CHAPTER 4

RESULTS AND DISCUSSION

The relationship analysis of morphometric and genetic diversity of fanged frogs, genus *Limnonectes* in Northern Thailand by using six molecular markers: Rhodopsin, Proopiomelanocortin A (POMC), Recombination activating protein 1 (RAG-1), 16S rRNA, Cytochrome Oxidase subunit 1 (CO1), and NADH dehydrogenase 2 (ND2). The results in this study are reported in the following order:

4.1 Exploring of the morphometric and meristic characteristics

4.2 Species identification based on BLAST and BOLD

4.3 Determination of genetic diversity

4.4 Calculation of genetic distance

4.5 Phylogenetic relationships

4.1 Exploring of the morphometric and meristic characteristics

All the measurements (L = length: measurements are mean ± SD, followed by the range in mm) were carried out according to McLeod (2008). Sixteen morphometric and seven ratios of morphological characteristics were examined in five *Limnonectes* species: *L. blythii, L. taylori, L. gyldenstolpei, L. jarujini* and *L. magastomias*. A total of 156 specimens (in Appendix) were used for the studies of morphometric and meristic characteristics. The differences between the mean values of the measured morphometric characteristics in the males and females were not statistically significant in each species (Table 3-7). There were differences in meristic characters between sexes. A comparison of the adult females and males in each species-group reveals that the males average larger than females in all five species-groups. There are similarities between the attitudes expressed by the meristic characters in this study and those described by Andulo and Icochea (2010) and Garda *et al*. (2010) that males are bigger than female, although a few studies showed that in some cases female are larger (Emerson and Ward, 1998; Tsuji and Matsui, 2002). It is also apparent from the results that significant differences were found in some morphometric and meristic characters, even though the ranges overlapped somewhat in all of the characters examined between the five groups. Thus, it was difficult to distinguish exactly between the five groups using only the morphological characters as can be seen from the similarity in both plumage and body size of the samples. Most morphological characteristics of the different species-group were less variable than for the species-groups.

Multivariate data analyses referred as principal component analysis (PCA) using seven morphological parameters (as a ratio) provides an independent assessment of adult dimorphism and the possibility of identifying variables with the discriminatory potential. PC1, PC2, PC3 and PC4 accounted for 38.67%, 23.67%, 42.49%, and 24.46% of the total variance, respectively (Table 8). In principal component analysis (PCA) plot based on the first two principal components (PC1, PC2) (Figure 9), there is overlap between males in all five species-group. Four species-groups aggregated on the mid-left side and were almost completely separate from *L. blythi* group which were scattered and mixed on the right side. The PCA plot of female samples (Figure 10) is comparable in the overlap pattern to that found in males PCA plot, although *L. blythii* group was completely separate from the others which overlap strongly on PC2. Similar results were also obtained after removing seven morphometric variables which shown in Table 8, could associated with sexual dimorphism in *Limnonectes*. Furthermore, the overlap results could be found in five *Limnonectes* species from all analyses, this suggests that morphometric variation is not mainly driven by species differentiation. Despite the observation of the overlap in the multivariate analyses, genus *Limnonectes* could be separated by the averages of a morphometric ratio (HW and SVL) and the modes of seven meristic variables (ED/HL, WH/HL, IN/IO, EN/IN, UEW/IO, TBL/SVL and FEL/SVL).

Table 3 Morphometric measurements for *L. blythii* (measurements in mm).

	Males (n = 20)				**Females (n = 11)**			
	Mean	SD	Max.	Min.	Mean	SD	Max.	Min.
IO	10.01	2.67	17.61	6.66	6.96	1.61	8.90	3.79
IN	1.89	1.87	14.38	5.87	6.77	0.88	7.77	5.47
UEW	8.77	2.08	16.11	6.63	8.55	0.66	9.06	6.96
HW	46.42	11.30	83.29	33.88	35.61	3.73	40.23	29.97
SVL	114.83	27.60	206.46	79.95	97.71	8.75	113.07	81.07
FOL	78.44	19.00	150.00	60.95	69.96	7.11	82.39	56.70
TBL	62.98	12.54	106.93	48.03	55.00	4.92	61.46	47.78
FEL	59.71	16.22	120.19	42.34	51.92	5.09	58.53	43.55
LAL	21.38	4.41	35.56	15.77	19.46	1.61	22.27	17.37
RL	21.31	4.59	35.77	15.27	17.71	1.51	20.58	15.46
EN	12.14	3.13	22.74	8.44	9.78	0.98	11.20	7.79
ED	12.75	2.44	19.71	9.22	11.76	1.12	13.31	10.11
TD	8.45	1.28	10.49	6.01	6.99	0.69	7.77	5.69
MN	49.44	12.80	87.24	33.83	38.04	3.48	42.88	32.23
HL	55.21	13.41	96.40	39.05	44.05	3.94	49.76	37.75
PAL	24.55	6.83	35.00	2.82	22.94	1.97	26.23	20.29

Remark: SVL = snout–vent length; FOL = foot length; TBL = Tibia length; FEL = femur length; UAL = upper arm length IO = interorbital distance; IN = internarial distance; UEW = upper eyelid width; HW = head width; RL = rostrum length; EN = eye–nostril distance; ED = eye diameter; TD = tympanum diameter; MN = mandible–nostril distance; HL = Head length; OH = odontoid height; MH = mandible height; PAL = palm length.

Table 4 Morphometric measurements for *L. gyldenstolpei* (measurements in mm).

	Males (n = 13)				**Females (n = 18)**			
	Mean	SD	Max.	Min.	Mean	SD	Max.	Min.
IO	6.17	0.71	7.85	5.27	4.01	0.59	4.95	2.90
IN	5.35	0.49	6.22	4.45	3.94	0.45	4.52	2.93
UEW	3.88	0.40	4.58	3.05	3.62	0.34	4.12	3.09
HW	25.88	2.40	29.6	21.45	19.11	1.74	21.34	15.94
SVL	58.41	4.95	68.18	51.08	47.42	3.79	53.88	41.70
FOL	37.59	3.12	44.35	33.07	32.04	3.26	38.94	26.70
TBL	30.44	2.40	34.93	26.89	24.69	2.56	29.33	21.04
FEL	30.96	3.08	36.71	26.37	24.64	2.18	28.34	21.24
LAL	11.31	1.33	13.07	8.48	8.87	1.07	10.49	7.15
RL	10.42	0.81	11.74	9.16	8.03	0.87	9.81	6.81
EN	6.16	0.51	7.16	5.43	4.52	0.59	5.60	3.46
ED	6.32	0.69	8.11	5.50	5.40	0.48	6.56	4.71
TD	5.71	0.79	7.09	4.45	4.27	0.68	5.76	3.38
MN	24.43	2.25	27.86	20.66	17.49	1.71	19.68	14.70
HL	27.84	2.15	30.55	24.09	20.53	1.87	23.50	17.71
PAL	14.51	1.04	16.13	12.65	11.63	1.31	14.07	9.35

Table 5 Morphometric measurements for *L. taylori* (measurements in mm).

	Males (n = 30)				**Females (n = 24)**			
	Mean	SD	Max.	Min.	Mean	SD	Max.	Min.
IO	5.79	1.69	9.38	3.00	4.50	0.91	6.72	3.03
IN	5.47	1.16	7.83	3.56	4.60	0.75	6.16	3.40
UEW	4.86	1.13	6.84	2.79	4.40	0.75	5.82	3.24
HW	33.99	8.89	51.16	20.25	24.87	4.12	36.98	16.54
SVL	69.41	15.21	93.64	37.66	58.93	7.84	78.92	45.96
FOL	40.10	7.51	56.45	23.36	34.08	4.34	43.77	25.16
TBL	31.36	5.70	41.76	23.08	26.49	3.85	37.69	20.22
FEL	34.10	6.44	44.16	24.20	28.69	3.99	39.16	19.80
LAL	13.29	4.14	21.79	6.98	10.54	1.59	12.76	5.85
RL	11.56	2.59	16.68	6.98	9.18	1.41	12.08	6.74
EN	5.82	1.24	8.52	4.12	4.68	0.91	6.91	2.95
ED	8.34	1.52	10.92	5.6	7.18	1.10	10.32	4.92
MN	31.57	8.21	49.84	17.15	24.76	4.88	35.21	14.86
HL	33.71	9.28	49.02	18.59	23.93	4.05	40.10	18.33
PAL	16.81	3.19	21.95	11.76	13.70	1.77	18.33	10.44
Data of tympanum diameter (TD) cannot be obtained as the natural physical difference of the species resulting in unable to do the measurement.								

Table 6 Morphometric measurements for *L. magastomias* (measurements in mm).

	Males (n = 12)				**Females (n = 8)**			
	Mean	SD	Max.	Min.	Mean	SD	Max.	Min.
IO	5.95	1.11	8.08	4.44	4.14	1.38	8.08	4.44
IN	5.70	0.93	7.01	4.11	4.56	0.89	7.01	4.11
UEW	5.06	1.11	6.53	3.55	4.25	0.83	6.53	3.55
HW	33.94	8.97	49.34	24.46	22.82	5.89	49.34	24.46
SVL	75.16	18.27	102.36	46.98	56.15	13.28	102.36	46.98
FOL	44.54	8.32	57.70	33.09	34.24	7.39	57.70	27.85
TBL	32.55	8.84	44.51	12.06	25.81	6.04	44.51	20.28
FEL	35.78	8.57	49.48	25.93	27.24	6.60	49.48	21.82
LAL	14.64	3.39	20.23	10.53	10.61	3.27	20.23	7.51
RL	12.05	2.83	16.37	8.48	8.71	1.75	16.37	6.68
EN	6.25	1.60	8.39	4.50	4.54	0.71	8.39	3.63
ED	9.27	2.18	11.88	6.63	7.04	1.53	11.88	5.15
TD	6.60	1.31	9.17	4.8	5.32	1.32	9.17	4.11
MN	31.79	7.24	43.97	21.63	21.48	7.24	43.97	16.51
HL	34.36	9.98	50.08	22.69	24.90	9.98	50.08	18.83
PAL	17.52	3.90	23.39	12.92	13.54	3.90	23.39	10.34

Table 7 Morphometric measurements for *L. jarujini* (measurements in mm).

	Males (n= 8)				**Females (n= 12)**			
	Mean	SD	Max.	Min.	Mean	SD	Max.	Min.
IO	5.60	1.82	9.91	3.92	3.70	0.71	5.09	2.42
IN	5.50	1.10	7.73	4.35	4.08	0.62	5.12	3.20
UEW	4.55	0.64	5.72	3.49	3.84	0.91	5.58	3.00
HW	30.78	5.85	41.61	24.85	21.50	3.78	27.27	16.82
SVL	67.56	9.35	85.28	56.26	52.72	9.55	65.98	39.65
FOL	39.50	6.71	50.56	31.44	32.43	4.52	39.10	26.21
TBL	30.97	5.09	41.61	25.49	24.18	3.88	29.88	19.03
FEL	33.28	4.33	40.35	26.83	25.90	4.32	33.23	20.18
LAL	12.37	2.44	16.94	9.12	9.72	2.27	13.59	6.99
RL	11.16	1.74	14.36	9.17	8.58	1.65	11.10	6.43
EN	5.95	1.03	8.00	4.98	4.58	0.79	5.27	3.27
ED	7.72	0.71	8.50	6.71	6.65	1.45	9.27	4.99
TD	5.19	1.10	6.99	3.59	4.20	0.78	5.46	3.21
MN	28.43	4.72	37.94	23.48	20.30	3.95	25.08	15.34
HL	32.20	5.22	42.57	26.66	22.35	4.15	29.03	17.36
PAL	17.59	3.78	26.68	15.06	13.38	1.94	16.25	11.09

Additional cryptic diversity may exist within several of these species (Matsui *et al*., 2010b), however, and further work must be done to determine whether the degree of divergence among these lineages merits recognition at the species level. Addressing these questions using morphological data may not be the best approach, as demonstrated by this study. The strong morphological conservatism among the taxa examined here and among other species of genus *Limnonectes* have found a stabilising selection on an ecologically efficient body form (e.g., Matsui *et al*., 2010b), which therefore is not useful for distinguishing species. The more effective approach, supported by the data presented here, would be to examine the degree of fine-scale behavioural, ecological, and genetic differences among lineages.

Table 8 Factor loading on the four principal components extracted from a correlation matrix of seven morphological parameters for five species male and female individuals of *Limnonectes* from Thailand.

Character	**PCA1**	**PCA2**	**PCA3**	**PCA4**
ED/HL	0.573	0.549	**-0.604**	0.178
WH/HL	**0.722**	-0.054	**-0.789**	-0.193
IN/IO	**0.727**	0.468	-0.007	**0.930**
EN/IN	**-0.746**	0.154	**0.718**	-0.220
UEW/IO	0.323	0.594	0.366	**0.836**
TBL/SVL	**-0.691**	**0.610**	**0.897**	-0.087
FEL/SVL	-0.437	**0.620**	**0.730**	-0.148
EIGENVALUES	2.707	1.657	2.975	1.712
% of variance	38.67	23.67	22.49	15.17

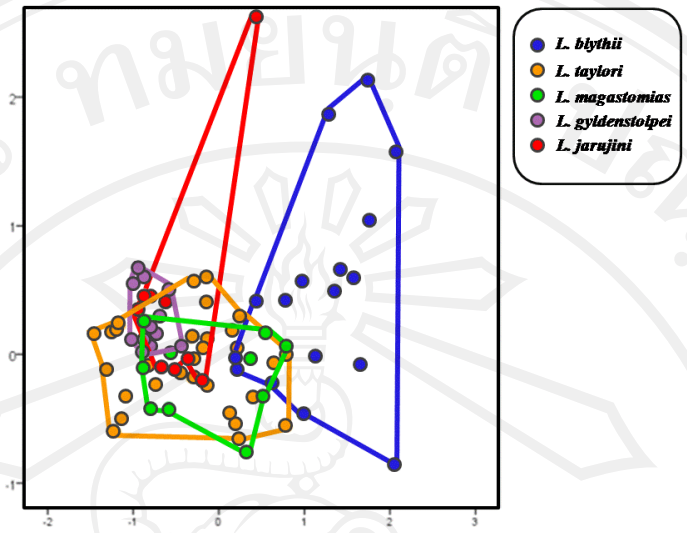

Figure 9 Scatterplot of principal component scores (PC1 and PC2) for male which are five *Limnonectes* species from Thailand.

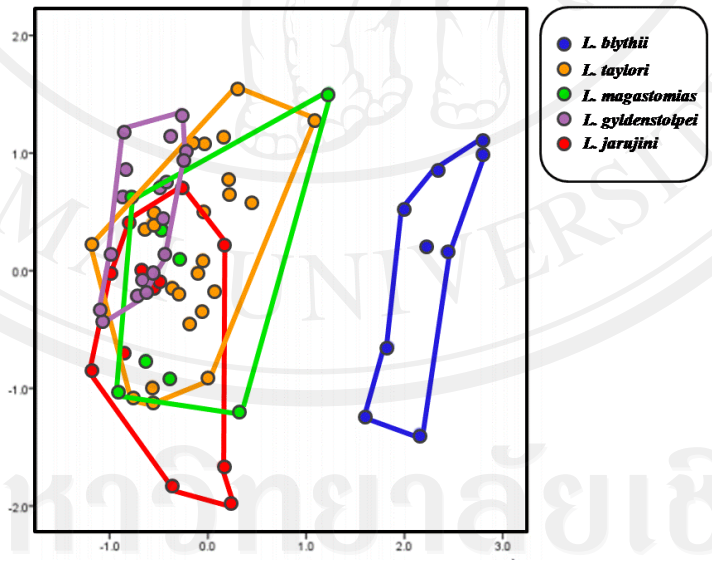

Figure 10 Scatterplot of principal component scores (PC1 and PC2) for female which are five *Limnonectes* from Thailand.

4.2 Species identification based on BLAST and BOLD

The mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA) regions of all samples were successfully amplified using PCR. Table 9 shows the comprehensive barcoding identification results based on GenBank and BOLD databases. Both databases revealed definitive identity matches in the range of 97%–100% for consensus sequences of six species (*L. blythii, L. taylori, L. gyldenstolpei, L. limborgi, L. jarujini* and *L. magastomias*). GenBank-based identification for all species yielded an alignment E-value of 0.0. BOLD-IDS results were in agreement with GenBank results in identification of these species, yielding 100% identity, except for *L. blythii, L. taylori* and *L. gyldenstolpei.* For examples, *L. gyldenstolpei* and *L. blythii* had 100% maximum identity in Genbank, whereas the percent similarity in BOLD database for this species was 99%. Similarly, *L. taylori* also showed 98% maximum identity in BOLD, whereas the percent similarity for this species in GenBank database was 99%. This study also highlighted, however, existing shortcomings in GenBank and BOLD databases for *Limnonectes* species.

The central goal and value of DNA barcoding remains the identification of candidate species, and to this extent, the barcoding data are very useful. For a given species, all specimens from different localities group together, i.e. they share the same maternal history. These clusters conform to morphological identifications made in the field. Because our study uses unambiguous species for the purpose of testing the primer pairs, it does not test the utility of DNA barcoding to either identify cryptic diversity or detect introgressed mtDNA and nuDNA (Che *et al.*, 2012).

Table 9 Summary of identification based on each species consensus barcodes sequence using BOLD Identification System (BOLD-IDS) and BLASTN search from GenBank.

Species studied	BOLD -IDS		GenBank (BLASTN)	
	Species identification	% similarity	Species identification	% Max identity
mtDNA				
L. blythii	***Limnonectes* sp.**	**99**	*L. blythii*	100
L. taylori	***L. kuhlii***	**98**	*L. taylori*	99
L. gyldenstolpei	***Limnonectes* sp.**	**99**	*L. gyldenstolpei*	100
L. limborgi	*L. limborgi*	100	*L. limborgi*	100
L. jarujini	*L. jarujini*	100	***L. kuhlii***	100
L. magastomias	*L. magastomias*	100	*L. magastomias*	100
nuDNA				
L. blythii	***Limnonectes* sp.**	**99**	***L. shompenorum***	**98**
L. taylori	***Limnonectes* sp.**	**100**	***L. fujianensis***	**97**
L. gyldenstolpei	***Limnonectes* sp.**	**99**	***L. shompenorum***	**98**
L. limborgi	*L. limborgi*	99	*L. limborgi*	100
L. jarujini	*L. jarujini*	99	*L. jarujini*	100
L. magastomias	*L. magastomias*	99	*L. magastomias*	99

4.3 Determination of genetic diversity

The results obtaining from a comparative genetic diversity analysis of the nucleotide sequences of the mtDNA and nuDNA genes in *Limnonectes* species are presented in Table 10. The mtDNA sequences (16s rRNA, ND2 and COI genes) define 53 haplotypes with haplotype diversity (*h*) 0.135, 0.095 and 0.084, respectively. On the other hand, the large haplotypes diversity (*h*) values of nuDNA genes (Rhodopsin, RAG-1 and POMC) were estimated to be 0.024, 0.028 and 0.033, respectively and these define 59 haplotypes. The nucleotide diversity (π) values of nuDNA are 0.018, 0.020 and 0.022, respectively. By contrast the lower nucleotide diversity (π) was found in mtDNA analyses which are 0.153, 0.112 and 0.102 respectively (Table 10). mtDNA genes partial sequences of 16s rRNA (512 bp), ND2 (604 bp) and COI (626 bp) were determined, compared and analysed, the result showed that the average nucleotide composition of A C G T in the coding area were 28.57%, 20.61%, 24.17%, 26.65% respectively (data not shown). The nucleotide composition of C was lowest, while A was highest, although the nucleotide composition of all four bases seems to be similar. The ts:tv ratio was 1.546 across the *Limnonectes* species of partial mtDNA gene sequences which demonstrates the close relationship between these species. The analysis results from three nuDNA genes partial sequences; Rhodopsin (360 bp), RAG-1 (406 bp) and POMC (573 bp) showed that the average nucleotide composition of A C G T in the coding area were 29.32%, 28.40%, 23.38%, 18.90% respectively (data not shown). The nucleotide composition of T was lowest with noticeable number, while A was highest.

The ts:tv ratio was 2.100 across the seven species of partial nuDNA gene sequences which demonstrates the close relationship between these species. The results obtaining from both nuDNA and mtDNA analyses showed that there is a genetic divergence found among the specimens. However, when using mtDNA, the fanged frogs species tend to have greater genetic diversity than using nuDNA data. Mitochondrial DNA has evolved faster than the nuclear genome as well as has limited repair ability, and therefore has a relatively high mutation rate which 5–10 times higher than that of nuDNA (Zhang *et al*., 2009). Therefore, mtDNA seems to be a powerful marker for inferring genetic relationships in low categorical levels like genera and species. This feature indicated that the analysis of mtDNA alone could be used for genetic diversity and structure study. 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. However, few studies have been previously reported on the genetic diversification of amphibians in Thailand (Stuart *et al*., 2006; Matsui *et al*., 2010b).The results strongly support these conclusions of a close phylogenetic relationship between *Limnonectes* specimens of this assessment.

Table 10 The nucleotide diversity parameters of mtDNA and nuDNA in *Limnonectes* species

Parameters	**mtDNA**			**nuDNA**		
	16S rRNA	**ND2**	**COI**	**Rhodopsin**	**RAG-1**	**POMC**
Haplotype diversity (*h*)	0.135	0.095	0.084	0.024	0.028	0.033
Nucleotide diversity (π)	0.153	0.112	0.102	0.018	0.020	0.022
DEP (±SD)	0.110±0.011	0.090±0.071	0.097±0.021	0.020±0.014	0.017±0.004	0.019±0.027
ID (±SD)	0.214±0.686	0.184±0.066	0.172±0.005	0.026±0.003	0.016±0.004	0.022±0.014
CD (±SD)	0.958±0.194	0.768±0.104	0.725±0.014	0.788±0.044	0.928±0.034	0.828±0.026
ts:tv	1.689	1.509	1.439	2.344	1.933	2.023

Remark: Haplotype (*h*), nucleotide (π) diversity, Compute Mean Diversity in Entire Population (DEP), Compute Mean Interpopulation Diversity (ID), Compute Coefficient of Differentiation (CD) and transition/transversion (ts:tv) estimates ± standard deviations (±SD).

4.4 Calculation of genetic distance

The results gaining from a comparative phylogenetic analysis of the nucleotide sequences of the mtDNA gene (16s rRNA, ND2 and COI) in six different species of the *Limnonectes* are shown in Table 11. The interspecific distances within the genus *Limnonectes* by Kimura 2-Parameter Model (K2P) distance between species ranging from 0.034 (3.4%) to a maximum value of 0.170 (17%) (Table 11). The overall K2P distance between the six species was 0.167 (16.7%) (data not shown). The K2P genetic distance was high 0.170 (17%) between *L. blythii* and *L. limborgi*. Very low K2P distance 0.034 (3.4%) was exhibited between *L. limborgi* and *L. jarujini*. Based on the nuDNA sequence data (POMC, RAG-1 and Rhodopsin genes) in six species belonging to different species of the *Limnonectes* species (Table 12), the K2P genetic distance was high 0.077 (7.7%) between *L. magastomias* and *L. limborgi*. The minimum level 0.019 (1.9%) of genetic distance was displayed between *L. magastomias* and *L. jarujini*. As shown in Table 13, 512 bp of mtDNA (16S rRNA) consensus barcodes for each species were treated as discrete units to estimate the pairwise level of genetic distance using the Kimura 2-parameter (K2P) correction model. The K2P distance matrix showed a relatively high overall mean interspecific divergence of 0.342 (34.2%) with a standard error of 0.027 (2.7%). The K2P distance between species ranged from a low 0.006 (0.6%) (between *L. gyldenstolpei* lineages1 and *L. gyldenstolpei* lineages2) to a maximum value of 16% (between *L. limborgi* and *L. magastomias*). All the studied species displayed low levels of conspecific divergence (Table 13).

Comparisons of the mean pair-wise distances within the *Limnonectes* complex were made using mtDNA and nuDNA and found to be 0.167 (16.7%) and 0.052 (5.2%), respectively (data not shown). Although extensive research has been carried out on amphibians, only a few studies exist which adequately covers the genetic diversity within the *Limnonectes* complex. McLeod (2010) reported that among species of the *L. kuhlii* complex from South East Asia, inter-and intra-specific genetic distances ranged 10.9% and 12.7%, respectively. Several other genetic diversity complexes were studies. Vences *et al*. (2005) found that among species of the family Mantellidae from Madagasgar, inter-and intra-specific genetic distances ranged from 1–16.5% and 0–5.1%, respectively. Ron *et al*. (2006) found that the distances between pairs of unambiguously recognised *Engystomops* species that ranged from 2.9% to 4.1%. The use of mtDNA for species delimitation is controversial. In addition, Wiens and Penkrot (2002) emphasised that employing genetic distances in species delimitation should be done in tandem with other evidence such as morphological differences, reproductive isolation (Vieites *et al*., 2009) and/or bioacoustic differences (Funk *et al*., 2012).

Table 11 Mean pairwise genetic distances between *Limnonectes* species of mtDNA in this study under Kimura 2-Parameter Model (K2P)

	L. blythii	***L. gyldenstolpei***	***L. magastomias***	***L. jarujini***	***L. taylori***	***L. limborgi***
L. blythii		0.019	0.020	0.021	0.021	0.020
L. gyldenstolpei	0.158		0.018	0.019	0.018	0.017
L. magastomias	0.160	0.152		0.012	0.012	0.017
L. jarujini	0.166	0.160	0.071		0.013	0.017
L. taylori	0.161	0.155	0.064	0.076		0.018
L. limborgi	0.170	0.139	0.139	0.034	0.141	

Remark: Pairwise congeneric divergence was denoted by number of base substitutions per site between species (below diagonal) with their corresponding standard error (above diagonal). Complete deletion of all codon position (1st, 2nd, 3rd and noncoding) was employed in this analysis.

Table 12 Mean pairwise genetic distances between *Limnonectes* Species of nuDNA in this study under Kimura 2-Parameter Model (K2P)

	L. blythii	***L. gyldenstolpei***	***L. magastomias***	***L. jarujini***	***L. taylori***	***L. limborgi***
L. blythii		0.010	0.011	0.009	0.010	0.011
L. gyldenstolpei	0.048		0.011	0.010	0.011	0.007
L. magastomias	0.056	0.064		0.006	0.008	0.012
L. jarujini	0.044	0.050	0.019		0.006	0.011
L. taylori	0.048	0.058	0.034	0.023		0.012
L. limborgi	0.066	0.034	0.077	0.064	0.075	

Remark: Pairwise congeneric divergence was denoted by number o f base substitutions per site between species (below diagonal) with their corresponding standard error (above diagonal). Complete deletion of all codon position (1st, 2nd, 3rd and noncoding) was employed in this analysis.

4.5 Phylogenetic relationships

mtDNA: Of a total of 1,742 analysed nucleotide sites (512 in the 16s rRNA, 604 in the ND2 and 626 in the COI data set), maximum-parsimonious tree was obtained**.** The phylogeny constructed from these data (Figure 11) provides stronger support for several familial groupings suggested earlier (McLeod 2010). Four distinct subclades which consist of species group A1 (*L. blythii* northern lineages (N), *L. blythii* southern lineages (S) and *L. shompenorum*, 2 species), group A2 (*L. limborgi, L. gyldenstolpei* lineages1 and *L. gyldenstolpei* lineages2, 2 species), group A3 (*L. megastomias* and *L. jarujini*, 2 species) and group A4 (*L. taylori* lineages1, *L. taylori* lineages2 and *L. bannaensis*, 2 species) were identified; supported by bootstrap values of 98%, 59%, 31% and 97% respectively. From data described above it can be indicated that samples of *Limnonectes* studied tended to form a monophyletic group with respect.

Table 13 Mean genetic distances between species of 16s rRNA gene in this study by the Kimura 2-parameter model (K2P)

	1	2	3	4	5	6	7	8	9
1 ***L. blythii* (S)**		0.008	0.019	0.020	0.020	0.017	0.018	0.017	0.017
2 ***L. blythii* (N)**	0.030		0.019	0.020	0.020	0.018	0.019	0.018	0.018
3 ***L. limborgi***	0.140	0.148		0.018	0.018	0.019	0.020	0.019	0.019
4 ***L. gyldenstolpei* (1)**	0.150	0.150	0.139		0.003	0.018	0.017	0.018	0.017
5 ***L. gyldenstolpei* (2)**	0.150	0.151	0.139	0.006		0.018	0.017	0.018	0.017
6 ***L. jarujini***	0.125	0.133	0.147	0.138	0.136		0.012	0.012	0.011
7 ***L. magastomias***	0.134	0.137	0.160	0.135	0.135	0.066		0.012	0.012
8 ***L. taylori* (1)**	0.131	0.141	0.153	0.149	0.147	0.068	0.067		0.007
9 ***L. taylori* (2)**	0.123	0.129	0.148	0.132	0.129	0.053	0.062	0.034	

Remark: Pairwise congeneric divergence was denoted by number o f base substitutions per site between species (below diagonal) with their corresponding standard error (above diagonal). Complete deletion of all codon position

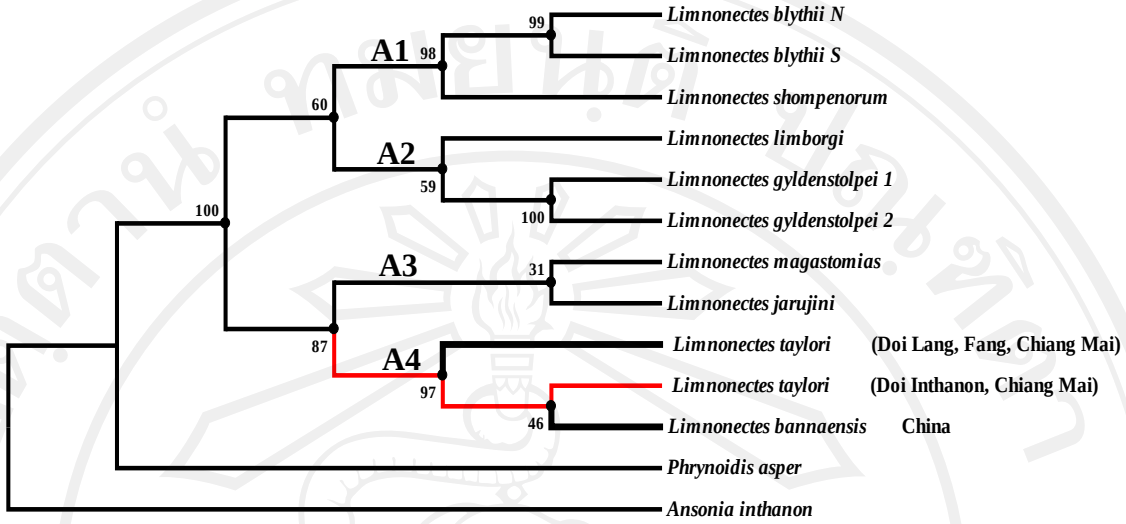

Figure 11 Phylogenetic consensus tree of eight *Limnonectes* species constructed using Maximum Parsimony (MP) Method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. *Phrynoidis asperwas* and *Ansonia inthanon* used as an outgroup.

Remark: Red line showed the type locality for *L. taylori.*

Phylogenetic analyses employing three different optimality criteria yielded similar topologies, and only the mtDNA phylogenetic tree is presented in Figure 12. The nucleotide sequence of part of the mtDNA gene (approx. 1,742 bp) was determined for *Limnonectes* specimens. Haplotypes were observed among the resultant sequences. The maximum likelihood (ML) trees of mtDNA sequences showed that four clades (A–D) are included within the clade. There is weak support for some deep nodes in the preferred tree (Figure 12). There is, however, strong support for the nodes of interest within the *L. taylori* and *L. blythii* complex. The clade composed of *Limnonectes* species (Clades A and C) is the sister taxon to the well-supported clade containing all members of the *L. blythii* and *L. taylori* complex

(Figure 12). The Genus *Limnonectes* complex comprises four major geographic clades (Clades A-D) consisting of 10 distinct lineages. Four clades (A, B, C and D) are recovered with robust support in all three analyses. Clade A comprises two Thailand lineages (Lineages 1 and 2) (Figure 12), contains the type locality for *L. blythii* (Myanmar), and the two sister taxon to the rest of the *L. blythii* (northern Thailand and southern Thailand) lineages in the complex. The northern Thailand lineages are most closely related to *L. shompenorum* (Lineages 3), (in Clade A) comprising lineages from Muara Siberut, Indonesia. Clade B and D comprises northern Thailand lineages (Lineages 4 and 10), contains the type locality for *L. gyldenstolpei* (Koon Tan Mountains, Lampang) and *L. limborgi* (Myanmar).

Clade C comprises two northern Thailand lineages (Lineages 7 and 9) (Figure 13 and 14), contains the type locality for *L. taylori* (Doi Inthanon, Chiang Mai) (Figure 15), and is the sister taxon to the rest of the *L. taylori* (Doi Lang, Chiang Mai) lineages in the complex. The northern Thailand lineages are most closely related to *L. bannaensis* (Lineages 8), *L. megastomias* (Lineages 5) and *L. jarujini* (Lineages 6) (in Clade C) comprising lineages from China, southern Thailand, and eastern Thailand respectively.

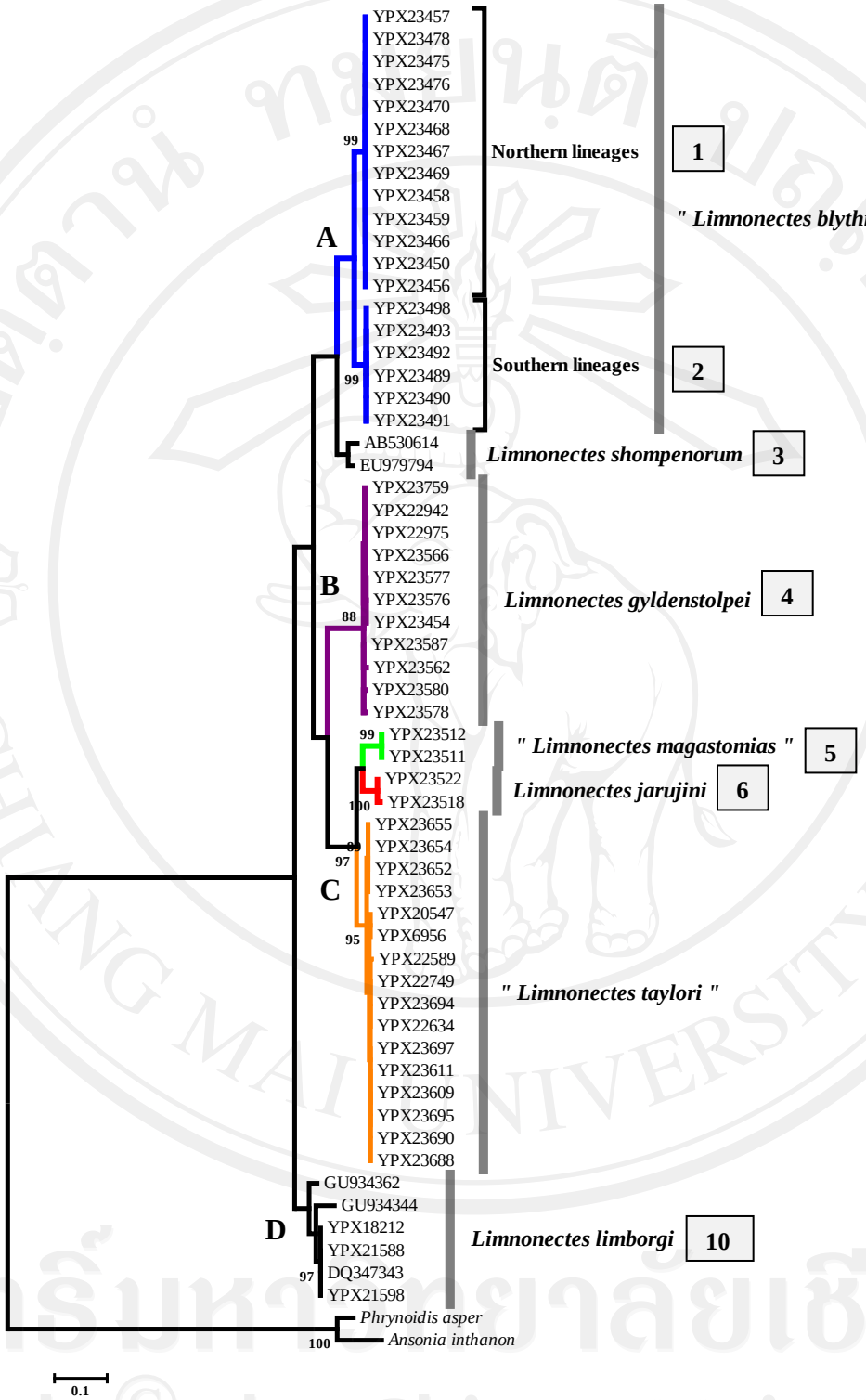

Figure 12 The maximum likelihood (ML) phylogenetic tree analysis of mitochondrial genes (mtDNA). Numbers indicates clades supported by bootstrap (2000 replicates).

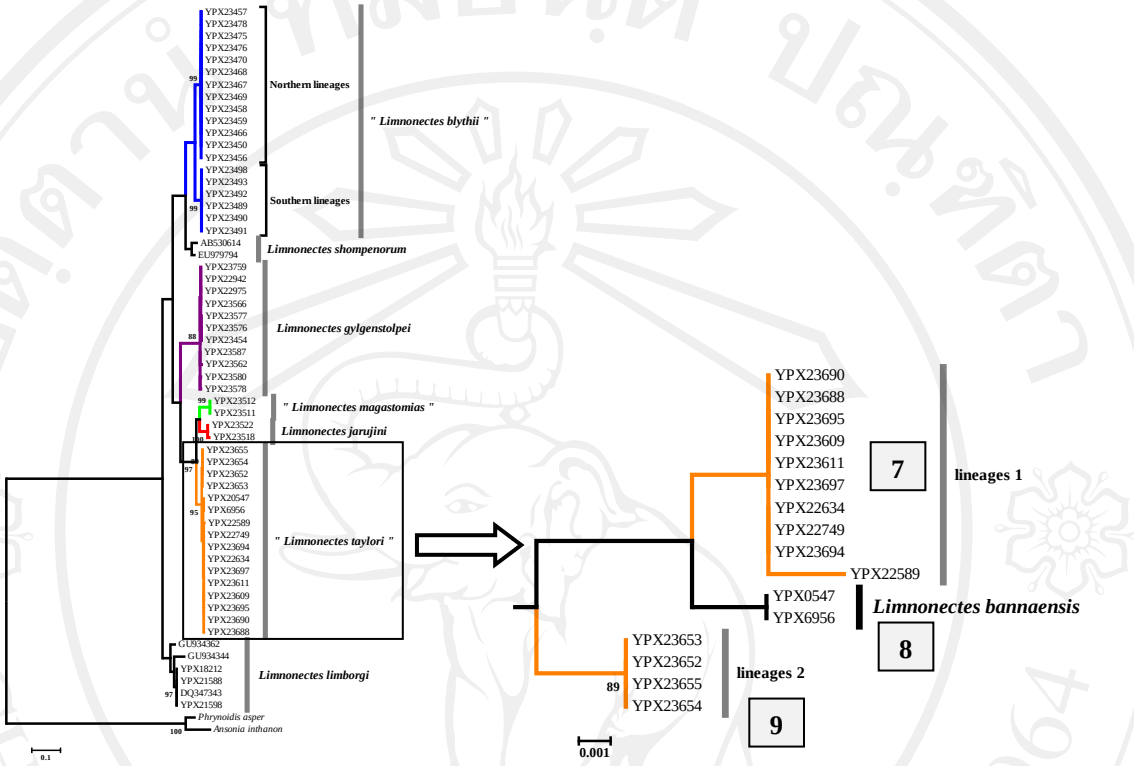

Figure 13 The maximum likelihood (ML) tree for mitochondrial genes (mtDNA) sequences of *L. taylori* clade. The ML tree was constructed under the K2P model. Bootstrap support values of > 80% from 2000 replicates are shown.

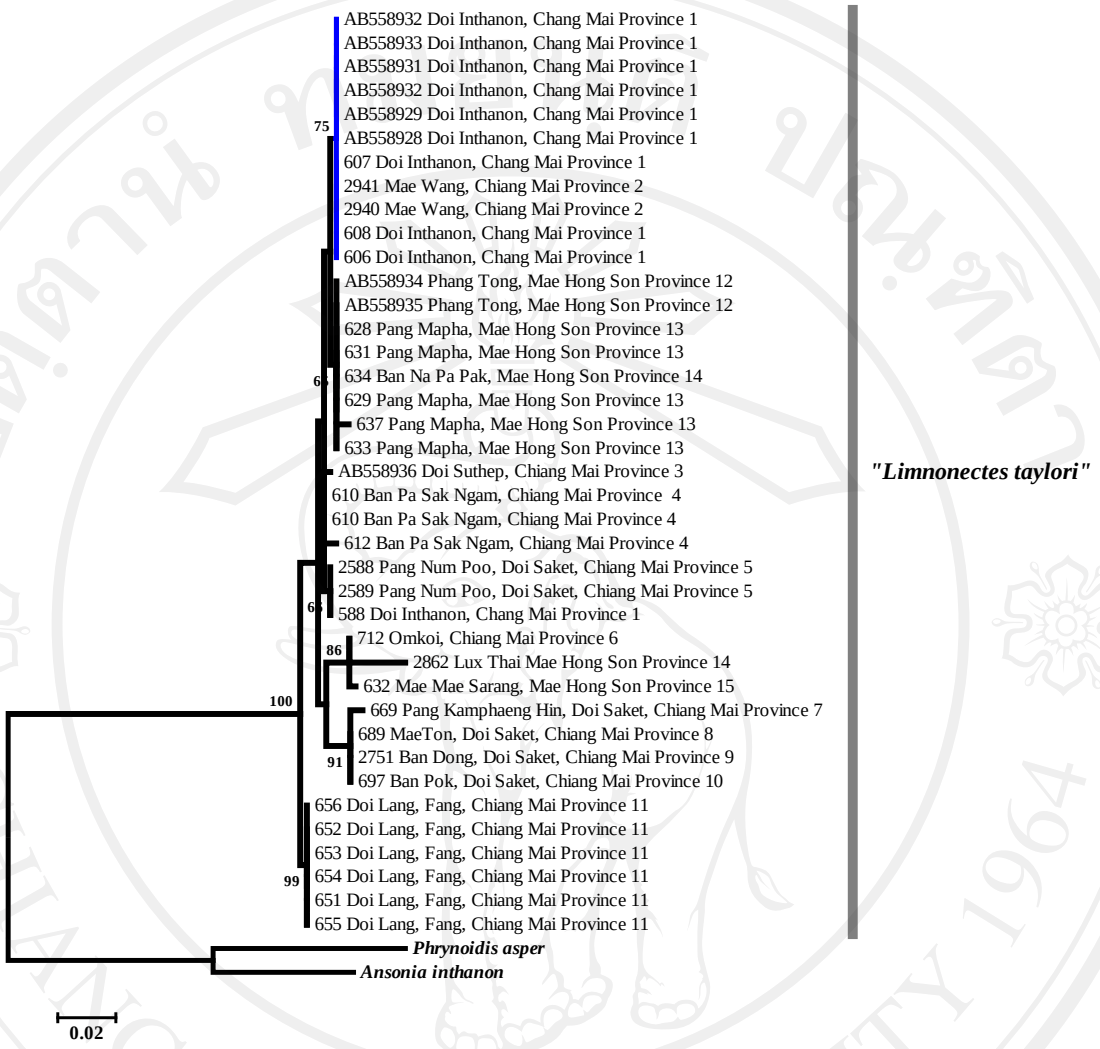

Figure 14 The maximum likelihood (ML) tree for mitochondrial genes (mtDNA) sequences of *L. taylori* in northern Thailand. Bootstrap support values of > 80% from 2000 replicates are shown.

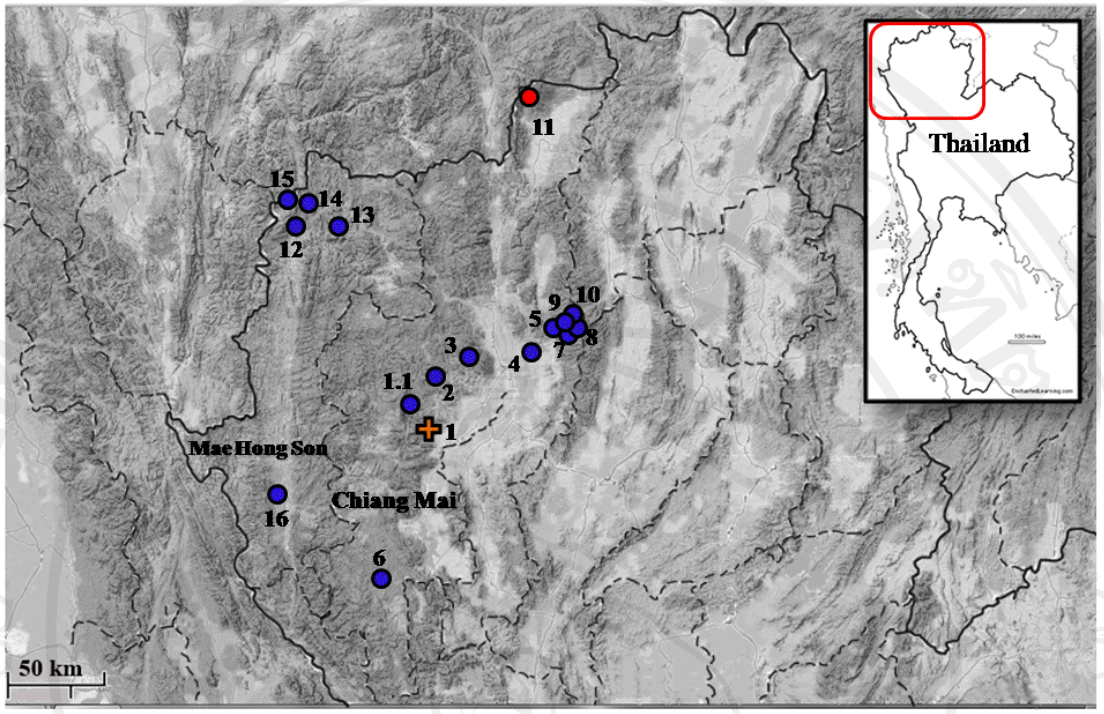

Figure 15 Map of northern Thailand showing the known distributions of the *L. taylori* complex (Plus = type locality; Red circle = Doi Lang locality).

nuDNA: The molecular analysis fully supports the finding from the previous mtDNA Maximum Parsimony (MP) tree study that the *Limnonectes* constitute four distinct subclades**.** According to the Maximum Parsimony (MP) tree (Figure 16) constructed from nuDNA data, the species in the present study were clustered independently within their corresponding genera. Four distinct subclades which consist of species group B1 (*L. taylori* lineages1, *L. taylori* lineages2 and *L. taylori* lineages3), group B2 (*L. megastomias* and *L. jarujini*), group B3 (*L. gyldenstolpei* lineages1 and *L. gyldenstolpei* lineages2) and group B4 (*L. blythii* northern lineages (N), *L. blythii* southern lineages (S)) were identified; supported by bootstrap values of 70%, 71%, 100% and 99% respectively. The results indicate that the studied

Limnonectes tended to form a monophyletic group with respect to the topotypic *L. blythii*, *L. gyldenstolpei* and *L. taylori.*

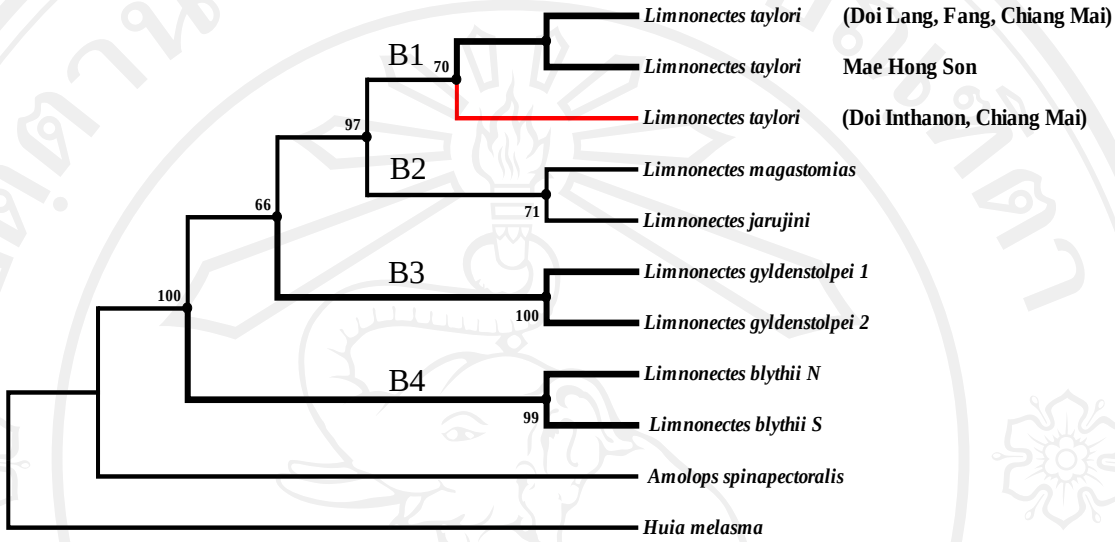

Figure 16 Phylogenetic consensus tree of eight *Limnonectes* species constructed using Maximum Parsimony (MP) Method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. *Amolops spinapectoralis* and *Huia melasma* used as an outgroup.

The phylogenetic relationships among these lineages were mostly consistent between the mtDNA and nuDNA genes as the resultant tree topologies for all phylogenetic analyses from mtDNA were basically identical and congruent with the phylogenetic analyses from nuDNA. The striking result to emerge from the nuDNA data is that (Figure 17) the *L. blythii* in clade A and *L. gyldenstolpei* in clade B, both comprised two distinctive genetic groups. In addition, *L. taylori* in clade D presented three genetically differentiated groups. These could be the by-product of adaptation to different geographical settings. Unlike the morphological results presented in a

previous section, phylogenetic analyses (based on two independent methods) of both mtDNA and nuDNA sequence data have revealed that *L. blythii* from the northern and southern populations are actually rather distinct and cannot represent a single monophyletic entity. Therefore there is a possibility of an uncovered cryptic species of the frogs in the group. However, it seems improbable to definitely that there is cryptic species escaped previous detection within the region sampled so further research is needed. Future studies on the microhabitat and detail morphology are therefore recommended which could make things more clearly understood.

The number of named species of frogs and toads from Thailand increased from 82 (Taylor 1962) to 125 (Khonsue and Thirakhupt, 2001) by the beginning of this century. The number is still steadily increasing and many unidentified forms have also been discovered (Chan-ard, 2003). This increase in faunal diversity is mainly due to extensive field surveys of researchers from this and other countries. In the course of these activities, some species formerly considered wide-ranging have been separated as distinct forms, and some cryptic species have been detected. Thus, high cryptic diversities have been demonstrated in various lineages such as *Rana archotaphus* (from *R. livida*: Inger and Chan-ard, 1997); *Leptobrachium smithi* (from *L. hasselti*: Matsui *et al*., 1999), *Leptolalax melanoleucus* and *L. fuliginosus* (from *L. pelodytoides*: Matsui, 2006), *Rana eschatia* (from *R. raniceps*: Inger *et al*., 2009), *L. megastomias* (from *L. kuhlii* McLeod, 2010) and *L. taylori* and *L. jarujini* (from *L. kuhlii*: Matsui *et al*., 2010).

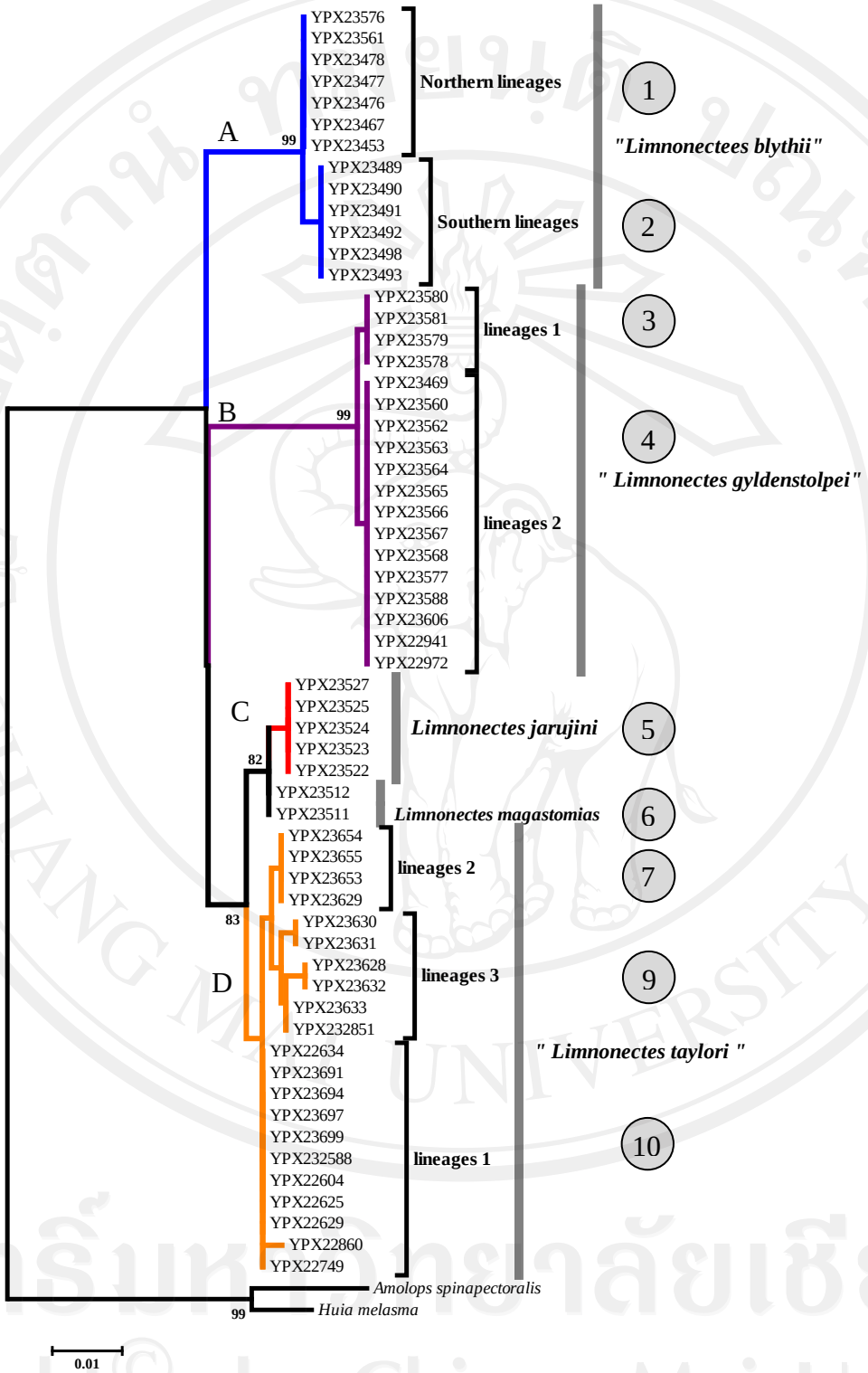

Figure 17 The maximum likelihood (ML) phylogenetic tree analysis of nuclear genes (nuDNA). Numbers indicates clades supported by bootstrap (2000 replicates).

The evidence of cryptic species discovery can be clearly seen in the case of genus *Limnonectes* as *L. taylori* and *L. jarujini* were separated from *L. kuhlii* (Matsui *et al*., 2010b) on the basis of phylogenetic relationship inferred from the mitochondrial gene sequences. In this study, an extensive molecular research was conducted on *Limnonectes* from northern Thailand and adjacent rejoins, and ascertained distinct status of *L.* "*taylori*" from Doi Lang. The results from the analyses showed that the possible sympatric occurrence of *L.* "*taylori*" from other locality in northern Thailand and *L.* "*taylori*" in Doi Lang, Chang Mai Province cannot be ruled out.

The findings of the current study are consistent with those of McLeod (2008) who proposed that *L. megastomias* should be separated from *L. kuhlii* on the basis of phylogenetic relationship inferred from the mitochondrial gene sequences. Without providing substantial diagnosis of his *L. megastomias*, McLeod (2008) briefly noted possible sympatric occurrence of *L. megastomias* and *L.* "*kuhlii*" in Loei Province.

The pattern of distribution within Thailand in the two new lineages of *L. taylori*, together with that of *L. megastomias* and *L. jarujini* are interesting. *L. megastomias* was originally described from two distinct regions of Dong Paya Yen Mountains between northeastern and central Thailand (McLeod, 2008). The new lineages of *L. taylori* undescribed together with that of *L. jarujini* here occur in the northern to southern mountain regions separated from the range of *L. megastomias* by a large gap of central Thai lowlands. Matsui *et al*. (2010b) also pointed out that on the Myanmar border, *L. taylori* occurs on Shan Hills extending from Tha Ton, Chiang Mai Province southward to Doi Hua Mot near Um Phang, Tak Province, which is close to the range of *L. jarujini*. Inclusion of the Tak population in *L. taylori* is based

on their morphological similarity as revealed by univariate and multivariate analyses. Taxonomic identity of this population of the *L. taylori* requires further morphological investigation. This study also illustrated a pattern of distribution within Thailand of the one lineage of *L. gyldenstopei* from northern Thailand together with that of one lineage of *L. gyldenstopei* from eastern population (Chachoengsao). The lineages undescribed here occur in the *L. gyldenstopei* found in mountain regions of northern Thailand which separated from the range of *L. gyldenstopei* from eastern population by a large gap of central Thai lowlands.

In this study, it is also found that a sample of *L. bannaensis* from Yunnan Province, China, is a sister taxon to *L.* "*taylori*" from north-western Thailand, which should be *L. taylori* (Matsui *et al*., 2010b). From the data of GenBank sequences analysis, it could be surmised that one of two *Limnonectes* species occurring in China is similar with those found in the north-western Thailand taxon of this study. *L. blythii* has a distributional range on Tenasserim Hills displacing to south of Thailand. Results of mtDNA is in agreement with the nuDNA analysis between the two lineages (northern and southern lineages), though an examination of the basic morphological characteristics did not indicate a morphological gradient across the boundary.

Notably, the results of the present study provide exception information to the rule that distinct populations (of *L. blythii, L. taylori* and *L. gyldenstopei*) represent distinct clade in molecular analyses, although morphologically comparison could not distinguish them apart. Interestingly, except the used of DNA and morphological data, investigations of bioacoustic differences might be possible to make this more clearly understood as a number of studies have found that calls evolve faster than morphology, perhaps owing to strong selection for species recognition or sexual

selection on calls or strong stabilising selection on morphology (Padial *et al*., 2008; Angulo and Icochea, 2010). Several studies have also revealed that very pronounced bioacoustic differences could be seen in amphibian (Funk *et al*., 2012). These results could explain why there are so many cryptic species: morphological differences among closely related species are generally subtle and are a weak indicator of reproductive isolation. By contrast, calls, which have known importance in causing and maintaining reproductive isolation (Boul *et al*., 2007; Vences and Wake, 2007; Guerra and Ron, 2008; Padial *et al*., 2010), show pronounced differences among closely related species and seem to be particularly useful for species delimitation.

Efforts to conserve globally important centres of biodiversity should take into account our finding that Thailand biodiversity is much greater than previously known, at least for some clades. Unfortunately, despite its unparalleled biodiversity, Thailand is vulnerable to several ongoing and increasing threats, including industrial agriculture and climate change (Laurance *et al*., 2002; Lewis *et al*., 2011). A first step in understanding the potential impacts of these threats would be to accurately characterise the magnitude and spatial distribution of biodiversity in additional clades from other amphibian families and more taxonomic groups.

Identification of cryptic diversity also has important implications for assigning conservation status to individual species. The three recognised species (*L. blythii, L. taylori* and *L. gyldenstopei*) in this study are considered of 'least concern' by the IUCN Red List because of their large ranges and abundance (downloaded July 2012). However, these analyses reveal that many species within these two genera have very small ranges, which is one factor that can put them in a higher risk category. Strong evidence of phylogeny trees reported above indicated that these three clades may

consist of several species of higher conservation concern, depending on additional factors such as evidence of population declines and threats. It can thus be stated that it seems inappropriate to mark these three species as ‘least concern’. The results predict that as cryptic species continue to be identified, more species of high conservation concern would be revealed. Improved species sampling, especially in tropical regions, is almost certain to exhibit that the percentage of amphibian species of conservation concern worldwide is even higher than the current estimate of 41 percent (Stuart, 2004; Funk *et al*., 2012).

Taking everything into account, the evidence presented here obtained from genetic data alone strongly suggests that there are probably numerous undescribed species hidden within the genus *Limnonectes* complex in Thailand. A growing concern among ecologists and other field biologists resulting inmaking attempts to resolve cryptic species groups with molecular evidence will yield species that cannot be identified in the field. Certainly, the use of molecular evidence alone to justify nomenclatural changes could create an environment of taxonomic chaos for field workers faced with sympatric species that are morphologically in distinguishable. The intent of this study is not to propose taxonomic changes to the lineages that constitute the genus *Limnonectes* complex, but rather to create a framework for a detailed morphological study of these frogs. Ultimately, the combination of molecular and morphological data should elucidate the full extent of the diversity within this group and permit appropriate application of names to the evolutionary lineages (species) recognised here.