รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาพันธุศาสตร์ประชากรของกบอกหนาม (*Paa fasciculispina*) สัตว์เฉพาะถิ่นในเขาสอยดาว, จังหวัดจันทบุรี

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

หัวหน้าโครงการขอขอบพระคุณ รศ.ดร. วราภรณ์ อัครปทุมวงศ์ และ ศ.ดร. วรเรณ บรอคเคลแมน แห่งสถาบันชีววิทยาโมเลกุล มหาวิทยาลัยมหิดล ศาลายา ที่เอื้อเฟื้อห้องปฏิบัติการให้หัวหน้าโครงการได้ทำงานวิจัยชิ้นนี้ได้สำเร็จ ขอขอบคุณหัวหน้าเขตรักษาพันธุ์สัตว์ป่าเขาสอยดาว จังหวัดจันทบุรี อินทนนท์ กลศาสตร์เสนี นักศึกษาปริญญาเอก ณัฐนรี ปิติมล นักศึกษาปริญญาโท มหาวิทยาลัยมหิดล ลุงวอนและคณะ สำหรับความช่วยเหลือในภาคสนาม ขอขอบคุณสมาชิกห้อง C212 สถาบันชีววิทยาโมเลกุล มหาวิทยาลัยมหิดล ศาลายา และ ทักษอร ภุมมะกสิกร นักศึกษาปริญญาโท มหาวิทยาลัยมหิดล สำหรับความช่วยเหลือในห้องปฏิบัติการ

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The spiny-breasted frog is an endemic species in Khao Soi Dao, Chantaburi Province. This population was previously recognized under the scientific name as *Paa fasciculispina* but later changed into *Quasipaa fasciculispina* due to its unique distribution and perhaps genetic identity. Due to its narrow range of distribution and current anthropogenic threats, the population of this species in Khao Soi Dao deserves urgent attention in conservation. Basic knowledge regarding population biology and genetics may help with the conservation planning. Field observation revealed relatively healthy population status and habitat quality. Population genetic study on this species was conducted using newly developed microsatellite DNA primers and other published primers in the literature. Although cross-species amplification was not successful, amplification using newly developed primers revealed considerate amount of genetic diversity of the population. Future investigation of other populations in nearby mountains may help better evaluate the status of this species under the pressure of urban expansion.

Keywords : Spiny-breasted frog, endemic species, genetic diversity, conservation
(คำหลัก)

Introduction

Many living organisms in the world currently are on the verge of extinction due to many factors, and one of them is due to anthropogenic activities. The most likely organisms to become extinct are usually endemic species that inhabit certain habitats or regions because they cannot be found anywhere else. Conservation and restoration of these species requires the comprehensive understanding of the nature of the species, especially reproduction, demography, and population structure. A lack of information on these matters in a species may lead to the mismanagements, especially the failure to recognize its existence and the misunderstanding about its distribution and persistence.

The spiny-breasted frog (*Qusipaa fasciculispina*) is endemic to the Cardamom region of southeastern Thailand and southwestern Cambodia (Inger 1970, Ohler et al. 2002). It has been found from 150 to 1,000 meters above sea level. This species generally inhabits headwater with strong current in lowland tropical dry forest. Because of its endemism, current habitat fragmentation and degradation exceedingly threatens the health and viability of the populations of this species (van Dijk and Swan 2004). Nothing regarding the life history of *Q. fasciculispina* is known. In addition, due to its vulnerable status, there is a great concern regarding the reduction in genetic diversity within species, which greatly influences its demography, persistence, and probability of extinction. The existence of the remaining populations of *Q. fasciculispina* as several small populations plays a significant role in the dynamics and persistence of this species. Small sizes of these populations are at higher risk of extinction than larger sizes as a result of genetic drift and inbreeding. Given the continued deforestation in the Cardamom region that creates unsuitable habitats, the understanding of the genetic structure of *Q. fasciculispina* deserves urgent attention.

Literature Review

- Taxonomy and phylogeny

The spiny-breasted frog in Khao Soi Dao, Chantaburi Province, has been recognized and described by Inger (1970). Its unique characteristic is the keratinized spines on the ventral size of the males. Since its first description, the scientific name of this species has been changed several times. The author first included this species in the family Ranidae, which is the true frog family, and under the genus *Rana*. Most of the frogs in this genus are distributed from Afghanistan-Pakistan to southern China and to the

Indochinese Mountains. When more specimens and more evidence became available, the classification of the family Ranidae did not reflect evolutionary relationships among species in the family. Therefore, the classification of this family was proposed, and the spiny-breasted frog in Khao Soi Dao is currently included in the family Dicroglossidae (tribe Paini), which are frogs known only in Asia (Frost 2011). Moreover, the scientific name of this species has been changed several times as the following.

Inger (1970) *Rana fasciculispina*

Dubois (1975) *Rana* → *Paa fasciculispina*

Dubois (1992) *Paa* → *Eripaa fasciculispina*

Chen et al. (2005) *Nanorana fasciculispina*

Frost et al. (2006) *Eripaa fasciculispina*

Ohler and Dubois (2006) *Quasipaa fasciculispina* (most recent and currently accepted name)

Frost (2011) *Quasipaa* → *Eripaa fasciculispina* (yet to be accepted but likely to be used as a new name of the spiny-breasted frog in Khao Soi Dao)

Name changing has been controversial for quite some time. When the molecular evidence becomes available and reliable, the genus *Rana* was no valid, and the new scientific name of the spiny-breasted frog from Khao Soi Dao was given as *Paa fasciculispina* (Dubois 1975). However, the genus *Paa* was again proven to be not monophyletic, which made the recognition of the species ambiguous (Chen et al. 2005; Jiang et al. 2005; Ohler and Dubois 2006; Che et al. 2009; Che et al. 2010). Although these studies provided very valuable information on the taxonomic validity of frogs in the tribe Paini, only two studies--Ohler and Dubois (2006) using morphological data and Che et al. (2010) using molecular data--included specimens of *Q. fasciculispina* in their analyses. These studies reveal that the taxonomic recognition of species in this group is also associated with their geographic distribution. The molecular evidence shows strong support of the hypothesis that the tribe Paini is monophyletic and can be divided into two monophyletic clades. One of the clade includes all species associated with the Himalaya-Tibetan region at high elevation (> 1,500 m above sea level). On the other hand, species in the other clade are found south-eastern of the Himalayans and at low elevation (< 1,500 above sea level). The southern-most population is the population of *Q. fasciculispina*. Such strong phylogenetic relationships of the tribe Paini are very useful to study the evolution and geology of the Himalayan and Indochina regions.

It is important to notice that the new taxonomic description is also associated with the geographic distribution of the organisms. Thus, this current scientific name and classification of the spiny-breasted frog in Khao Soi Dao may be valid based on current available evidence. With this validation of species identification, many studies regarding this species can be conducted without generating the confusion of mistakenly considering different species as the same species.

- Available biological information of this species

It is not surprising that not much information regarding the biology of *Q. fasciculispina* is available because of the difficulty to get access to its habitat. Its type locality is in Khao Soi Dao Wildlife Sanctuary, Chantaburi Province, Thailand (Inger 1970). Recently, Chuaynkern et al. (2011) have documented the current distribution of this species with the new record in the adjacent province (Trat Province) near the border between Thailand and Cambodia. Based on their survey, *Q. fasciculispina* is distributed in several small areas in the mountains in Chantaburi Province. Although these areas are protected by law, they appear to be fragmented and isolated from one another. The distribution of this species is very small (less than 20,000 km^2), and it is now listed by IUCN as vulnerable (van Dijk and Swan 2004).

Thorough understanding of the biology of this species has not been conducted yet. However, it is commonly known that this species is closely tied to aquatic habitat since many species of the tribe Paini are also found to be associated with aquatic habitats (Che et al. 2009; Ohler et al. 2002). They inhabit high gradient streams in the mountainous areas and perhaps are restricted to specific drainages.

- The study of population genetics using microsatallite DNA markers

A study of population genetics concerns about the dynamics of a population at molecular scales, which implies the microevolution of the population. Microevolution can be observed using molecular techniques to measure any changes, such as genetic diversity and other related parameters. Appropriate markers should be neutrally selected and showing adequate variation so that fine-scale measurement, namely at the population level, can be quantified. Markers such as functional nuclear genes or mitochondrial genes may not be appropriate since they are subjected to selection and do not show adequate variations at population scales. Allozymes are used to be employed in population genetic studies but no longer popular because they may be also be subjected to selections. Microsatellite DNAs are repetitive sequences in genome of an organism. This type of

sequences can be found in nuclear genome and does not appear to have any function hence not subjected to selections. Variations are mostly caused by slippage during replication. This type of markers is also Mendelian-inherited and can be easily scored using available software. Currently, microsatellite DNAs become popular for the studies of population genetics and other forensic studies since they are highly polymorphic. However, microsatellite DNAs are highly species-specific. Primers developed specifically using tissue of one species may not be applicable to other species even closely-related species. Therefore, the development of microsatellite DNAs for one species may be required and so costly for just one study.

Objectives

1. To collect information on the biology of the spiny-breasted frog (*Q. fasciculispina*) in Kao Soi Dao, Chantaburi Province, Thailand. The biological information included the examination of body size, sex ratio, habitat use, diet analysis, and any biological aspects that could be observed without too much disturbing and sacrificing individual frogs
2. To isolate microsatellite DNA primers as tools to study the genetics of *Q. fasciculispina* population in Khao Soi Dao
3. To determine genetic variation, bottleneck effect, and effective population size of this species

Methods

- Study site and DNA collection

This study site was Khao Soi Dao Wildlife Sanctuary in Chantaburi Province (Figure 1). The sampling locations were chosen because of their accessibility. Locations where the frogs were found and some habitat characteristics were observed and recorded. Buccal swabs of each individual frog were stored in 95% ethanol and brought to the laboratory in Bangkok. Gender and size of the captured frogs were also recorded.

- DNA extraction and isolation of microsatellite DNA primers

DNA extraction was conducted using QIAGEN tissue extraction kit. However, when the yield was low, phenol-chloroform extraction was employed to obtain optimal concentration. One of the samples with high DNA yield was used to develop microsatellite DNA primers following the modified protocol of Glenn and Schable (2005), and the screening of colonies with repetitive motif inserts was conducted as described in Kongrit et al. (2008).

Genomic DNA of the frog was cut using two restriction enzymes (Rsa I, Xmn I). Then, the DNA fragments were ligated to the SNX linkers and amplified by PCR. DNA fragments were hybridized to probes and captured the products with magnetic beads. The products were captured using magnetic particle concentrator, and washed. These enriched products were recovered by PCR. The products were ligated into plasmids and transformed to *Escherichia coli* competent cells. These cells were grown on LB agar plates containing ampicilin and X-gal. Colonies with insertion of enriched products appeared white and those without appeared blue. White colonies were selected and boiled for 10 minutes in TE buffer.

To select the colony containing microsatellite DNA, the boiled colony was used as a template in two PCR reactions—one using two primers (M13 forward and M13 reverse), and the other one using three primes (M13 forward, M13 reverse, and either oligo repeats $(AG)_{10}$ or oligo repeats $(GT)_{10}$). PCR reactions were carried out following the protocol in Kongrit et al. (2008). The amplified products were screened in a 1.5% agarose gel stained with ethidium bromide. Products from reactions using two primers (M13 forward and M13 reserve) appeared as single bands while those with three primers appeared as multiple bands with or without smear. If the size of the bands from three-primer PCR reactions equaled the size from two-primer PCR reactions, the insert, in this case, was not useful because it did not contain the microsatellite tandem repeat. On the other hand, if

the size of the bands from three-primer PCR reactions was smaller than the size from two-primer PCR reactions by approximately 200 base pairs (bp) but still larger than the size of the template with no insert, this colony was assumed to contain microsatellite DNA repeats with enough flanking regions to develop primers and selected and sequenced in an ABI PRISM 3730 automated DNA sequencer. The microsatellite DNA primer pairs were developed using PRIMER 3 (Rozen and Skaletsky 2000). Genetic polymorphism was screened using 30 samples of the spiny-breasted frog. Only microsatellite DNA primers that showed polymorphism were used for population genetic analyses.

- Population genetics

Selected microsatellite DNA primers were used to quantify genetic variation of all samples in addition to the 30 samples used to screen polymorphism in the previous process. The number of alleles per locus, expected heterozygosity, the deviation from Hardy-Weinberg equilibrium, the average gene diversity, and F-statistics were determined using Genepop and ARLEQUIN (Excoffier et al. 2005). Long-term population sustainable size was determined using the Heterozygosity-based method (Ohta and Kimura 1973). The model assumes that under mutation-drift equilibrium under the stepwise mutation model (SMM), N_e may be calculated as $[(1/1-H)^2-1]/8\mu$, where H is average heterozygosity across all loci examined, and $\mu = 5x10^{-4}$, which is the mutation rate applied in fish (Jarne and Lagoda 1996, Estoup and Angers 1998). This calculation provides the high estimate of N_e relative to other methods (Lippe et al. 2006).

Results

- Habitat Description

The spiny-breasted frogs were found in the head water streams in Khao Soi Dao. The areas were rich in many floras. Microhabitat within streams consisted of riffles and pools alternatively. Substrates consisted of large boulder, gravel, pebbles, and occasionally sand. Epiphytes were found covering these big stones. Average water temperature in the dry season was 24^{o}C. The water in streams was about 15-30 cm deep, but the depth of pool areas could be greater than 1.5 m. Water was very clear. Water current was moderately except after the rain the current became much stronger.

The frogs inhabited the stream banks and on boulders. Most of them were caught near the waterfalls. The frogs were difficult to see because their body color camouflaged with the background. Moreover, they were found at night from dusk to dawn feeding on noctural insects and animals. Because of this noctural behavior, catching these frogs became problematic for people who were expert.

- Population Biology

The population of *Q. fasciculispina* in Khao Soi Dao was abundant although the actual count was not conducted due to the difficulty of accessing the areas. Based on the survey, it was found that the sex ratio of the population was about 1:3 in favor of females. Size (head to anus length) ranged from 6.8 to 13.3 cm with the average of 10.98 cm. Caught individual males were slightly bigger than Caught individual females with the average size of the males were 11.07 cm and the average size of the females was 10.91 cm.

As moving closer to the waterfalls or in higher elevation, more individuals of the spiny-breasted frogs were found. The frogs were not aggregate but mostly solitary. They fed on noctural insects and animals, such as centipedes, and scorpions, which were found in the frogs' mouth during the collection of buccal tissue for genetic analyses.

- Population Genetics
  - Newly isolated microsatellite DNA primers

Over 1,000 colonies were screened, and only 294 colonies contained recombinant plasmids. Of all these 294 colonies, only 39 clones could be used to design primers. Eight primer pairs could be amplified and showed allelic polymorphism (Table 1).

Therefore, they were selected to screen genetic variation of the population of *Q. fasciculispina* in Khao Soi Dao.

Thirty DNA samples were used to screen genetic variation at each locus. The number of alleles per locus varies from 6 (PA004, PG177) to 29 alleles per locus (PG214) whiles other loci contain more than 10 alleles per locus. At each locus, some alleles were only observed once, especially loci with high numbers of alleles. The average number of alleles per locus was 15.6. Gene diversity at loci with high number of alleles showed higher values than loci with low number of alleles. The observed heterozygosity values at almost all studied loci were less than the expected heterozygosity values except at Locus PG177. The average heterozygosity across all loci was 0.8078. The F_{IS} of all loci showed slight heterozygote deficiency except at Locus PG177, which showed higher observed heterozygosity value than the expected heterozygosity value. Significant linkage disequilibrium was observed between the pair of Locus PA001 and PA237 ($P < 0.05$). Significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$) was detected at Locus PA001, PG169, PG177, and PG214. The estimate of long term sustainable size was 6,518 individuals.

- Cross-species amplification

1. Using primers of *Rana*

Seven pairs of microsatellite DNA primers of frogs from the genus *Rana* (Julian and King 2003; Richter and Broughton 2005) were randomly selected and amplified using tissue of 20 individuals of *Q. fasciculispina*. The results were shown in Table 2. Four microsatellite DNA loci failed to amplify while three of them were successfully amplified. Although the size of the microsatellite DNA fragments was comparable, the number of alleles found in *Q. fasciculispina* was fewer than those found in the species whose primers were developed using their tissue.

2. Using primers of *Paa*

Five pairs of microsatellite DNA primers of frogs from the genus *Paa* in which *Q. fasciculispina* was previously included were chosen. Loci with highest number of alleles per locus were preferable and selected. Results were shown in Table 3. Three loci failed to amplify while two of them were successfully amplified. Similar to the previous results, the number of alleles found in *Q. fasciculispina* was much fewer than those found in the species whose primers were developed using their tissue.

Discussion

- Habitat and population biology

Khao Soi Dao appeared to be in good condition. It rained all year even in the dry season although the effect of El Niño in 2010 reduced the amount of water in streams in Khao Soi Dao (personal communication). The decrease in amount of rainfall may have some effects on the population of *Q. fasciculispina*, and further investigation is necessary. Currently, the population status of the species in Khao Soi Dao appeared to be abundant. In addition to Khao Soi Dao, the most recent study on the distribution of this species revealed several other habitats of this species (Chuaynkern et al. 2011). However, the distribution appeared to be limited and likely to be threatened by land development (personal observation). Because of its endemism, the issue of protection of the population and its habitat should be raised.

- Population genetics

The spiny-breasted frog in Khao Soi Dao was previously included in the family Ranidae, which included many species of true frogs, and under the genus *Rana*. After taxonomic re-description, this species was classified in the family Dicroglossidae (tribe Paini), which are frogs known only in Asia (Frost 2011). However, there were many microsatellite DNA primers developed for species of the genus *Rana*, and cross-species amplification was worth a try. The amplification was not successful and those with successful amplification did not show acceptable results. In contrast, amplification using primers developed from tissue of the studied species revealed much satisfactory outcomes by showing considerable amount of variation at all eight studied loci. Some loci contained high number of alleles while some showed fewer. The number of alleles at each newly developed microsatellite DNA loci in this study was not unusual. The report from Zheng et al. (2009) showed comparable numbers from the frog in the genus *Paa*. Because of the fine-scale population study, microsatellite DNA loci with high number of alleles are better appropriate than those with low number of alleles; otherwise, diversity may not be determined.

Significant deviation from Hardy-Weinberg equilibrium was observed at four loci (PG001, PG169, PG177, PG214), which may be due to null alleles commonly found in microsatellite loci. However, Locus PG169 was the only locus showing higher value of observed heterozygosity than expected heterozygosity. This usual outcome may be responsible for the deviation from Hardy-Weinberg equilibrium unlike other loci. Potential

linkage disequilibrium was observed between Locus PA1 and PA237. This means that one of the two loci will be chosen for further analysis of population structure. However, it is also possible that the identification of alleles at these loci may be mistaken. As a result, it is better to re-examine the allele identification by performing additional amplification to confirm the results.

Long term sustainable population size of this population requires greater than 6,000 individuals. It has been suggested that Ne in wildlife populations is about 10% of the total population (Frankham 1995). However, another has suggested Ne to be 25-50% of the total population (Nunney and Campbell 1993, Nunney 1995). As a result, the ideal total population should be 60,000 individuals using the 10% ratio and 12,000-24,000 individuals using the 25-50% ratio. Unfortunately, the rough estimate of the existing population of the spiny-breasted frog in Khao Soi Dao could not be determined due to the difficulty in accessing the area. The given number in this study may provide a guideline of future population monitoring. At least, minimum number of population size can be set as a goal for sustainable population.

- Phylogenetic implication and conservation aspect

Taxonomic confusion of frogs in the tribe Paini has been problematic for quite some time. Phylogenetic studies clarify this confusion and confirm the validity of *Q. fasciculispina* (Che et al. 2010). On-going research regarding taxonomy is still conducting, and there is no need to be concerned about this matter. All studies come to the same conclusion about the endemism of frogs in this tribe and perhaps their genetic uniqueness. Under the current pressure of land development and urban expansion; together with climate change, there is a serious issue about extinction of endemic frogs. Because of their genetic uniqueness and narrow distribution range, the loss of habitats can seriously jeopardize the existence of the population of this frog.

Most research on frogs of the tribe Paini focuses on the phylogenetic and evolutionary aspects. Although one study (Zheng et al. 2009) attempted to step into the population genetic realm, further advance has been reported. One of their problems is the taxonomic confusion of the populations under the same species name that needs to be clarified. Furthermore, the study on population genetics is not popular if the target species are not listed as “endangered” or “threatened”. This is unfortunate because endemic species are one of the most vulnerable organisms to become extinct. Their distributions are very specific and in many cases narrow. The distribution of *Q.*

fasciculispina in Thailand is narrow and also fragmented (Chuaynkern et al. 2011). Therefore, it is at high risk of extinction and deserves urgent attention. While taxonomic clarification is necessary, the study at small scale can be conducted by aiming at conservation goals at the population level.

- Future research

Sampling of populations of frogs currently described as *Q. fasciculispina* in nearby mountains, especially Khao Kitchagoot and Khao Sa Bab, will help elucidate population structure of this species. Based on the study of Chuaynkern et al. (2011), distribution of *Q. fasciculispina* in Thailand appears to be fragmented and the presence of this species in some areas is questionable. Thus, additional sampling in the nearby mountains not only clarifies the existence of this species but also provides additional samples for population genetic study of this species. The population of this species in Khao Sa Bab is very interesting considering that this area is completely isolated from other mountains. It is possible that the population of this frog in this area may be subjected to genetic drift or related factors. With the use of microsatellite DNA primers developed from this study, the status of the population of *Q. fasciculispina* in Khao Sa Bab can be determined, and conservation issue may be raised.

In addition, *Q. fasciculispina* is the southern-most group of frogs in the tribe Paini and endemic to the Cardamom region. The sample of *Q. fasciculispina* in the phylogenetic studies came from the population in Cambodia. It is interesting to investigate the genetic difference between frogs currently described as *Q. fasciculispina* between populations in Cambodia and Thailand. This investigation can be easily conducted using the universal primers as listed in Che et al. (2010). It may be possible that the population of this frog in Thailand is different from that in Cambodia due to its high endemism as seen in *Q. spinosa*. This species previously described several populations in China but now are recognized as more than one species due to their genetic differences (Che et al. 2009). The current phylogenetic tree of frogs in the tribe Paini still includes several undescribed species. Additional information of the population of *Q. fasciculispina* in Thailand may fill in the blank of the evolution of this group of frogs.

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Figure 1 Map of the sampling site

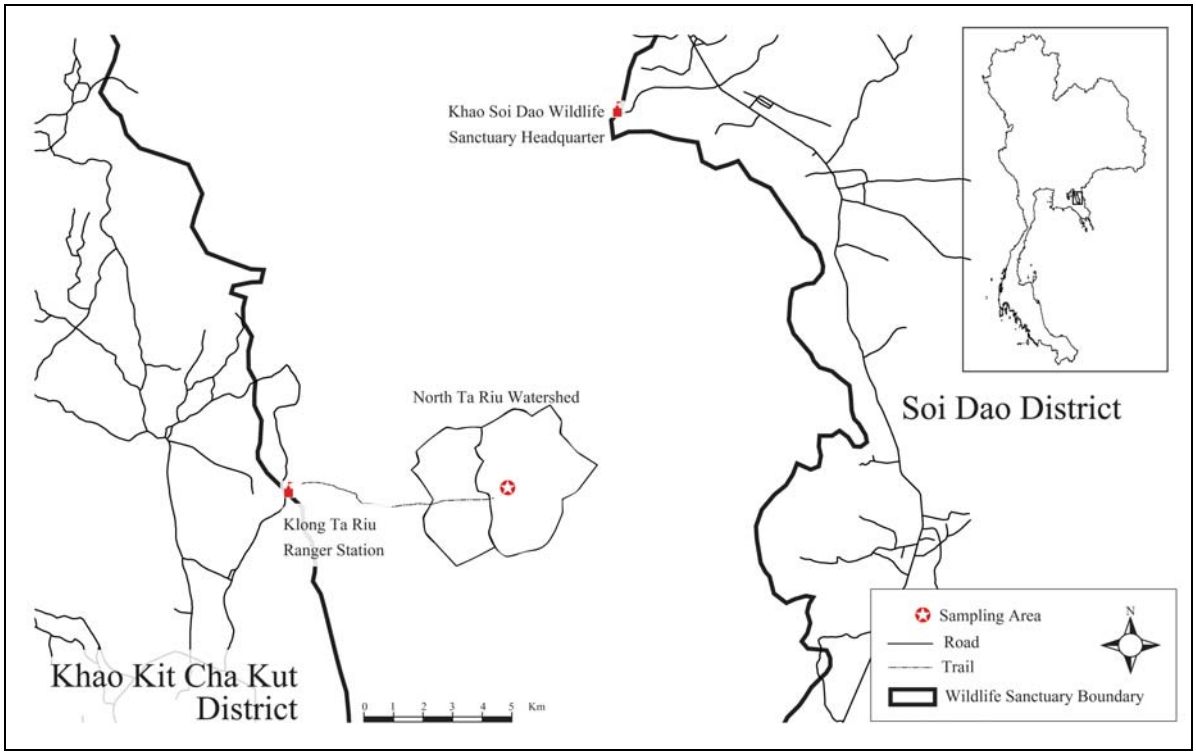

Table 1 Eight newly developed microsatellite DNA primers from *Q. fasciculispina* tissue

Locus	Repeat motif	Primer sequence (5' → 3')	Size ranges (bp)	Number of alleles	Observed heterozygosity	Expected heterozygosity	Gene diversity
PA001	$(TC)_{27}$	F: GGAAACAGCAGACAGGCTTC R: CCGCTCCTCCTTCTACTACC	204-248	18	0.8667	0.9288	0.930
PA004	$(GA)_7GG(GA)_5GG$ $(GA)_7$	F: TGCTGCTTTCTAGCGATTCA R: CCTCACTGACTGCCACATCA	229-245	6	0.5333	0.6684	0.671
PA115	$(CT)_{23}$	F: CAATTTGGGGGCATTATCTG R: ACTCAGGTGTGATGGTGCAA	191-229	12	0.8000	0.8723	0.874
PA126	$(GA)_{21}$	F: ATGCCTGTGGCTGTCTGAGT R: CAGCGTATGGCCAACCTAAT	171-251	19	0.8333	0.9299	0.932
PA237	$(GATA)_{11}GATCAA$ $(GA)_{25}(TAGA)_{14}$	F: GGTTGGAGATGGATGGATTG R: GAAGCATTTAGGACCCACCA	251-315	24	0.8621	0.9558	0.960
PG169	$(TC)_{11}TATACACACAA$ $(TATC)_{12}$	F: CTGCGTAAATTGTCGGCTCT R: TCTTGCATTGTGACCTTTGC	254-298	11	0.7333	0.8780	0.878
PG177	$(GT)_{21}$	F: ACAAAATCTGCAGGGGATCA R: GTGACAAACAGGCAAAGCAA	136-182	6	0.8667	0.6797	0.661
PG214	$(TG)_{26}$	F: CATGTTGCAAGAAGCCTTGA R: TGGGATGGAAAGGTAGTTGG	197-291	29	0.9667	0.9672	0.967

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Table 2 Microsatellite DNA primers developed from frog tissues from the genus *Rana*

Locus	**Repeat motif**	**Primer sequence (5' → 3')**	**Size ranges (bp)**	**Number of alleles (publication)**	**Number of alleles (found)**
RsyC11*	$(TACA)_9$	F: TTACTTTCAGTTTCAAAAGGCAG R: TACACAGTGCTTCACAAGTTCC	105-185	24	Failed to amplify
RsyC41*	$(TACA)_8$	F: GTCAAAACACAGATGCACAATC R: ACAAAACAGGAATCGGTCATAC	80-135	16	Failed to amplify
RsyD20*	$(TAGA)_{18}$	F: GTTACTGTGGAGGTGATGTCTG R: TTCTATATCAAGCACCCATCTG	200-280	23	Failed to amplify
RsyD32*	$(TAGA)_{17}$	F: GGACACACAATTCCTTGGTTC R: GAGGAGATTTCCAAAACAATCC	160-230	16	Failed to amplify
RsevG11†	$(TCTA)_n$	F: GTCTTCCATTACAAGGCTGC R: ACTTCTGACAGTCTAGTTAA	226-276	8	2
RsevE03†	$(GA)_n$	F: ATCTCGGCTTCACTGATTGC R: GCCTACTATGTAACTACTAT	276-304	6	4
RsevMs3†	$(CA)_n$	F: ATGTAAGCAATGCTTGTCC R: AAGGACATTGCCACTCAGGC	274-306	6	1

Citation *Julian and King (2003): sample size ranging from 102-112 individuals

†Richter and Broughton (2005): sample size = 46 individuals

Table 3 Microsatellite DNA primers developed from frog tissues from the genus *Paa*

Locus	**Repeat motif**	**Primer sequence (5' → 3')**	**Size ranges (bp)**	**Number of alleles (publication)**	**Number of alleles (found)**
Psp2	$(GT)_7T$ $(TG)_6$	F: AACAGTGAAAGAACCGAAAC R: CCCACAATGGAATGGACACG	142-178	15	Failed to amplify
Psp5	$(TG)_6CG$ $(TG)_3CG$ $(TG)_7$	F: TAAATAAACCCATGCGTAGG R: GGTAATTCCATCTTCCCAAA	186-229	19	1
Psp8	$(CA)_{12}$	F: TGCTTGGTAGTTTGCGATT R: CGTGACCGGAGTGATGTC	314-358	11	2
Psp11	$(GT)_{18}$	F: GGACAGGGTGAAGGCAGTAT R: CCTGTGAGGCAATATGAAAA	224-274	18	Failed to amplify
Psp14	$(TG)_{11}$	F: ATGGCTGGTGGAAAAAGACT R: TAGGAGGGGCAACGGAG	228-300	22	Failed to amplify

Citation Zheng et al. (2009)