Original Article

Discordant morphological and genetic pattern in *Leptobrachium hendricksoni* (Anura: Megophryidae) from southern Thailand, revealed by non-parametric ANOVA with Monte Carlo simulations

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Abstract

In this study, we analysed patterns of intraspecific structuring between populations of *Leptobrachium hendricksoni* from southern Thailand using qualitative and quantitative morphological characters. Furthermore, we compared the phenotypic divergence with neutral genetic variation based on the concatenated mtDNA 12S, 16S and ND4 sequences (2,452bp). Non-parametric ANOVA analyses in combination with Monte Carlo simulations and Chi-square test for qualitative characters, showed differences in phenotypical variation between populations of *L. hendricksoni* from southern Thailand. UPGMA clustering based on size-and-shape variation and size variation, and simulations under scenario where each population was represented by 100 individuals (structuring in consensus tree p = 0.5), yielded a similar pattern of population distribution. Our results highlight the role of ecological mechanisms of adaptation to local habitat types and climate conditions as a driving force affecting the distribution of morphological characters within *L. hendricksoni* rather than genetic differentiation due to random drift and/or genetic flow.

Keywords: Megophryidae, southern Thailand, morphometric analyses, np-ANOVA, Monte Carlo simulations

1. Introduction

Leptobrachium hendricksoni Taylor (1962), is a frog species endemic to Southeast Asia and is distributed in southern parts of the Thai-Malay Peninsula, Sumatra and Borneo. In southern Thailand its distribution is limited to the low elevation mountain streams and swampy areas along Nakhon Si Thammarat and Titiwangsa mountain ranges (Draškić, Wangkulangkul, Martínez-Solano & Vörös, 2018; Matsui, Nabhitabhata & Panha, 1999; Taylor, 1962) (Figure 1). Previous molecular studies based on mitochondrial 12S and 16S genes showed the presence of deeply diverged lineages within *L. hendricksoni* across its distribution range (Draškić *et al.*, 2018; Matsui *et al.*, 2010*et al.*). Additionally, Draškić *et al.* (2018) reported that these lineages correspond to different mountain ranges or different parts of mountain ranges in Thai-Malay Peninsula and that populations from southern Thailand had independent evolutionary history from

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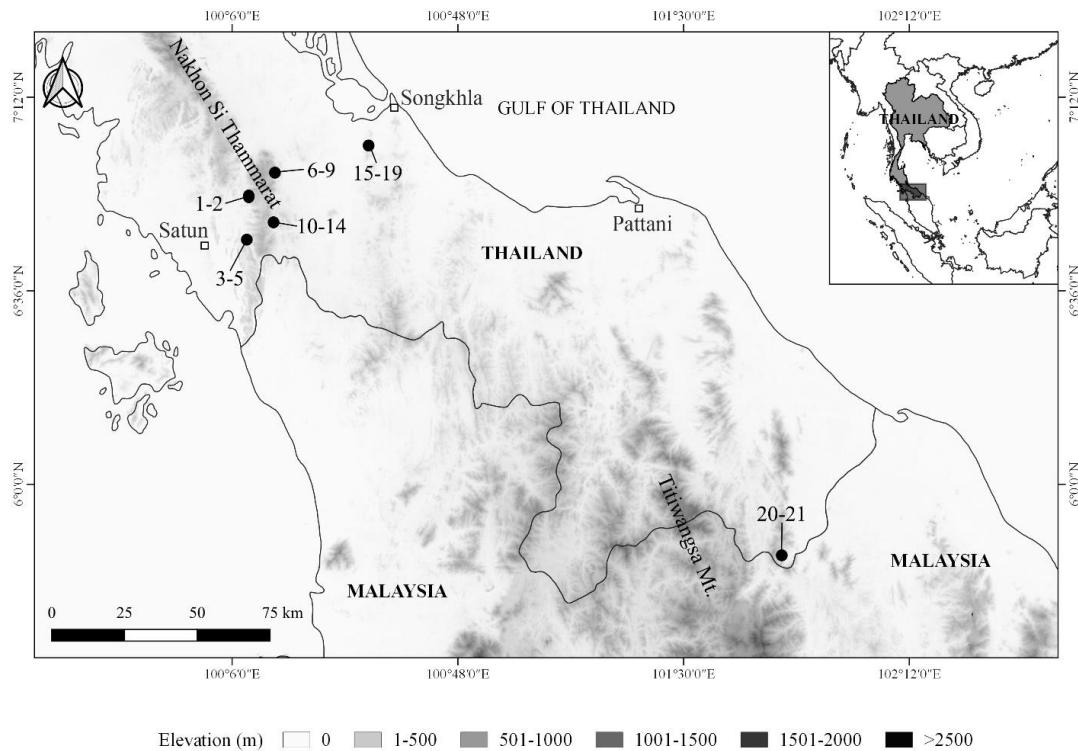Email address: gordon.draskic@gmail.com

Figure 1.   Map of southern Thailand showing sampling localities of *L. hendricksoni*: 1-2 Ton Pliu, 3-5 Thaleban, 6-9 Ton Nga Chang, 10-14 Kaichon stream, 15-19 Kho Hong hill, 20-21 Hala-Bala. Sample codes for molecular analyses as in Table 1. Shaded area represents mountain ranges.

conspecific populations in Malaysia and Sumatra. Sublineages from southern Thailand were very closely related and these studies did not detect an effect of the mountain ranges on mitochondrial DNA differentiation (Draškić *et al.*, 2018). However, studies on some other species from this genus showed the effect of fragmented habitat on genetic subdivision of populations (e.g. *within L. ailaonicum* (Zhang, Rao, Yang, Yu & Wilkinson, 2009) and *L. nigrops* (Hamidy, Matsui, Nishikawa & Belabut., 2012)).

Interpopulation variation studies on morphological traits are useful for evaluating divergence patterns, because intraspecific populations presumably developed unique characters in relatively recent times (Simões, Lima & Mangusson, 2008). There is a number of evidences reported in a large number of organisms that optimal trait values change with environmental variation to adapt to local conditions (Conover & Schultz, 1995; Huey, Gilchrist, Carlson, Berrigan & Serra, 2000; Rogell, 2009). Nakhon si Thammarat and Titiwangsa mountain ranges lay on different sides of the historical plant transition zone (Kangar – Pattani line: c. 7° N latitude along the Thai–Malay border) (Wikranmanayake *et al.*, 2002) and in different climate zones (Hughes, Satasook, Bates, Bumrungsri & Jones, 2011). Additionally, Nakhon si Thammarat range separates the central peninsula into East and West Coast, influenced by different monsoons and rainfall peaks causing different patterns of climate transition on each side of the peninsula (Woodruff, 2003).

Even though previous molecular studies solved confusion in species determination within *Leptobrachium hendricksoni* in southern Thailand, no quantitative morpho-

logical analyses have been conducted on this species. Taking into consideration restriction to forested environments along two mountain ranges in southern Thailand, this study aimed to analyse an intraspecific structuring between populations of *L. hendricksoni* using qualitative and quantitative morphological characters and genetic data based on partial mtDNA sequences.

2. Material and Methods

Specimens from a set of 80 live and dead individuals of *Leptobrachium hendricksoni* collected from field and museum collections were included in following morphometric and molecular analyses.

2.1 Morphometric analyses

1) Linear measurements and transformation of the Data

For morphometric analyses, 18 linear body measurements were taken from a subset of 66 male specimens from six localities (Figure1) only by a single observer (G.D.) to minimize interobserver measurement error (Hayek, Heyer & Gascon, 2001), using Vernier calliper to the nearest of 0.1 mm: 1) Snout-vent length (SVL), 2) Head length (HL), 3) Head width (HW), 4) Snout length (SL), 5) Inter-nostril distance (IN), 6) Snout-nostril length (SNL), 7) Nostril-eye distance (N-EL), 8) Eye length (EL), 9) Interorbital distance (IOD), 10) Tympanum-eye length (T-EL), 11) Tympanum

diameter (TD), 12) Forelimb length (FLL), 13) Hand length (HAL), 14) Inner palmar tubercle length (IPTL), 15) Outer palmar tubercle length (OPTL), 16) Tibia length (TL), 17) Foot length (FL), and 18) Inner metatarsal tubercle length (IMTL). In order to investigate and separate allometric effect in the morphometric data, the matrix of 18 linear body measurements was transformed with base 10 logarithm transformations (Huxley, 1932) and afterwards centred prior to further analyses to investigate and separate allometric effect in the data (Kerkhoff & Enquist, 2009). Moreover, first principal component performed on log-transformed linear distances usually captures size-related variation and can be considered as general size axis (Jolicoeur, 1984; Jolicoeur & Mosimann, 1960).

2) Statistical analyses

Excluding simulations, all the analyses were performed on three levels: 1) total variation – log-transformed measurements were considered as size-and-shape variation; 2) size variation – PC1 axis from PCA conducted on log-transformed measurements was considered as a general size; 3) shape variation – PC1-corrected trait values were obtained by applying Burnaby back-projection (Burnaby, 1966) following the procedure outlined in Claude (2008).

Relationships of six populations were statistically analysed with non-parametric ANOVA (np-ANOVA) - a robust non-parametric method which can partition variation based on any distance measurement in any ANOVA design, but lacks formal assumptions concerning distribution of variables (Anderson, 2001). This method is specifically adopted for designs where the number of variables is greater than the number of individuals (Collyer, Sekora & Adams, 2015). Statistical evaluation was based on 999 permutations and np-ANOVA on combined traits was performed in Geomorph package v.3.0.3 (Adams & Otárola-Castillo, 2013) in R statistical environment v.3.4.1 (R Development Core Team, 2017). Relationship among population-specific grand means was visualized with clustering methods based on UPGMA algorithm in R statistical environment.

3) Simulations of uncertainty due to sampling

Due to small and unequal sample size across populations, we performed Monte Carlo simulations in order to assess whether and to which extent sampling error influenced patterns found in ANOVA and UPGMA. Random samples (datasets) were generated from multivariate Gaussian distribution with parameters μ and Σ, using MASS package v.7.3.47 (Venables & Ripley, 2002) in R. μ represents k-dimensional mean vector and Σ represents k x k covariance matrix (k equals to the number of traits, which for this study is 18). Since sampled sites are relatively close to each other, simulations were performed assuming two scenarios: 1) each population has unique μ and Σ which were equal to observed population (treating sampled sites as unique populations); and 2) all populations have the same μ and Σ which were equal to observed μ and Σ of the whole dataset (treating whole sample as one population). The first scenario mimics uncertainty due to sampling assuming that populations have population-specific parameters whereas the second one mimics uncertainty due to sampling assuming all six populations are

actually subsets of larger panmictic population.

For each scenario, we generated 1000 datasets with 1) observed number of individuals per population, and 2) assumption that each population had 100 individuals. In other words, we performed four independent runs of Monte Carlo simulations and in each we simulated 1000 datasets with specified μ and Σ parameters. Afterwards, each simulated dataset was transformed by Burnaby back-projection and np-ANOVA and UPGMA were performed as outlined above. We compared observed np-ANOVA parameters such as effect size (Z, sensu Collyer *et al.*, 2015) and coefficient of determination (R^2) with distribution of those parameters obtained through Monte Carlo simulations. If random sampling error is negligible, it is expected that observed values of the two np-ANOVA parameters are within the distribution of those parameters generated under scenario 1. Likewise, if populations have their own population-specific parameters (e.g. trait mean values and traits covariance structure) it is expected that observed values of two np-ANOVA parameters are outside the distribution of those parameters generated under scenario 2. On the other hand, if observed np-ANOVA parameters are within the distributions generated with Monte Carlo simulations under scenario 2, the possibility that the observed pattern of populations differences is a consequence of sampling error from one larger, panmictic population cannot be ruled out.

Finally, consensus UPGMA tree was computed for the set of trees found through simulations and compared with the observed UPGMA topology. Results of the sampling error simulations are reported only for shape data as differences in shape among populations are more consistent with genetic data than size. It is important to highlight that the estimation of sampling uncertainty with the approach outlined here assumes that there is no measurement error in the data. Consensus UPGMA tree was calculated with ape package v.4.1 (Paradis, Claude & Strimmer, 2004) for R statistical language and the code to reproduce the analyses of sampling error is available on request from the fourth co-author.

4) Qualitative morphological analyses

For qualitative morphological analyses, 63 live specimens were studied due to the lack of colour in museum specimens. Following attributes were coded: 1) Eye colour (1 = orange, 2 = upper half or third orange, 3 = scarlet, 4 = upper half or third scarlet, 5 = yellow and 6 = upper half or third yellow), 2) Orange markings on the head (1 = moderately present, 2 = markedly present and 3 = absent), 3) Dorsal markings (1 = blotched, 2 = spotted, 3 = reticulated, 4 =partly absent 5 =present only on the head, 6 =partly present only on the head and 7 = absent), 4) Tympanic dark mask (1 = present all over, 2 = present on upper half to two thirds, 3 = partly present and 4 = absent), and 5) Hindlimb marking (1 = lined, 2 = blotched, 3 = spotted and 4 = absent). We performed non-parametric Chi-Square test for independence integrated in SPSS v.22 (IBM) on combined dataset of both male and female specimens.

5) Molecular analyses

For molecular analyses, a subset of 21 individuals of *L. hendricksoni* was sampled from six sites on the Nakhon

Si Thammarat and Titiwangsa mountain ranges in southern Thailand between February 2014 and January 2016 (Figure 1, Table 1). We sequenced mitochondrial ND4 gene and complemented our dataset with already published sequences of mitochondrial 12S and 16S genes from same individuals from our previous study (Draškić *et al.*, 2018). Additionally, we used sequences of *L. boringii* (Liu, 1945) from GenBank as an outgroup (see accession number in Table 1). Tissue samples for molecular analyses were obtained from either liver of dead or toe clips from live specimens. Sampling was authorized by the National Park, Wildlife and Plant Conservation Department, Thailand. All tissue samples were preserved in 95% ethanol and kept in freezer. The specimens are stored in the reference collection of Prince Maha Chakri Sirinthorn Natural History Museum at Prince of Songkla University Hat Yai for future reference.

6) DNA extraction, PCR and sequencing

DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN) or using phenol chlorphorm and following the DNA extraction method of Collins *et al.* (1987). PCR was run in a total volume of 25 µl or 50 µl and PCR protocol was 94ºC for 5 min, 35 cycles at 94ºC for 60 sec, 44-49ºC for 30 sec and 72ºC for 60 sec, and final extension at 72ºC for 5 min. Primers used in PCR were ND4 and Leu (Arévalo, Davis & Sites, 1994). Some of the amplified double strand products were purified using High Pure PCR Product Purification Kit (Roche) and directly sequenced in both directions following the ABI Prism BigDye Terminator Cycle sequencing protocol on an ABI 3130 Genetic Analyser (Applied Biosystems).

Other PCR products were purified using Favorgen Gel/PCR Purification Mini Kit and sent to Macrogen Inc., Korea for sequencing.

7) Alignment and genetic diversity analysis

BioEdit Sequence Alignment Editor (Hall, 1999) was used to edit and align the sequences. For all downstream analyses the ND4, 12S and 16S sequences were trimmed and combined into a single alignment. The number of haplotypes (N) and estimates of haplotype diversity (h; Nei, 1987) and nucleotide diversity (π; Nei & Tajima, 1981) were computed using DnaSP 5.1 (Librado & Rozas, 2009).

8) Phylogenetic analysis

Phylogenetic analyses based on Bayesian inference were run with Mr.Bayes 3.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist, Huelsenbeck & Teslenko, 2011). We used software jModelTest 2.1.7 (Darriba, Taboada, Doallo & Posada, 2012) to find the best DNA substitution model for each gene, using default settings and the Akaike Information Criterion (AIC). We set a 4-by-4 model for all genes with the general time reversible (GTR) nuclear substitution model for 12S gene, GTR with proportion of invariant sites (GTR+I) for 16S gene, and Hasegawa-Kishino-Yano nuclear substitution model with a gamma distribution (HKY+G) for ND4 gene. Phylogenetic reconstruction was performed running Metropolis-coupled Markov chain Monte Carlo sampling with four chains for 3×10^6 generations, sampling every 100th tree.

Table 1.   List of samples of *L hendricksoni* and outgroups used in phylogenetic analyses. The list includes samples from this study and from previous studies, with information on vouchers, GenBank accession numbers and sampled localities.

No	Species	Voucher	GenBank			Locality	Haplotypes
			12S	16S	ND4		
01	*L. hendricksoni*	Herp.A 01502	MF686827	MF686854	MK770804	THA, SA, Ton Pliu	H1
02	*L. hendricksoni*	Herp.A 01504	MF686829	MF686856	MK770805	THA, SA, Ton Pliu	H2
03	*L. hendricksoni*	Herp.A 01497	MF686832	MF686859	MK770806	THA, SA, Thaleban	H3
04	*L. hendricksoni*	Herp.A 01499	MF686834	MF686861	MK770807	THA, SA, Thaleban	H4
05	*L. hendricksoni*	Herp.A 01501	MF686836	MF686863	MK770808	THA, SA, Thaleban	H1
06	*L. hendricksoni*	Herp.A 01508	MF686837	MF686864	MK770809	THA, SO, Ton Nga Chang	H5
07	*L. hendricksoni*	Herp.A 01511	MF686838	MF686865	MK770810	THA, SO, Ton Nga Chang	H6
08	*L. hendricksoni*	Herp.A 01517	MF686840	MF686867	MK770811	THA, SO, Ton Nga Chang	H7
09	*L. hendricksoni*	Herp.A 01518	MF686841	MF686868	MK770812	THA, SO, Ton Nga Chang	H5
10	*L. hendricksoni*	Herp.A 01471	MF686842	MF686869	MK770813	THA, SO, Sadao, Kaichon	H1
11	*L. hendricksoni*	Herp.A 01472	MF686843	MF686870	MK770814	THA, SO, Sadao, Kaichon	H5
12	*L. hendricksoni*	Herp.A 01473	MF686844	MF686871	MK770815	THA, SO, Sadao, Kaichon	H1
13	*L. hendricksoni*	Herp.A 01475	MF686845	MF686872	MK770816	THA, SO, Sadao, Kaichon	H1
14	*L. hendricksoni*	Herp.A 01485	MF686846	MF686873	MK770817	THA, SO, Sadao, Kaichon	H1
15	*L. hendricksoni*	Herp.A 01489	MF686847	MF686874	MK770818	THA, SO, Kho Hong Hill	H5
16	*L. hendricksoni*	Herp.A 01490	MF686848	MF686875	MK770819	THA, SO, Kho Hong Hill	H5
17	*L. hendricksoni*	Herp.A 01491	MF686849	MF686876	MK770820	THA, SO, Kho Hong Hill	H5
18	*L. hendricksoni*	Herp.A 01492	MF686850	MF686877	MK770821	THA, SO, Kho Hong Hill	H5
19	*L. hendricksoni*	Herp.A 01493	MF686851	MF686878	MK770822	THA, SO, Kho Hong Hill	H5
20	*L. hendricksoni*	Herp.A 0742	MF686852	MF686879	MK770823	THA, NA, Hala-Bala	H8
21	*L. hendricksoni*	Herp.A 01520	MF686853	MF686880	MK770824	THA, NA, Hala-Bala	H9
22	*L. boringii*	SCUM120630		NC024427		CHN, SI, Emei Mt.	

No: sample codes as in Figures 1 and 2.

Countries and provinces: THA: Thailand, CHN: China; SA: Satun, SO: Songkhla, NA: Narathiwat, SI: Sichuan.

References: *L. hendricksoni* 12S and 16S: Draškić *et al.*; *L. hendricksoni* ND4: this study; *L. boringii* 12S, 16S, ND4: Xu *et al.* (2014).

Additionally, a Maximum Likelihood (ML) phylogenetic analysis on the combined dataset was performed using MEGA 6 (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). We set the general time reversible nuclear substitution model with a gamma distribution and proportion of invariant sites (GTR+G) as calculated by jModelTest under the AIC. Gaps and missing data were excluded, and 1000 non-parametric bootstrap replicates were performed to evaluate clade support.

A minimum spanning haplotype network (Bandelt, Forster & Röhl, 1999) was constructed using Popart 1.7 (Leigh & Bryant, 2015) to visualize relationships among haplotypes, their relative frequencies, and patterns of haplotype sharing and geographical extent.

Analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) on the combined dataset was performed using ARLEQUIN 3.5 (Excoffier & Lischer, 2010). F-statistics were used to estimate the proportion of genetic variability found among populations (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). For these analyses, populations were grouped according to the clades found in the phylogenetic analyses. Additionally, we determined levels of genetic differentiation among populations using F_{ST} (Weir & Cockerham, 1984) in ARLEQUIN 3.5 (Excoffier & Lischer, 2010).

3. Results

3.1 Morphometric analyses

1) Observed differences among populations

Non-parametric ANOVA consistently showed that the six populations phenotypically differed at all three levels (Table 2). Based on coefficient of determination (R^2), the differences among populations accounted for 33%, 24% and 18% of the overall variation of size, size-and-shape and shape data, respectively. PC1 axis (general size) accounted for 42.15 % of the total size-and-shape variation and the size of each population (i.e., the PC1 scores averaged by population) is depicted in Figure 2. PC1 exhibited the greatest correlation with SVL variable (0.92) and lowest with IN variable (0.35). All PC1 loadings had the same sign and the range of loadings was 0.11 (IN) – 0.3 (OPTL). Theoretical value of isometry (Jolicoeur, 1963) for our dataset was 0.23 and loadings of two variables approached this threshold (HW, TL) while the others exhibited slightly greater or lesser values relative to the theoretical expectation. Np-ANOVA showed lack of interaction between overall size (PC1) and population factor (p = 0.244) indicating that allometry is homogenous across the populations, and this further allowed size correction using Burnaby back-projection. UPGMA clustering based on size-and-shape variation and size variation yielded a similar pattern of population distribution (Figure 3).

2) Differences among populations after simulations

The distributions of Z and R^2 values from np-ANOVA obtained after simulations under scenario 1 and scenario 2 are presented in Figure 4, whereas mean, standard error and range of Z and R^2 values are reported in Table 3. Except for the distribution of Z values generated under scena-
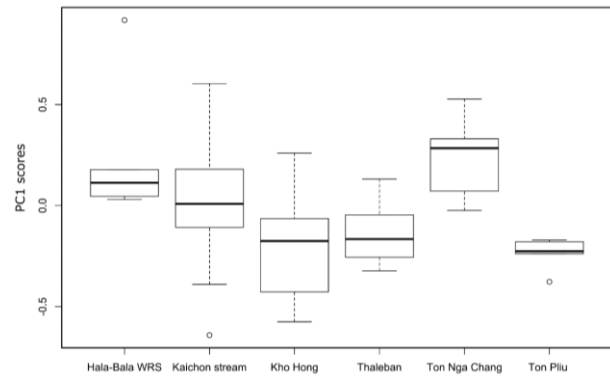

Figure 2. Box plot shows the distribution of population-specific overall size. Bold, ticker black line within each box represents the median of population-specific PC1 scores, the boxes represent lower and upper quartiles, and the whiskers range from minimal to maximal values excluding outliers (empty circles).
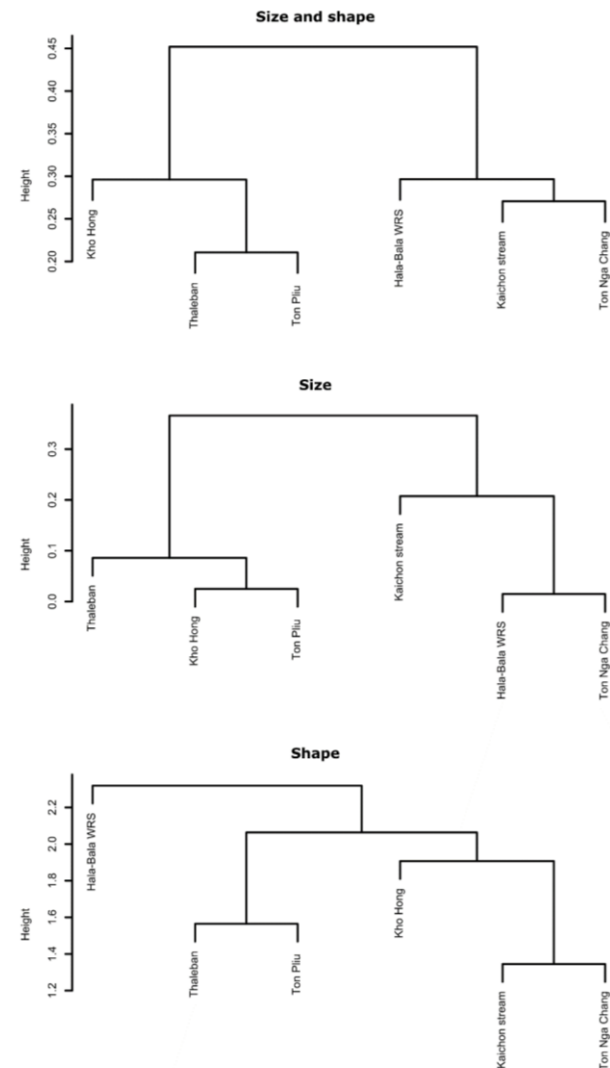

Figure 3. Clustering of populations based on size-and-shape, size, and shape variation, using UPGMA algorithm.

Table 2.   Distance based ANOVAs performed on size-and-shape, size, and shape variation. Significance was estimated with 999 iterations.

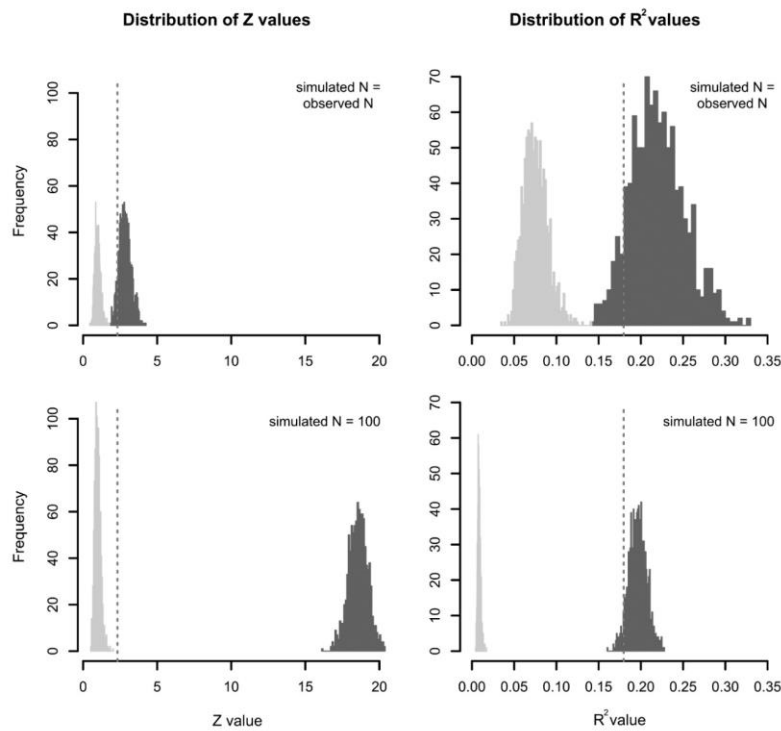|	Terms	Df	SS	MS	R^2	F	Z	Pr(>F)
Size and shape	Pop.	5	3.399	0.680	0.24	3.91	3.09	0.001
	Residuals	61	10.607	0.174				
	Total	66	14.007					
Size	Pop.	5	1.950	0.390	0.33	6.02	3.76	0.001
	Residuals	61	3.954	0.065				
	Total	66	5.904					
Shape	Pop.	5	96.330	19.267	0.18	2.67	2.32	0.001
	Residuals	61	439.820	7.210				
	Total	66	536.150					

Figure 4.   Distributions of effect size (Z) and coefficient of determination (R^2) obtained after performing np-ANOVAs on the 1000 simulated datasets from multivariate Gaussian distribution under scenario 1 (each population has unique mean and covariance, μ and Σ, respectively –dark gray distributions), and under scenario 2 (populations have the same mean and covariance, μ and Σ, respectively – light gray distributions), and transformed with Burnaby back-projection (shape data). Sample size (N) for graphs (simulations) in the first and second row was equal to observed number of individuals per population (Hala-Bala WRS: 6, Kaichon stream: 27, Kho Hong: 12, Thaleban: 6, Ton Nga Chang: 10 Ton Pliu: 6) and 100 individuals per population, respectively. Black dashed lines represent observed values for Z and R^2.

Table 3.   Mean, standard error (SE) and range of Z and R^2 values after Monte Carlo simulations under scenario 1 and scenario 2. Observed values for Z and R2 for shape data were 2.32 and 0.18, respectively.

Scenario	Sample size	Z			R^2		
		Mean	SE	Range	Mean	SE	Range
Scenario 1	N = observed	2.83	0.41	1.80-4.23	0.22	0.03	0.14-0.33
	N = 100	18.57	0.65	16.16-20.38	0.18	0.01	0.16-0.23
Scenario 2	N = observed	0.97	0.19	0.46-1.80	0.07	0.01	0.03-0.14
	N = 100	0.98	0.20	0.52-2.01	0.008	0.002	0.004-0.017

rio 1 (assuming that each population had 100 individuals), it was obvious that the observed Z and R^2 were within the minimal and maximal distributions generated under scenario 1, but not under scenario 2 (see the range in Table 3). As

regards scenario 1, the observed Z and R^2 values appeared a bit lower relative to the means of the distributions of those values from the simulations (Figure 4).

Consensus trees constructed from the set of trees obtained after simulations under scenario 1 are presented in Figure 5. The maximum frequency of bipartitions was observed for Kaichon stream and Ton Nga Chang populations (they appeared 583 times as monophyletic group) when simulations were conducted under scenario 1 and sample size was the same as observed number of individuals per population (see majority-rule consensus tree in the Figure 5). Likewise, the other populations did not show any clustering at all and the trees appeared comb-like. However, after simulations under scenario 1 where each population was represented with 100 individuals, structuring in the consensus trees (p = 0.5; p = 0.6) appeared more evident. All consensus trees constructed after simulations under scenario 2, regardless of the sample size, were comb-like, showing lack of any structure in the data.

3) Qualitative morphological analyses

Chi-Square test showed significant difference between localities in all five qualitative characters ($p<0.05$). In the eye colouration, most specimens had upper half or third of eye orange (χ^2 (df=10) = 44.19, $p < 0.001$). However, individuals from Kaichon stream and Hala-Bala differed from others in having only upper half or third of eye orange, whereas most individuals from Ton Pliu had orange eyes. In addition, one individual from Ton Pliu had yellow eyes. Orange markings on the head were only found in two lo-

calities (χ^2 (df=10) = 44.66, $p < 0.001$). They were moderately present in most individuals from Thaleban and in half of individuals from Ton Pliu, whereas one specimen from Thaleban had markedly present orange markings. In tympanic dark mask, individuals from Kaichon differed from others in mostly having dark mask present on upper half to two-thirds of tympanum whereas individuals from other localities mostly had partly present tympanic dark mask (χ^2 (df=10) = 19.19, $p = 0.038$). Only one individual from Ton Nga Chang had blotched dorsal marking (χ^2 (df=25) = 45.03, $p = 0.008$). In contrast, most samples from Kho Hong hill lacked any dorsal markings. In hindlimb markings, samples from Kaichon stream and Ton Pliu differed from other localities in having mostly blotched hindlimbs whereas all samples from Hala-Bala and most samples from Kho Hong and Ton Nga Chang had lined markings (χ^2 (df=10) = 25.73, $p = 0.004$). Additionally, spotted hindlimbs were present only in one sample from Ton Nga Chang.

4) Genetic diversity and population subdivision

Combined sequences of mitochondrial 12S, 16S and ND4 genes consisted of 2452 sites (761 bp of 12S, 943 bp of 16S and 748 bp of ND4 sequences). Excluding outgroup individuals, there were 20 polymorphic sites. We identified nine haplotypes (H1-H9) among 21 individuals (Table 1) with haplotype diversity of (h) = 0.795 and nucleotide diversity of (π) = 0.0023.
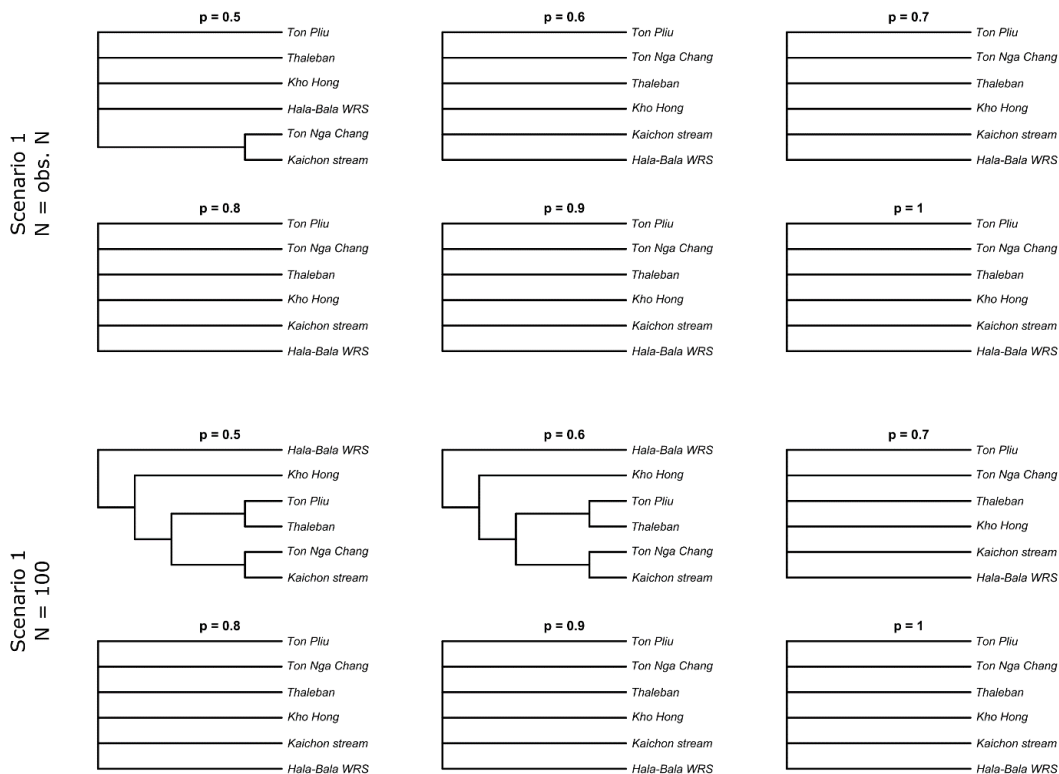

Figure 5.   Consensus trees from morphological data with a set of different probabilities (p) for a clade to be represented in the consensus tree found after simulations under scenario 1 (sampled sites treated as unique populations). The majority-rule consensus tree is given with p = 0.5, while the strict-consensus tree is computed with p = 1.

From six locations where multiple sequences were sampled, four localities contained private haplotypes (H2 Ton Pliu, H3 and H4 Thaleban, H6 and H7 Ton Nga Chang, and H8 and H9 Hala-Bala) while two haplotypes (H1 and H5) were shared among populations. Haplotype H1 was found in Ton Pliu, Thaleban and Kaichon whereas H5 was shared between Kaichon, Kho Hong Hill and Ton Nga Chang (Table1, Supplementary Figure S1).

The results of AMOVA confirmed the presence of phylogeographic structure in our data. Most of the molecular variation was distributed among groups (58.07%, p = 0.016) rather than within populations (37.04%, p = 0.000) and among populations within groups (4.88%, p = 0.19). We found high levels of genetic differentiation among populations with F_{ST} range -0.17 – 0.92 (Table 4).

5) Phylogenetic analyses

Both Bayesian inference and Maximum Likelihood analyses resulted in similar tree topologies (Figure 6) in which *L. hendricksoni* haplotypes formed monophyletic group. This monophyletic group was split in two well supported but closely related major clades including 1) individuals from two localities on east side of the Nakhon Si Thammarat mountain

Table 4.   Estimates of pairwise F_{ST} based on partial mtDNA loci. Boldfaced values were statistically significant at p = 0.05.

	Thaleban	Ton Pliu	Kaichon	Kho Hong Hill	Ton Nga Chang
Thaleban	-				
Ton Pliu	0.00000	-			
Kaichon	-0.16883	0.25319	-		
Kho Hong Hill	0.55224	0.65517	0.75000	-	
Ton Nga Chang	0.28713	0.45631	0.58283	0.17526	-
Hala-Bala	0.62500	0.06667	0.78583	0.91597	0.80000

Figure 6.   Maximum likelihood (ML) phylogram of 2452bp of combined 12S rRNA, 16S rRNA and ND4 mitochondrial genes for samples of *L. hendricksoni* and an outgroup. Sample codes and localities as in Table 1. Numbers on nodes represent Bayesian posterior probabilities and ML bootstrap support values, respectively (BPP/ML). Scale bar on bottom represents number of substitutions per site.

range together with individuals from the Titiwangsa mountain range (Lineage A, including all samples from Ton Nga Chang, Kho Hong Hill and Hala-Bala, plus one sample from each Thaleban, Kaichon stream and Ton Pliu); and 2) west and east side of the Nakhon si Thammarat range (Lineage B, most of samples from Thaleban and one sample from Ton Pliu on the west, plus most individuals from east side locality Kaichon stream). In addition, samples from Titiwangsa mountain range (Hala-Bala) together with one sample from Ton Pliu formed a not well supported sublineage within Lineage A. Average *p*-distance between lineages was 0.3 %.

4. Discussion

Non-parametric ANOVA analyses showed pheno-typic differentiation between populations of *L. hendricksoni* from southern Thailand. Based on a shape variation, all populations from Nakhon si Thammarat mountain range clustered together whereas Hala-Bala, located on Titiwangsa mountain range, was the most distinct from other populations. This pattern corresponds to Kangar – Pattani transition zone, similarly to some species of bats (Ith, 2014). Even though these results are based on a small sample size, Monte Carlo simulations based on a majority rule consensus tree (p=0.5) showed similar structure as observed in UPGMA shape topology with the only difference in the position of Kho Hong hill clade. Additionally, two qualitative characters, orange markings on the head and yellow eyes (found only in Ton Pliu), were restricted to the localities on the west side of the Nakhon si Thammarat mountain. On the other hand, individuals from Hala-Bala located on the Titiwangsa mountain differed from others in having only lined hindlimb markings whereas in populations from the Nakhon si Thammarat mountain these markings had certain level of variation. The difference between UPGMA and majority rule consensus tree, and maximum likelihood tree topologies suggests that these morphological patterns were not well supported by molecular analyses, neither from this nor from previous studies (Draškić *et al.*, 2018). Although the subclade from southernmost Hala-Bala appeared to be closely related to other eastern populations from Lineage A, the haplotype distribution and F_{ST}-based genetic differentiation between populations showed that Hala-Bala is highly isolated from populations from Nakhon si Thammarat mountains. Likewise, AMOVA results were congruent with the phylogenetic analyses showing that the among group variance received highest support. This implies that ancestors of both southern and eastern sublineages once co-existed before dispersing to colonize available habitats and later genetic and geographic isolation. Furthermore, neutral markers can be used to assess the significance of ecological and phenotypic differences due to only genetic drift within populations or drift and gene flow between populations (Gay *et al.*, 2009; Nosil, Egan & Funk, 2008). This suggests that the subdivision occurred in a more recent timeframe with the underlying cause associated with adaptive responses to different microhabitats and climates, which corresponds with minor diversification events within *L. hendricksoni* from 0.7-0.02 million years ago (Draškić *et al.*, 2018).

The distribution of anurans is mostly influenced by habitat type. The sampled populations from Nakhon si Thammarat range are not far from each other (up to 30 km) and the present time geomorphological zone between sampled localities does not show any physical barriers (elevation up to 700 m) that could prevent individuals of *L. hendricksoni* from crossing. However, Monte Carlo simulations showed that the observed populations can be treated as unique and not as a part of a larger panmictic population. Individuals of *L. hendricksoni* were all found in low elevations and in lower courses of streams with the water depth up to 0.5 m, yet in different habitat types. In Thaleban, *L. hendricksoni* was found in a swampy area on a muddy ground covered with litter leaves, whereas in Ton Pliu and in Kaichon, these frogs were found on sandy depositions in wide (around 4–5 m) mountain streams near slow flowing water. In contrast, in Ton Nga Chang and on Kho Hong hill these frogs were found near small mountain streams, on the ground covered with rocks and litter leaves. Additionally, in Hala-Bala *L. hendricksoni* was found on ground near slow-flowing-water parts of a wide (around 5 m), stony mountain stream. All this suggests that the distribution of some morphological characters might be in direct correlation with the position of localities within mountain ranges in southern Thailand, and that these differences are more likely associated with the adaptations to local habitat types and climate conditions rather than genetic isolation with genetic drift and/or gene flow. Considering apparent restriction of *L. hendricksoni* in Thailand to the lowlands and low elevation mountain streams along Nakhon si Thammarat and Titiwangsa mountain ranges (Matsui *et al.*, 2010; Taylor, 1962) it is likely that an ecological mechanism of adaptation to a discrete environmental gradient across geological domains (*sensu* Endler, 1982) is the driving force for a distribution of morphological characters in *L. hendricksoni* rather than natural barriers.

5. Conclusions

This is one of the first studies of *L. hendricksoni* to examine morphological differences between populations in southern parts of peninsular Thailand. We confirmed iris variation between and within population from previous studies and found significant differences in morphometric traits between populations. These differences were not supported by mtDNA phylogenetic analyses suggesting that they were possibly shaped by the habitat and climate patterns, although this should be further explored in a larger sample with more sensitive methods, such as geometric morphometrics, and with additional possibly more variable molecular markers, such as microsatellites.

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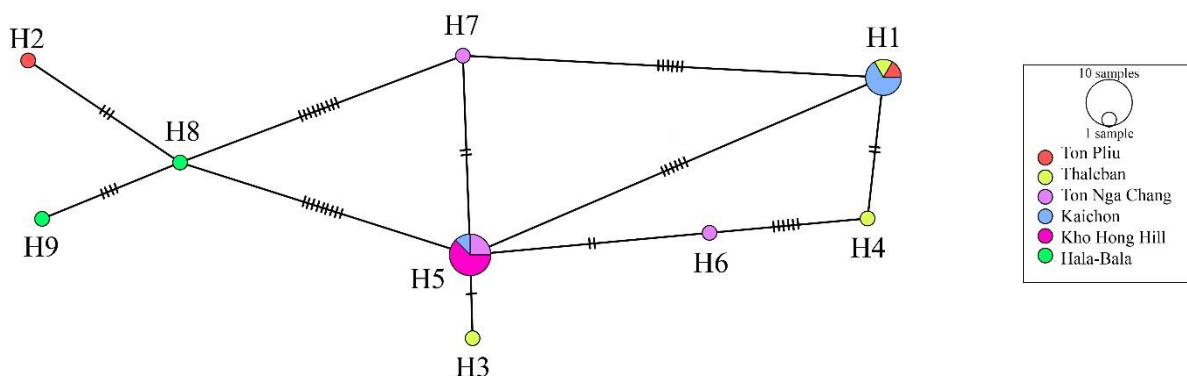
Appendix

Figure S1. Minimum spanning haplotype network of combined mitochondrial 12S, 16S and ND4 sequences showing the relationships among haplotypes of *L. hendricksoni*. Circles represent haplotypes, with sizes proportional to the number of individuals sharing that haplotype. Hatch marks on the branches represent the number of mutations distinguishing haplotypes.