

CHAPTER 2

REVIEW OF THE LITERATURE

Biology and Ecology of *Limnonectes*

The *Limnonectes* (Fitzinger, 1843) inhabiting tropical and subtropical Asia, are unusual among anurans in that the normal sexual size dimorphism is reversed and males are larger than females (Inger, 1966; Emerson and Berrigan, 1993). Males *Limnonectes* also have an unusual suite of secondary sexual characteristics: odontoid processes (fangs) in the lower jaw, enlarged heads and forelimb flexors, an absence of developed nuptial pads, vocal sacs, and advertisement calls (Inger, 1966; Emerson and Berrigan, 1993).

The evolutionary processes of unusual sexually dimorphic features in rapid *Limnonectes* have been studied by Emerson (Emerson, 1994, 1996, 2001; Emerson and Berrigan, 1993; Emerson and Ward, 1998; Emerson *et al.*, 2000). The evolution of large male body size in *Limnonectes* involves proximate and ultimate factors. For a proximate cause, the large male body size could be the result of the enlargement of the male head (Pope, 1931); a growth pattern of head size in sexually matured individuals is positive allometry for males but isometry for females (Emerson, 1994; Matsui, 1979; Tsuji and Matsui, 2002).

An ultimate factor is more likely sexual selection rather than natural selection for ecological divergence between the sexes, because in *L. leporina* (as *Rana blythii*), a *Limnonectes* from Borneo, there is no strong support for intraspecific dietary divergence (Emerson and Voris, 1992). Additionally, the evolution of large male body size in the *Limnonectes* might have resulted from intrasexual selection (male-male competition) rather than intersexual selection (female choice), As can be seen in *L. kuhlii* from Taiwan and *L. blythi* from Vietnam, male-male agonistic behavior for breeding territory has been reported (Orlov, 1997; Tsuji and Matsui, 2002). To confirm the contribution of intrasexual selection to large male body size in the *Limnonectes*, selective pressure on male body size should be assessed (i.e., correlation between body size and reproductive success for males).

However, the reproductive ecology and mating behaviour of male *Limnonectes* have never been described in detail (Emerson, 1992; Emerson and Inger, 1992; Orlov, 1997; Pope, 1931; Tsuji and Matsui, 2002), and male mating success has never been studied in any species of *Limnonectes*.

Biogeography Distribution of *Limnonectes*

The *Limnonectes* of Asia are a moderately species-rich group of 58 described taxa distributed across much of Southeast Asia (AmphibiaWeb, 2012; Che *et al.*, 2009). Species of the genus *Limnonectes* have been recorded from as far west as India and China, through the Malaysian Peninsula and the Sunda Shelf Islands of Indonesia, the Philippines, and as far east as the Indonesian islands of the Malukus, the Lesser Sundas, and Papua New Guinea (Daudin, 1802; Duellman, 1993; Frost, 1985; Inger, 1999; Inger and Tan, 1996).

Many cryptic species of amphibian and reptile have been identified worldwide and widely distributed of species complexes are commonly discussed in geographically and morphological taxonomic summaries (Evans *et al.*, 2003; Inger, 1999; Iskandar and Colijn, 2000). In Southeast Asia geographical history and species groups have emphasised the complex geological history of the region (Hall, 1996, 1998; Brown *et al.*, 2008) as a potential generator of amphibian and reptile species diversity in the Southeast Asia, many studies have highlighted the impact that the formation of Pleistocene Aggregate Island complexes (PAICs) may have had on the evolutionary process of speciation.

The few available molecular phylogenetic studies of *Limnonectes* suggest that numerous cryptic species may exist (Emerson, 1996; Emerson *et al.*, 2000; Evans *et al.*, 2003), especially in “widespread” species (complexes) like the *L. kuhlii* and *L. blythii* groups; however, taxonomists have been reluctant to describe these taxa on the basis of molecular sequence data alone and revisionary studies have lagged far behind molecular work. Nevertheless, some of the undescribed species are morphologically distinct and readily diagnosable on the basis of morphological characters. The *Limnonectes* of Northern Thailand (Figure 1 to 4) are a moderately species-rich group of four species distributed across much of Northern Thailand (IUCN downloaded July 2012) follows:

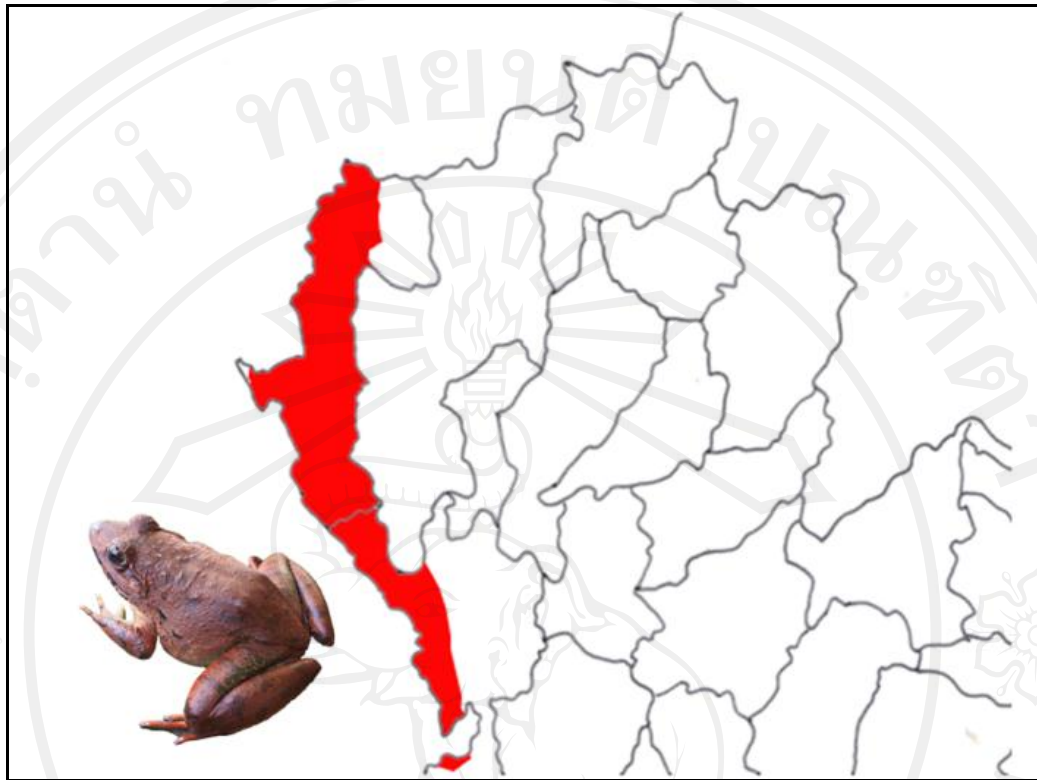


Figure 1 Map showing distribution of *L. blythii* (Boulenger, 1920) in northern Thailand.

Description of Giant frog (*Limnonectes blythii*) (Figure 1)

Range Description: In adults, male are significantly larger than females in SVL (mean = 114.83 mm vs. 97.71 mm). This species ranges widely in Southeast Asia, from Viet Nam and the Lao People Democratic Republic, to Thailand (from Salawin, Mae Hong Son to West Thailand) and Peninsular Malaysia, Singapore and Sumatra, the Anambas Islands and the Natuna Islands (Indonesia). It is also present on the islands of Phuket, Langkawi, Penang and Tioman. There is no record from the Cardamom Mountains in Cambodia, though it has been found at heights up to 1200 m above sea level.

Habitat and Ecology: It inhabits streams with gravel and boulders in primary and secondary evergreen forest. Males build a nesting hollow in a sandy streambed area, and the larvae develop in streams.

Population: The population status of this species is locally variable, ranging from uncommon in Western Thailand to very common in areas where harvesting does not occur in Peninsular Malaysia. It is generally less common in easily accessible areas near human habitation than in similar inaccessible areas, indicative of depleted populations due to harvesting. Cessation of harvesting leads to population recovery in 5 to 10 years.

Population Trend: Decreasing

Major Threats: The major threat to this species is intensive collection for consumption at subsistence levels, and for local, national and international trade. Some populations have also probably been eliminated locally due to forest clearance.

Conservation Actions: This species inhabits a great number of protected areas throughout much of its range, and is protected by the Wild Animals Reservation and Protection Act (WARPA), 1992, in Thailand. There is a need to ensure that the harvesting of this species from the wild is managed in a sustainable manner (this species could benefit from commercial farming). Clarification of the taxonomy of the *blythii* complex is necessary to identify cryptic species of conservation concern.

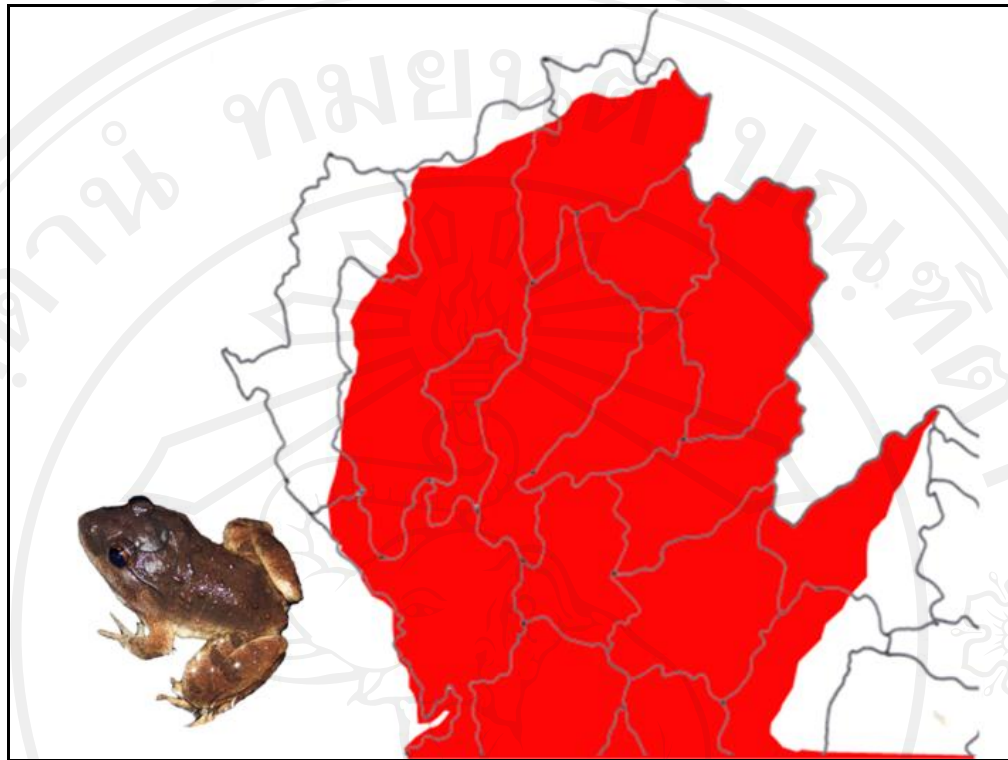


Figure 2 Map showing distribution of *L. gyldenstolpei* (Andersson, 1916) in northern Thailand.

Description of Capped frog (*L. gyldenstolpei*) (Figure 2)

Range Description: In adults, male are significantly larger than females in SVL (mean = 58.41 mm vs. 47.42 mm). This species is known from most of mainland Thailand and Cambodia, and from northern Lao People's Democratic Republic (Taylor, 1962; Stuart, 1999). It has been recorded at altitudes ranging from 200 (Inger and Colwell, 1977) to 800 m asl, although Taylor (1962) recorded the species at 2,200 m above sea level.

Habitat and Ecology: It inhabits small streams in evergreen or dense deciduous forest. It breeds in streams by larval development.

Population: It is generally a relatively common species in appropriate habitat.

Population Trend: Decreasing

Major Threats: The main threats involve forest degradation processes, which open up the canopy and interfere with the hydrological cycle, for example agricultural encroachment, logging and fires.

Conservation Actions: It is known or suspected to inhabit most protected areas, in mainland Thailand and adjacent Lao People's Democratic Republic and Cambodia, which contain streams in evergreen or mixed deciduous forest in hill or mountain areas.

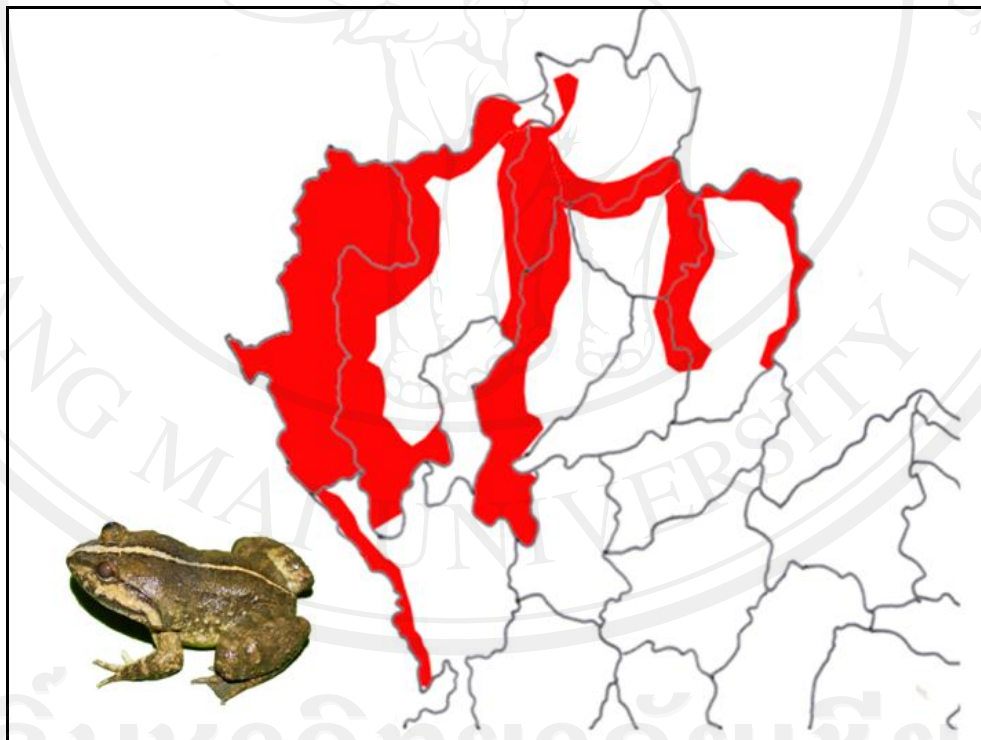


Figure 3 Map showing distribution of *L. taylori* (Matsui, Panha, Khonsue, and Kuraishi, 2010) in northern Thailand.

Description of Taylor's Stream Frog (*L. taylori*) (Figure 3)

Range Description: In adults, male are significantly larger than females in SVL (mean = 66.0 mm vs. 51.9 mm). This species ranges widely in Mae Hong Son, Chiang Mai, Lampang, Chiang Rai Provinces, and Northern Thailand.

Habitat and Ecology: It is found in small, clear streams in evergreen forest in hilly areas. It breeds in small tributary streams and the larvae develop in quiet pools along these streams. It does not wander from the vicinity of streams.

Population: It is a common and locally abundant frog.

Population Trend: Decreasing

Major Threats: The major threat to this species is deforestation, and although the species complex as a whole is not at risk, some of its component cryptic taxa might be threatened by local threatening processes. It is locally collected for consumption.

Conservation Actions: It occurs in many protected areas, and the priority is to safeguard these. Clarification of its taxonomic status and studies of its conservation biology and harvest levels are needed.

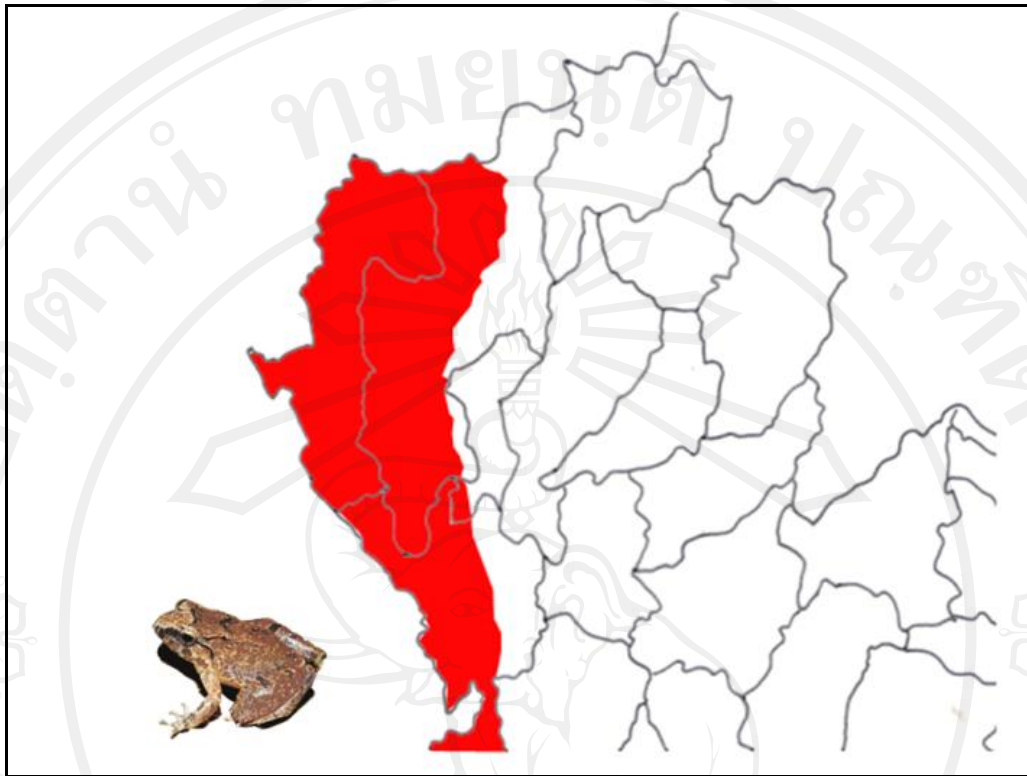


Figure 4 Map showing distribution of *L. limborgi* (Sclater, 1892) in northern Thailand.

Description of Limborg's frog (*L. limborgi*) (Figure 4)

Range Description: In adults, male are significantly larger than females in SVL (mean = 33.0 mm vs. 31.7 mm). This species ranges widely in Northern and Western Thailand.

Habitat and Ecology: Data deficient

Population Trend: Decreasing

Major Threats: The major threat to this species is deforestation and being consumed.

Conservation Actions: It occurs in many protected areas, and the priority is to safeguard these. Clarification of its taxonomic status and studies of its conservation biology and harvest levels are needed.

Systematic of *Limnonectes*

Most living frogs across the world belonged to the family Dicroglossidae. Presumed to have had an African origin sometime in the early Tertiary, Dicroglossidae currently show areas of high species richness in both Southeast Asia and Africa. A special interest was drawn to the Southeast Asian species group as they occur throughout the Malay Archipelago a region with a well-studied and particularly complex biogeographic history. Nonetheless, few studies have examined phylogenetic relationships among these frogs. Among the Southeast Asian Dicroglossidae is a cluster of species variously characterised by unusual, derived, sexually dimorphic features, including fangs (out-growths of the lower jaw bone) (Figure 5), male voicelessness, males being larger than females, and male parental care. Many of these taxa show great phenotypic similarity (Kiew, 1978, 1984; Inger, 1954, 1958, 1966) and even recognising individual species has proven to be a challenge (e.g., Emerson and Ward, 1998).

Dubois (1987) placed the fanged frogs and their relatives in one tribe, two genera, and five subgenera, five years later (Dubois, 1992) redistributing them into three tribes, four genera, and three subgenera but without any type of systematic analysis (Inger, 1996). Emerson and Berrigan (1993), using a morphological data set (40 characters) performed a preliminary cladistic analysis on 18 species. The results of that study (1) confirmed that the fanged frogs were a monophyletic group and (2)

recognised at least two main species groups within the clade. The morphological data set could not resolve the majority of species relationships within the clade.

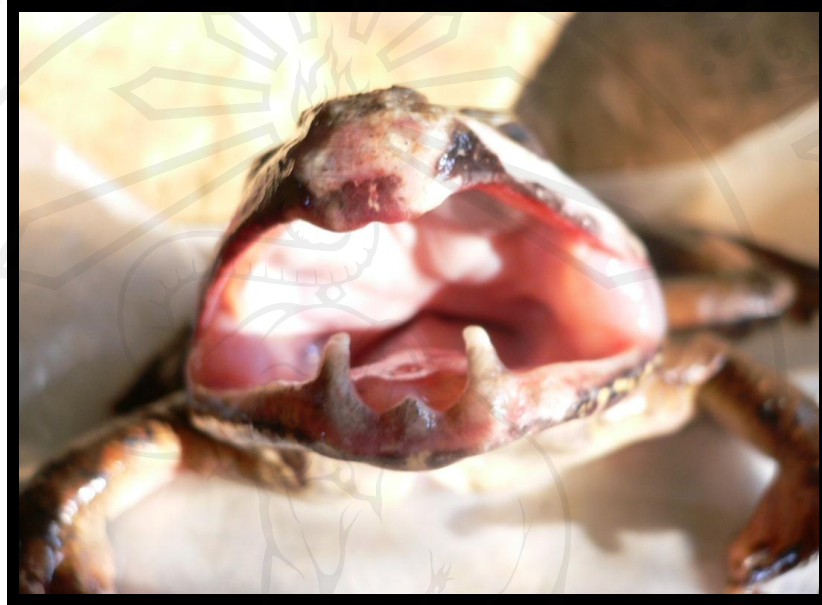


Figure 5 Out-growths of the lower jaw bone is one of a specific characteristic frog of family Dicroglossidae.

Recent developments in the field of molecular biology have a huge impact on various areas of biology particular in population genetics, evolutionary biology, and phylogenetic systematic. At present many further technological innovations, for example, automated sequencers have significantly simplified the technical procedures; sequencing DNA is now a standard and routine method. For the first time it became possible to analyse any genomic DNA of amphibians and reptiles without the requirement of isolating large amounts of ultra-pure genomic DNA or to DNA cloning segments in amphibians and reptiles. Dramatic advances in program of computational technology have allowed processing large datasets and applying the

complex mathematical algorithms and sophisticated statistical procedures that are needed to analyse phylogenetic relationships and Biogeography of amphibians and reptiles (Nei and Kumar, 2000; Arbogast *et al.*, 2002).

Breakthrough of molecular genetics has also had an enormous impact on the systematic of recent amphibians. Initial molecular investigations in the 1950s and 1960s were concerned with aspects of population genetics and genetic polymorphisms within and among species. Pioneer work was carried out by Guttman and collaborators on serum protein polymorphisms in toads of the genus *Bufo* (reviewed by Guttman 1973). In the following decades genetic markers, mostly mitochondrial DNA and nuclear DNA, were successfully applied in population genetics for the reconstruction of genetic and phylogenetic relationships of closely related species, for the estimation of divergence times in the context of historical geography, and for simple taxonomic purposes, e.g., to detect and differentiate species that are morphology indistinguishable (e.g., Hotz and Uzzell, 1982; Nishioka *et al.*, 1987; Nishioka *et al.*, 1992).

In biology, the study of nucleotide sequences has becoming increasingly popular in amphibian and reptile systematics since the early 1990s. For other animal groups, systematists almost exclusively used nuclear DNA and mitochondrial DNA sequences. One of the early, more comprehensive studies is that of Hedges and Maxson (1993), who studied the relationships among higher-ranked amphibian and reptile groups ("family") on the basis of a relatively short fragment (333 bp) of the 12S ribosomal RNA (rRNA) gene in mitochondrial DNA.

After a few years, Hay *et al.* (1995) employed a dataset of about 1300 bp of mitochondrial DNA from amphibian species representing 28 of the 40 recognised

amphibian families. More recent studies have gone further in analysis complete mitochondrial genomes (Mueller *et al.*, 2004; Zhang *et al.*, 2005). Whereas mitochondrial DNA (mtDNA) genes are used to infer relationships among closely related taxa, nuclear DNA (nuDNA), with a slower rate of evolution, are more useful for deciphering older relationships (Simmons *et al.*, 2002, 2004). Nuclear genes are also less influenced by base pair compositional bias (Simmons *et al.*, 2004). Therefore, a large data set consisting of single-copy protein-coding nuclear genes is desirable to assess the phylogeny of the family Dicroglossidae. Nowadays, nuclear genes, such as the Rhodopsin gene, recombination-activating gene 1 (RAG1) and Proopiomelanocortin A (POMC), play an important role in systematic studies on amphibians and reptiles (San Mauro *et al.*, 2005). Recent work on a phylogeny of the living amphibians based on a combined analysis of mitochondrial and nuclear genes from 522 species representing all families within the Amphibian was analysed on a program computer duster, the result showed that in the most comprehensive hypothesis on the phylogenetic relationships within the amphibian available to the present time (Frost *et al.*, 2006).

Conservation and Genetic diversity

Conservation genetics or the application of genetics to the preservation of species has received increasing attention in recent years (Allendorf and Luikart, 2007; Frankham, 2003). In conservation genetics, knowledge of the relatedness between individuals is particularly important in captive breeding programs that seek to reduce incestuous mating in order to minimize inbreeding and the loss of genetic variation (Frankham *et al.*, 2002). It is well established that a decline in genetic

variation reduces the ability of a population to adapt to environmental changes and therefore decreases its long term survival. The loss of genetic diversity also results in lower individual fitness and poor adaptability (Lande, 1988). The fate of small populations is linked to genetic changes. The captive breeding of endangered wildlife animals is often necessary for their conservation; however, this strategy potentially increases the chances of inbreeding that, in turn, causes poor fitness of these populations (Ralls and Ballou, 1983; Crnokrak and Roff, 1999). Inbreeding is known to decrease genetic diversity and to reduce reproductive and survival rates leading to increased extinction risk. Genetically impoverished endangered populations often fail to exhibit signs of recovery until crossed with individuals from other populations (Land and Lacy, 2000; Westemeier *et al.*, 1998). Moreover, wildlife populations with lower genetic diversity are at greater risk of extinction (Saccheri *et al.*, 1998). Knowledge and studies on genetics can reduce the extinction risk by helping to develop appropriate population management programs that can minimise the risks implied through inbreeding. Breeding programs are often started assuming that the wild founders initiating the captive population are unrelated. However, threatened animals brought into captivity often have small population sizes and therefore the founders may be related to each other (Geyer *et al.*, 1993; Haig *et al.*, 1994). Assessment and preservation of biodiversity of wild populations is crucially important to minimise the loss of initial genetic variation as a consequence of inbreeding (Russello and Amato, 2004).

Mitochondrial DNA

The mitochondrial DNA comprises a covalently closed circular duplex histone-free chromosome (Fig. 5), present in one or more copies in every mitochondrion (Zhang *et al.*, 2009). The mt genome is maternally and cytoplasmically inherited. Until recently it was thought that mtDNA does not undergo recombination. Current studies, however, showed that mitochondrial DNA recombination might be a common phenomenon (Kraytsberg *et al.*, 2004; Tsaousis *et al.*, 2005).

In amphibians, especially in the *Limnonectes*, a substantial size variability of mitochondrial genomes has been recognised with values ranging from about 14.4 to 22.2 kb. Size polymorphisms of mitochondrial DNA also occur among conspecific individuals, for example, in frogs of the genus *Rana* (Sumida *et al.*, 2001) and newts of the *L. kuhlii* complex (McLeod, 2010).

The mitochondrial genome typically contains two ribosomal RNA coding genes (12S rRNA, 16S rRNA), 22 transfer RNA (tRNA) coding genes, 13 protein coding genes, and a control region (CR) (Figure 6). Eight tRNAs and one mRNA are encoded on the light (L) strand, and 14 tRNAs, 12 mRNAs, and two rRNAs are encoded on the heavy (H) strand, as shown, is a mitochondrial genome of *L. bannaensis* (Zhang *et al.*, 2009).

Mitochondrial DNA has been used extensively in the last three decades to infer the systematics of living organisms and even of fossils. Compared to nuclear DNA, mtDNA has a fourfold lower effective population size (N_e) and therefore tends to coalesce and become monophyletic relatively quickly (Moore, 1995), which in turn makes it a reliable tool for estimating relationships among closely related and even incipient species (Wiens and Penkrot, 2002). On the other hand, substitutions become

saturated more quickly in mitochondrial genes, which diminish the resolution of deeper phylogenetic splits. The substitution rate varies among different functional parts of the mitochondrial genome also must be considered when using mtDNA for estimating phylogeny.

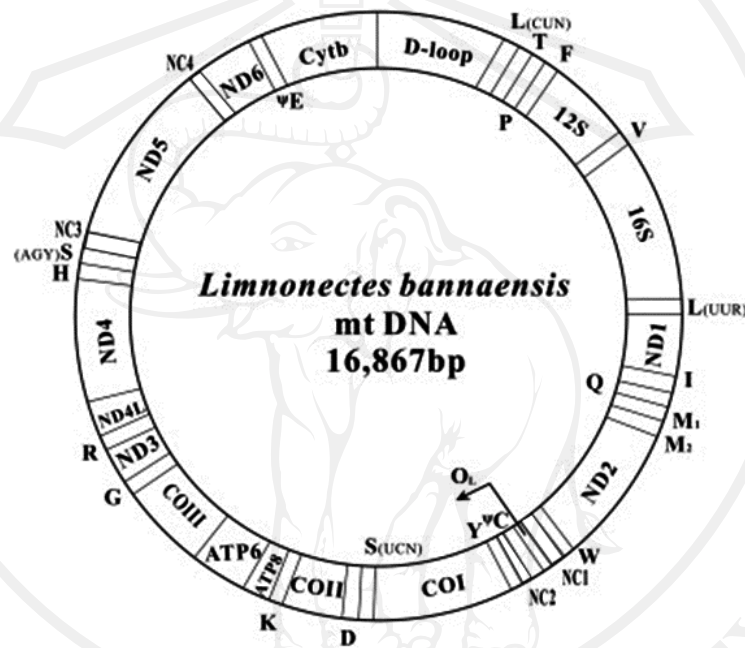


Figure 6 Gene organisations of the *L. bannaensis* the mitochondrial genome. (Zhang *et al.*, 2009)

Nuclear DNA

The size of the nuclear genome is indicated by the C-value, which is a measure of the amount of DNA per haploid genome. Compared to other vertebrate taxa the size of amphibian genomes is relatively large and can vary greatly among species. For example the *gymnophionan* species, were quite variable. C-values of 4.69 pg and 13.95 pg were found in the, *Geotrypetes seraphini* and *Siphonops annulatus*, respectively (Olmo, 1973). In *Rana ridibunda*, a member of the western Palearctic

water frog group, C-values ranged between 7.5 pg and 9 pg (Mazin and Borkin, 1979; Borkin *et al.*, 1987; Vinogradov and Borkin, 1993); this is more than twice the size of the *Homo sapiens* genome. More information on the size of animal genomes is available at the animal genome size database. Nuclear genes are being used much more commonly in amphibian systematic. Most of the interest has been at relatively deep levels, addressing relationships among genus and species. What has been less well evaluated is the utility of nuclear genes, particularly exons, for more recent divergences, where many more nodes are waiting for resolution and where the lower evolutionary rates of nuclear genes may not provide many characters. More recently, family-level domain is also approximately where the utility of rapidly evolving mitochondrial genes decreases because of saturation. Here report on the first broad application of three nuclear genes, Rhodopsin, POMC and RAG1 genes, to a within genus and species level question in amphibian.

The Rhodopsin, POMC and RAG1 genes were found to be a single-copy gene with three exons of approximately Rhodopsin (360 bp), RAG-1 (406 bp) and POMC (573 bp), and their structure and function are well known because of their role in tumor development (Braun *et al.*, 1985; Cole, 1986). To date, each of the gene has proved useful in resolving mammal (Miyamoto *et al.*, 2000), crocodylian (Harshman *et al.*, 2003), and passerine bird (Irestedt *et al.*, 2001) phylogenies. Nearly the entire exon has been used to resolve ordinal phylogenetics among birds (Barker *et al.*, 2002; Ericson *et al.*, 2002) and amphibian (Vences *et al.*, 2004; Wiens *et al.*, 2005; Shimada *et al.*, 2011), and smaller portions of the conserved region have been applied to deep level questions on relationships in mammals (Madsen *et al.*, 2001; Murphy *et al.*, 2001), bats (Teeling *et al.*, 2002), and sharks (Martin and Burg, 2002). So far,

however, there has been little discussion about the application of combining three nuclear genes (Rhodopsin, POMC and RAG1 genes) in which seem to have a potential to reveal divergences in the species level (Inger and Stuart, 2010; Matsui *et al.*, 2010b).

Species complex

Amphibian and reptile species complex are genetically distinct, but similar morphological, taxa that are currently, or were historically, classification as same species. Cryptic species are neither taxonomically biogeographical unique, but instead are common among many taxa each species and we can found in all parts of the globe (Pfenninger and Schwenk, 2007).

For a long time, the complex of genus *Limnometes* was classified in genus *Rana*. Nikolai *et al.* (2002), but Emerson *et al.* (2000) suggested that the group of frogs usually referred to as the *Limnometes* complex should be regarded as belonging to *Limnometes* within the family Dicroglossidae. The type species of the family Dicroglossidae genus *Limnometes* is the widely distributed frog in Southeast Asia. *Limnometes* comprises 55 currently recognised species (AmphibiaWeb, 2012) found throughout East and Southeast Asia; these anurans are characterised by fanglike odontoid processes on the lower jaw, male-biased size dimorphism, and a great amount of phenotypic similarity (Emerson *et al.*, 2000). One of the main goals of conservation biology is the description of existing biodiversity; in Southeast Asia, however, the amphibian biodiversity is not well documented due to the limited number of studies conducted.

In an mtDNA study, Kotaki *et al.* (2010) found that phylogenetic relationships of genus *Fejervarya* from Southeast Asia “*F. multistriata*” is a junior synonym of *F.*

limnocharis, or that only some of the populations now recognized as “*F. multistriata*”. Recently, Matsui *et al.* (2010b) classified *L. kuhlii* in terms of mitochondrial (12S rRNA, tRNA^{Val}, 16S rRNA genes, nuclear POMC and RAG-1 genes) and found that *L. kuhlii* could be classified into two types: *L. taylori* as northern lineage and *L. jarujini* as southern lineage. In the study of Matsui, locale of the study was in Northern Thailand during 1984 - 1994 which is almost 20 years ago. Therefore, adding locality records of correctly identified specimens should be included to increase accuracy of the results. Besides, further field surveys in Northern Thailand and some areas of Myanmar are clearly needed in order to understand the distribution pattern, genetic structure, population genetic and to infer the evolutionary history of Thai amphibians of *Limnonectes* in this study. Numerous authors have included *L. kuhlii* in phylogenetic analyses at the level of class (Frost *et al.*, 2006), family (Chen *et al.*, 2005; Che *et al.*, 2007; McLeod, 2010; Matsui *et al.*, 2011) and genus (Emerson *et al.*, 2000; Matsui *et al.*, 2010a; Hong *et al.*, 2011).