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Genetic diversity of the grass frog (*Fejervarya limnocharis*, Gravenhorst, 1829) in northeastern Thailand using PCR-RFLP

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Abstract

The genetic diversity of the grass frog (*Fejervarya limnocharis*) in northeastern Thailand was studied by using PCR-RFLP technique. The total of 120 specimens, consisting of 106 females and 14 males, were collected from 12 provinces. Genomic DNA was extracted from Gastrocnemius muscle of specimens with CTAB-Phenol: Chloroform Proteinase K method. Appropriate primers were screened for PCR and COI primer was selected. The PCR product of COI primers showed 710 bp in all the samples. These PCR products were digested with *Alu* I, *Dde* I and *Taq* I restriction enzymes and found patterns of single haplotypes as 2, 1 and 1 respectively. The composite haplotypes were constructed from single haplotypes of 3 enzymes and showed all 2 patterns as AAA and BAA. The highest percentages of composite haplotypes patterns were 98.33 and 1.67 respectively. The UPGMA dendrogram of restriction haplotype was constructed using NTSYS PC version 2.1p. The polymorphism of *Alu* I, *Dde* I and *Taq* I digested COI products were evaluated and divided into 2 groups: group 1 divided clearly from the *F. limnocharis*, Group 2 consisted of all *F. limnocharis* which can be divided into 2 subgroups. The similarity coefficient between 2 subgroups exhibited approximately 77.5 percentages. From this study *F. limnocharis* of UD and MH showed clearly genetic difference, maybe a new species or not. In future study, we need to collect more samples in this area.

Keywords: grass frog, genetic diversity, COI, *Fejervarya limnocharis*, PCR-RFLP

1. Introduction

The grass frog *Fejervarya limnocharis*, Gravenhorst, 1829 [1], is previously known as *Rana limnocharis*. This frog is also known as rice frog, common pond frog, ricefield frog, paddy frog, Indian cricket frog and marsh frog (Figure 1). Grass frog is important to many peoples in northeastern Thailand. It can be food resource and make income. Recently regarded as belonging to *Fejervarya* [2] is represented by 45 species worldwide [3]. This species is often regarded as one of the most widely distributed species of Asian frogs. It can be found in every country of East, Southeast, and South Asia in a range that extends across western Japan, Taiwan, China, the Malay Peninsula, Bangladesh, Nepal, the Philippines, Indonesia, Sri Lanka, and India to Pakistan [4–6]. Being widely distributed, *F. limnnocharis* is an ideal subject for population genetics and phylogeographic investigations and the mechanisms or forces most likely to have been involved in shaping their population patterns [7]. Thailand has nine species [8], including *F. chiangmaiensis* [9], *F. andamanensis* [10], *F. cancrivora* [1], *F. limnocharis* [1], *F. moodiei* [11], *F. multistriata* [12], *F. orissaensis* [13], *F. triora* [14], and new species is *F. muangkanensis* [8]. Except for *F. andamanensis*, which belongs to the South Asian group, all other Thai species are assigned to the East and Southeast Asian group [15,9].

The *F. limnocharis* group to be identified should be called the *F. limnocharis* complex [16]. Furthermore, there are few morphological differences and few morphological characteristics usable for classification throughout this genus, not only for the *F. limnocharis*, and so it is difficult to correctly identify species. Therefore, in some cases, even a systematically and greatly different lineage might be included in the *F. limnocharis* [17]. However, differences in behaviour of organisms may be important on survival and adaptation to the wild [18]. The external characteristics (e.g. color, size) are influenced by habitat and the environment. Allopatric populations may show ecomorphological variations and a questionable species status [19–21].

2. Objectives

The objectives of this study were to determine the levels of genetic diversity of the *F. limnocharis* in northeastern Thailand, and to identify molecular genetic markers capable of facilitating the taxonomic identification of *F. limnocharis* by using restriction analysis of cytochrome oxidase subunit I (COI).

3. Materials and methods

3.1 Sample collection

One hundred and twenty grass frogs (*F. limnocharis*) were collected for this study. They

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Figure 1 Grass frog *Fejervarya limnocharis* [1] from northeastern Thailand

consisted of both male and female individuals from twelve different locations in northeastern Thailand (Figure 2). Identification of all frogs was done by referring to Nutpun [22] and Chan-ard [23]. Dorsal ground color in life varied from mid-dorsal line. The mid-dorsal line run from snout to the vent showed three-character states; (I) present as a broad line; (II) present as a narrow line; and (III) present as absent line. Their frequencies did not differ sexually (Figure 3) (Table 1).

3.2 DNA extraction

The total DNA of each frog was extracted from gastrocnemius muscle using a phenol-chloroform-proteinase K method [24]. Approximately, 10 mg of tissue was taken in a 1.5 ml microcentrifuge tube, to which CTAB and proteinase K were added. After homogenization, the tubes were incubated at 60 °C for 2.30–3 hours in a water bath. Then, an equal volume of phenol: chloroform: isoamyl alcohol (24: 25: 1) mixture was added to the lysis tissue in the tube. The contents were mixed gently and centrifuged at 13,000 rpm for 10 minutes. The top aqueous layer was then transferred to a new 1.5 ml microcentrifuge tube and added to an equal volume of chloroform: isoamyl alcohol (24:1). The tube was again centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to a new 1.5 ml microcentrifuge tube. The DNA was precipitated by the addition of an equal volume of cold absolute alcohol 100% and then stored at −20 °C. Thereafter centrifuge again 13,000 rpm 10 minutes then the solution was removed using micropipette. The pellet was then washed again of chilled 70% ethanol, air-dried and added of TE buffer. DNA concentration was determined spectrophotometrically and extracted DNA was stored at 4 °C until required.

3.3 PCR, restriction enzyme digestion, and agarose gel electrophoresis

The mitochondrial cytochrome oxidase subunit I (COI) gene segment of each grass frog (710 bp) was amplified using primers LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' [25]. Amplification was carried out in a 50 µl reaction volume containing 10x buffer, 1 mM of each dNTP, 50 mM $MgCl_2$, 0.5 µM of each primer, 1 unit of Taq DNA Polymerase, and DNA template.

PCR was performed in a thermocycler and consisted of predenaturation at 94 °C for 3 minutes followed by 10 cycles of denaturation at 94 °C for 1 minute, annealing at 54 °C for 1 minute and extension at 72 °C for 1 minute, and an additional 35 cycles at higher stringency using the same conditions except an annealing temperature of 54 °C. Final extension was carried out at 72 °C for 7 minutes. The PCR product five microliters of the amplified products were size-fractionated through 1.0% agarose gels using 0.5% TBE buffer to determine whether the expected fragment had been successfully amplified. A 100 bp DNA ladder was used as standard marker. Amplified DNA fragment was visualized under a UV transilluminator.

The PCR-RFLP analysis was approximately 200 ng of the amplified COI was separately digested with *Alu* I, *Dde* I, and *Taq* I. The digestion reaction mixture 15 µl comprised buffer, BSA, spermidine, enzyme and PCR product. The tubes were incubated at 37 °C for 3 hours (Except for *Taq* I were incubated at 65 °C for 4 hours). The digests were electrophoretically analyzed using 2.0% agarose gels with 1% TBE buffer and then visualized under a UV transilluminator.

3.4 Data analysis

Restriction profiles of COI were alphabetically coded in order of appearance. Each frog was assigned a three letter code (that of COI digested with *Alu* I, *Dde* I and *Taq* I, respectively) to describe the composite haplotypes. The UPGMA dendrogram of restriction haplotype was constructed using NTSYS PC version 2.1p. This research used *Microhyla pulchra* as an out group.

4. Results and discussion

4.1 PCR-RFLP

In this study, the mtDNA was amplified using the primers COI. The result of primer screening found that COI primers were appropriate for PCR-RFLP of the grass frogs. PCR RFLP was inexpensive, fast and non-invasive method for DNA based methods [26]. COI primers were universal primers for DNA barcoding amphibians [27]. The PCR product of COI primers in all samples of *F. limnocharis* and out group showed 710 bp (Figure 4). The complete mtDNA sequence of *F. limnocharis* is 17,717 bp in length containing 13 protein-coding genes, 2 rRNA genes and 23 tRNAs genes (including an extra copy of tRNAMet), and noncoding regions (including the control region) [28].

The COIs of the grass frogs (*F. limnocharis*) and the out group (*M. pulchra*) were subjected to restriction analysis using *Alu* I, *Dde* I, and *Taq* I and generated 3, 2, and 2 digestion profiles, respectively. Figure 5 showed PCR-RFLP pattern of amplified PCR products cleaved by *Alu* I (a), *Dde* I (b), and *Taq* I (c) from *F. limnocharis* form I, II and III. In *F.limnocharis* I, II and III digestion profiles of *Dde* I and *Taq* I were found

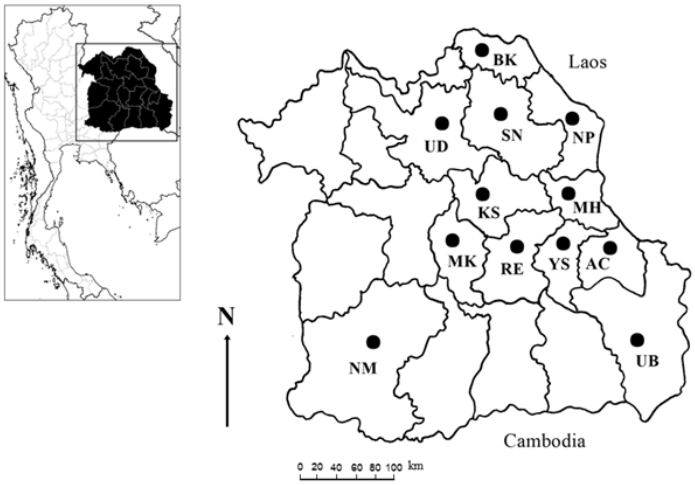

Figure 2 Map of northeastern Thailand indicating the grass frog (*F. limnocharis*) sample collection sites. Dots represent geographic locations from which at least one grass frog was collected.

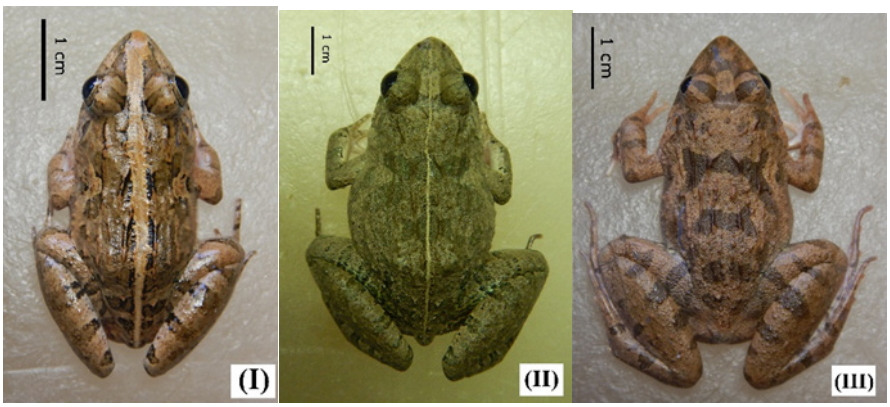

Figure 3 Three-character states, (I) a broad line, (II) a narrow line and (III) absent line

Table 1 Collecting localities of specimens used in this study

	Species	Localities	Abbrev*	Total (*N*)	I (%)	II (%)	III (%)
ingroup	*F. limnocharis*	Kalasin	FlKS	13	3(23.07)	2(15.40)	8(61.53)
		Nakhon Phanom	FlNP	10	1(10.00)	5(50.00)	4(40.00)
		Nakhon Ratchasima	FlNM	7	3(42.85)	3(42.85)	1(14.30)
		Bueng Kan	FlBK	3	1(33.33)	1(33.33)	1(33.33)
		Mukdahan	FlMH	10	1(10.00)	2(20.00)	7(70.00)
		Maha Sarakham	FlMK	5	1(20.00)	3(60.00)	1(20.00)
		Yasothon	FlYS	10	1(10.00)	7(70.00)	2(20.00)
		Roi Et	FlRE	17	1(5.90)	12(70.58)	4(23.52)
		Sakon Nakhon	FlSN	15	-	4(26.67)	11(73.33)
		Amnat Charoen	FlAC	12	3(25.00)	3(25.00)	6(50.00)
		Udon Thani	FlUD	9	4(44.44)	5(55.56)	-
		Ubon Ratchathani	FlUB	9	1(11.11)	1(11.11)	7(77.78)
			Total	120	20(16.67)	48(40.00)	52(43.33)
outgroup	*M. pulchra*	Nakhon Ratchasima	MpNM				

* Species names (Fl, Mp) are followed by two capital letters to illustrate names of localities.
* I = present as a broad line, II = present as a narrow line, III = absent line

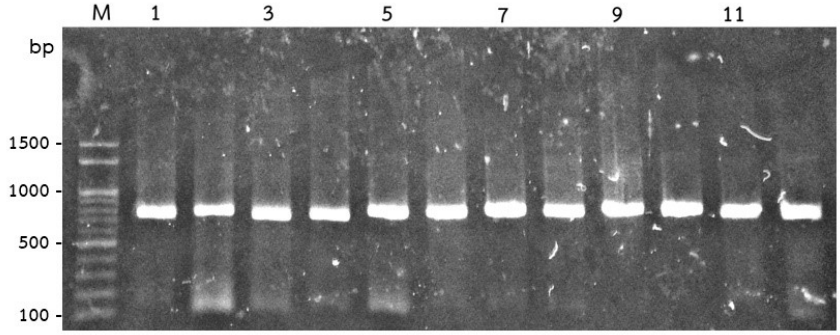

Figure 4 PCR products of frogs using COI primers. lane M = 100 bp DNA ladder, lanes 1-11 = *F. limnocharis*, lane 12 = out group (*M. pulchra*)

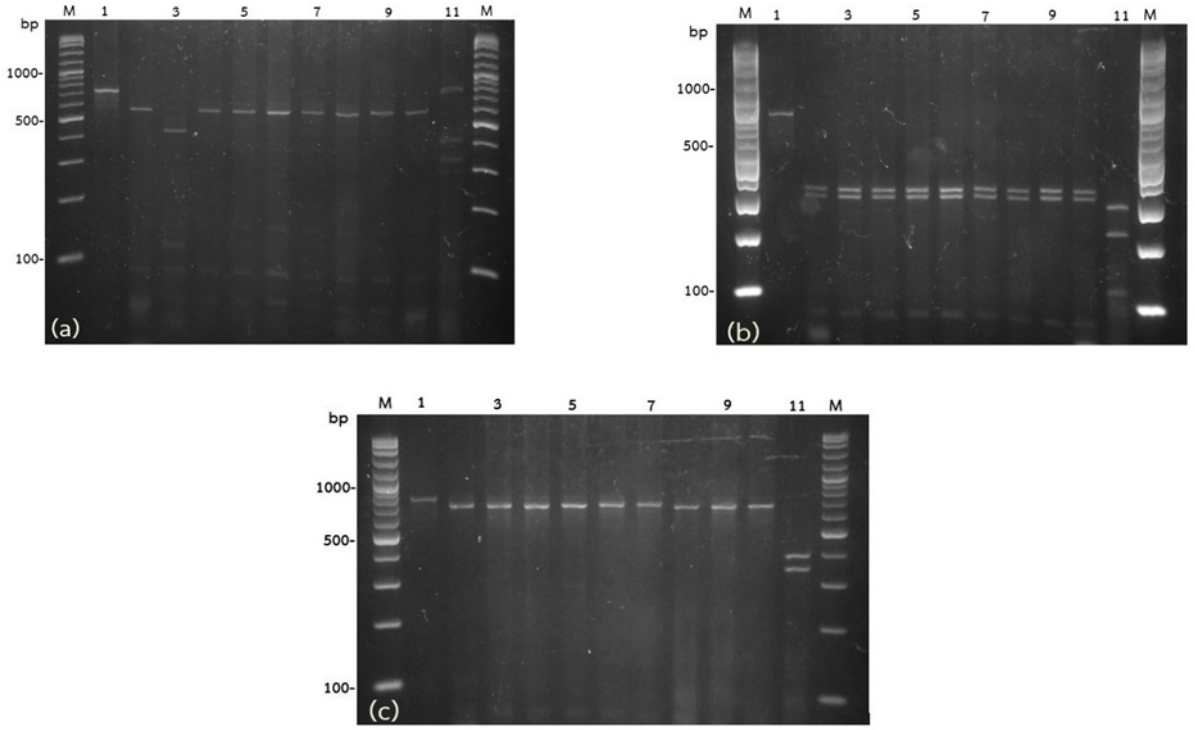

Figure 5 PCR-RFLP patterns of COI gene in frog digested with three restrictions enzyme: *Alu* I (a), *Dde* I (b) and *Taq* I (c) (lane M =100 bp DNA ladder, lane 1=uncut, lanes 2-10 = *F. limnocharis* and lane 11 = *M. pulchra*

only one single haplotype but *Alu* I was found 2 single haplotype so single restriction enzyme as *Alu* I was capable of distinguishing all *F.limnocharis* form I, II and III by providing different DNA fragment sizes. A single haplotype of *Alu* I pattern observed (A) had 3 fragments bands include 580, 90, 40 bp sizes and pattern observed (B) had 4 fragments bands include 450, 130, 90, 40 bp sizes (Figure 5 a). On the other hand, restriction pattern of *Dde* I had 3 fragments bands include 330, 310, 70 bp sizes (Figure 5 b) and restriction pattern of *Taq* I had 1 fragment band include 680 bp sizes (Figure 5 c).

The COI amplified products digested with *Alu* I showed percentages of restriction patterns (A) as 100, 97.91 and 98.07 in *F. limnocharis* form I, II and III, respectively. Thus, *Alu* I digested COI provided a species-specific RFLP profile (B) for only *F. limnocharis* II and III as 2.08 and 1.92 percentages, respectively. However, digestion profiles of *Dde* I and *Taq* I were not different in all *F. limnocharis* samples, but they were different clearly from out group (Table 2).

In total, 3 composite haplotypes of *F. limnocharis* were found AAA and BAA as 98.33 and 1.67 percentages, respectively and composite haplotypes of out group (*M. pulchra*) was CBB as 100 percentages (Table 3). No composite haplotypes were shared among species.

Composite haplotypes AAA was found in *F. limnocharis* form I, II and III and BAA was found in *F. limnocharis* form II and III. Composite haplotypes BAA as 1.67 percentages as 2 samples of *F. limnocharis* consisting *F. limnocharis* II from

Table 2 Obtained size of digested DNA fragments of grass frog and out group upon digestion with three restriction enzymes, *Alu* I, *Dde* I and *Taq* I.

Enzyme	Pattern observed (bp)	*F. limnocharis* complex			Out group
		I (%)	II (%)	III (%)	(%)
Alu I	A: 580, 90, 40	+(100)	+(97.91)	+(98.07)	-
	B: 450, 130, 90, 40	-	+(2.08)	+(1.92)	-
	C: 410, 300	-	-	-	+(100)
Dde I	A: 330, 310, 70	+(100)	+(100)	+(100)	-
	B: 290, 240, 140	-	-	-	+(100)
Taq I	A: 680	+(100)	+(100)	+(100)	-
	B: 400, 350	-	-	-	+(100)

*+, found in investigated species and in parenthesis represent percentages.

Table 3 Molecular taxonomic key for identification of *F. limnocharis* complex based on composite haplotypes of COI of *F. imnocharis* complex digested with *Alu* I, *Dde* I and *Dde* I.

Species	Composite haplotypes
Fejervarya limnocharis complex	AAA (98.33 %)
Fejervarya limnocharis complex (FlaMH3, FlnUD3)	BAA (1.67 %)
Microhyla pulchra (MpNM)	CBB (100 %)

Udon Thani province and *F. limnocharis* III from Mukdahan province so these samples had more difference genetic than the other places, they maybe new species or not. In this study the grass frog from Mukdahan and Udon Thani are interested in genetic variation. Thus, we need to collect more samples in this area for further study.

The polymorphism of *Alu* I, *Dde* I and *Taq* I digested COI products were evaluated and divided into 2 groups: group 1 was MpNM (*Microhyla pulchra*) that divided clearly from the *F. limnocharis*. Group 2 consisted of all *F. limnocharis* which can be divided into 2 subgroups: subgroup 1 consisted FlbKS, FlnKS, FlaKS, FlbNP, FlnNP, FlaNP, FlbNM, FlnNM, FlaNM, FlbBK, FlnBK, FlaBK, FlbMH, FlnMH, FlaMH, FlbMK, FlnMK, FlaMK, FlbYS, FlnYS, FlaYS, FlbRE, FlnRE, FlaRE, FlbSN, FlnSN, FlbAC, FlnAC, FlaAC, FlbUD, FlnUD, FlbUB, FlnUB and FlaUB. Subgroup 2 consisted FlaMH and FlnUD. The similarity coefficient between 2 subgroups exhibited approximately 77.5 percentages (Figure 6).

Amphibians are very sensitive to environmental and climatic changes, and thus the genetic diversity of their population can provide us useful information for tracking historical environmental variation [5].

As a common, widely distributed species, the grass frog exhibits extensive diversity in body size, skin color, and mid-dorsal line pattern. Investigation of the species' genetic diversity at the population level should thus be very interesting. F. limnocharis is generally abundant in human habitation and this may increase the opportunity for the frog to be transported accidentally or deliberately into new areas [6].

In the present investigation, we collected samples from northeastern Thailand. The body length of these brown frogs generally ranges from 42 to 65 mm, and their skin color varied from dark brown to gray to olive gray, with dark markings and scattered longitudinal skin folds. A dark V-shaped spot located between the eyes and several dark, vertical stripes are clearly visible along the edge of both lips, and in some specimens a bright longitudinal stripe stretches along the midline of the back. Variation in these characters is closely associated with the ecological niche of the frogs; however, no morphological traits obviously correlated with sampling localities were observed in this investigation.

Recent molecular phylogenetic studies indicate that the genus *Fejervarya* is divided into two main groups: The *F. limnocharis* complex group distributed in East and Southeast Asia group and South Asia group [9].

5. Conclusions

Molecular genetic evidence from this study indicated that two group of the *F. limnocharis* consisting group 1 were 98.33 percentage and group 2 were 1.67 percentage. Group 2 include *F. limnocharis* from Udon Thani and Mukdahan province both had similar morphological in all samples but genetic different. From the result showed similarity coefficient between 2 subgroups exhibited circa 77.5 percentages. Although the digestion of COI with 3 restriction enzymes was sufficient for species identification, more restriction enzymes and more samples would be needed to provide more accurate estimates of genetic diversity in these taxa.

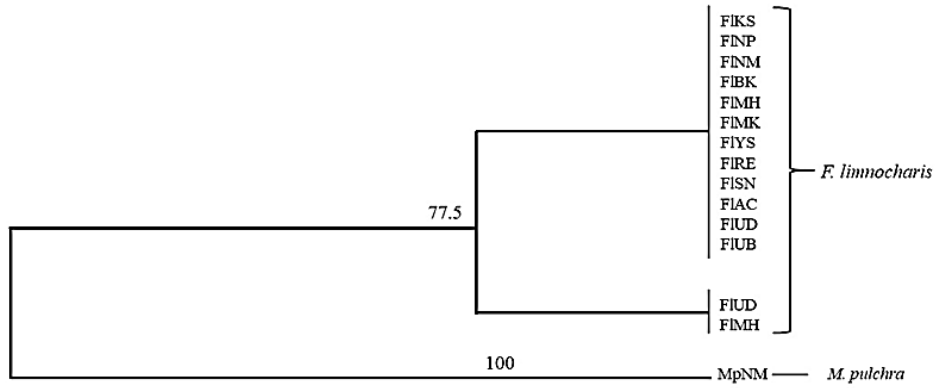

Figure 6 UPGMA dendrogram showing the genetic relationships of COI gene in *F. limnocharis* and *M. pulchra* after *Alu* I, *Dde* I and *Taq* I digestion analyzed by NTSYSpc version 2.10p.

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