

Evaluation of Hatchery Stock of Giant Frog (*Limnonectes blythii*) in Thailand

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Abstract: The aims of this work were to study the relationship between temperature and hatching rate and to evaluate genetic diversity of giant frog (*Limnonectes blythii*) as parental stock in Mae Hong Son Inland Fisheries Station, northern Thailand. The results showed that the optimal temperature for development of giant frog hatching ranged from 19 to 27°C, based on total hatch rate, viability of newly hatched larvae post-hatch, and total mortality rate of eggs. Moreover, from November to February, the greatest percentage of hatching was in January, with 40%. The outcome of determining the genetic diversity of parental generation by using seven microsatellite loci was moderately high levels.

Key words: Genetic diversity, giant frog, morphology, relationship

INTRODUCTION

Southeast Asia, one of the world regions with species richness distribution of amphibians is now facing threats on species extinction. Giant frog *Limnonectes blythii*, has a wide range in South-East Asia from Vietnam, Laos, Thailand, Peninsular Singapore, Malaysia, Sumatra, Anambas and Natuna Islands (Indonesia). Giant frogs in Thailand are distributed in and around high mountain streams. In Thailand, they were found in the Tanao Sri Range, Tak, Mae Hong Son, Kanchanaburi and Yala Provinces. The populations in Mae Hong Son province are considered to be Near Threatened (NT) (IUCN, 2010) as a vulnerable animal according to the Wildlife Protection Department of Thailand. In recent years natural populations of the *L. blythii* in this region, have been heavily impacted by habitat destruction, and human consumption. To ease the problem restocking has been widely practiced for conservation and enhancing production of the species. Despite the ecologic and economic importance of *L. blythii* in Thailand, to date little is known not only about impacts of this practice but also genetic diversity of the species. Thus, it is difficult to adopt scientifically guided management measures to ensure their conservation. We observed the hatch production rate in females of *L. blythii* in the cool season breeding period to examine the effects of temperature on the survival of tadpoles, and spawning of females were examined in this study. As the temperature is a variable known to affect the rate of egg development in many

amphibian species, the relationship between the temperature and the egg incubation period was also studied to predict the hatching date of tadpoles as can be useful for mass production of *L. blythii* for reintroduction in many areas of northern Thailand. Evaluation of both biological and physical of stocks and understanding levels of genetic variability of *L. blythii* provide information on the range of the genetic resources available and may allow improvement on management to sustain the hatchery stocks. As this information is one of the keys for the management and conservation of this species, this work then focus on the finding of the relationship between temperature and hatching rate and genetic diversity of giant frog (*L. blythii*).

MATERIALS AND METHODS

Study site: During November 2010 to February 2011, males and females of giant frog were captured from Mae Hong Son province for parent stock (Fig. 1). Tissue samples of 69 individuals (44 tadpoles and 25 adults) for genetic analysis from Mae Hong Son Inland Fisheries Station populations, Thailand were collected at (18°44' N and 97°52' E), tissue samples were obtained from either toe clips in adults or tail tips in larvae, and stored in 95% ethanol until DNA extraction.

Reproductive analyses: Data were monthly collected from parent stock including, Total Length (TL) and body Weight (W) which measured to the nearest 0.1 cm and 0



Fig. 1: Stages in the development of hatchery stock from inland fisheries station (Mae Hong son province). A: Simulated habitat; B: Parent stock (male); C: Collecting eggs; D: Water flow for the eggs; E: Hatched eggs frog; F: Nursery ponds for tadpoles

0.01 g, respectively. The length-weight relationships were estimated from the allometric formula: $W = aL^b$, where W is total body weight (g), L the total length (cm), a and b are the coefficients of the functional regression between W and L (Ricker, 1973). The relationships between air temperature and amount of eggs, water temperature and amount of eggs, and percent hatching each month were determined using statically analysis.

Genetic analyses: Tissue samples were digested in a homogenising solution with proteinase-K and incubated overnight at 60°C (Jiwam *et al.*, 2005; Bruford *et al.*, 1992). DNA was then extracted following a standard phenol-chloroform protocol (Sambrook *et al.*, 1989). Microsatellite analyses were performed using the following seven polymorphic loci: AF257481, AF257482, AF257478, AF297972, AF297975, D78590 and X64324. PCR amplification was performed according to the following protocol: 25 µL, DNA amplification was performed with a predenaturation at 94°C for 5 min followed by 35 cycles for 30 s at 94°C, 30 s at annealing temperature (*Ta*) 55 to 60°C, 30 s at 72°C and final extension at 72°C for 5 min. Genetic diversity within the hatchery stock was measured following the parameters of Schneider *et al.* (2000) and was independently calculated for each locus by Arlequin, version 3.1.

RESULTS

Reproductive study: The length-weight relationships of males and females during the study period were determined using ordinary least square regression with 95% confidence. The linear relationship between length and weight is shown in Fig. 2 and the result show that are

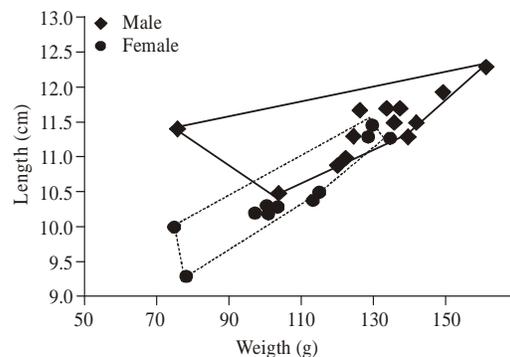


Fig. 2: Relationships between total length and weight in giant frog samples of the Mae Hong Son inland fisheries station of the *Limnonectes blythii* from Thailand. Males (square) and females (circle)

larger than females of difference morphology between length and weight (Fig. 2).

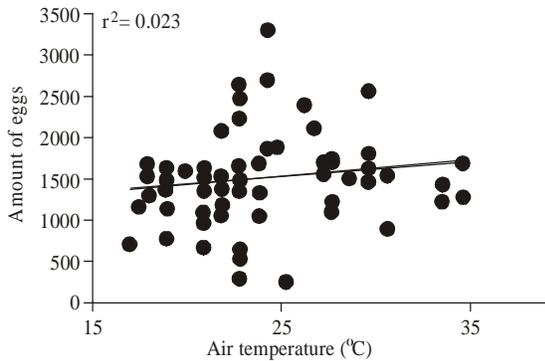
The results show that there was a lower relationship in the survival of giant frog eggs at air and water temperatures of 15 to 35°C this suggests that egg incubation at or below this temperature range is not for this species. Although there was no significant difference in total hatch rate and total mortality rate among the three high temperatures (°C), trend towards lower total hatch rate and higher total mortality rate existed at the warmer temperature of 27°C (Fig. 3). Therefore, the optimal temperature for incubating obscure puffer eggs is about 19 to 27°C according to the results of total amount eggs and hatch rate.

Genetic diversity: The mean number of alleles (*Na*) (±SE) at seven microsatellite loci in the Mae Hong Son

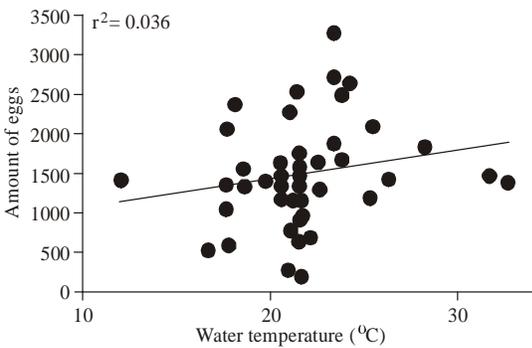
Table 1: Levels of genetic diversity of seven microsatellite loci

Loci (n = 69)	Rtempμ4	RECALQ	RRD590	Rt-U4	Rt-U7	Rt-SB14	Rtempμ1	Mean±SE
Na	3.0	4.0	3.0	4.0	4.0	3.0	3.0	3.42±0.20
Ne	2.458	2.230	2.843	2.545	2.803	2.715	2.433	2.57±0.08
H _O	0.826	0.739	0.870	0.522	0.754	0.884	0.739	0.76±0.04
H _E	0.593	0.552	0.648	0.607	0.643	0.632	0.589	0.60±0.01

N: Sample size; Na: Observed number of alleles; Ne: Effective number of alleles; H_O: Observed heterozygosity; H_E: Expected heterozygosity; ±SE: Standard error of mean



(a)



(b)

Fig. 3: Graphs showing the air temperature and the amount of eggs (A), water temperature and the amount of eggs (B) in hatchery stocks of Thailand (N = 72)

Inland Fisheries Station populations is 3.42 (±0.20) and the effective number of alleles (Ne) is 2.576 (±0.08). The average observed heterozygosity (H_O) and expected heterozygosity (H_E) values are 0.76 (±0.04) and 0.609 (±0.01), respectively (Table 1).

DISCUSSION AND CONCLUSION

Interactions in the physical environment can have profound impacts in amphibians. For example, in the terrestrially breeding frog *Cophixalus ornatus*, Brooke *et al.* (2000) showed that such interactions may be important as a trigger in chorus initiation. The relationship between air and water temperature and the amount eggs produced by female giant frog in our study was not

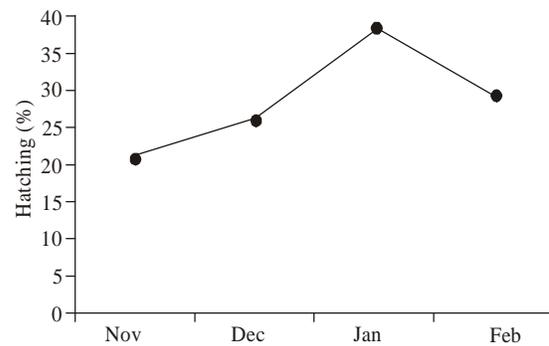


Fig. 4: Percent hatching (%) in four months of mating success of *L. blythii* in hatchery stocks

significant for the amount of eggs produced. This study is consistent with the findings of Wong *et al.* (2004). This studied the interaction of temperature and environment affecting call rate in a frog (*Crinia signifera*). Their results show that the effect of water temperature on call rate was not significant for frogs calling in duets or groups. Our study found giant frogs spawn in Thailand between the end of November and end of February each year. The results show that in the January the maximum hatching rate (40%) was observed. (Fig. 4). For our future studies various factors that affect the hatching rate and foods for giant frog tadpoles are envisioned. The assessment of genetic diversity showed a moderately high level. We are concerned about the genetic diversity of other populations which require conservation. Loss of genetic diversity due to environmental changes, habitat degradation and anthropogenic activities adversely affects the structure of natural populations. Differences in genetic diversity and genetic structure of parent stock should be taken into account when planning conservation strategies for giant frog populations.

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