CHAPTER 3

MATERIALS AND METHODS

3.1 Study area and Sample collection

Field work surveyed 28 general areas located inside or on the periphery of National Park on three occasions spanning 2-year period, each during the rainy, winter and summer seasons. Samples in this study were collected from as many distinct microhabitats as possible at each general survey area including streams, rivers, ponds and waterfall areas in Northern Thailand from June 2010 to July 2011.

A total of 503 specimens of adult *Limnonectes* spp. were analysed in this study. All individuals were collected from 28 collection sites in Northern Thailand; others were collected from Southern Thailand. Species identification followed the keys of Taylor (1962), Chan-Ard *et al*. (1999) and Khonsue and Thirakhupt (2001). Identification of all specimens was kindly provided by the amphibian taxonomist, Mr. Tanya Chan-Ard of the Natural Science Research Division, National Science Museum of Thailand.

The geographic locations and detail of various study sites are shown in Figure 7 and Table 1.

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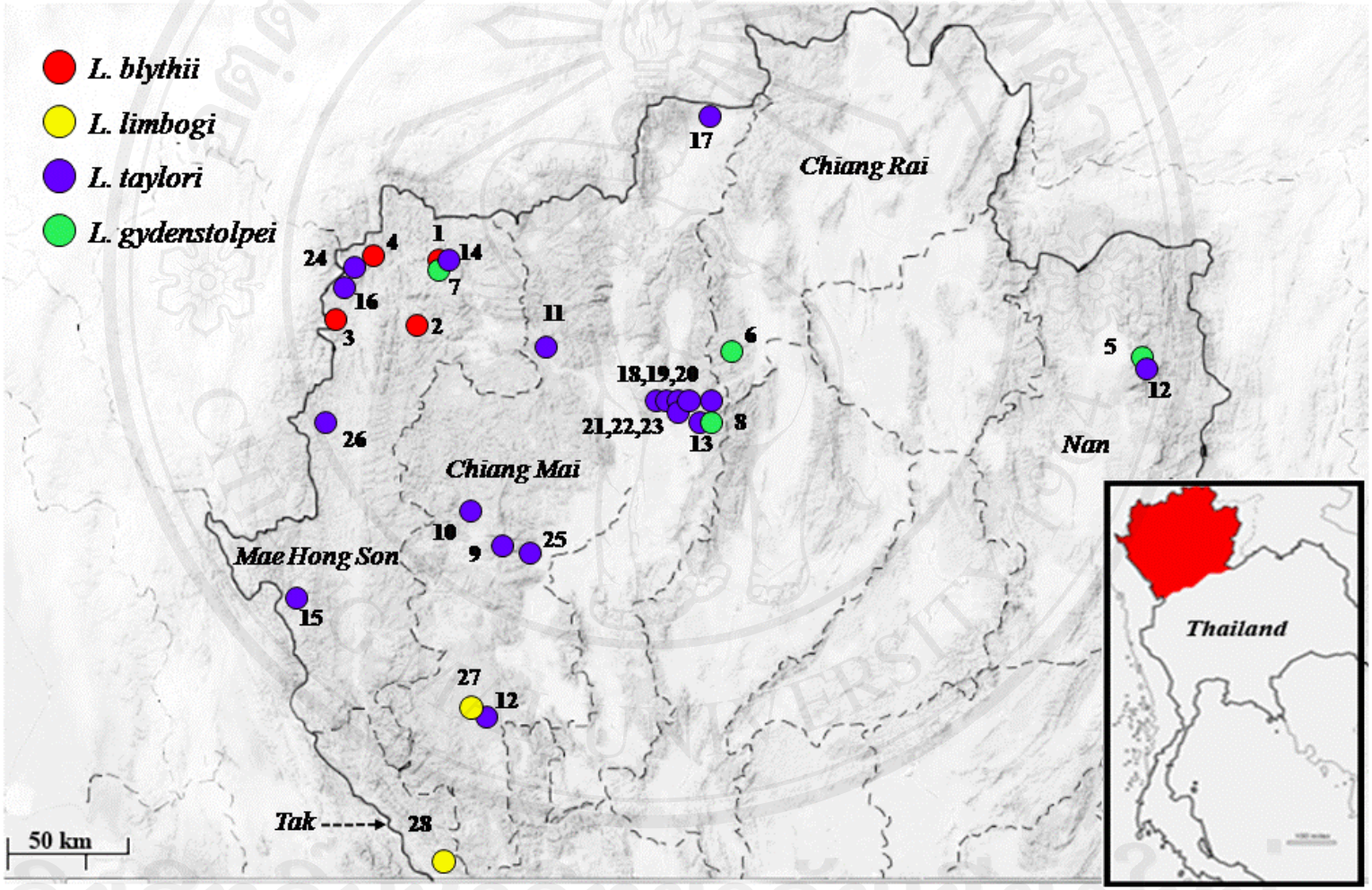

Figure 7 Map showing the collecting stations for frogs used in the present study.

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Table 1 Samples of *Limnonectes* spp. collected in the present study.

Locality no.	Species	Locality	No. of samples	Latitude	longitude
1	*Limnonectes blythii*	Pang Mapha, **Mae Hong Son Province**	5	19°47'N	98°12'E
2		Mae Hong Son district, **Mae Hong Son Province**	20	19°21'N	97°96'E
3		Thai - Myanmar border, **Mae Hong Son Province**	20	19°38'N	98°87'E
4		Pang Aung, **Mae Hong Son Province**	15	19°50'N	97°87'E
5	*Limnonectes gyldenstopei*	Doi Phu Cha, **Nan Province**	2	19°17'N	100°91'E
6		Wiang Pa Pao, **Chiang Rai Province**	5	19°16'N	99°53'E
7		Pang Mapha, **Mae Hong Son Province**	10	19°47'N	98°12'E
8		Ban Pa Sak Ngam, **Chiang Mai Province**	5	18°92'N	99°14'E
9	*Limnonectes taylori*	Chom Thong, **Chiang Mai Province**	3	18°41'N	98°67'E
10		Doi Inthanon, **Chiang Mai Province**	10	18°54'N	98°58'E
11		Huay Nam Dang, **Chiang Mai Province**	5	19°27'N	98°60'E
12		Omkoi, **Chiang Mai Province**	10	17°80'N	98°36'E
13		Ban Pa Sak Ngam, **Chiang Mai Province**	30	18°92'N	99°14'E

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Table 1 Samples of *Limnonectes* spp. collected in the present study. (Continued)

Locality no.	Species	Locality	No. of samples	Latitude	longitude
14	*Limnonectes taylori*	Pang Mapha, **Mae Hong Son Province**	15	19°47'N	98°12'E
15		Mae Sarang, **Mae Hong Son Province**	1	18°20'N	97°91'E
16		Ban Na Pa Pak, **Mae Hong Son Province**	45	19°61'N	98°00'E
17		Doi Lang, Fang, **Chiang Mai Province**	25	20°09'N	99°27'E
18		Pang Kamphaeng Hin, , **Chiang Mai Province**	30	18°97'N	99°34'E
19		Mae Ton, **Chiang Mai Province**	36	19°00'N	99°32'E
20		Ban Pok, **Chiang Mai Province**	25	18.87'N	99.27'N
21		Pang Num Poo, **Chiang Mai Province**	27	18°96'N	99°23'E
22		Pang Bong, **Chiang Mai Province**	35	18°98'N	99°33'E
23		Ban Dong, **Chiang Mai Province**	45	18°92'N	99°33'E
24		Lux Thai, **Mae Hong Son Province**	35	19°58'N	97°94'E
25		Mae Wang, **Chiang Mai Province**	20	18°65'N	98°68'E

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Table 1 Samples of *Limnonectes* spp. collected in the present study. (Continued)

Locality no.	Species	Locality	No. of samples	Latitude	longitude
26	*Limnonectes taylori*	Khun Yuam, **Mae Hong Son Province**	5	18°81'N	97°90'E
27	*Limnonectes limborgi*	Omkoi, **Chiang Mai Province**	3	17°80'N	98°36'E
28		Umphang, **Tak Province**	1	16°71'N	98°57'E
29	*Limnonectes blythii*	**Kanchanaburi Province**	1	17°46'N	98°64'E
30		**Surat Thani Province**	1	8°80'N	99°36'E
31		**Pattani Province**	1	6°68'N	101°13'E
32		**Nakhon Si Thamarat Province**	1	8°75'N	99°77'E
33		**Phatthalung Province**	1	7°50'N	100°02'E
34		**Phuket Province**	1	8°02'N	98°35'E
35		**Narathiwat Province**	1	5°79'N	101°73'E
36		**Phetchaburi Province**	1	12°90'N	99°63'E
37		**Ranong Province**	1	9°44'N	98°48'E

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Table 1 Samples of *Limnonectes* spp. collected in the present study. (Continued)

Locality no.	Species	Locality	No. of samples	Latitude	longitude
38		**Chumphon Province**	1	9°88'N	98°86'E
39	*Limnonectes megastomias*	**Nakhon Ratchasima Province**	1	14°70'N	101°52'E
40		**Loei Province**	1	17°14'N	101°66'E
41	*Limnonectes jarujini*	**Ranong Province**	1	9°84'N	98°62'E
42		**Phetchabun Province**	1	12°91'N	99°64'E
43		**Prachuap Khiri Khan Province**	1	11°59'N	99°50'E
44	*Limnonectes gyldenstopei*	**Chachoengsao Province**		13°58'N	101°55'E

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3.2 Morphological data analyses

3.2.1 Morphometric characters

Measurements were made with digital calipers to the nearest 0.01 mm. Sixteen morphometric characters of post metamorphic individuals used here are modified from those of McLeod (2008) Table 2 and are illustrated in Figure 8. Modifications include the addition of five measurements, which are defined as follows:

Table 2 Morphological parameters used in this study.

Abbreviation	Character
1. IO	Inter-orbital width
2. IN	Internarial distance (distance between 2 nostrils)
3. UEW	Upper eyelid width
4. HW	Head width (left side back of mandible to right side back of mandible)
5. SVL	Snout–vent length
6. FOL	Foot length (from base of tarsus to tip of fourth toe)
7. TBL	Tibia length
8. FEL	Thigh (femur) length
9. LAL	Lower arm length (from elbow to base of outer palmer tubercle)
10. RL	Rostrum length-distance from the level of the anterior corner of the eye to the anterior-most point of the head (“snout” is most appropriately applied to the portion of the head anterior of the nostrils, but is frequently misused to refer to the region anterior of eye-level)

Table 2 Morphological parameters used in this study. (Continued)

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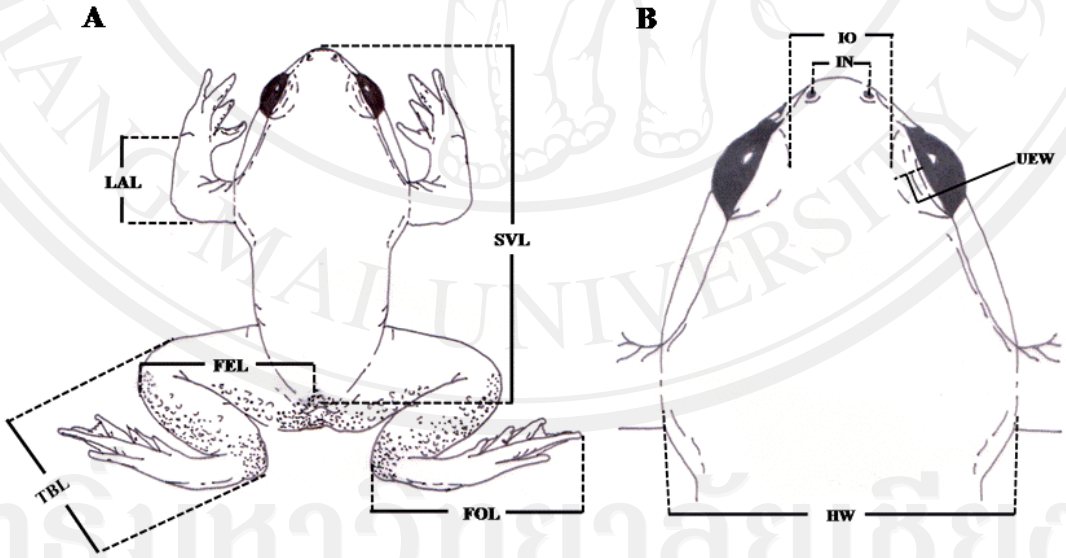
Abbreviation	Character
11. EN	Eye-nostril distance (Distance from front of eyes to nostril)
12. ED	Eye diameter (greatest diameter of the eye including upper eye-lids)
13. TD	Tympanum diameter (Maximum diameter)
14. MN	Mandible–nostril distance
15. HL	Head length (from back of mandible to tip of snout)
16. PAL	Palm length (from base of outer palmer tubercle to tip of third finger)



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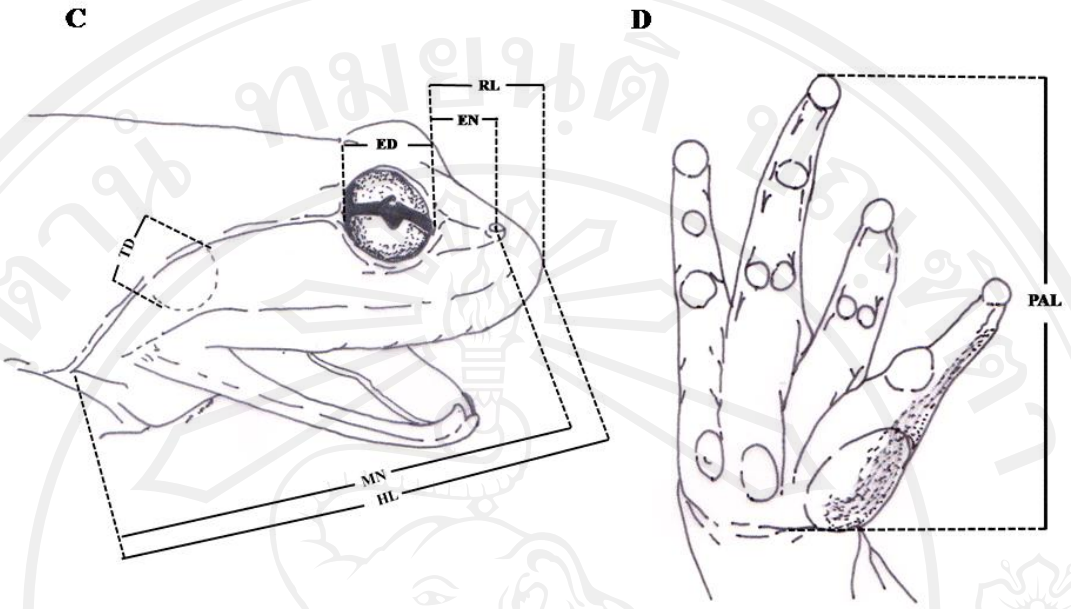

Figure 8 Morphological characters measured in this study. Dorsal aspect of *Limnonectes* Abbreviations: SVL = snout–vent length; FOL = foot length; TBL = Tibia length; FEL = femur length; UAL = upper arm length; IO = interorbital distance; IN = internarial distance; UEW = upper eyelid width; HW = head 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.

3.2.2 Morphometric Analyses

Morphological variation within the genus *Limnonectes* was quantified using morphometric and meristic analyses. Measurements were taken with either a ruler or with dial calipers, both recorded to the nearest 0.01 millimeter (mm). Sixteen measurements representing truss homologous points were made on 31 individuals of *L. blythii*, 31 individuals of *L. gyldenstolpei*, 54 individuals of *L. taylori*, 20 individuals of *L. magastomias* and 20 individuals of *L. jarujini*. (Table 3-7). Principal

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component (PC) analyses were conducted to estimate morphological variation by reducing the dimensionality of the dataset while retaining as much variation as possible (Jolliffe, 2002). The PC analyses were performed on a covariate matrix of log-transformed measurements in the SPSS 17.0 was used for statistical analyses. Univariate analyses were conducted to examine potential morphometric differences between the two lineages by plotting two measurements with opposite polarity (as identified by PC analyses). Counts for meristic analyses were obtained from ED/HL, WH/HL, IN/IO, EN/IN, UEW/IO, TBL/SVL and FEL/SVL on 156 individuals of five *Limnonectes* species.

3.3 Tissues sampling and DNA extraction

3.3.1 Tissues sample collection

Muscular tissue samples were preserved in 99.5% ethanol and stored at -20 °C for laboratory analyses. Most *Limnonectes* spp. specimens used in this study have been deposited in the Natural Science Research Division, National Science Museum of Thailand (THNSM) and Kunming Institute of Zoology (KIZ) (Table 1). Three related *Limnonectes* were also including in the analysis and the voucher numbers are listed in Table 1. Sequences of *Phrynoidis asper* and *Ansonia inthanon* were applied as outgroup in this study.

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3.3.2 DNA extraction protocol

Total genomic DNA was extracted from liver or muscle tissue using the standard phenol-chloroform protocols (Sambrook *et al*., 1989) as followed:

1. Take a small amount of tissue (~ 0.5 cm^2) and chop with a sterile scalpel blade. Transfer the sample to a labeled 1.5 ml microcentrifuge tube.

2. Add 500 µl of TNES buffer and 10 µl of Proteinase-K (20 mg/ml). Mix the sample by inverting the tube several times.

3. The extract was homogenised and incubated at 65 °C for 12 h

4. Add 500 µl of standard phenol : chloroform : isoamyl (25 : 24 : 1) and the tube was centrifuged for 10 min at 13,680 g.

5. Remove supernatant to a new, labeled 1.5 ml microcentrifuge tube and add 500 µl of chloroform to each tube. The tube was mixed well by inverse back and forth. The tube was centrifuged for 10 min at 13,680 g.

6. Remove supernatant to a new, labeled 1.5 ml microcentrifuge tube and add an equal volume 50 µl of 6 M NaCl and 900 µl of 95 % ethanol and gently mix by inverting the tube a couple of times. White DNA precipitate out of solution should visible at this point, store at -20 °C for 12 - 24 h.

7. Centrifuge the sample at full speed 13,680 g for 15 min at 4ºC. and supernatant was discarded.

8. DNA pellet was washed with 1 ml of 70 % ethanol at 13,680 g for 5 min, air dried and resuspended in 50 µl of TE buffer.

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3.3.3 Nuclear DNA sequencing

1. Rhodopsin gene

DNA amplification of nuclear DNA Rhodopsin gene gene sequences was obtained with the rhod1-a and rhod1-d primers designed by Vences *et al*. (2004)

rhod1-a: 5′-ACC ATG AAC GGA ACA GAA GGY CC-3′

rhod1-d: 5′-GTA GCG AAG AAR CCT TCA AMG TA-3′

The amplification was performed in a 25 µl volume reaction containing 1 µl (50 ng) of genomic DNA, 17.5 µl of dH_2O, 2.8 µl of 10x PCR buffer, 0.6 µl (10 pmol) of each primer, 1.2 µl (0.1 mg/ml) of BSA, 1.2 µl (10 mM) of dNTP and 0.12 µl (5U/µl) of *Taq* polymerase with the following procedures: initial denaturation step with 5 min at 95 °C, 35 cycles of denaturation 1 min at 95 °C, annealing for 1 min at either 55 °C for primer set or 45 °C for set , extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 7 min. PCR products were purified with Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai) and sequenced in both directions with BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems) and a ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The sequence data were translated into amino acids to confirm the absence of premature stop codons, i.e. to preclude the sequencing of nuclear DNA pseudogenes, and also submitted to a BLAST search in GenBank to confirm that the required sequences had been amplified.

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2. Proopiomelanocortin A (POMC)

DNA amplification of nuclear DNA POMC gene sequences was obtained with the POMC1 and POMC-7 primers designed by Wiens *et al*. (2005).

POMC1: 5′-GAATGTATYAAAGMMTGCAAGATGGWCCT-3′

POMC-7: 5′-TGGCATTTTTGAAAAGAGTCAT-3′

The amplification was performed in a 25 µl volume reaction containing 1 µl (50 ng) of genomic DNA, 17.5 µl of dH_2O, 2.8 µl of 10x PCR buffer, 0.6 µl (10 pmol) of each primer, 1.2 µl (0.1 mg/ml) of BSA, 1.2 µl (10 mM) of dNTP and 0.12 µl (5U/µl) of *Taq* polymerase with the following procedures: initial denaturation step with 5 min at 95 °C, 35 cycles of denaturation 1 min at 94 °C, annealing for 1 min at either 55 °C for primer set or 45 °C for set , extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 10 min. PCR products were purified with a Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai) and sequenced in both directions with BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems) and a ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The sequence data were translated into amino acids to confirm the absence of premature stop codons, i.e. to preclude the sequencing of nuclear DNA pseudogenes, and also submitted to a BLAST search in GenBank to confirm that the required sequences had been amplified.

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3. Recombination activating protein 1 (RAG-1)

DNA amplification of nuclear DNA gene RAG-1 gene sequences was obtained with the RAG_1F and RAG_1R primers designed by Shimada *et al*. (2011)

RAG_1F: 5′-GCMTTGCTSCCRGGGTATCA-3′

RAG_1R: 5′-AGRCARAGKGGTTTGCAGCA-3′

The amplification was performed in a 25 µl volume reaction containing 1 µl (50 ng) of genomic DNA, 17.5 µl of dH_2O, 2.8 µl of 10x PCR buffer, 0.6 µl (10 pmol) of each primer, 1.2 µl (0.1 mg/ml) of BSA, 1.2 µl (10 mM) of dNTP and 0.12 µl (5U/µl) of *Taq* polymerase with the following procedures: initial denaturation step with 5 min at 95 °C, 35 cycles of denaturation 1 min at 94 °C, annealing for 1 min at either 46 °C for primer set or 45 °C for set , extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 10 min. PCR products were purified with a Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai) and sequenced in both directions with BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems) and a ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The sequence data were translated into amino acids to confirm the absence of premature stop codons, i.e. to preclude the sequencing of nuclear DNA pseudogenes, and also submitted to a BLAST search in GenBank to confirm that the required sequences had been amplified.

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3.3.4 Mitochondrial DNA sequencing

1. 16S rRNA gene

DNA amplification of partial mitochondrial 16S rRNA gene sequences was obtained with the 16Sar and 16Sbr primers designed by Simon *et al*. (1994)

16Sar: 5′-GCCTGTTTAACAAAAACAT-3′

16Sbr: 5′-CCGGTCTGAACTCAGATCACGT-3′

The amplification was performed in a 25 µl volume reaction containing 1 µl (50 ng) of genomic DNA, 17.5 µl of dH_2O, 2.5 µl of 10x PCR buffer, 0.5 µl (10 pmol) of each primer, 1 µl (0.1 mg/ml) of BSA, 1 µl (10 mM) of dNTP and 0.2 µl (5U / µl) of *Taq* polymerase with the following procedures: initial denaturation step with 5 min at 95 °C, 35 cycles of denaturation 1 min at 95 °C, annealing for 1 min at either 55 °C for primer set, extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 7 min. PCR products were purified with a Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai) and sequenced in both directions with BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems) and a ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The sequence data were translated into amino acids to confirm the absence of premature stop codons, i.e. to preclude the sequencing of nuclear DNA pseudogenes, and also submitted to a BLAST search in GenBank to confirm that the required sequences had been amplified.

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2. Cytochrome Oxidase subunit 1 (CO1)

DNA amplification of partial mitochondrial COI gene sequences was obtained with the LCOCI1490 and HCOCI2198 primers designed by Folmer *et al*. (1994).

LCO1490: 5′-GGTCAA-CAAATCATAAAGATATTGG-3′

HCO2198: 5′-TA-AACTTCAGGGTGACCAAAAAATCA-3′

The amplification was performed in a 25 µl volume reaction containing 1 µl (50 ng) of genomic DNA, 17.5 µl of dH_2O, 2.5 µl of 10x PCR buffer, 1 µl (3 pmol) of each primer, 1 µl (0.1 mg/ml) of BSA, 1 µl (10 mM) of dNTP and 0.2 µl (5U/µl) of *Taq* polymerase with the following procedures: initial denaturation step with 5 min at 95 °C, 35 cycles of denaturation 1 min at 95 °C, annealing for 1 min at either 46 °C, extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 7 min. PCR products were purified with a Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai) and sequenced in both directions with BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems) and a ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The sequence data were translated into amino acids to confirm the absence of premature stop codons, i.e. to preclude the sequencing of nuclear DNA pseudogenes, and also submitted to a BLAST search in GenBank to confirm that the required sequences had been amplified.

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3. NADH dehydrogenase 2 (ND2)

DNA amplification of partial mitochondrial ND2 gene sequences was obtained with the H4980 and L4221 primers designed by Doughty *et al*. (2009).

H4980: 5′-ATT TTTCGTAGTTGGGTTTGRTT-3′

L4221: 5′-AAGGACCTCCTTGATAGGGA-3′

The amplification was performed in a 25 µl volume reaction containing 1 µl (50 ng) of genomic DNA, 17.5 µl of dH_2O, 2.8 µl of 10x PCR buffer, 0.6 µl (10 pmol) of each primer, 1.2 µl (0.1 mg/ml) of BSA, 1.2 µl (10 mM) of dNTP and 0.12 µl (5U/µl) of *Taq* polymerase with the following procedures: initial denaturation step with 5 min at 95 °C, 35 cycles of denaturation 1 min at 94 °C, annealing for 1 min at either 46 °C for primer set or 45 °C for set , extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 10 min. PCR products were purified with a Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai) and sequenced in both directions with BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems) and a ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The sequence data were translated into amino acids to confirm the absence of premature stop codons, i.e. to preclude the sequencing of nuclear DNA pseudogenes, and also submitted to a BLAST search in GenBank to confirm that the required sequences had been amplified.

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3.4 Molecular data analysis

Sample identification based on the sequence similarity approach was carried out using two databases; BOLD and GenBank. The highest percent pairwise identity of the consensus sequence from each species blasted (BLASTN) against NCBI were compared to the percent specimen similarity scores of the consensus sequence from each species within the BOLD-IDS (BOLD Identification System) (Ratnasingham and Hebert, 2007). To test the efficiency of DNA barcoding as a species identification tool, a blind sampling test was conducted, in which samples, identity unknown except to the submitting individual, were selected and sequenced. Nucleotide sequences were aligned using Clustal X 1.81 (Thompson *et al*., 1997) with default parameters, and then optimised by eye in MEGA 5.0 software (Tamura *et al*., 2011). 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) and MEGA 5.0 software (Tamura *et al*., 2011).

Phylogenetic analyses among haplotypes were conducted using maximum likelihood (ML) and Maximum Parsimony (MP) analyses were performed in MEGA 5.0 software. Each analysis used four heated Markov chains (using default heating values) that were run for 4 million generations. Trees were sampled every 1,000 generations and calculating a consensus tree was calculated after omitting the first 1,000 trees as burn-in. MP analyses were implemented using MEGA 5.0 software (Tamura *et al*., 2011). The heuristic MP searches were executed for 1,000 replicates with all characters treated as unordered and equally weighted. Tree searching used

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tree bisection reconnection (TBR) branch swapping. To assess nodal reliabilities, bootstrap analysis (BBP) was conducted using 2,000 replicates.

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