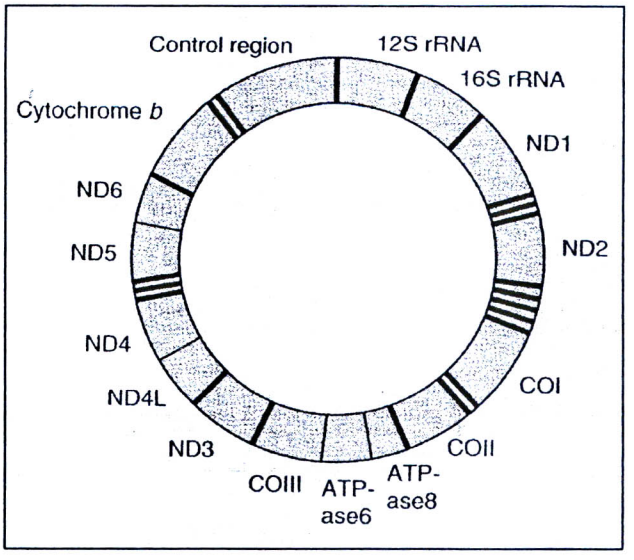

Figure 4.1 Typical gene organization of vertebrate mtDNA. Unlabelled dark bands represent each of the 22 transfer RNAs (tRNAs). Gene abbreviations starting with ND are subunits of NADH dehydrogenase, and those staring with CO are subunits of cytochrome *c* oxidase (Freeland, 2005)

Over the years the markers of choice, at least when studying animals, have been mitochondrial sequences that were obtained through either direct sequencing or RFLP analysis. In fact, prior to 2000, approximately 70 percent of all phylogeographic studies were based on the analyses of animal mitochondrial DNA (Avise, 2000). For phylogeographic analyses of animal populations, direct sequencing of mtDNA retains several advantages. First, versatile PCR primers now enable amplification of mtDNA sequences without mtDNA purification, thus avoiding any need for destructive sampling to obtain sufficient mitochondria rich tissues for their purification. Second, because of the high mtDNA copy number in most tissues, successful PCR amplifications can be achieved from museum material and even from some archaeological remains, such as bones and teeth. Third, the generally high mutation rate of mtDNA compared with the nuclear genome usually results in genetic variation in all but the most inbred or bottlenecked populations. Fourth, intraspecific

nucleotide polymorphism in mtDNA is considered, for the most part, to be effectively neutral so haplotype distribution is influenced more by demographic events in the population history than by selection. Fifth, the effective population size of mtDNA is one quarter that of diploid nuclear genes (in a sexual diploid organism with uniparental mitochondrial inheritance) so haplotype frequencies can drift rapidly, creating genetic differences among populations in relatively short times. Finally, because there is no recombination between animal mtDNA molecules, each uniparentally inherited haplotype form a (maternally) clonal lineage in sexually reproducing organisms (Beebee and Rowe, 2008). They are thus amenable to standard phylogenetic analysis (since recent recombination is not a problem) and clonal analysis, such as e-burst. From these advantages, the analyses of mtDNA can often provide useful information on the population variability, intraspecific phylogeography, historical biogeography, hybridization, gene flow and species boundaries, patterns and rates of molecular evolution, conservation and phylogenetic biology.

The two rRNA genes (12S rRNA and 16S rRNA) found in the genome of vertebrate animal mitochondria are considered unique sequences (Graur and Li, 2000). Since rRNA constitutes the non-translated structural components of the ribosome that functions in translation of protein from mRNA (Hillis et al., 1996; Smith and Szathmary, 1999; Campbell and Reece, 2002), rRNA genes have very specific functional and three-dimensional structural requirements that restrict their primary nucleotide sequence evolution (Graur and Li, 2000). The analysis of 12S and 16S rRNA sequences is therefore useful and often employed for the study of the inter- and intra-specific relationships and historical biogeography in many amphibians species, such as the phylogenetic relationship of *Hynobius naevius* (Igawa et al.,

2006), between the family Ranidae and Dicroglossidae for Chinese ranids (Che et al., 2007), and the phylogeography and historical demography of *Polypedates leucomystax* in Indonesia and Philippines (Brown et al., 2010), amongst other studies.

The product of the cytochrome-*b* (Cyt-*b*) gene is involved the electron transport in the respiratory chain of mitochondria, and is the most widely used gene sequence for phylogenetic and phylogeographic studies. Although it evolves slowly in terms of non-synonymous substitutions, the rate of evolution in silent positions is relatively fast (Irwin et al., 1991). Cyt-*b* is thought to be variable enough for population and phylogenetic relationship studies. However, cyt-*b* gene is under strong evolutionary constraints because some parts of the gene are more conserved than others due to functional restrictions (Meyer, 1994). Moore and DeFilipps (1997) suggested that it could be a suitable marker for resolving relatively recent evolutionary history. Cyt-*b* gene has been the most used source of sequence data for phylogeographic studies in amphibian species, including in the two sub-species of California newt; *Taricha torosa torosa* and *T. t. sierrae* (Tan and Wake, 1995), Malagasy poison frog (*Mantella bernhardi*) (Vieites et al., 2006), and the sword-tailed newt (*Cynops ensicauda*) (Tominaga et al., 2010).

The genus *Hoplobatrachus* is represented in Asia and Africa (Frost, 2010) and currently is recognized to consist of four species, namely *H. occipitalis, H. rugulosus, H. tigerinus* and *H. crassus*. The proposed hypothesis that this genus dispersed to Africa from Asia was clarified using the analysis of three mtDNA sequences (12S rRNA, 16S rRNA and cyt-*b*) (Kosuch et al., 2001). The result showed that *H. occipitalis*, now found in Africa, was the sister group of *H. rugulosus, H. tigerinus* and *H. crassus* that are found in Asia (Figure 4.2).

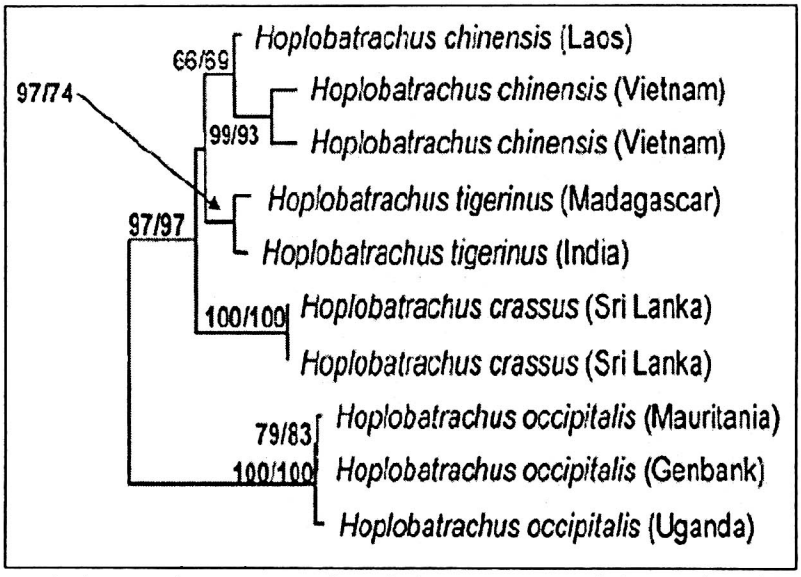

Figure 4.2 Phylogenetic relationship of the genus *Hoplobatrachus* (Kosuch et al., 2001).

H. rugulosus is the only species of this genus that can be found throughout Thailand. A high degree of genetic divergence of this species was found among populations in Thailand (average divergences of 13.4%, 5.5% and 2.7% for the cyt-*b*, 12S and 16S genes, respectively) (Alam et al., 2008), which may suggest the separation of *H. rugulosus* into more than one cryptic species. However, this study was incomplete in that the samples were collected from only three populations and did not cover all of Thailand. Thus, it is still unclear about the species status of *H. rugulosus* in Thailand.

In this chapter, we investigated the phylogenetic relationships of *H. rugulosus* from 18 distinct natural populations across the six geographical regions of Thailand using partial fragments of three mitochondrial DNA genes (cytochrome-*b*, 12S rRNA and 16S rRNA) in order to elucidate the phylogenetic relationship among populations and to clarify the species level of *H. rugulosus* in Thailand.

Materials and methods

Tissue sampling

A total of 73 individual adult rice field frogs (*H. rugulosus*) were collected using the VES technique from 18 geographically separate localities (populations) across the six biogeographic regions of Thailand, namely the North, Northeast, Central, West, East and South (Nabhitabhata and Chan-ard, 2005) (Figure 3.2 in chapter III; Table 4.1). All tissue samples (toe clip or liver) were immediately placed into absolute ethanol and were stored at -20 °C until required.

DNA extraction and genomic DNA analysis

Total DNA extraction and quality testing was performed as detailed in Chapter III.

PCR Amplification

The total DNA extract (20–30 ng/μl) was used as the template for amplifying the partial mtDNA fragments genes (cytochrome-*b*, 12S rRNA and 16S rRNA) by PCR. PCR primers L-14841 (5'-CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC TTC G-3') and CB3-H (5'-GGC AAA TAG GAA GTA TCA TTC TG-3' (Kosuch et al., 2001) were used to amplify the partial fragment of the Cyt-*b* gene. Primers FS01 (5'-AAC GCT AAG ATG AAC CCT AAA AAG TTC T-3') and R16 (5'-ATA GTG GGG TAT CTA ATC CCA GTT TGT TTT-3') (Sumida et al., 1998) were used to amplify the partial fragment of the 12S rRNA gene, and primers F51 (5'-CCC GCC TGT TTA CCA AAA ACA T-3') and R51 (5'-GGT CTG AAC TCA GAT CAC GTA-3') (Sumida et al., 2002) were used to amplify the partial fragment of the 16S rRNA gene.

Table 4.1 Details of the sampling sites and numbers of specimens of *H. rugulosus* used in this study.

Region	Locality[1]	Locality code	No. of Samples
North	Nan (1)	NAN	3
Northeast	Udon Thani (2)	UDN	5
	Sakon Nakhon (3)	SKN	5
	Mukdahan (4)	MDH	4
	Nakhon Ratchasima (5)	NKR	6
	Wang Nam Khiao (6)	WNK	4
	Ubon Ratchathani (7)	UBR	5
Central	Lopburi (8)	LOP	2
	Nakhon Nayok (9)	NKN	5
West	Tak (10)	TAK	6
	Phetchaburi (11)	PCB	5
East	Chonburi (12)	CBR	4
	Sa-Kaeo (13)	SKO	4
	Chanthaburi (14)	CTR	4
	Trad (15)	TRA	1
South	Chumphon (16)	CHP	3
	Phang-nga (17)	PNA	5
	Songkhla (18)	SKL	2
TOTAL	18		73

[1]*Numbers refer to the indicated locality on the map of Figure 3.2 (Chapter III)*

Similar PCR amplification reagents were used for all primer pairs. 25 µl of PCR amplification reagent contained 20-30 ng total DNA, 250 µM of each dNTPs

(Fermentas™), 1 μM of each primer, 2.5 mM of $MgCl_2$, 1 x PCR buffer and 1 unit *Taq* DNA polymerase (Fermentas™). The thermal cycling conditions were an initial denaturation at 94 °C for 3 minutes followed by 35 cycles of 94 °C for 45 seconds, XX °C for 45 seconds and 72 °C for 1 minute, with a final 72 °C for 10 minutes, where XX was 53 °C for Cyt-*b* and 16S rRNA genes and 55 °C for the 12S rRNA gene. The quality of PCR products were checked by 1.0% (w/v) agarose-TBE gel electrophoresis based resolution of the amplicons in the presence of 0.6 μg /ml ethidium bromide for visualization by uv transillumination. To this end 2 μl of each PCR reaction was mixed with the loading buffer and dye (see chapter III) and loaded into one well of the gel. Samples were coresolved with a 100 bp DNA marker set (Fermentas™) in two flanking wells of the gel to allow determination of the molecular weight (for quality) and estimation of size of the PCR products.

PCR Purification

The remaining PCR products were purified to remove the residual primers, salts and enzyme, using the MACHEREY-NAGEL™ kit according to the manufacturer's instructions. The PCR products were eluted from the silica resin in 20 μl of TE (65 °C for 2 min).

DNA Sequencing and Phylogenetic Analysis

The purified PCR products were outsourced for commercial direct sequencing at the contract sequencing facility (Macrogen, Seoul, Korea) on an AB3100 automatic DNA sequencer. PCR products were directly sequenced in both directions, using the same primers as used for their PCR amplification. All chromatograms were checked by eye for quality, loss of resolution, miscalled bases and evidence of multiple heterozygous templates. Then, the accepted sequences from

both strands were used to compile a consensus sequence, and these consensus sequences were aligned using the default parameters of the alignment program CLUSTAL X (Thompson et al., 1997). All sequences were searched for similar sequences in the GenBank database using the BLASTn program from http://www.ncbi.nlm.nih.gov. The alignments with homologs from the data base were also used to help confirm that the sequences were correct, including the removal of degenerate pseudogene sequences. In case of the same haplotype being represented in different populations, the haplotypes were also submitted at the DDBJ/EMBL/GenBank nucleotide sequence databases.

Phylogenetic analyses were conducted from two data sets (Cyt-*b* sequences and the combined data of 12S rRNA and 16s rRNA sequences). These data sets were imported to MacClade version 4.06 (Maddison and Maddison, 2000) for creating NEXUS format. Phylogenetic relationships were constructed using maximum parsimony (MP), maximum likelihood (ML) and the neighbor joining (NJ) distance based analyses. MP, ML and NJ were performed using a heuristic search setting with random-addition sequences and tree bisection-reconnection (TBR) branch swapping in PAUP* 4.0b10 (Swofford, 2003). For the MP analysis, all characters were weighted equally, and gaps were treated as missing data. For the ML analysis, the best-fit model of sequence evolution was determined using Akaike Information Criterion (AIC; Akaike, 1974) in Modeltest version 3.7 (Posada and Crandall, 1998). The bootstrap technique was used to test the reliabilities of the MP, ML and NJ trees with 1000, 100 and 1000 replicates, respectively. Tree topologies with bootstrap values of 70% or greater were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993), and those between 50 and 70% as weakly supported pairwise comparisons of corrected sequence divergences [Kimura-2 parameter (K2p) distances

(Kimura, 1980)]. The data sequence of cyt-*b* (AB 274044), 12S rRNA (AB 273157) and 16S rRNA (AB 272589) of *H. tigerinus* (Alam et al., 2008) was used as the respective outgroup to construct the phylogenetic trees.

Results

DNA Extraction

Total DNA was extracted from the dissected tissue sample (toe clip or liver) of each *H. rugulosus* using standard protocols of proteinase K digestion followed by phenol/chloroform extraction (Hillis *et al.*, 1996). The quality and quantity of extracted DNA were determined by resolving a 2 µl aliquot of the PCR product, in comparison with a λ/*Hind* III DNA marker, though a 0.8% (w/v) agarose-TBE gel with visualization by ethidium bromide staining and uv transillumination. The concentration of extracted genomic was approximately 45 - 200 ng/µl and appeared to be mostly high molecular weight DNA (~23 kb) without too much sheared low molecular weight DNA (Figure 4.3).

The extracted DNA was thus deemed to be suitable for PCR amplification of these three amplicons and so was adjusted to approximately 20 - 30 ng/µl for using in PCR amplification.

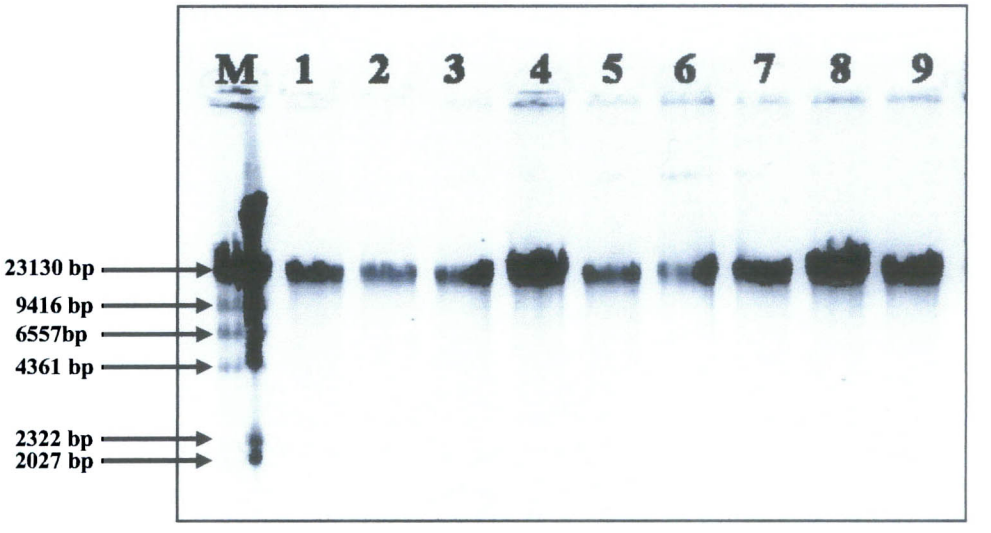

Figure 4.3 The extracted genomic DNA was carried out on 0.8% (w/v) agarose-TBE gel and stained with ethidium bromide. Lane M = the λ*Hind* III DNA marker (Fermentas™); Lanes 1 - 9 represent the total liver genomic DNA extraction of nine representative *H. rugulosus* individuals.

PCR Amplification

The partial fragments of the cyt-*b*, 12S rRNA and 16S rRNA mtDNA genes were successfully amplified by the L-14841 / CB3-H, FS01 / R16 and F51 / R51 primer pairs, respectively. The expected PCR product sizes of cyt-*b*, 12S rRNA and 16S rRNA genes were approximately 750 bp, 450 bp and 600 bp, respectively (Figures 4.4 - 4.6).

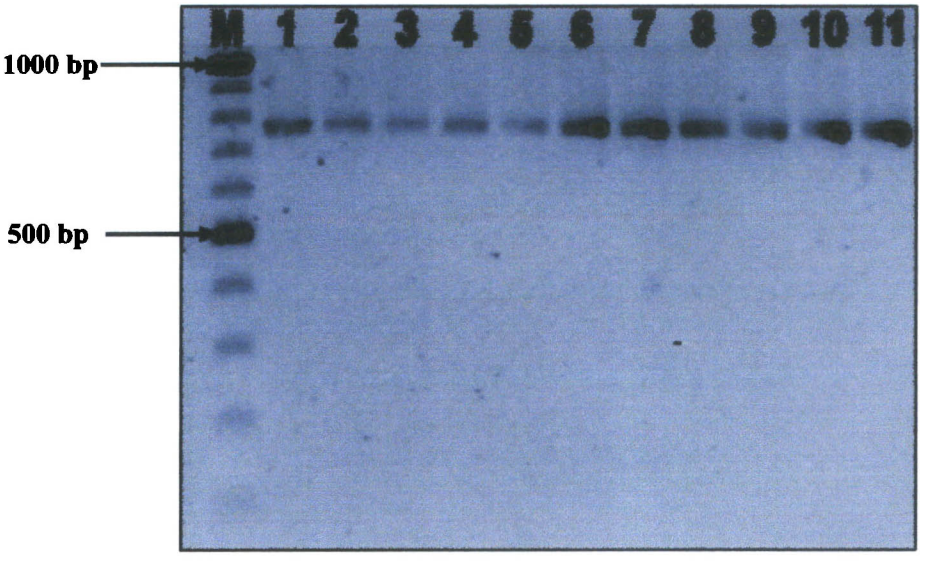

Figure 4.4 1.0% (w/v) agarose-TBE gel carried out PCR products of the partial fragment of the cyt-*b* mtDNA gene, as amplified by the L-14841 and CB3-H primers. Lane M = 100 bp DNA marker (Fermentas™); Lanes 1 - 11 are the purified PCR products (cyt-*b* gene fragment) from each of 11 representative *H. rugulosus* individuals.

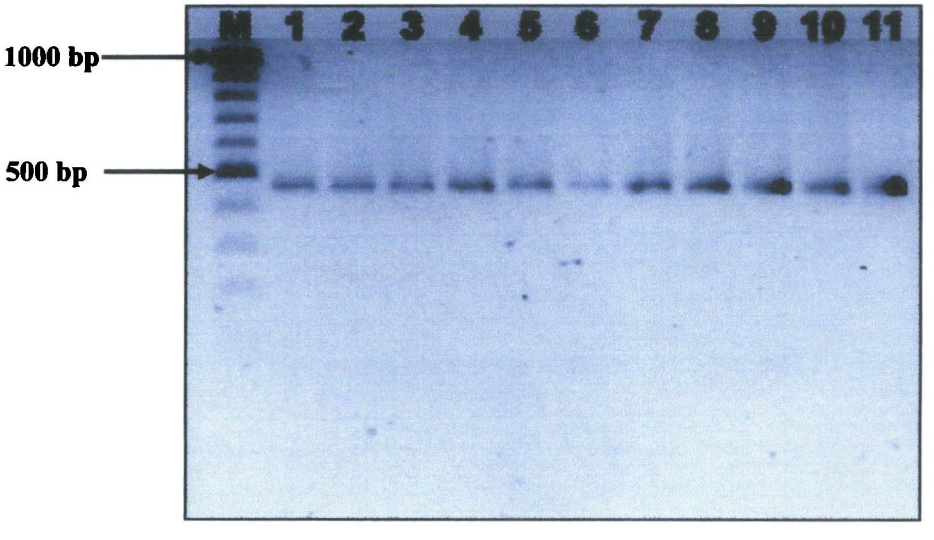

Figure 4.5 1.0% (w/v) agarose-TBE gel carried out PCR products of the partial fragment of the 12S rRNA mtDNA gene, as amplified by the FS01 and R16 primers. Lane M = 100 bp DNA marker (Fermentas™); Lanes 1 - 11 are the purified PCR products (12S rRNA gene) from each of 11 representative *H. rugulosus* individuals.

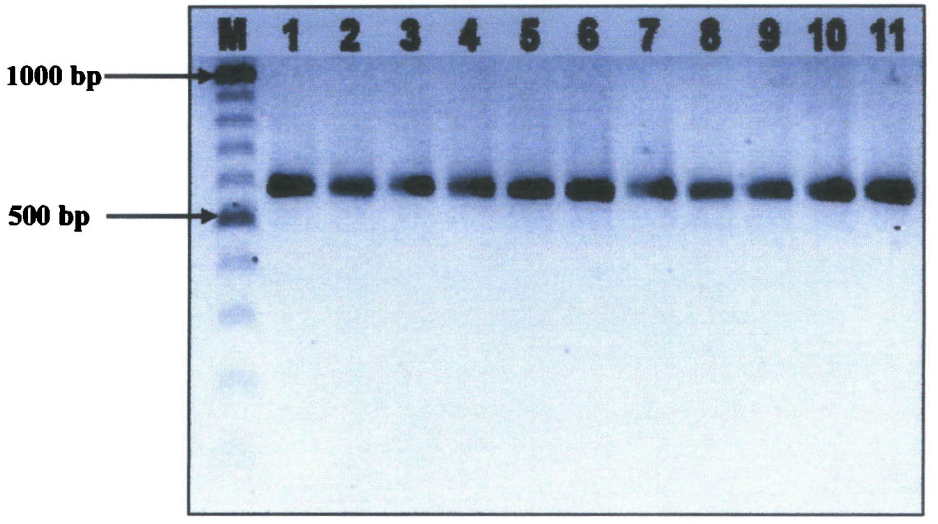

Figure 4.6 1.0% (w/v) agarose-TBE gel carried out PCR products of the partial fragment of the 16S rRNA mtDNA gene, as amplified by the F51 and R51 primers. Lane M = 100 bp DNA marker (Fermentas™); Lanes 1 - 11 are the purified PCR products (16S rRNA gene) from each of 11 representative *H. rugulosus* individuals.

Sequence Analysis

The PCR products of the cyt-*b*, 12S rRNA and 16S rRNA genes from the 73 *H. rugulosus* samples were purified and then commercially direct sequenced in both the forward and reverse directions. The quality of the sequence data was checked using Chromas version 1.45 (Zajec, 1986), which show the most likely base for each interval (Figures 4.7-4.9), and then manually checked for miscalls, noise and secondary smaller peaks (heterozygotes). In most cases (69/73, 70/73 and 72/73 samples for cyt-b, 12S and 16S rRNA, respectively), the forward and reverse sequence data were consistent. After the sequence data was modified, the length of partial cyt-*b*, 12S rRNA and 16S rRNA genes were 564 bp, 362 - 363 bp and 449 bp, respectively.

The consensus sequence data for each partial cyt-*b*, 12S rRNA and 16S rRNA gene fragment were searched for similar sequence data in the GenBank database using the BLASTn algorithm from http://www.ncbi.nlm.nih.gov.

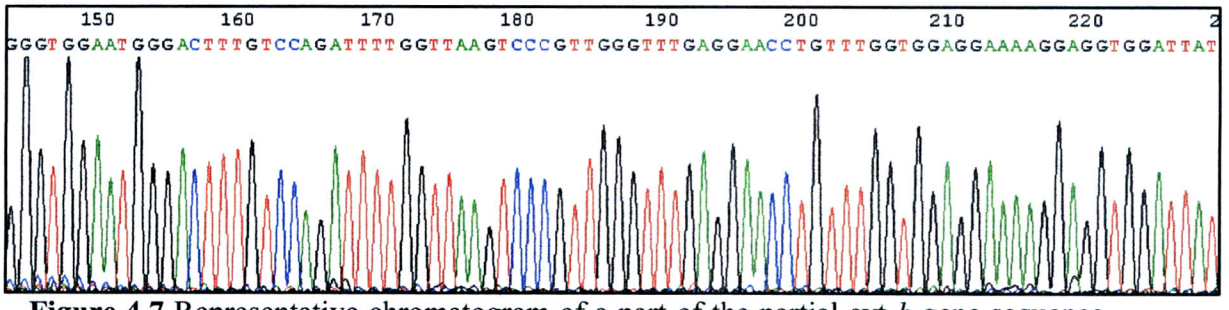

Figure 4.7 Representative chromatogram of a part of the partial cyt-*b* gene sequence of *H. rugulosus* from Chanthaburi. Green, blue, black and red colors show Adenine (A), Cytosine (C), Guanine (G) and Thymine (T), respectively.

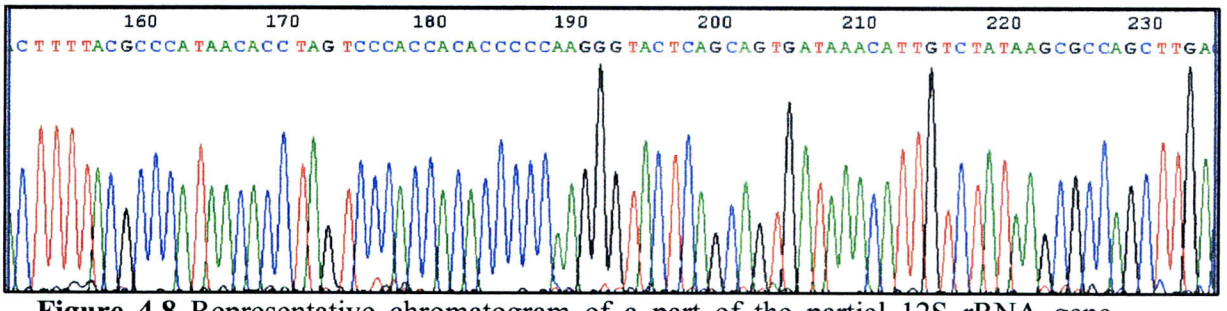

Figure 4.8 Representative chromatogram of a part of the partial 12S rRNA gene sequence of *H. rugulosus* from Udon Thani. Green, blue, black and red colors show Adenine (A), Cytosine (C), Guanine (G) and Thymine (T), respectively.

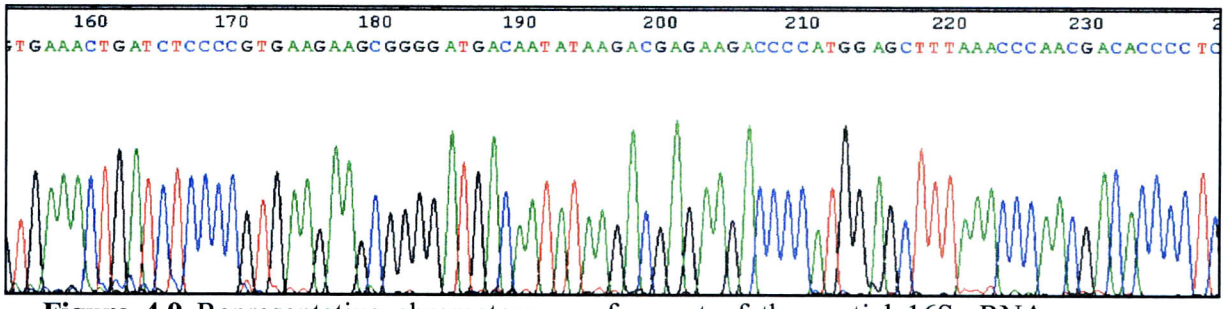

Figure 4.9 Representative chromatogram of a part of the partial 16S rRNA gene sequence of *H. rugulosus* from Tak. Green, blue, black and red colors show Adenine (A), Cytosine (C), Guanine (G) and Thymine (T), respectively.

The sequence data of the partial cyt-*b*, 12S rRNA and 16S rRNA genes in this present study were slightly similar with the sequence data of cyt-*b*, 12S rRNA and 16S rRNA genes of the other species in genus *Hoplobatrachus*, such as *H. crassus*. Thus, the sequenced PCR products were accepted as likely to be genuine.

The consensus sequence data for each partial cyt-*b*, 12S rRNA and 16S rRNA gene fragment were searched for similar sequence data in the GenBank database using the BLASTn algorithm from http://www.ncbi.nlm.nih.gov. The sequence data of the partial cyt-*b*, 12S rRNA and 16S rRNA genes in this present study were slightly similar with the sequence data of cyt-*b*, 12S rRNA and 16S rRNA genes of the other species in genus *Hoplobatrachus*, such as *H. crassus*. From these results the sequenced PCR products were designated as real sequence data of cyt-*b*, 12S rRNA and 16S rRNA genes.

All 73 sequences of cyt-*b* gene, and the combined data of the partial 12S rRNA and 16S rRNA genes, were aligned using the default parameters of the alignment program CLUSTAL X (Thompson et al., 1997) (Appendix C). From the 564 bp of the partial cyt-*b* gene, 12 haplotypes were found in the 73 samples, representative of 18 localities (populations). These haplotypes were represented in

different populations, and are shown, with their DDBJ/EMBL/GenBank nucleotide sequence database accession numbers in Table 4.2 and Figure 4.10.

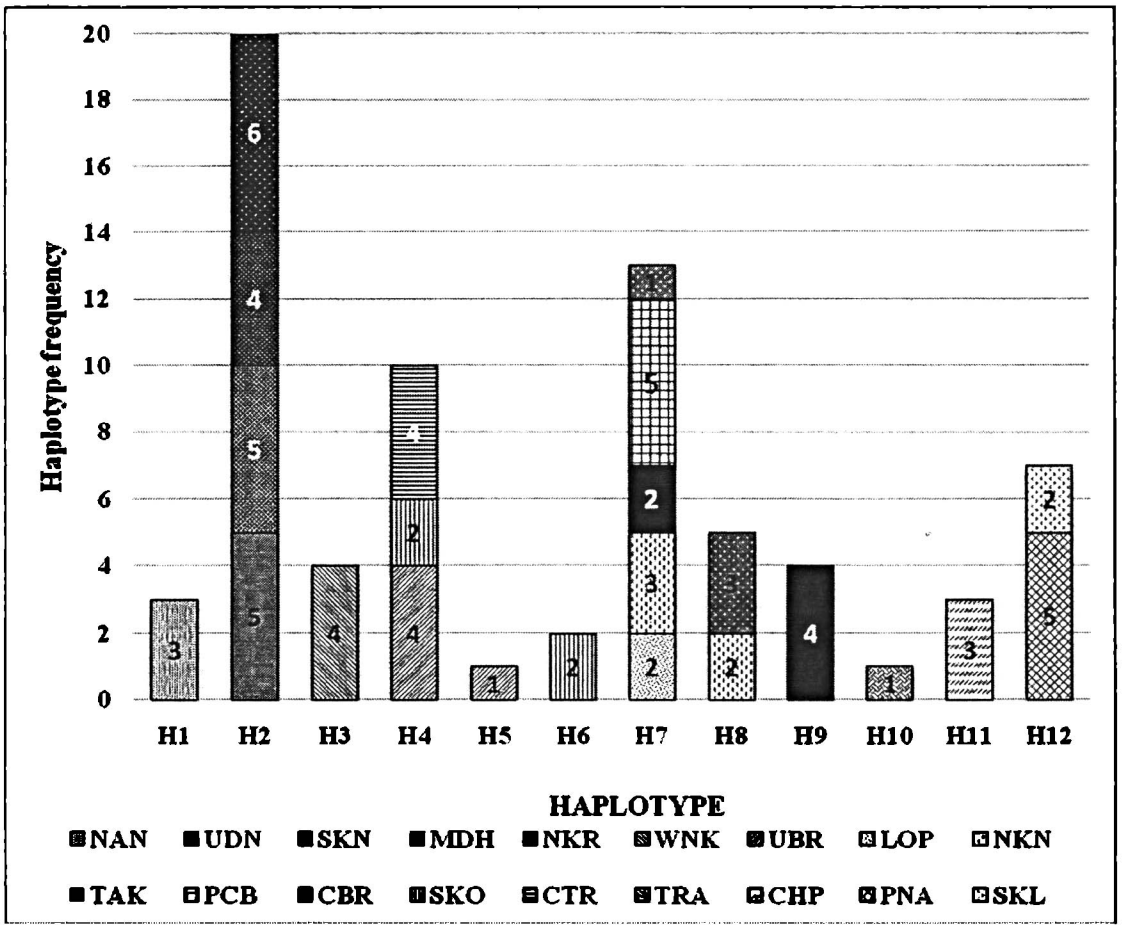

Figure 4.10 Haplotype frequencies of the 564 bp cyt-*b* gene fragment in 18 populations of *H. rugulosus*

Table 4.2 Haplotypes and GenBank accession numbers of the 564 bp partial cyt-*b* gene fragment sequences in 73 individuals (from 18 populations) of *H. rugulosus*.

Population (Locality code)	Haplotype (No. of samples)	Accession number
Nan (NAN)	H1 (3)	AB 514482
Udon Thani (UDN)	H2 (5)	AB 514494
Sakon Nakhon (SKN)	H2 (5)	AB 514489
Mukdahan (MDH)	H2 (4)	AB 514481
Nakhon Ratchasima (NKR)	H2 (6)	AB 514485
Wang Nam Khiao (WNK)	H3 (4)	AB 514495
Ubon Ratchathani (UBR)	H4 (4), H5 (1)	AB 514492, AB 514493
Lopburi (LOP)	H7 (2)	AB 539960
Nakhon Nayok (NKN)	H7 (3), H8 (2)	AB 514483, AB 514484
Tak (TAK)	H7 (2), H9 (4)	AB 514490, AB 514491
Phetchaburi (PCB)	H7 (5)	AB 514486
Chonburi (CBR)	H7 (1), H8 (3)	AB 514477, AB 514478
Sa-Kaeo (SKO)	H4 (2), H6 (2)	AB 514475, AB 514476
Chanthaburi (CTR)	H4 (4)	AB 514480
Trad (TRA)	H10 (1)	AB 514496
Chumphon (CHP)	H11 (3)	AB 514479
Phang-nga (PNA)	H12 (5)	AB 514487
Songkhla (SKL)	H12 (2)	AB 514488

From the 12 haplotypes, five haplotypes (H2, H4, H7, H8 and H12) represented in two or more of the 18 populations, whilst the others (H1, H3, H5, H6, H9, H10 and H11) were distinct haplotypes for Nan, Wang Nam Khiao, Ubon Ratchathani, Sa-Kaeo, Tak, Trad and Chumphon, respectively. H2 was the most frequent haplotype, being found in 20 / 73 individuals (27.4%) and in 4 of the 18 localities, all of which were located in the northeastern region. Moreover, H1, H2, H3, H5, H6 and H12 were found in only one locality each, being the northern, northeastern, eastern and southern regions of Thailand, respectively (Figure 4.10)

On the other hand, the alignment of the partial 12S rRNA (363 bp) and 16S rRNA gene (449 bp) sequences revealed a total of 16 haplotypes from 18 populations. Of these, 11 haplotypes (H1, H2, H5, H7, H8, H9, H10, H13, H14, H15 and H16) were distinct haplotypes and were found in Nan, Udon Thani, Mukdahan, Nakhon Ratchasima, Wang Nam Khiao, Ubon Ratchathani (2), Chanthaburi, Chumphon (2) and Phang-nga, respectively (Table 4.3 and Figure 4.11). H11 was the most abundant haplotype (in terms of frequency occurrence), occurring in 16 of 73 individuals (21.9%), in the four localities (populations) of Lopburi, Nakhon Nayok, Phetchburi and Chonburi (Table 4.3).

Table 4.3 Haplotypes and GenBank accession numbers of the 813 bp combined 12S rRNA and 16S rRNA gene fragment sequences of 73 individuals from 18 populations of *H. rugulosus*.

Population (Locality code)	Haplotype (No. of samples)	Accession number	
		12S rRNA	16S rRNA
Nan (NAN)	H1 (3)	AB 514825	AB 514553,
Udon Thani (UDN)	H2 (1), H3 (4)	AB 514837	AB 514553/4
Sakon Nakhon (SKN)	H3 (4), H4 (1)	AB 514832/3	AB 514548/9
Mukdahan (MDH)	H3 (2), H5 (1), H6 (1)	AB 514823	AB 514539/40
Nakhon Ratchasima (NKR)	H3 (1), H4 (2), H7 (3)	AB 514827/8	AB 514543/4
Wang Nam Khiao (WNK)	H6 (3), H8 (1)	AB 514838/9	AB 514555
Ubon Ratchathani (UBR)	H4 (1), H9 (1), H10 (3)	AB 514835/6	AB 514551/2
Lopburi (LOP)	H11 (2)	AB 539961	AB 539962
Nakhon Nayok (NKN)	H11 (5)	AB 514826	AB 514542
Tak (TAK)	H12 (6)	AB 514834	AB 514550
Phetchaburi (PCB)	H11 (5)	AB 514829	AB 514545
Chonburi (CBR)	H11 (4)	AB 514818	AB 514536
Sa-Kaeo (SKO)	H4 (4)	AB 514817	AB 514535
Chanthaburi (CTR)	H4 (3), H13 (1)	AB 514821/2	AB 514538
Trad (TRA)	H4 (1)	AB 514840	AB 514556
Chumphon (CHP)	H14 (1) H15 (2)	AB 514819/20	AB 514537
Phang-nga (PNA)	H16 (5)	AB 514830	AB 514546
Songkhla (SKL)	H12 (2)	AB 514831	AB 514547

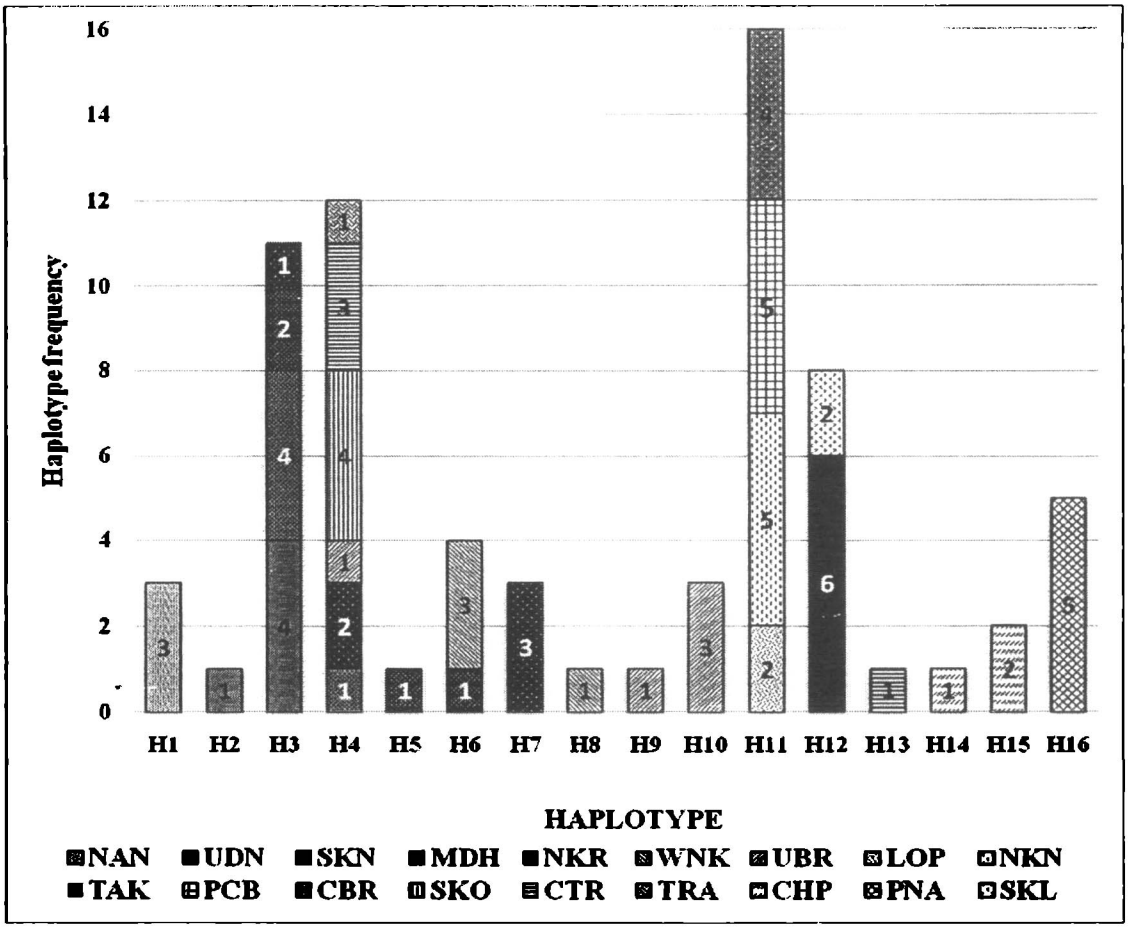

Figure 4.11 Haplotype frequencies of the combined partail of 12S rRNA and 16S rRNA gene fragments (813 bp) in 18 populations of *H. rugulosus*

Phylogenetic Analysis

The 12 haplotypes of the 564 bp cyt-*b* gene fragment found here, plus the outgroup taxa, were used for phylogenetic analyses. Of the 564 sites of cyt-*b*, 63 of these positions were variable and 80 were parsimony-informative. Modeltest suggested the HKY + G model as the best-fit model for our data, with the proportion of invariable sites (I) as 0.0000, a gamma distribution shape parameter (G) of 0.2555, a transition/transversion (Ti/Tv) ratio of 5.9860, and equilibrium base frequencies of A = 0.2406, C = 0.3099, G = 0.1599 and T = 0.2896. The maximum likelihood (ML)

analysis under the HKY+G model produced a topology with -lnL = 1500.2120. Maximum parsimony (MP) analysis of the cyt-*b* data resulted in seven equally parsimonious trees [166 steps in length, consistency index (CI) = 0.964, retention index (RI) = 0.978], and, along with ML and NJ analyses, revealed two well-supported basal clades among the 12 haplotypes of *H. rugulosus* (Figure 4.12).

The first clade (Clade A) consisted of four haplotypes (H7, H8, H9 and H12) from the western, central, southern regions (except Chumphon) plus Chonburi from the eastern region to be a monophyletic group (bootstrap support of 100% for MP, ML and NJ). Haplotype H12 from the southern region (except Chumphon) was sister to the other three haplotypes of H7 from Lopburi (LOP), Nakhon Nayok (NKN), Tak (TAK), Phetchaburi (PCB) and Chonburi (CBR), H8 from Nakhon Nayok (NKN) and Chonburi (CBR) and H9 from Tak (TAK), with high bootstrap values (93, 93 and 97% for MP, ML and NJ, respectively).

The second clade (Clade B) included all the populations from the northern, northeastern and eastern regions (except Chonburi) plus Chumphon from the southern region, and was divided into two groups with high bootstrap support (100, 80 and 93% for MP, ML and NJ, respectively). Group 1 was composed of only one haplotype (H1) from Nan (NAN) and it was sister to the seven haplotypes (H2, H3, H4, H5, H6, H10 and H11) from Sa-Kaeo (SKO) Chanthaburi (CTR), Trad (TRA), Chumphon (CHP) and all populations from the northeastern region (96, -, and -% for MP, ML and NJ, respectively).

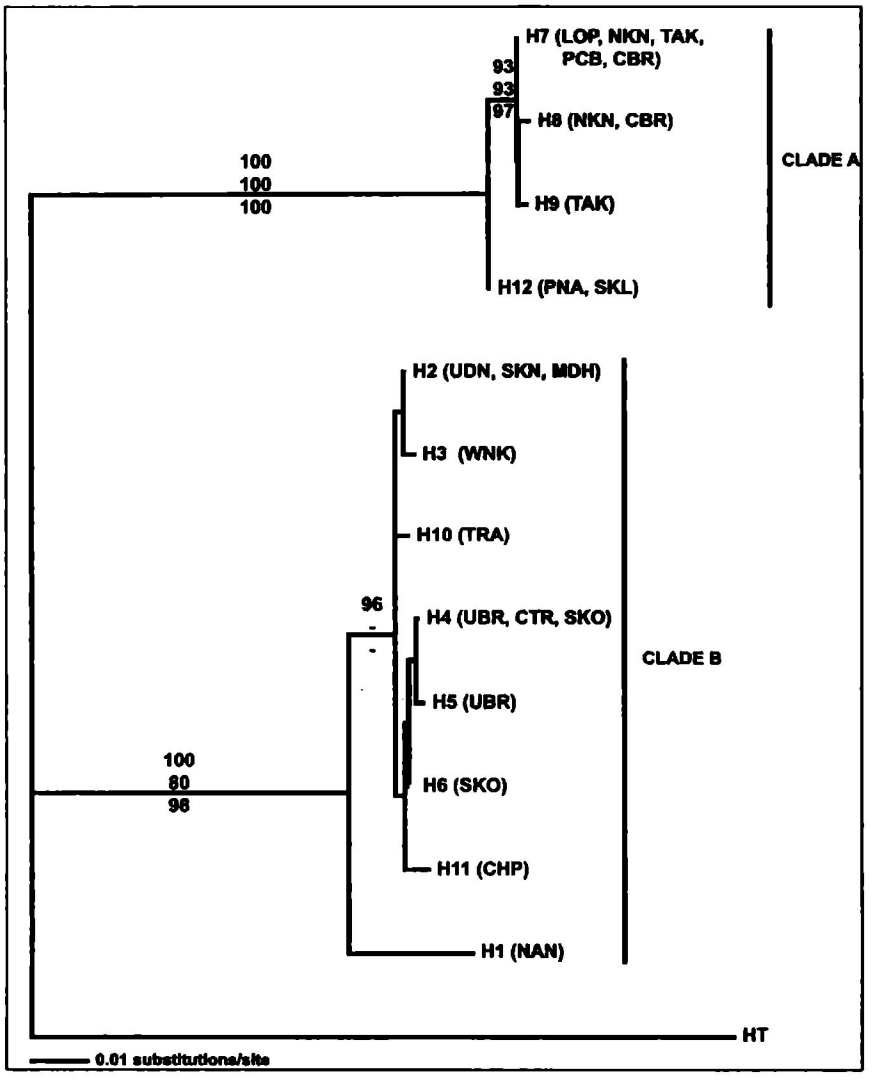

Figure 4.12 Maximum likelihood tree of the 564 bp partial cyt-*b* gene fragment for the 12 haplotypes of *H. rugulosus* plus that for *H. tigerinus* (HT) as an outgroup. Bootstrap supports are given in the order for MP, ML and NJ (1000, 100 and 1000 replicates, respectively).

As expected from the phylogenetic analysis, the intrapopulational sequence divergences were small (0.18%) within Ubon Ratchathani (UDN), Nakhon Nayok (NKN), Tak (TAK), Chonburi (CBR) and Sa-Kaeo (SKO). The sequence divergences between the 12 haplotypes within clade A and clade B were also small (0.36 - 0.72% and 0.54 - 0.71%, respectively), except that the sequence divergences between Nan and the other populations within clade B were somewhat larger (3.11 – 3.67%). In contrast, the sequence divergences between clade A and B were large (14.8 - 17.0%), and that between *H. rugulosus* and *H. tigerinus* (outgroup) were extremely large (19.3 – 21.8%) (Table 4.4).

On the other hand, the 16 haplotypes of the combined data of the 12S rRNA and 16S rRNA fragments (813 bp) revealed 32 variable sites and 43 parsimony informative sites if the outgroup taxa is included. The phylogenetic trees were constructed from the combined 12S rRNA and 16S rRNA sequences using the GTR+I+G model following the parameter settings: the proportion of invariable sites(I) = 0.6930; shape parameter of the gamma distribution (G) = 0.7795; the substitution matrix R(a) [A-C] = 6295419.5000, R(b) [A-G] = 13679331.0000, R(c) [A-T] = 8724214.0000, R(d) [C-G] = 344212.8438, R(e) [C-T] = 49708444.0000 and R(f) [G-T] = 1.0000; base frequencies: A = 0.2972, C = 0.2651, G = 0.2128 and T = 0.2249. The ML analysis under the GTR+I+G model generated a topology with –lnL = 1612.1141 whilst the MP analysis generated 463 equally parsimonious trees (89 steps in length, CI = 0.921 and RI = 0.940). The topology was very similar to the topology derived from the cyt-*b* gene. In all MP, ML and NJ analyses, the 16 haplotypes of *H. rugulosus* observed in the combined dataset formed two distinct clades that were supported with high bootstrap values (100% each for MP, ML and NJ) (Figure 4.13).

Clade A included three haplotypes (H11, H12 and H16) from the western, central and southern regions (except Chumphon) plus Chonburi from the eastern region as a monophyletic group. Within clade A, haplotype H16 from Phang-nga was sister to the other haplotypes, H11 from Lopburi (LOP), Nakhon Nayok (NKN), Phetchaburi (PCB) and Chonburi (CBR), and H12 from Tak (TAK) and Songkhla (SKL), although the bootstrap support was very weak (64, 56 and 64% for MP, Ml and NJ, respectively).

Moreover, 13 haplotypes (H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H13, H14 and H15) from the northern, northeastern and eastern regions (except Chonburi), plus Chumphon from the southern region were placed in the second clade (clade B). Haplotype H1 from Nan (NAN) was sister to the monophyletic group consisting of the haplotypes H2, H3, H4, H5, H6, H7, H8, H9, H10, H13, H14 and H15 from Sa-Kaeo (SKO) Chanthaburi (CTR), Trad (TRA), Chumphon (CHP) and all populations from the northeastern region (89, 72 and 88% for MP, Ml and NJ, respectively).

Table 4.4 Percent sequence divergences, as calculated using the Kimura-2 parameter (K2p) method, among haplotypes of the 564 bp partial cyt-*b* gene sequences of *H. rugulosus* and the outgroup *H. tigerinus* (HT)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	HT
H1	****												
H2	3.11	****											
H3	3.30	0.18	****										
H4	3.49	0.36	0.54	****									
H5	3.67	0.53	0.71	0.18	****								
H6	3.30	0.54	0.71	0.18	0.36	****							
H7	16.73	15.30	15.54	15.78	15.75	15.54	****						
H8	16.98	15.54	15.78	16.01	15.99	15.78	0.18	****					
H9	16.98	15.54	15.78	16.01	15.99	15.78	0.18	0.37	****				
H10	3.48	0.36	0.53	0.71	0.89	0.89	15.05	15.28	15.28	****			
H11	3.67	0.53	0.71	0.53	0.71	0.71	15.99	16.23	16.23	0.89	****		
H12	16.49	15.07	15.30	15.07	15.05	14.84	0.54	0.71	0.71	14.81	15.28	****	
HT	19.82	19.57	19.82	19.57	19.79	19.32	21.54	21.80	21.29	19.79	19.60	20.78	****

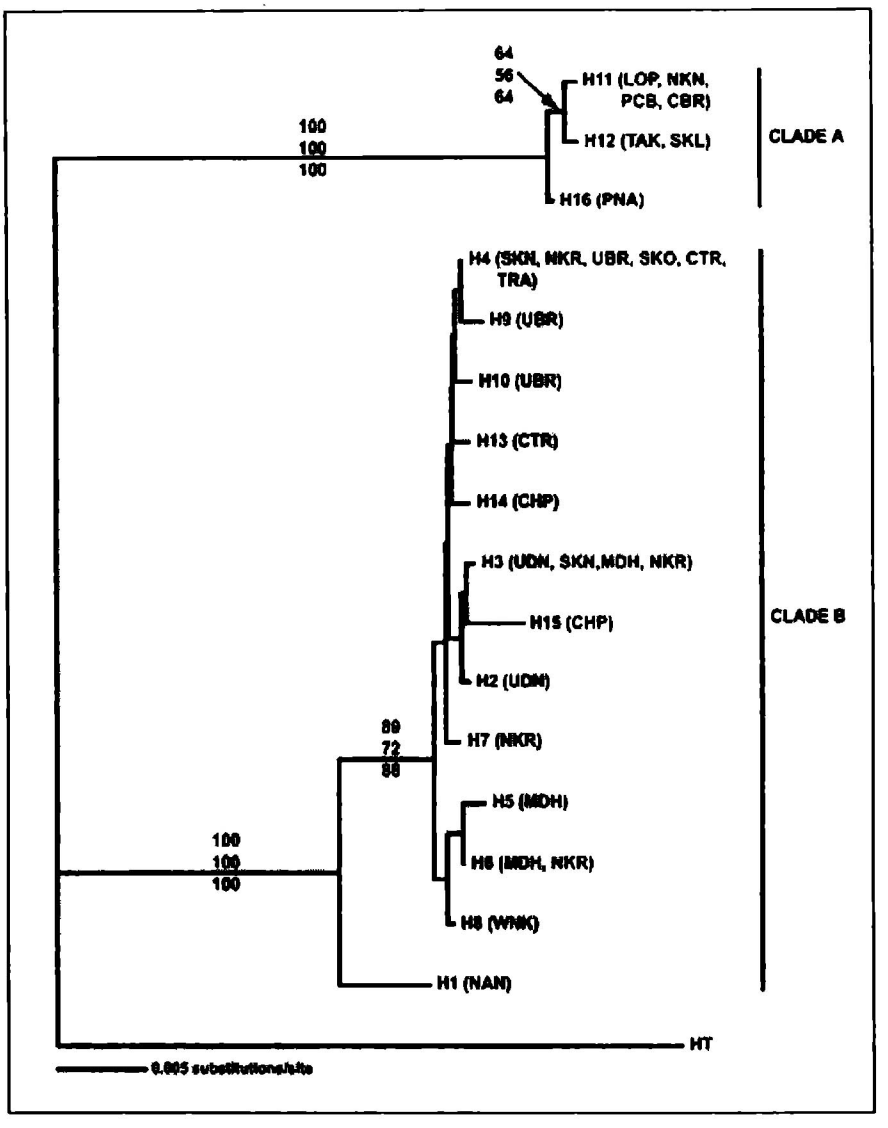

Figure 4.13. Maximum likelihood tree of the combined 12S rRNA and 16S rRNA gene fragments (813 bp) for the 16 haplotypes of *H. rugulosus* plus *H. tigerinus* (HT) as the outgroup. Bootstrap supports are given in the order for MP, ML and NJ (1000, 100 and 1000 replications, respectively).

As expected from the phylogenetic analysis, the intrapopulational sequence divergences were small (0.12 - 0.37%) within Udon Thani (UDN), Sakon Nakhon (SKN), Mukdahan (MDH), Wang Nam Khiao (WNK), Ubon Ratchathani (UBR), Chanthaburi (CTR) and Chumphon (CHP). The sequence divergences between the 16

haplotypes within clade A and clade B were small (0.12 - 0.25% and 0.12 - 0.62%, respectively), except the sequence divergences between Nan and the other populations within clade B, which were somewhat larger (1.00 – 1.63%). In contrast, the sequence divergences between clade A and B were large (5.01 – 5.82%), and that between *H. rugulosus* and *H. tigerinus* (outgroup) were extremely large (5.80 – 6.62%) (Table 4.5).

Table 4.5 Percent sequence divergences, as calculated using the Kimura-2 parameter (K2p) method, among haplotypes of the combined 812 bp partial 12S rRNA and 16S rRNA gene fragment sequences of *H. rugulosus* and the outgroup *H. tigerinus* (HT)

	H1	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**H9**	**H10**	**H11**	**H12**
H1	****											
H2	1.37	****										
H3	1.25	0.12	****									
H4	1.25	0.12	0.25	****								
H5	1.25	0.37	0.25	0.50	****							
H6	1.12	0.25	0.12	0.37	0.12	****						
H7	1.12	0.25	0.12	0.12	0.37	0.25	****					
H8	1.00	0.37	0.25	0.25	0.25	0.12	0.12	****				
H9	1.37	0.25	0.37	0.12	0.62	0.50	0.25	0.37	****			
H10	1.37	0.25	0.37	0.12	0.62	0.50	0.25	0.37	0.25	****		
H11	5.15	5.42	5.55	5.28	5.55	5.42	5.42	5.28	5.42	5.15	****	
H12	5.15	5.42	5.55	5.28	5.55	5.42	5.42	5.28	5.42	5.15	0.12	****

Table 4.5 (cont.)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
H13	1.37	0.25	0.37	0.12	0.62	0.50	0.25	0.37	0.25	0.24	5.15	5.15
H14	1.25	0.25	0.25	0.12	0.50	0.37	0.12	0.25	0.25	0.24	5.41	5.41
H15	1.63	0.37	0.37	0.50	0.62	0.50	0.50	0.62	0.62	0.62	5.82	5.82
H16	5.01	5.28	5.42	5.15	5.42	5.28	5.28	5.15	5.28	5.01	0.12	0.25
HT	5.80	5.80	5.93	5.93	6.07	5.93	6.07	6.07	6.07	5.93	6.62	6.62

Table 4.5 (cont.)

	H13	H14	H15	H16	HT
H13	****				
H14	0.25	****			
H15	0.62	0.37	****		
H16	5.01	5.28	5.69	****	
HT	5.80	6.06	6.20	6.49	****

Discussion

In this study, 12 haplotypes of the 564 bp cyt-*b* gene fragment and 16 haplotypes of the combined (813 bp) 12S rRNA and 16S rRNA gene fragments were found in 73 specimens of *H. rugulosus* from 18 geographic localities. The phylogenetic analysis, based upon two parts of the mitochondrial DNA (cyt-*b* gene and the combined 12S rRNA and 16S rRNA gene fragments) showed that *H. rugulosus* can be divided into two distinct clades. The first clade consisted of populations from the western, central and southern regions [except Chumphon (CHP)] plus Chonburi (CBR) from the eastern region. The second clade consisted of populations from the northern, northeastern and eastern regions [except Chonburi (CBR)], plus one population [Chumphon (CHP)] from the southern region. A similar result was found in a previous study, which showed the separation between *H. rugulosus* collected from the southern region (Phang-nga) from those in the northeastern (Nong Khai) and the eastern (Ko Chang, Trad) regions based on the sequence divergence of the same mitochondrial DNA fragments (cyt-*b*, 12S and 16S rDNA genes) (Alam et al., 2008).

Based on the geography of Thailand, the northeastern region is separated from the central and eastern regions by four mountain ranges. The Phetchabun, Dong Phya yen and the western part of the San Kampheng mountain ranges separate the northeast from the central regions, while the Phanom Dong Rak and the eastern part of San Kampheng mountain ranges separate the northeast and the east regions (Figure 4.14). However, the result in this study showed that the *H. rugulosus* populations in Sa-Kaeo (SKO), Chanthaburi (CTR) and Trad (TRA), which are located near Chonburi (CBR), were placed in the same clade with the northeastern populations. The Chanthaburi mountain range could be the cause of this separation, by serving as

natural barrier between the regions (Figure 4.14) so gene flow between *H. rugulosus* populations in Chonburi and the other eastern populations may not occur. In contrast, the altitude of Phanom Dong Rak mountain range is considerably lower (600 m asl) than the Chanthaburi mountain range, so *H. rugulosus* populations in Sa-Kaeo (SKO), Chanthaburi (CTR) and Trad (TRA) could potentially interchange with the northeastern populations.

As far as the role of mountain ranges in the obstruction of gene flow is concerned (Hagemann and Pröhl, 2007; Zhang et al., 2010), the migration of animals has important consequences for the genetic pools of populations, tending to increase the genetic variation in any population. However, the movement of amphibians is reported to commonly be only over a short-distance (< 0.5 km) (Zug, 1993). In this study, the phylogenetic tree showed a genetic structure of *H. rugulosus* that could be (cor)related to the geographical structure, as described above (see Figures 4.12, 4.13 and 4.14). Therefore, these mountain ranges could be natural barriers obstructing gene flow among the regions. Further work with polymorphic nuclear markers, such as SSR, will be required to attempt to address this issue.

In the case of the Chumphon (CHP) samples (population), they are located in the same region of Thailand (southern region) as the Phang-nga (PNA) and Songkhla (SKL) populations, yet the phylogenetic analysis in this study showed that these populations are more closely related to the northeastern populations, and some populations from the eastern region [Sa-Kaeo (SKO), Chanthaburi (CTR), and Trad (TRA)], than to the other southern populations. Chumphon is more than 500 km distance from the east of Thailand and is separated by the Gulf of Thailand (< 100 m deep). It is plausible that both regions may have been connected by land bridges through the continent in the past (Hall, 1998; Voris, 2000; Sathiamurthy and Voris,

2006) so the gene flow between these populations could have occurred though a route over the present Gulf of Thailand in the past. This assumption is supported by the observed genetic diversity of *R. nigrovittata*, where the southern population of *R. nigrovittata* is reported to be genetically closely related to the eastern populations, based on allozyme analysis (Matsui et al., 2001). However, alternatively this might simply reflect more recent human transport (artificial introductions) from the other locations to Chumphon via local people since this species is an edible and economic animal.

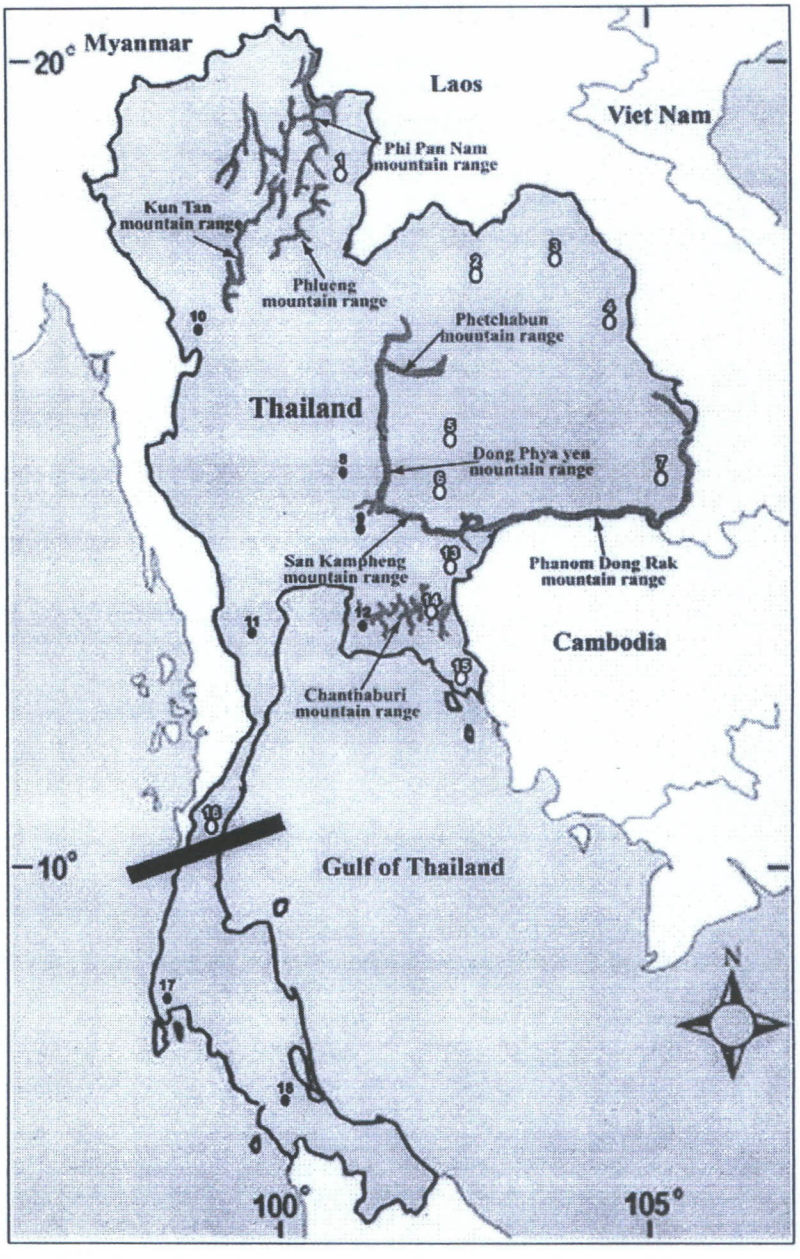

Figure 4.14 Map showing the relevant mountain ranges that could act as potential barriers to gene flow and immigration between different *H. rugulosus* populations in Thailand. The locality numbers and black and white symbols refer to the populations that are grouped into clade A (black) and clade B (white), respectively. The solid line indicates the location of the Isthmus of Kra.

Within clade A, most populations from the southern region [Phang-nga (PNG) and Songkhla (SKL)] except Chumphon (CHP) could be grouped in the same clade as those from the central and the western populations, but they were separated into a different group. Phang-nga and Songkhla are located to the South of the Isthmus of Kra (Fig 4.14), a zoogeographic barrier in Southeast Asia located on the Thai-Malay peninsula that obstructs the faunal transition between Indochinese (north) and Sundaic (south) subregions (Hughes et al., 2003; de Bruyn et al., 2005; Woodruff and Turner, 2009). Recently, it has been hypothesized that this land bottleneck may have produced the observed animal and floral distribution patterns (Woodruff, 2003a; 2003b), including the distribution and divergence of anuran fauna between the southern and the other regions of Thailand (Inger, 1999; Inger and Voris, 2001).

Molecular analysis has been used to suggest that the values of intra- and inter-specific sequence divergence of DNA can help to identify cryptic anuran species. Graybeal (1997) reported on conspecific cyt-*b* haplotypes of up to 15% pairwise distance in bufonid frogs. Matsui et al. (2005) provided evidence that isolated cryptic species of *Microhyla* can be separated by a pairwise distance of 16% for cyt-*b* gene sequences. On the other hand, for the slower evolving rRNA genes, Fouquet et al. (2007) suggested that the sequence divergence of rRNA in inter-species comparison was more than 3%. In this study, the interpopulation comparisons showed large sequence divergences between clade A and B (14.8 – 17.0% and 5.01 – 5.82% for cyt-*b* gene and the combined 12S and 16S rRNA gene fragments, respectively), and related to the sequence divergence of *H. rugulosus* between the southern population and the other populations in Thailand (13.4%, 5.5% and 2.7% for cyt-*b*, 12S rRNA and 16S rRNA genes). Thus, our results imply that *H. rugulosus* as currently recognized may in fact contain (at least) two distinct species in Thailand;

one species might occupy the northern, northeastern and some parts of the eastern regions, and the other seems to inhabit the remaining regions of Thailand. This is congruent with the morphological data of Taylor (1962). However, specimens from the type locality were not available, so it is difficult to specify which haplotype group corresponds to the nominal species and the other locations should be verified to confirm this suggestion.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

The results from this study can be divided into two main parts, (i) the morphometric differences (Chapter II) and (ii) the genetic diversity (Chapters III and IV) of *H. rugulosus* in Thailand. In this chapter, the results from these three previous chapters will be discussed.

With respect to the morphological differences, the size sexual dimorphism of *H. rugulosus* was clearly seen in all six regions as well as within localities across Thailand. This is in congruence with a previous report which showed a sexual dimorphism in the SVL size of *H. rugulosus* in four localities (Taiwan, Hong Kong, Thailand and Myanmar), although these authors also reported a general decrease in the SVL size from east to west (Schmalz and Zug, 2002). In this study presented here the adult males and females of *H. rugulosus* from the central region of Thailand had, on average, a relatively longer SVL than individuals from the other regions, but no clear east to west trend within the Thailand populations was evident. The results did, however, segregate individuals on the basis of size on the first component for adult males and females using PCA, but this difference did not display any significant concordant patterns of geographic regions. For the cluster analysis, the dendrograms represented two major groups for adult males and females. Nevertheless, these groups also did not display any significant concordant patterns with their geographic region, but rather the morphological variation in populations at the regional level revealed very little difference between all six regions. Usually, the morphological differences of amphibians are related to the geographic variation (environmental factors), such as relative altitude (Sotiropoulos et al., 2008), temperature (Castellano and Giacoma,

1998) and humidity (Alexandrino et al., 2005). Because the morphological differences do not correlate with the patterns of geographic region, then these morphological differences are probably affected more by ecological (*e.g.*, effects of coexisting species) than by physical factors. Moreover, *H. rugulosus* is an economically important species because it is a favourite dish among Thai people, and additionally is used as a pet and an experimental animal. Thus, it has been transported to diverse cities across the country for human utilization and some will likely have either escaped or been released and establish a new population, or integrate into existing populations, if the environmental conditions are suitable. These may in turn then integrate with other neighboring populations and so negate the expected morphological variations between regions whilst perhaps maintaining that within populations.

With respect to the genetic diversity of *H. rugulosus*, a high level of genetic diversity within regions was revealed using ISSR mitochondrial DNA sequence analysis. The genetic diversity of *H. rugulosus* from natural habitats was found to be higher than in the populations of farmed *H. rugulosus* (Jiwyam et al., 2006). That a relative high level of genetic diversity of *H. rugulosus* was revealed is likely to be because *H. rugulosus* is widely distributed in Thailand (Chan-ard, 2003). In general, widespread species have a higher level of genetic variability than narrowly distributed ones (Hamrick and Godt, 1996). Nevertheless, the genetic distances did not relate to the geographic distances. A high level of inter-population gene flow (Nm = 1.2584) was evident among populations of *H. rugulosus* in different regions of Thailand that confirmed the high level of genetic diversity was revealed within regions.

With respect to the mitochondrial DNA sequence based analysis, the phylogenetic analysis of *H. rugulosus* in Thailand, based upon the mitochondrial

DNA sequence of a 564 bp fragment of the cyt-*b* gene and the 812 bp combined fragments of the 12S rRNA and 16S rRNA genes, also revealed a clear division of Thai samples into two distinct clades, and that the genetic structure of *H. rugulosus* is related to the geographical structure. The first clade consisted of populations from the western, central and southern regions [except Chumphon (CHP)] plus Chonburi (CBR) from the eastern region of Thailand. The second clade consisted of populations from the northern, northeastern and eastern regions of Thailand [except Chonburi (CBR)], plus one population [Chumphon (CHP)] from the southern region. The sequence divergences between the two clades were large (14.8 – 17.0% and 5.01 – 5.82% for cyt-*b* gene and the combined 12S rRNA and 16S rRNA gene fragments, respectively). It is likely that mountain ranges have played an important role in obstructing gene flow among the regions because the movement of amphibians is commonly only over a short-distance (< 0.5 km) (Zug, 1993). The observed sequence divergences can support the notion that *H. rugulosus* as currently recognized may in fact be two distinct species in Thailand.

From the results of this study, it is clear that the morphological data is not congruent with the mitochondrial DNA based molecular data. The morphological data reveals a low level of difference among regions whilst the molecular data reveals a high level of sequence divergence between regions that can be clearly divided into two clades. We suggest that *H. rugulosus* in Thailand is in fact a cryptic species complex that superficially are morphologically indistinguishable and can only be separated with molecular data or by assays for reproductive isolation (Bickford et al., 2006). Most of the morphologically 'cryptic' species identified by molecular approaches are allopatrically or parapatrically distributed (Hillis et al., 1983; Green et al., 1997; Narins et al., 1998; Gower et al., 2005). The application of molecular

genetics in systematic studies has been particularly effective at revealing morphologically 'cryptic' species complex within taxa that were previously considered to be a single species (Stuart et al., 2006). One good example is the study of biochemical evolution within what was considered to be a single, geographically widespread species of salamander, *Plethondon glutinosus* (Highton, 1989). There are at least 16 genetically differentiated population systems identified within *P. glutinosus* sensu lato, representing full species or subspecies. However, we suggest that additional bioacoustic, physiological, ecological and behavioral characters will further elucidate the two clades of *H. rugulosus*.

Conservation and management proposition

Management units are usually defined based on the significant difference in allele frequency of nuclear DNA and/or mitochondrial DNA, regardless of the occurrence of systemic differentiation between populations or between distribution regions (Moritz 1994). As such, management units are essential for the preservation of the genetic diversity of any species. In this study, ISSR molecular markers clearly offer the ability to investigate the genetic diversity, population genetic structure and the level of gene flow between populations of *H. rugulosus* in Thailand. However, more studies on the life history, tagging and advanced genetics of this species / cryptic species complex are recommended to gain a better understanding of the biology of this species.

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APPENDICES

APPENDIX A

THE DESCRIPTION OF CHARACTER DIMENSION

1. **Snout-vent length (SVL or body length):** the distance from the tip of the snout to the anterior margin of the vent (measured dorsally on flattened body).
2. **Head length (HL):** the distance from the tip of the snout to hind border of the angle of the jaw (not parallel with the median line and measured ventrally).
3. **Snout-nostril length (S-NL):** the distance from the center of an external naris to the tip of snout (not parallel with body axis).
4. **Nostril-eye length (N-EL):** the distance from the corner of the external naris to the anterior margin of the eye (not parallel with body axis).
5. **Snout length (SL):** the distance from the tip of the snout to the angle formed by snout and upper eyelid (not parallel with body axis).
6. **Eye length (EL):** the greatest diameter of the eye.
7. **Tympanum-eye length (T-EL):** the minimum distance from the posterior corner of upper eyelid to the anterior border of tympanum.
8. **Tympanum diameter (TD):** the greatest diameter of the tympanum.
9. **Head width (HW):** the width of the head measured at the angles of the jaws and ventrally.
10. **Internarial distance (IND):** the distance between centers of the external nares.
11. **Intercanthal distance (ICD):** the distance between anterior edges of canthus.
12. **Forelimb length (FLL):** the distance from the axilla to the tip of the longest finger (the 3rd finger) measured with the forelimb stretched perpendicular to the body axis.

13. **Lower arm length (LAL):** the distance from the elbow joint to the tip of the longest finger (the 3rd finger), measured with the forearm stretched straight and flexed perpendicular to the upper arm.
14. **Third finger length (TFL):** the distance from the base point between third and fourth fingers to the tip of the third finger, measured dorsally with the fingers stretched straight.
15. **First finger length (FFL):** the distance from the base point between first and second fingers to the tip of the first finger, measured with the finger fully stretched.
16. **Hand length (HAL):** the distance from the proximal end of the wrist to the tip of the longest finger (the 3rd finger).
17. **Hindlimb length (HLL):** the distance from the center of anus to the tip of the longest (fourth) toe, measured dorsally with the hindlimb fully stretched perpendicular to the axis.
18. **Tibia length (TL):** the greatest length of the tibia, measured with the hindlimb positioned in a Z pattern.
19. **Foot length (FL):** the distance from the proximal end of the heel to the tip of the longest toe (the 4th toe).
20. **Fourth toe length (FTL):** the distance from the end of the third phalanx to the tip of the fourth toe.

APPENDIX B

CHEMICAL AND REAGENTS

10x Tris boric acid EDTA (TBE) buffer

Tris base	108	g
Boric acid	55	g
0.5 EDTA (pH 8.0)	40	ml
Deionized H_20 adjust to	1,000	ml

Tris-EDTA (TE) buffer pH 8.0

1M Tris (pH 8.0)	10	ml
0.5 EDTA (pH 8.0)	2	ml
Deionized H_20 adjust to	1,000	ml

1M Tris pH 8.0

Tris base	121.14	g
Deionized H_20	800	g
Adjust pH to 8.0 with concentrated HCl		
Mix and add Deionized H_20 to	1,000	ml

0.5 M EDTA pH 8.0

$Na_2EDTA.2H_2O$	18.61	g
H_2O	80	ml
Adjust pH to 8.0 by NaOH (~1 g)		
Add H_2O to	100	ml

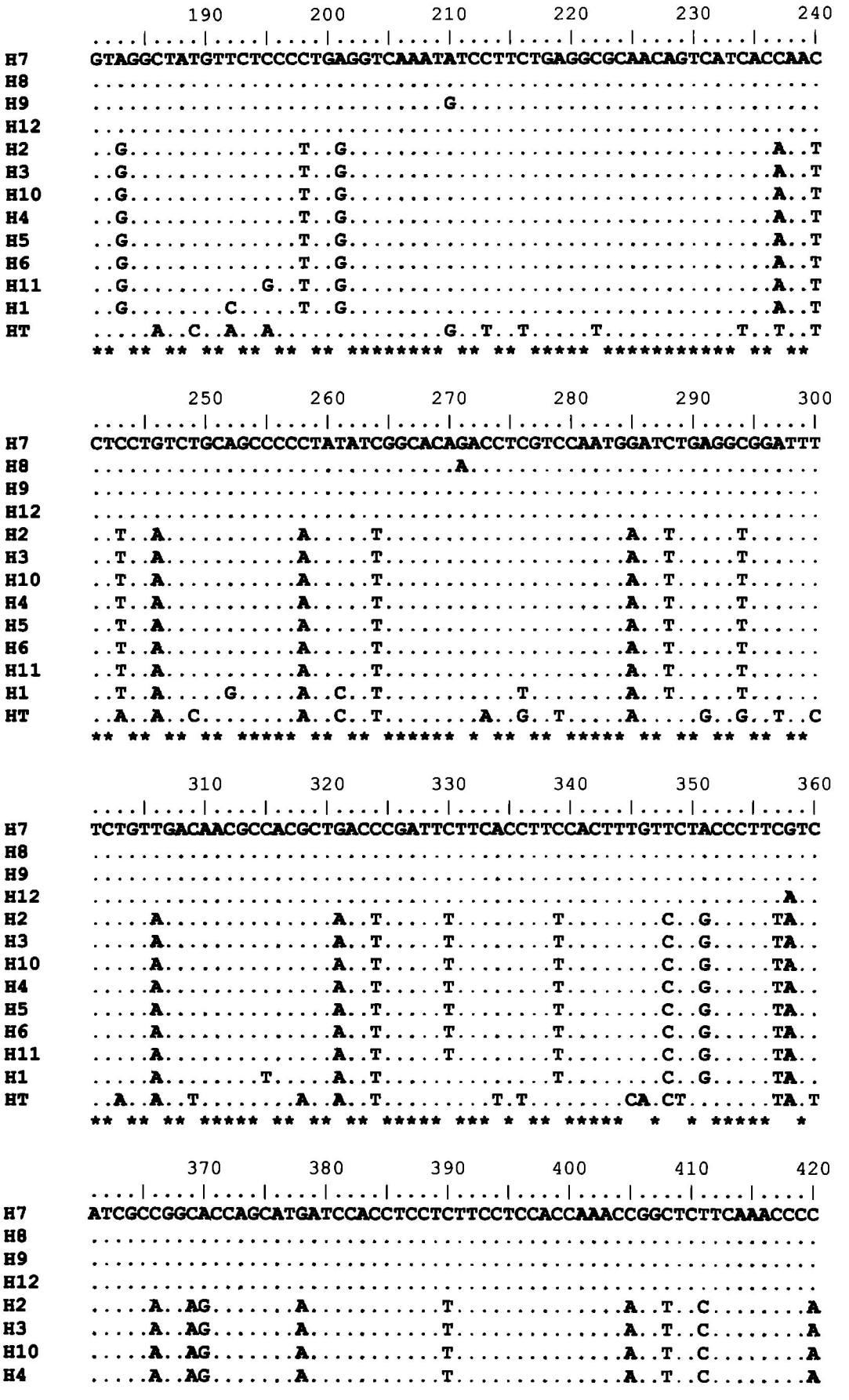
APPENDIX C

Appendix C.1 The 564 character matrix of 12 haplotypes of *H. rugulosus* and the outgroup *H. tigerinus* (HT) based on partial cyt-*b* gene sequences. Asterisks (*) represent conserved nucleotide residues across all samples.

```
                10        20        30        40        50        60
        ....|....|....|....|....|....|....|....|....|....|....|....|
H7      ATTTGCCGCGACGTCAATAGCGGCTGACTACTACGCAACCTTCATGCAAATGGAGCATCA
H8      ............................................................
H9      ............................................................
H12     ............................................................
H2      ........T.....T..C.A.........G..T..T..T..C.....T..C..C......
H3      ........T.....T..C.A.........G..T..T..T..C.....T..C..C......
H10     ........T.....T..C.A.........G..T..T..T..C.....T..C..C......
H4      ........T.....T..C.A.........G..T..T..T..C.....T..C..C......
H5      ........G.....T..C.A.........G..T..T..T..C.....T..C..C......
H6      ........T.....T..C.A.........G..T..T..T..C.....T..C..C......
H11     ........T.....T..C.A.........G..T..T..T..C.....T..C..C......
H1      ........T.....T..C.A.........G..T..T..T........T..C..C......
HT      ........T.........CCAT..T.....T..T..A.........C..C..........
        ******** ***** **     ** ***** ** ** ** ** ** ** ** ** ******

                70        80        90        100       110       120
        ....|....|....|....|....|....|....|....|....|....|....|....|
H7      TTTTTTTTCATCTGCATCTACCTCCACATTGGACGGGGCCTATACTACGGGTCCTTCCTA
H8      ............................................................
H9      ............................................................
H12     ............................................................
H2      ..C.....T..............T.....C.....................A........G
H3      ..C.....T..............T.....C.....................A........G
H10     ..C.....T..............T.....C.....................A........G
H4      ..C.....T..............T.....C.....................A........G
H5      ..C.....T..............T.....C.....................A........G
H6      ..C.....T..............T.....C.....................A........G
H11     ..C.....T..............T.....C.................T..A........G
H1      ..C.....T..............T.....C.....................A........G
HT      ..C..C..T.....T.....T..T..TG.C.....A.....C.........C..T......
        ** ** ** ***** ***** ** **  * ***** ***** ***** ** ** *****

                130       140       150       160       170       180
        ....|....|....|....|....|....|....|....|....|....|....|....|
H7      TTCAAAGAGACCTGAAACATCGGCGTTGTCCTTCTCTTCTTAGTTATAGCCACAGCTTTC
H8      ............................................................
H9      ............................................................
H12     ............................................................
H2      .A......A........T.....T...A.T..C..T........A...............
H3      .A......A........T.....T...A.T..C..T........A...............
H10     .A......A........T.....T...A.T..C..T........A...............
H4      .A......A........T.....T...A.T..C..T........A...............
H5      .A......A........T.....T...A.T..C..T........A...............
H6      .A......A........T.....T...A.T.....T........A...............
H11     .A......A........T.....T...A.T..C..T........A...............
H1      .A......A........T.........A.T.....T........A........G......
HT      .AT.....A..T.....T..T.....AA.T.....T.....G..G...............
        *  ***** ** ***** ** ** **  * ** ** ***** ** ******** ******
```


190 200 210 220 230 240
H7 GTAGGCTATGTTCTCCCCTGAGGTCAAATATCCTTCTGAGGCGCAACAGTCATCACCAAC
H8
H9
H12
H2
H3
H10
H4
H5
H6
H11
H1
HT
250 260 270 280 290 300
H7 CTCCTGTCTGCAGCCCCCTATATCGGCACAGACCTCGTCCAATGGATCTGAGGCGGATTT
H8
H9
H12
H2
H3
H10
H4
H5
H6
H11
H1
HT
310 320 330 340 350 360
H7 TCTGTTGACAACGCCACGCTGACCCGATTCTTCACCTTCCACTTTGTTCTACCCTTCGTC
H8
H9
H12
H2
H3
H10
H4
H5
H6
H11
H1
HT
370 380 390 400 410 420
H7 ATCGCCGGCACCAGCATGATCCACCTCCTCTTCCTCCACCAAACCGGCTCTTCAAACCCC
H8
H9
H12
H2
H3
H10
H4

```
H5      .....A..AG.......A..........T.............A..T..C........A
H6      .....A..AG.......A..........T.............A..T..C........A
H11     .....A..AG.......A..........T.............A..T..C........A
H1      .....A..GG.T.....A..........T.............A..T..C..G.....A
HT      ..T..A...G.......A........T.....................C........A
        ** ** **  * ***** ******** ** ************** ** ** ** *****

                 430        440        450        460        470        480
        ....|....|....|....|....|....|....|....|....|....|....|....|
H7      ACCGGACTTAATCAAAACCTAGACAAAGTCCCATTCCACCCCTACTTCTCCTACAAAGAT
H8      ............................................................
H9      ............................................................
H12     ............................................................
H2      ..G........C.....T..G.....................T..T.............C
H3      ..G........C.....T..G.....................T..T.............C
H10     ..G........C.....T..G.....................T..T.............C
H4      ..G........C.....T..G.....................T..T..T..........C
H5      ..G........C.....T..G.....................T..T..T..........C
H6      ..G........C.....T..G.....................T..T..T..........C
H11     ..G........C.....T..G.....................T..T.............C
H1      ..G.....C..C.....T..G...........G.........T..............C
HT      ..T.....A..C..G.....T..T.....G.............................C
        ** ***** ** ** ** ** ** ***** ** ******** ** ** ***********

                 490        500        510        520        530        540
        ....|....|....|....|....|....|....|....|....|....|....|....|
H7      GCCCTGGGCTTTGCCATCATAATCGGTGCTCTGGCAAGTCTTTCCACTTTTGCACCCAAT
H8      ............................................................
H9      ............................................................
H12     ..........................................................C.....T...
H2      .....A.....C...G..T....T..C...........C...........C..........
H3      .....A.....C...G..T....T..C...........C....T......C..........
H10     .....A.....C...G..T....T..C...........C...........C..........
H4      .....A.....C...G..T....T..C...........C...........C.....T...
H5      .....A.....C...G..T....T..C...........C...........C.....T...
H6      .....A.....C...G..T....T..C...........C...........C.....T...
H11     .....A.....C...G..T....T..C...........C...........C.....T...
H1      .....A.....C...G..T....T..C...........C...........C..........
HT      .....A.....C.........G.T.....C..T........A.....C..C.....T...
        ***** ***** *** ** ** * ** ** ** ***** ** * *** ** ***** ***

                 550        560
        ....|....|....|....|....
H7      CTTCTGGGAGACCCAGACAACTTT
H8      ........................
H9      ........................
H12     ........................
H2      .....A..G........T..T...
H3      .....A..G........T..T...
H10     .........G........T..A...
H4      .....A..G........T..T...
H5      .....A..G........T..T...
H6      .....A..G........T..T...
H11     .....A..G........T..T...
H1      .....A..G........T..T..C
HT      ..C..A..................
        ** ** ** ******** ** **
```

Appendix C.2 The 813 character matrix of 16 haplotypes of *H. rugulosus* and the outgroup *H. tigerinus* (HT) based on the combine data of 12S rRNA and 16S rRNA gene sequences. Asterisks (*) represent conserved nucleotide residues across all samples.

```
                10        20        30        40        50        60
        ....|....|....|....|....|....|....|....|....|....|....|....|
H11     CCCGTGAGGACGCCCTCTACCCCTACCCCAGGCTTAGGAGCTGGTATCAGGCACA TTTC
H12     ..................................................
H16     ..................................................  ....
H4      ................T.....................................G..AC..T
H9      ................T.....................................G..AC..T
H10     ................T.....................................G..AC..T
H13     ................T.....................................AC..T
H14     ................T.....................................G..AC..T
H3      ................T.....................................G..AC..T
H15     ................T.....................................G..AC..T
H2      ................T.....................................G..AC..T
H7      ................T.....................................G..AC..T
H5      ................T.....................................G..AC..T
H6      ................T.....................................G..AC..T
H8      ................T.....................................G..AC..T
H1      ................T.....................................G..AC..T
HT      ................T.....................................AC.AT
        ***************** ************************************ **  *

                70        80        90        100       110       120
        ....|....|....|....|....|....|....|....|....|....|....|....|
H11     CCCGCCCATTACACCTAGTCCCACCACGCCCCCAAGGGTACTCAGCAGTGATAAACATTG
H12     ............................................................
H16     ............................................................
H4      TA.......A.................A.............................
H9      TA.......A.................A.............................
H10     TA.......A.................A.............................
H13     TA.......A.................A.............................
H14     TA.......A.................A.............................
H3      TA.......A.................A.............................
H15     TA.......A.................A.............................
H2      TA.......A.................A.............................
H7      TA.......A.................A.............................
H5      TA.......A.................A.............................
H6      TA.......A.................A.............................
H8      TA.......A.................A.............................
H1      TA.......A.................A.............................
HT      T T......A.................A............T..............T....
          ****** ****************** ************ ************** ****

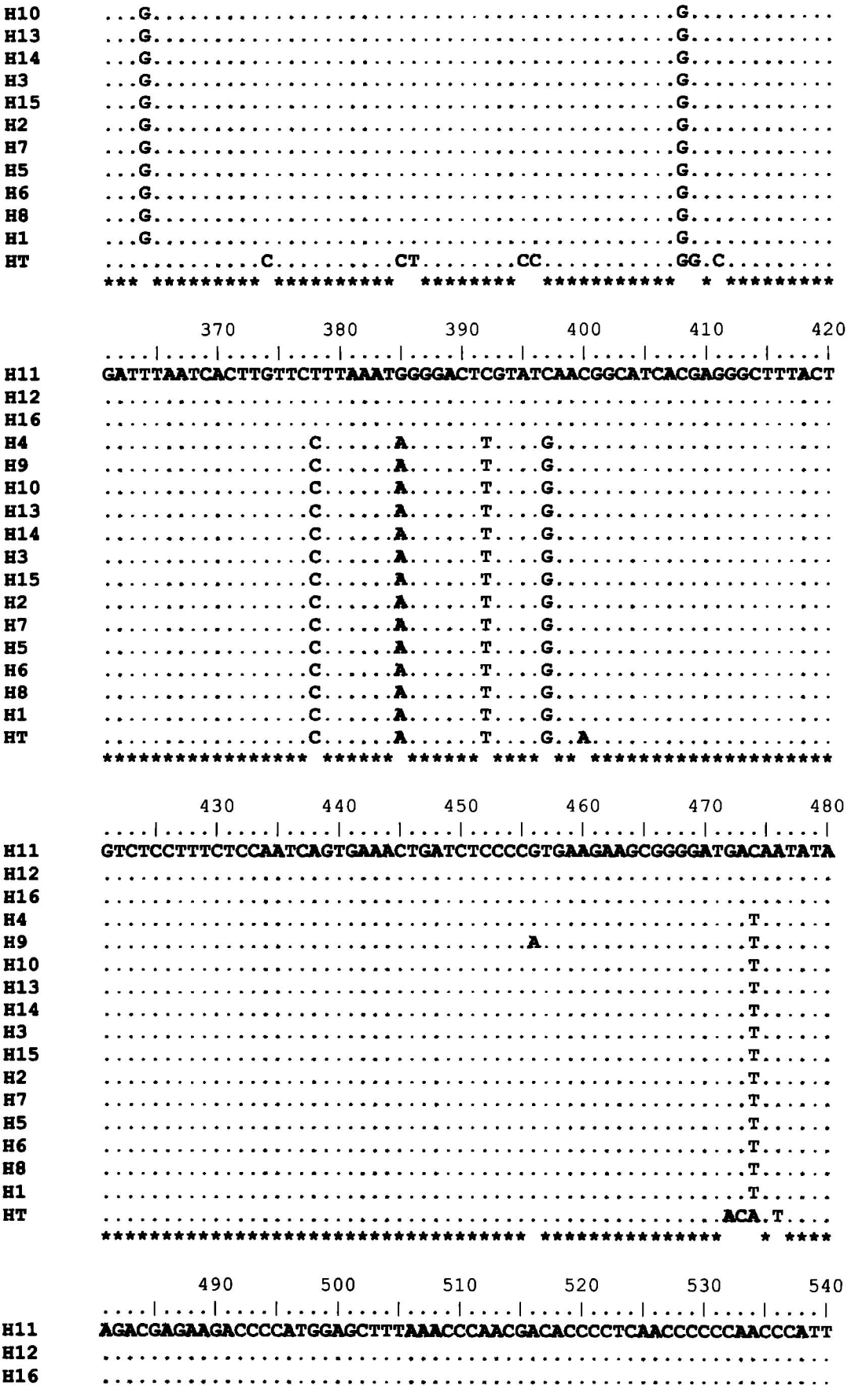
                130       140       150       160       170       180
        ....|....|....|....|....|....|....|....|....|....|....|....|
H11     TATATAAGCGTCAGCTTGACCCAGTTAAAGAGAAGAGAGCCGGCCAACTTGGTGCCAGCC
H12     .G..........................................................
H16     ..........C.................................................
H4      .C........C................T.A.......G.....................
H9      .C........C................T.A.......G.....................
H10     .C........C................T.A.......G.....................
H13     .C........C................T.A.......G.....................
```

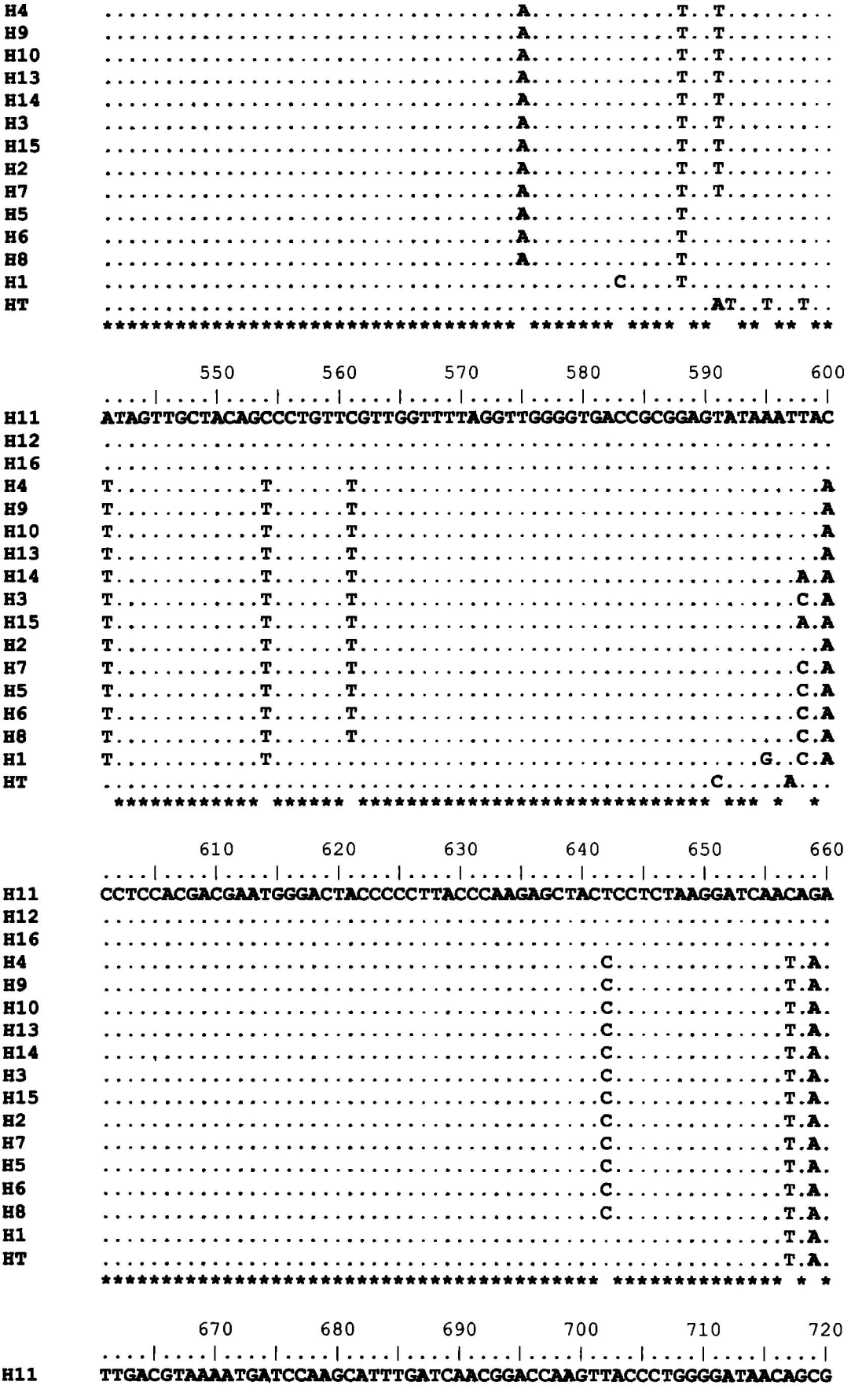
```
H14     .C........C.................T.A.......G.....................
H3      .C........C.................T.A.......G.....................
H15     .C........C......A.........T.A.......G.....................
H2      .C........C.................T.A.......G.....................
H7      .C........C.................T.A.......G.....................
H5      .C...G....C.................T.A.......G.....................
H6      .C........C.................T.A.......G.....................
H8      .C........C.................T.A.......G.....................
H1      .TC.......C.................T.A.......G.....................
HT      .T........CT................T.....................................
        *  ** ****  ***** ********* * ******* ************************

                 190        200        210        220        230        240
        ....|....|....|....|....|....|....|....|....|....|....|....|
H11     GCCGCGGCTAAACCAACTGGACTCAAATTGATACCCCCCCGGCGTTAAGCGTGATTAAAG
H12     ............................................................
H16     ............................................................
H4      ..........G.....................ATT... ....................
H9      ..........G.....................ATT... ....................
H10     ..........G.....................ATT... ....................
H13     ..........G.....................ATT... ....................
H14     ..........G.....................ATT... ....................
H3      ..........G.....................ATT... ....................
H15     ..........G.....................ATT... ....................
H2      ..........G.....................ATT... ....................
H7      ..........G.....................ATT... ....................
H5      ..........G.....................ATT... ....................
H6      ..........G.....................ATT... ....................
H8      ..........G.....................ATT... ....................
H1      ..........G.....................ATT... ....................
HT      ..........G.....................A..... ....................
        ********** ********************   *** ********************

                 250        260        270        280        290        300
        ....|....|....|....|....|....|....|....|....|....|....|....|
H11     CCACCTACTAATTAGAGTTAAACCTAACGTTGAGCAGTGAAAAGCACACGACAGGGAAAC
H12     ............................................................
H16     ............................................................
H4      ....AC..C.................. T.......................T........
H9      ....AC..C.................. T.......................T........
H10     ....A...C.................. T.......................T........
H13     ....AC..C.................. T.......................T........
H14     ....AC..C.................. T.......................T........
H3      ...TAC..C.................. T.......................T........
H15     ...TAC..C.................. T.......................T.......T
H2      ...TAC..C.................. T.......................T........
H7      ....AC..C.................. T.......................T........
H5      ...TAC..C.................. T.......................T........
H6      ...TAC..C.................. T.......................T........
H8      ....AC..C.................. T.......................T........
H1      ....AC..C.................. T.......................T........
HT      ...TAA.TA.................. ........................T........
        ***   *  ****************  ************************ *******

                 310        320        330        340        350        360
        ....|....|....|....|....|....|....|....|....|....|....|....|
H11     CCAAAAACGAAAGTTACTCTAATCTCTGCTTGAATACACGACAGCTAAAAAACAAACTGG
H12     ............................................................
H16     ............................................................
H4      ...G.....................................................G.....
H9      ...G.....................................................G.....
```


H10 ...G.....G.....
H13 ...G.....G.....
H14 ...G.....G.....
H3 ...G.....G.....
H15 ...G.....G.....
H2 ...G.....G.....
H7 ...G.....G.....
H5 ...G.....G.....
H6 ...G.....G.....
H8 ...G.....G.....
H1 ...G.....G.....
HT .....C.....CT.....CC.....GG.C.....
370 380 390 400 410 420
H11 GATTTAATCACTTGTTCTTTAAATGGGGACTCGTATCAACGGCATCACGAGGGCTTTACT
H12 .....
H16 .....
H4 .....C.....A.....T....G.....
H9 .....C.....A.....T....G.....
H10 .....C.....A.....T....G.....
H13 .....C.....A.....T....G.....
H14 .....C.....A.....T....G.....
H3 .....C.....A.....T....G.....
H15 .....C.....A.....T....G.....
H2 .....C.....A.....T....G.....
H7 .....C.....A.....T....G.....
H5 .....C.....A.....T....G.....
H6 .....C.....A.....T....G.....
H8 .....C.....A.....T....G.....
H1 .....C.....A.....T....G.....
HT .....C.....A.....T....G..A.....
430 440 450 460 470 480
H11 GTCTCCTTTCTCCAATCAGTGAAACTGATCTCCCCGTGAAGAAGCGGGGATGACAATATA
H12 .....
H16 .....
H4 .....T.....
H9 .....A.....T.....
H10 .....T.....
H13 .....T.....
H14 .....T.....
H3 .....T.....
H15 .....T.....
H2 .....T.....
H7 .....T.....
H5 .....T.....
H6 .....T.....
H8 .....T.....
H1 .....T.....
HT .....ACA.T....
490 500 510 520 530 540
H11 AGACGAGAAGACCCCATGGAGCTTTAAACCCAACGACACCCCTCAACCCCCCAACCCATT
H12 .....
H16 .....


H4 ..........A..........T..T.....
H9 ..........A..........T..T.....
H10 ..........A..........T..T.....
H13 ..........A..........T..T.....
H14 ..........A..........T..T.....
H3 ..........A..........T..T.....
H15 ..........A..........T..T.....
H2 ..........A..........T..T.....
H7 ..........A..........T..T.....
H5 ..........A..........T.....
H6 ..........A..........T.....
H8 ..........A..........T.....
H1 ..........C....T.....
HT ..........AT..T..T..
550 560 570 580 590 600
H11 ATAGTTGCTACAGCCCTGTTCGTTGGTTTTAGGTTGGGGTGACCGCGGAGTATAAATTAC
H12 .....
H16 .....
H4 T.....T.....T.....A
H9 T.....T.....T.....A
H10 T.....T.....T.....A
H13 T.....T.....T.....A
H14 T.....T.....T.....A.A
H3 T.....T.....T.....C.A
H15 T.....T.....T.....A.A
H2 T.....T.....T.....A
H7 T.....T.....T.....C.A
H5 T.....T.....T.....C.A
H6 T.....T.....T.....C.A
H8 T.....T.....T.....C.A
H1 T.....T.....G..C.A
HT .....C.....A...
610 620 630 640 650 660
H11 CCTCCACGACGAATGGGACTACCCCCTTACCCAAGAGCTACTCCTCTAAGGATCAACAGA
H12 .....
H16 .....
H4 .....C.....T.A.
H9 .....C.....T.A.
H10 .....C.....T.A.
H13 .....C.....T.A.
H14 .....C.....T.A.
H3 .....C.....T.A.
H15 .....C.....T.A.
H2 .....C.....T.A.
H7 .....C.....T.A.
H5 .....C.....T.A.
H6 .....C.....T.A.
H8 .....C.....T.A.
H1 .....T.A.
HT .....T.A.
670 680 690 700 710 720
H11 TTGACGTAAAATGATCCAAGCATTTGATCAACGGACCAAGTTACCCTGGGGATAACAGCG

```
H12     ..................................................
H16     ..................................................
H4      .......T..........................................
H9      .......T..........................................
H10     .......T..........................................
H13     .......T..........................................
H14     .......T..........................................
H3      .......T..........................................
H15     .......T..........................................
H2      .......T..........................................
H7      .......T..........................................
H5      .......T..........................................
H6      .......T..........................................
H8      .......T..........................................
H1      ..................................................
HT      ......................T.A.........................
        ******* ************* * ******************************************

                 730        740        750        760        770        780
        ....|....|....|....|....|....|....|....|....|....|....|....|....|
H11     CAATCCATTTCAAGAGCTCCTATCGACAAATGGGTTTACGACCTCGATGTTGGATCAGGG
H12     ..................................................
H16     ..................................................
H4      ..................C...............................
H9      ..................C...............................
H10     ..................C...............................
H13     ..................C...............................
H14     ..................C...............................
H3      ..................C...............................
H15     ..................C...............................
H2      ..................C...............................
H7      ..................C...............................
H5      ..................C...............................
H6      ..................C...............................
H8      ..................C...............................
H1      ..................C...............................
HT      ..................C...............................
        ****************** ***********************************************

                 790        800        810
        ....|....|....|....|....|....|...
H11     TACCCAAGTGGTGCAGCCGCTACTAATGGTTTG
H12     .................................
H16     .................................
H4      .................................
H9      .................................
H10     .................................
H13     .................................
H14     .................................
H3      .................................
H15     .................................
H2      .................................
H7      .................................
H5      .................................
H6      .................................
H8      .................................
H1      .................................
HT      ..T..............................
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BIOGRAPHY

Mr. Anusorn Pansook was born on March 9th, 1979 in Prachuap Khiri Khan province, Thailand. He received the Bachelor's degree of Science in biology in 2001 from the Department of Biology, Faculty of Science, Silpakorn University. He continued his study for mater degree in genetics at the Department of Botany, Faculty of Science, Chulalongkorn University and received the Master's degree of Science in genetics in 2005. At present, he is a Ph. D. candidate in the Ph. D. program in Biological Sciences at Chulalongkorn University.