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Genetic Structure and Diversity of the Giant Frog (*Limnonectes blythii*) in Northern Thailand

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Abstract: The aim of this study is to analyse genetic diversity, structure and differentiation of the giant frogs (*Limnonectes blythii*). One hundred and sixty four individuals from 4 populations in Mae Hong Son Province, Thailand were used for the analysis of genetic polymorphism at 7 microsatellite loci. The collection showed considerable polymorphism with observed number of alleles per locus ranging for seven different loci, with an average of 3.4 alleles per locus. Mean genetic diversity of the four populations with moderate level, but in populations with lower genetic diversity. Furthermore, the NJ tree approach clustering confirmed the results of PAM is more differentiated than the others. The significant levels of genetic structure among the sites were found in which could be resulting from isolation by distance rather than a position relative to habitat. The results of this study indicate that genetic structure could be useful for evaluation of neutral genetic variation particularly as the basis for inferring population and species capacity for species conservation and management decisions.

Keywords: Genetic diversity, genetic relationship, heterozygosities, Thailand

INTRODUCTION

The Giant frog, *Limnonectes blythii*, is widely found in mountain streams in South-East Asia, from Viet Nam and Laos, Thailand, the Malay Peninsula, Singapore, Sumatra and the Anambas Islands and the Natuna Islands (Indonesia). In Thailand, the distribution of this frog species ranges from the Tanao Sri Tak, Mae Hong Son, Kanchanaburi and Yala provinces (Taylor, 1962). Populations from Mae Hong Son province are considered to be Near Threatened (NT) (IUCN, 2010) as a vulnerable animal according wildlife protection group. These natural frogs rapidly decrease because of habitat destruction and human disturbance. Nevertheless, basic information regarding patterns of genetic diversity of these populations is unknown, making it difficult to adopt scientifically guided management measures to ensure their conservation. Among vertebrates, amphibians' genetic differentiation across small geographic scales can be either low or high (Driscoll, 1998; Burrowes and Joglar, 1999; Storfer, 1999; James and Moritz, 2000; Shaffer *et al*., 2000). Additionally, dispersal has been shown to be an important factor in amphibian local population survival by increasing overall population size and allowing populations to reestablish (Gill, 1978). The objective of this investigation is to evaluate levels of genetic diversity in populations of giant frogs in Mae Hong Son province and to study their genetic structure using microsatellite DNA. The result is discussed with particular reference to the conservation of giant frogs in there and development of effective management of their populations.

MATERIALS AND METHODS

Sample collection and DNA extraction: During October 2009 to May 2010 tissue samples of 164 individuals were collected from four populations located in Mae Hong Son province and the geographical location of the populations is shown in listed in Table 1 and Fig. 1. Tissue samples were obtained from either toe clips in adults or tail tips in larvae and stored in 95% ethanol until DNA extraction. All frogs were immediately released at the place of capture. Prior to DNA extraction, tissues were digested in homogenizing solution with proteinase-K incubated over night at 60°C (Bruford *et al*., 1992; Jiwyam *et al*., 2005). DNA was then extracted following a standard phenol-chloroform procedure (Sambrook *et al*., 1989).

Microsatellite analysis: Microsatellite analyzes were performed using the following seven polymorphic loci:

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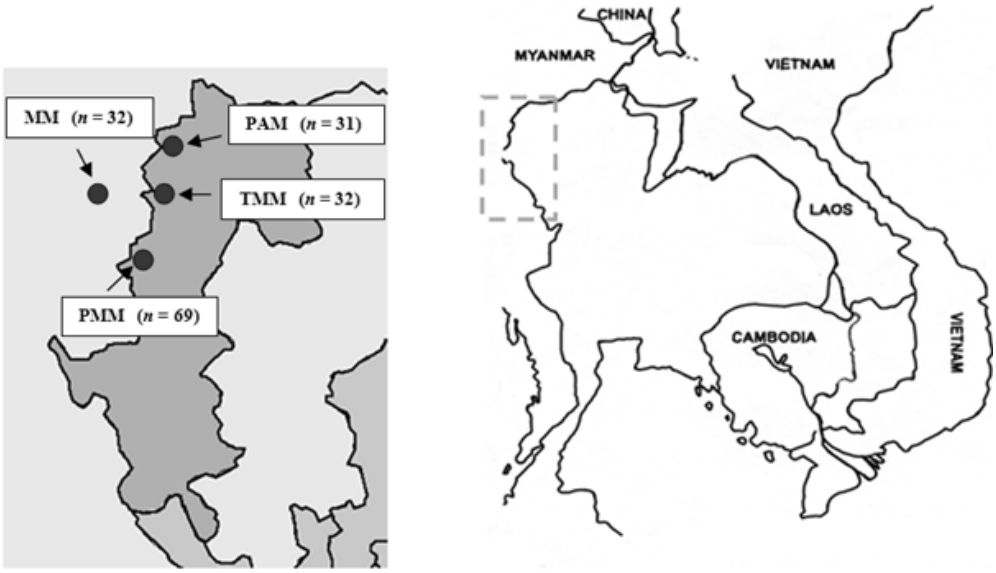


Fig. 1: Sample sites of the giant frog (*L. blythii*) in different regions in Mae Hong Son Province, Thailand and Myanmar

Table 1: Sample code, sample size and the collecting sites of giant frogs in this study

Area code	Locality	Latitude, longitude	Sample size
TMM	Thai-Myanmar border	19º 22′ N, 97º 52′ E	32
PMM	Mae Hong Son inland fisheries station	18º 44′ N, 97º 52′ E	69
MM	Myanmar	19º 16′ N, 97º 38′ E	32
PAM	Pang Aung	19º 29′ N, 97º 53′ E	31

AF257481, AF257482, AF257478, AF297972, AF297975, D78590 and X64324. PCR amplification was conducted containing the following components: 50 ng of genomic DNA, 1X PCR buffer, 0.2 μM of each forward and reversed primers, 0.2 μM dNTP, 5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Vivantis, Malaysia) and ddH_2O to a final volume of 25 μL. DNA amplification was performed with a predenaturation at 94ºC for 5 min followed by 35 cycles for 30 s at 94ºC, 30 s at annealing Temperature (*Ta*) 55-60ºC, 30 s at 72ºC and final extension at 72ºC for 5 min. The PCR products were size fractionated through 6% polyacrylamide gel electophoresis. The sizes of microsatellite alleles were determined by comparing with 100 bp DNA markers (Fermentas, USA).

Data analyses: Genetic diversity within the four populations was measured using the following parameters: the observed number of alleles (*na*), effective allele number (*ne*), observed (H_O) and expected (H_E) heterozygosities and deviations from Hardy-Weinberg Equilibrium (HWE) were independently calculated for each locus by Arlequin, version 3.1 and GenAlEx6 software (Peakall and Smouse, 2006). Multivariated relationships among individual frogs were examined by Principal Coordinate Analysis (PCoA) employing GenAlEx. The distance matrix of F_{st} was then used to generate an unrooted Neighbour-Joining (NJ) tree employing MEGA4 (Tamura *et al*., 2007).

The population genetic structure was determined using STRUCTURE 2.3 (Pritchard *et al*., 2000) and assigned individuals to inferred population clusters based on multilocus genotypes. Four independent runs of K = 1-4, 10 run were performed at 200,000 Markov Chain Monte Carlo (MCMC) repetitions and a 100,000 burn-in period. The optimum number of clusters was determined by evaluating the values of K as the highest mean ln Pr(X|K) (Pritchard *et al*., 2000) and DK (Evanno *et al*., 2005). Each cluster identified in the initial STRUCTURE run was analysed separately using the same settings to identify potential within-cluster structure (Evanno *et al*., 2005).

RESULTS

Genetic diversity: Variation at seven microsatellite loci was examined in 164 individuals from four populations of giant frogs and showed moderate polymorphism with observed number of allele per locus (*na*) ranging from 2 to 4 with an average of 3.357 per locus. Mean observed and expected heterozygosities ranging from 0.429 (±0.049) to 0.762 (±0.046) and 0.429 (±0.045) to 0.609 (±0.013), respectively and other parameters of genetic diversity for the four populations are presented in Table 2. The Hardy-Weinberg equilibrium show that the Pang Aung population was not included in the Hardy-Weinberg equilibrium and there for was a mixture

Table 2: Genetic diversity and differentiation at 7 microsatellite loci

Allele	Locality	TMM (n = 32)	PMM (n = 69)	MM (n = 32)	PAM (n = 31)
Rtemμ4	na	2.000	3.000	2.000	3.000
	ne	1.969	2.458	1.983	1.879
	H_O	0.875	0.826	0.719	0.613
	H_E	0.492	0.593	0.496	0.468
	F	-0.778	-0.393	-0.450	-0.310
RECALQ	na	4.000	4.000	3.000	3.000
	ne	1.514	2.230	2.024	1.476
	H_O	0.406	0.739	0.531	0.323
	H_E	0.339	0.552	0.506	0.323
	F	-0.197	-0.340	-0.050	0.000
RRD590	na	3.000	3.000	3.000	4.000
	ne	2.860	2.843	2.677	3.008
	H_O	0.844	0.870	0.781	0.484
	H_E	0.650	0.648	0.626	0.668
	F	-0.297	-0.341	-0.247	0.275
Rt-U4	na	4.000	4.000	4.000	4.000
	ne	2.149	2.545	3.230	1.544
	H_O	0.844	0.522	0.594	0.290
	H_E	0.535	0.607	0.690	0.352
	F	-0.578	0.141	0.140	0.176
Rt-U7	na	4.000	4.000	4.000	4.000
	ne	2.723	2.803	2.373	1.857
	H_O	0.781	0.754	0.750	0.581
	H_E	0.633	0.643	0.579	0.461
	F	-0.235	-0.172	-0.296	-0.258
Rt-SB14	na	3.000	3.000	3.000	3.000
	ne	1.990	2.715	2.062	1.614
	H_O	0.719	0.884	0.750	0.355
	H_E	0.498	0.632	0.515	0.380
	F	-0.445	-0.400	-0.456	0.067
Rtemμ1	na	3.000	3.000	3.000	4.000
	ne	2.550	2.433	2.304	1.543
	H_O	0.688	0.739	0.719	0.355
	H_E	0.608	0.589	0.566	0.352
	F	-0.131	-0.255	-0.270	-0.009
Mean±SE	na	3.286±0.286	3.429±0.202	3.143±0.261	3.571±0.202
	ne	2.251±0.182	2.576±0.084	2.379±0.169	1.846±0.203
	H_O	0.737±0.061	0.762±0.046	0.692±0.035	0.429±0.049
	H_E	0.536±0.041	0.609±0.013	0.568±0.027	0.429±0.045
	F	-0.380±0.088	-0.251±0.07	-0.233±0.081	-0.008±0.081

H_O: Observed heterozygosity; H_E: Expected heterozygosity; na: Observed number of alleles; ne: Effective number of alleles; F: Fixation index; n: Sample size; SE: Standard error of the mean

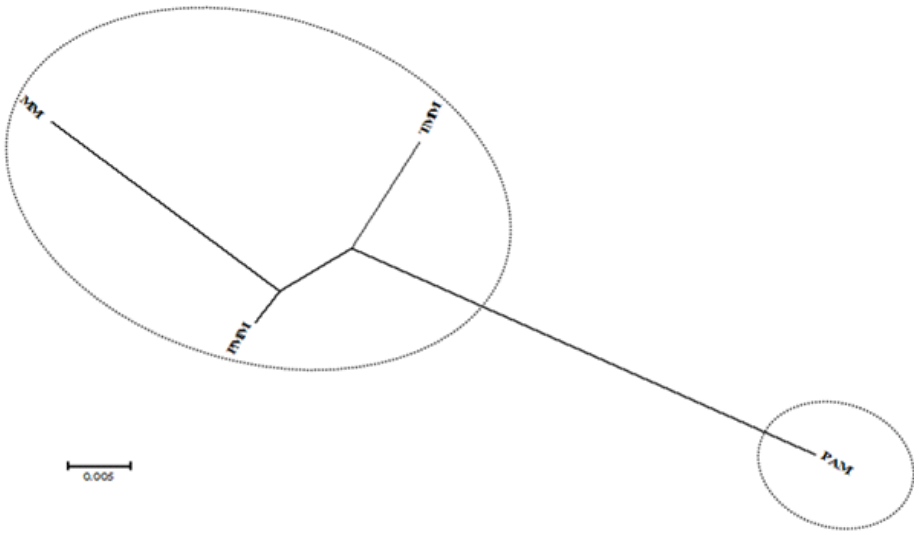


Fig. 2: Neighbour-joining tree of *L. blythii* populations based on nei'unbias distance

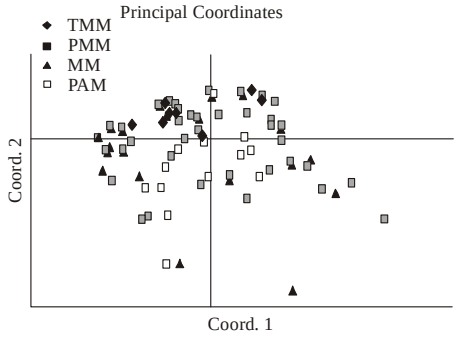
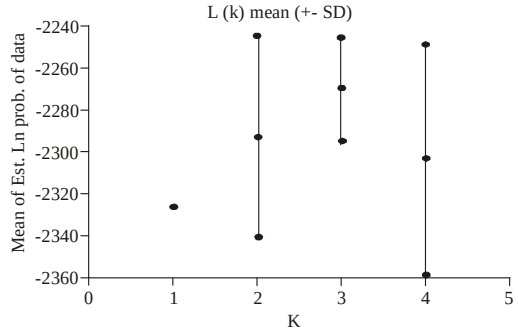
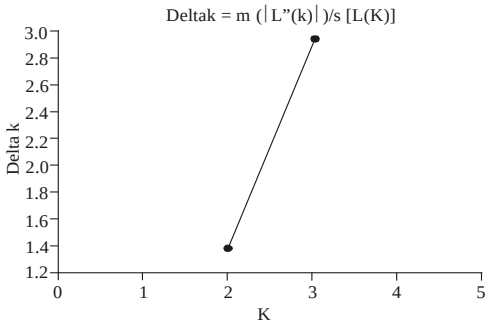


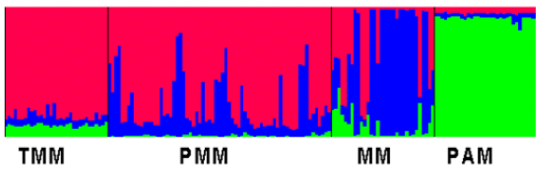
Fig. 3: Principal Coordinates Analysis (PCoA) of genetic variation in 7 microsatellite loci of genetic structuring among all 164 individuals in 4 populations



(a)



(b)



(c)

Fig. 4: STRUCTURE analysis results: (a) Value of ln P(D) from four independent runs for K = 1-4. (b) Value of delta K as a function of K based on ten runs. (c) Distribution of the four genetic clusters generated by STRUCTURE 2.3

from other populations. This might be caused by many factors. For instance, the transportation of the giant frog populations from one place to another can lead to accidental release. This might cause the genetic characteristics of the giant frogs in each place to be mixed. Other factors are the destruction of giant frogs habitat and subsequent mixture among combined populations within its group or the as well as to experience inbreeding. In this case, it might cause a decrease of genetic diversity in of giant frog populations.

Microsatellite analysis of all loci was parsimoniously informative. The unrooted Neighbour-Joining (NJ) tree analyses revealed a phylogeny which was consistent with two genetic groups of giant frogs species (MM, PMM and TMM populations) and (PAM population) (Fig. 2). The Principal Component Analysis (PCoA) of the microsatellite data showed that multivariated relationships among frog individuals were examined by (PCoA) (Fig. 3).

Genetic structure: Analysis of the genetic structure of the complete data set used the program STRUCTURE 2.3, following Pritchard *et al*. (2000) and the most appropriate value of K given for our data was 3. Using the L(K) and delta K method of Evanno *et al*. (2005) our data was best represented by K = 3. Regardless of whether a K of 2 or 3 is chosen as the most appropriate value of K (Fig. 4), a general pattern was observed that individuals in the MM, PMM and TMM populations tended to be similar and somewhat different from those in PAM population, yet this pattern is more apparent in the K = 3 plot.

DISCUSSION

Despite the large biodiversity of giant frogs in which many of them are importance amphibians wildlife of Thailand, there are a few studies of population genetics using microsatellites. Giant frogs are moderately variable in the seven microsatellite loci. Within-population genetic diversity is similar to or greater than that found for microsatellite loci in several other amphibians (Brede and Beebee, 2004; Martinez-Solano *et al*., 2005; Arens *et al*., 2006; Beauclerc *et al*., 2010). The presence of moderate heterozygosity in the other loci suggests a possible reduction in the population size of giant frogs in our study. Considering a significant departure from equilibrium based on the differences in standardised test outcomes under the assumption that all loci fit the equilibrium model was detected in the MM, TMM and PMM populations. Our findings revealed genetic differences between giant frogs populations of northern Thailand, as was expected because of currently differences of habitats and geographic limited isolation among them. Wild populations of giant frogs are subjected to continuous habitat alteration. The structure analysis was congruent between the DK and the ln(K)

estimators used to assess the number of independent genetic clusters. The increase of standard deviation of ln(K) at K = 3 (Fig. 4) seems to support our interpretation that each of the four populations represent a local population agrees with Evanno *et al*. (2005). The recovery of giant frog populations depends on two main factors. The first measure is to conserve or reverse habitat and environmental degradation so that natural recovery of wild populations of giant frogs can happen. The second is to promote stock enhancement giant frogs populations, locally in where populations are extremely reduced, as is the case in Mae Hong Son province, Thailand (E. Jalernsiriwongthna, unpublished data). To implement such a measure, the genetic diversity found among different populations of giant frogs should be taken into account to establish conservation strategies. The genetic variability can be maintained by keeping these populations in captivity under appropriate management aimed at reducing the risks of genetic regression.

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