

Phylogenetic Relationship of *Limnonectes* (Anura: Dic平glossidae) in Thailand

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Abstract: The aims of this study was to relationship between systematic of fanged frogs are assessed by using the nuclear POMC gene from twenty three samples. It is suggested that there are three monophyletic groups in which the *L. gyldenstolpei*, *L. hascheanus* and *L. limborgi* were clustered together (group I); *L. blythii* (group II) and *L. taylori*, *L. jarujini* and *L. megastomias* (group III). Sequence analyses indicated that *Limnonectes* in Thailand has genetic diversity ($h = 0.96$, $\pi = 0.045$). Analysis of genetic distance on the basis of sequence differences for the POMC gene showed significant genetic difference data from phylogenetic analysis and revealed a very large genetic difference between species for the POMC gene sequences. It was also confirmed that the POMC gene is an effective unit for studying relationships in *Limnonectes* species.

Key words: Genetic diversity, *Limnonectes*, phylogenetic tree

INTRODUCTION

In the past two decades, increased attention has been given to the need for conservation measures that protect populations of amphibians across the globe (McLeod, 2010). Amphibian populations face numerous threats to their survival, including habitat loss, environmental degradation and competition with invasive species, pathogens and diseases and a variety of detrimental anthropogenic factors (Mendelson *et al.*, 2006). Tolerance to disturbance does not include a single species, but instead are collectives of several cryptic species. Cryptic species are genetically distinct, but morphologically similar taxa that are currently or were historically classified as a single species. Recent discoveries of cryptic species were achieved by employing not only the conventional morphological approach, but also use of various molecular analyses (Matsui *et al.*, 2010a). In the case of *Limnonectes kuhlii*, several molecular studies have suggested the presence of many cryptic species (Evans *et al.*, 2003), and recent descriptions of Chinese and Thai populations each as a distinct species (McLeod, 2008; Matsui *et al.*, 2010a; Matsui *et al.*, 2010b). These studies were often done largely or exclusively by utilizing GenBank sequence data that are often different in regions and lengths, or include much missing data. The classification of Thai *Limnonectes* McLeod (2008) performed phylogenetic analyses based on 2518 bp of 12S and 16S rRNA genes. *L. kuhlii* samples from Laos and northwestern Thailand formed a monophyletic group having a sister relation with respect to his new species *L. megastomias* from eastern Thailand. McLeod noted that populations of *L. kuhlii* from the northern Thailand

might not be conspecific with it based on the phylogenetic tree he obtained. McLeod (2010), while further strengthening this idea, also suggested recognizing two taxa within *L. megastomias*. More recently, (Matsui *et al.*, 2010b) described the new species of the northern lineage as *L. taylori* and the southern lineage as *L. jarujini*. In our study, we did a comparison of the sequences of the POMC gene in seven species of Thai *Limnonectes*; *L. blythii*, *L. gyldenstolpei*, *L. hascheanus*, *L. limborgi*, *L. taylori*, *L. jarujini*, and *L. megastomias*. Data were compared to assess the nuDNA genetic diversity of the populations and the phylogenetic relationship to other Thai populations.

MATERIALS AND METHODS

During October 2009 to May 2010 tissue samples of fanged frogs were collected from located in Mae Hong Son province. For DNA sequencing, we used twenty three specimens in addition to the results of Inger and Stuart (2010) and Matsui *et al.* (2010b) as an out group species *Kurixalus idiootocus* (Table 1). DNA was then extracted following a standard phenol-chloroform protocol (Sambrook *et al.*, 1989) from tissue samples (muscle preserved in 95% ethanol). We used the primers POMC (POMC-1: 5'-GAATGTATYAAAGMMTGCA AGATGGWCCT-3) and (POMC-7: 5'-TGGCATTTGAAAAGAGTCAT-3) according to Wiens *et al.* (2005) to amplify a section of the partial sequences of nuclear exons of proopiomelanocortin A (POMC). Every 25 μ L reaction volume consisted of 50 ng of extracted DNA, 1X PCR buffer, 0.2 μ M of each forward and primers, 0.2 μ M dNTP, 5 mM MgCl₂ and 1

Table 1: Samples and information of *limnonectes* used in this study and genbank accession numbers

Species	Locality	Accession No.	Source
<i>L. blythii</i>	Mae Hong Son (PAM113)	JQ180448	This study
<i>L. blythii</i>	Mae Hong Son (PMM147)	JQ180449	This study
<i>L. blythii</i>	Mae Hong Son (PMM153)	JQ180450	This study
<i>L. blythii</i>	Mae Hong Son (TMM6)	-	This study
<i>L. blythii</i>	Mae Hong Son (PMM45)	-	This study
<i>L. blythii</i>	Prachuap Kirikan	GU934414	Inger and Stuart (2010)
<i>L. gyldenstolpei</i>	Sa Kaeo	GU934417	Inger and Stuart (2010)
<i>L. hascheanus</i>	Nakhon Si Thammarat	GU934422	Inger and Stuart (2010)
<i>L. hascheanus</i>	Prachuap Kirikan (1)	GU934423	Inger and Stuart (2010)
<i>L. hascheanus</i>	Prachuap Kirikan (2)	GU934452	Inger and Stuart (2010)
<i>L. limborgi</i>	Nan	GU934421	Inger and Stuart (2010)
<i>L. limborgi</i>	Nong Khai (1)	GU934433	Inger and Stuart (2010)
<i>L. limborgi</i>	Nong Khai (2)	GU934434	Inger and Stuart (2010)
<i>L. taylori</i>	Chang Mai (Doi Inthanon)	AB568548	Matsui <i>et al.</i> (2010a, b)
<i>L. taylori</i>	Mae Hong Son (Pang Tong)	AB568549	Matsui <i>et al.</i> (2010a, b)
<i>L. taylori</i>	Chang Mai (Doi Suthep)	AB568550	Matsui <i>et al.</i> (2010a, b)
<i>L. taylori</i>	Chang Mai (Tha Ton)	AB568551	Matsui <i>et al.</i> (2010a, b)
<i>L. jarujini</i>	Kanchanaburi (Sangkhla Buri)	AB568552	Matsui <i>et al.</i> (2010a, b)
<i>L. jarujini</i>	Phetchaburi (Kaeng Krachan)	AB568554	Matsui <i>et al.</i> (2010a, b)
<i>L. jarujini</i>	Surat Thani (Khlong Sok)	AB568555	Matsui <i>et al.</i> (2010a, b)
<i>L. megastomias</i>	Phu Luang Loei (1)	AB568556	Matsui <i>et al.</i> (2010a, b)
<i>L. megastomias</i>	Phu Luang Loei (2)	AB568557	Matsui <i>et al.</i> (2010a, b)
<i>L. megastomias</i>	Phu Luang Loei (3)	AB568558	Matsui <i>et al.</i> (2010a, b)
<i>K. idiootocus</i>	-	GQ285719	Matsui <i>et al.</i> (2010a, b)

Table 2: Mean genetic distances between species in this study by the Kimura 2-parameter model (K2P)

	1	2	3	4	5	6	7
<i>L. blythii</i>							
<i>L. hascheanus</i>	0.069						
<i>L. limborgi</i>	0.068	0.017					
<i>L. gyldenstolpei</i>	0.041	0.033	0.031				
<i>L. taylori</i>	0.048	0.074	0.076	0.051			
<i>L. jarujini</i>	0.048	0.067	0.070	0.047	0.023		
<i>L. megastomias</i>	0.052	0.069	0.073	0.053	0.027	0.015	

unit *Taq* DNA polymerase (Vivantis, Malaysia). Amplification was conducted under the following conditions: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 min and at 72°C for 5 min. All PCR products were purified using the Qiagen PCR Purification Kit (Qiagen, Germany) and finally sequenced (BIO BASIC, Canada).

Data analysis: The phylogenetic tree was elucidated by sequences aligned using the computer program ClustalX v1.64 (Thompson *et al.*, 1994; Thompson *et al.*, 1997). Genetic diversity was quantified as the number of distinct haplotypes per population and number of haplotypes, nucleotide diversity (π), and haplotype diversity (h) (Nei, 1987) computed using the program DnaSP vers.5.1 (Librado and Rozas, 2009). A Phylogenetic tree was constructed and Maximum-Likelihood (ML) analysis was performed. The ML tree was constructed with MEGA5 software (Tamura *et al.*, 2011).

RESULTS AND DISCUSSION

The nucleotide sequence of part of the proopiomelanocortin A (POMC) gene (approx. 528bp) was determined for twenty three *Limnonectes* specimens.

Seventeen haplotypes were observed among the resultant sequences. The ML trees of POMC DNA sequences showed that three monophyletic groups are included within the clade. Species in group I are related to *L. hascheanus* that from Prachuap Kirikan and Nakhon Si Thammarat. Another part of group I includes specimens from Sa Kaeo that are related to *L. gyldenstolpei* and *L. limborgi* that from Nan and Nong Khai. Group II, with *L. blythii*, occurs only in Mae Hong Son and Prachuap Kirikan. Species in group III are restricted to *L. taylori* from Chang Mai and Mae Hong Son. Another part of group III includes species from Kanchanaburi, Phetchaburi, and Surat Thani related to *L. jarujini* and *L. megastomias*, only from Loei. A sister species relationship of *L. taylori*, *L. jarujini*, and *L. megastomias* from Thailand was supported by Matsui *et al.* (2010b) (Fig. 1). Most of the clades shown in this tree are supported by high bootstrap values (>70%). Only maximum-likelihood results are presented (Fig. 1) and a comparison of the ML methods of analysis is discussed. The large nucleotide diversity (π) is 0.045 and large haplotypes diversity (h) is 0.96 by Dnsp 5.1. The genetic distance between seven species by Kimura 2-parameter model (K2P) is 0.015 to 0.076 (Table 2). The average nucleotide composition across all the species was T = 15.1, A = 28.3, C = 29.2 and G = 27.4%, respectively. The ts: tv ratio was 2.406 across the seven species of

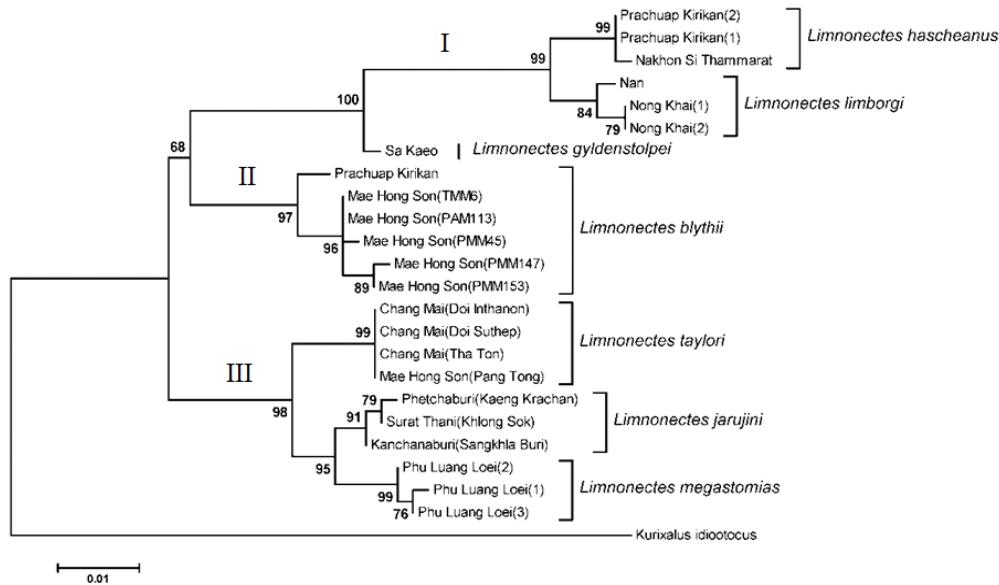


Fig. 1: The maximum likelihood (ML) phylogenetic tree analysis of POMC nuclear genes. Numbers indicates clades supported by bootstrap (2000 replicates)

partial POMC gene sequences (Wiens *et al.*, 2005) which demonstrates the close relationship between these species. Our results strongly support these conclusions of a close phylogenetic relationship in three monophyletic groups between twenty three *Limnonectes* specimens of seven species this assessments.

CONCLUSION

This study has shown the genetic relationships between twenty three specimens from seven species of *Limnonectes* obtained from the proopiomelanocortin A (POMC) gene. The POMC gene has proven useful in genetic diversity studies and relationships. Although molecular methods are easy to apply, considerable DNA data is required covering as many related member species as possible for establishing reliable phylogenetic relationships. In order to make an effective management plan to protect this genus, more information on the biology, demography and ecology of Thai *Limnonectes* spp. are desperately needed in the future.

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