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Phylogenetic Patterns of the Southeast Asian Tree Frog *Chiromantis hansenae* in Thailand

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Abstract Although landscape features such as mountains and rivers are recognized often as limiting factors to amphibian dispersal and gene flow, a limited number of studies have investigated such patterns across Southeast Asia. A perfect example of this is Thailand, located in one of the world's biodiversity hotspot regions. Thailand represents the corridor between mainland Asia and the Sunda Shelf, a famous and widely recognized biogeographic region, and yet there are few studies on the genetic structure among populations of amphibian species distributed across Thailand. The Southeast Asian tree frog, *Chiromantis hansenae* has been reported to possess a geographic range that is restricted to Thailand and, presumably, Cambodia. Here, we investigate phylogenetic relationships among *C. hansenae* populations using partial sequences of the mitochondrial 16S rRNA gene and nuclear POMC gene. Our results reveal two distinct evolutionary lineages within *C. hansenae* populations in Thailand. The genetic divergence among populations between these two clades is considerable, and results support inter-population divergence, and high genetic differentiation (pairwise $F_{ST} = 0.97$), between two localities sampled in western Thailand (TK1 and TK2), separated from each other by 40 kilometers only. The results suggest that landscape features across Thailand may have a profound impact on patterns of diversification in the country, underscoring the urgent need for fine-scale investigations of genetic structure of endemic and "widespread" species.

Keywords 16s rRNA, Genetic diversity, Isthmus of Kra, Landscape barriers, POMC, Southeast Asia

1. Introduction

The biodiversity of many complex landscapes is shaped by geologic events and climatic changes (Zink, 2002). Knowledge of the degree to which these environmental changes and conditions impact putatively widespread species provides critical information on the evolutionary trajectories of lineages (Hickerson *et al*., 2010) and the regional distribution of diversity. Today, a necessary first step in exploring the impact these potential forces have on species diversification is to study phylogeographic diversity across a species distribution. From this baseline information, multi-taxon, or comparative phylogeographic studies based on ecological, demographic, and molecular data (e.g., Zink, 2002; Lessa *et al*., 2003; Feldman and Spicer, 2006), can then allow for more robust inferences about barriers to gene flow, concordance between population genetic structures, and the influences of geography and climate on the evolution of the ranges of species (Arbogast and Kenagy, 2001; Knowles and Alvarado-Serrano, 2010).

Southeast (SE) Asia has quickly become an intriguing system for investigating the effects of ecological, tectonic,

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and climatic processes on vertebrate diversification because it has a complex geological history and diverse geography (Evans *et al.*, 2003; Jansa *et al.*, 2006; Brown *et al.*, 2013). Within this region, the Thai-Malay Peninsula has been a classic system for studies investigating patterns and processes of diversification and faunal transitions, particularly the Isthmus of Kra (reviewed by Woodruff and Turner, 2009). The Isthmus of Kra (~10°30' N) covers a transition between two distinct zoogeographic subregions, the Indochinese and Sundaic (Woodruff and Turner, 2009). Researchers have documented high percentages of species turnover across this region; examples include birds (greater than 50% turnover; Hughes *et al.*, 2003; Round *et al.*, 2003; Woodruff, 2003a,b), plants (Wikramanayake *et al.*, 2002; Woodruff and Turner, 2009), and mammals (for review, see Woodruff and Turner, 2009). Although the regional position of floral and faunal turnover across the Isthmus of Kra coincides with a major north-south climate gradient, recent studies indicate the factors driving these species transitions are more complex, and likely involve dramatic sea-level changes resulting from historical climate oscillations (Woodruff, 2003b; Pimvichai *et al.*, 2014). These findings are consistent with a nascent body of literature focused on other regions of Southeast Asia (for review, see Siler *et al.*, 2014; Brown *et al.*, 2013). To date, few phylogenetic studies have focused on population- and species-level diversity across this historically famous ecotone (Pimvichai *et al.*, 2014); however, arboreal frogs of the family Rhacophoridae represent an ideal group for such investigations.

Although our understanding of species-level diversity among frogs of the family Rhacophoridae has improved greatly over the last decade, largely as a result of numerous phylogenetic studies aimed at elucidating genetic, ecological and morphological diversity (Yu *et al.*, 2009; Brown *et al.*, 2010; Li *et al.*, 2011, 2013; Hertwig *et al.*, 2013; Gonzalez *et al.*, 2014), it is clear that many genera within this family remain poorly understood. A prime example includes the enigmatic frogs of the genus *Chiromantis* Peter, 1854. Currently, 15 species are recognized in the genus *Chiromantis*, with four species, *C. vittatus* (Boulenger, 1887), *C. doriae* (Boulenger, 1893), *C. hansenae* (Cochran, 1927) and *C. nongkhorensis* (Cochran, 1927), recognized to occur in Thailand (Taylor, 1962; Chan-ard, 2003; Frost, 2013).

Of these species, *Chiromantis hansenae* is a poorly understood, small-bodied (21–24 mm SVL) rhacophorid frog with brown or light lavender body coloration, and parallel cream, yellow or white dorsolateral stripes. This species is distributed in northern, east central and southeastern Thailand, and presumably occurs in adjacent Cambodia and possibly Myanmar (Figure 1). The type locality of *C. hansenae* is recognized as Nong Khor, Chonburi Province, southeastern Thailand (Cochran, 1927; Taylor, 1962; Frost, 2013). This species has been recorded up to mid-elevations (ranging up to 900 m asl; Stuart *et al.*, 2004), and is arboreal, inhabiting secondary and primary growth forests and breeding in small rain pools or ponds (Taylor, 1962; Sheridan and Ocock, 2008; Chan *et al.*, 2011). *Chiromantis hansenae* deposits green eggs in gelatinous masses on plants above lentic water bodies, and display parental care behavior, with eggs guarded by the parental female until hatched (Sheridan and Ocock, 2008; Poo and Bickford, 2013).

The validity of *C. hansenae* as a unique species, distinct from *C. vittatus*, had been questioned by earlier studies (Wilkinson *et al.*, 2002; Stuart and Emmet, 2006; Chan *et al.*, 2011), however, Aowphol *et al.* (2013) presented robust data supporting the recognition of *C. hansenae* on the basis of several distinct datasets: molecular, morphological, and bioacoustics. Furthermore, Aowphol *et al.* (2013) presented preliminary evidence supporting the presence of two distinct evolutionary lineages of *C. hansenae* within Thailand. However, prior to this study, sufficient geographic sampling to elucidate cryptic lineage diversity and describe inter-population genetic structure within the species has not been available. In this study, we use newly collected and vouchered specimens from across Thailand, and novel molecular genetic datasets, to investigate patterns of genetic diversity among populations of *C. hansenae.*

2. Materials and Methods

2.1 Sampling *Chiromantis hansenae* was sampled at nine distinct localities across Thailand during 2011–2013 field expeditions, representing the geographic range of this species (Figure 1, Table 1). The sampled localities include: Mae Hong Son (MHS), Tak1 (TK1), Tak2 (TK2), Loei (LE), Kanchanaburi (KCB), Nakhon Ratchasima (NRS), Chonburi and Chanthaburi (CB), Prachuap Khiri Khan (PK) and Surat Thani (SRT). Individuals were collected by locating calling males and by visual encounter surveys. Specimens were euthanized using MS-222, fixed in 10% formalin, and subsequently preserved in 70% ethyl alcohol. Liver or muscle tissues were removed from each individual prior to formalin preservation, and preserved in 95% ethyl alcohol and stored at 4°C for DNA extraction. Voucher specimens are deposited in the

herpetological collection, Zoological Museum, Kasetsart University, Bangkok, Thailand (ZMKU; Appendix A).

Based on these surveys, 135 individuals of *Chiromantis hansenae* were available as ingroup samples. Sequence data for some ingroup samples were already available on GenBank and included in this study (GenBank accession numbers for 16S: KC357625–KC357634, KC357636–KC357645, KC357647–KC357669; Aowphol *et al*., 2013; Appendix A). Outgroup samples were chosen based on recent phylogenetic studies, and included *Rhacophorus kio* and *Polypedates leucomystax* (GenBank accession numbers: 16S, EU215532, AB728137; POMC, GQ285734, AB728240; Li *et al*., 2008, 2009; Kuraishi *et al*., 2013). Samples of *C. vittatus* was not included in the phylogenetic analyses following the results of Aowphol *et al*. (2013), which provided robust support for *C. hansenae* as a distinct species from *C. vittatus* based on the mitochondrial 16S ribosomal RNA gene.

2.2 DNA extraction, amplification and sequencing

Total genomic DNA was extracted from liver or muscle tissue samples using the DNeasy Blood & Tissue Kit (Qiagen, Inc.) following the manufacturer's instructions. A partial fragment of the mitochondrial 16S ribosomal RNA (16S) gene was amplified via polymerase chain

Table 1 Summary of *Chiromantis* sampling (N), number of haplotypes (N_h), number of unique haplotype (N_u), haplotype diversity (h), and nucleotide diversity (π) based on the mitochondrial 16S rRNA dataset.

Localities	N	N_h	N_u	Haplotype distribution	Haplotype diversity ($h \pm$ SD)	Nucleotide diversity ($\pi \pm$ SD)
LE	2	2	1	H24, H16	1.000 ± 0.500	0.00704 ± 0.00352
NRS	21	15	12	H5, H15, H16, H30, H32, H33, H34,H39, H40, H48, H49, H50, H51, H52, H53	0.943 ± 0.039	0.00538 ± 0.00066
CB	19	12	10	H17, H18, H19, H25, H31, H35, H36, H37, H38, H39, H40, H57	0.930 ± 0.038	0.00492 ± 0.00083
TK1	20	5	5	H6, H7, H8, H9, H10	0.674 ± 0.098	0.00180 ± 0.00042
SRT	21	7	7	H1, H2, H26, H27, H28, H29, H47	0.952 ± 0.028	0.00352 ± 0.00048
MHS	26	8	8	H20, H21, H23, H41, H42, H43, H54,H55	0.871 ± 0.044	0.00351 ± 0.00057
KCB	19	6	6	H3, H4, H22, H44, H45, H46	0.743 ± 0.085	0.00293 ± 0.00063
TK2	4	4	4	H11, H12, H13, H14	1.000 ± 0.177	0.00796 ± 0.00192
PK	3	1	1	H56	0.667 ± 0.314	0.00078 ± 0.00037
All	135	57	54		0.975 ± 0.004	0.03561 ± 0.00101

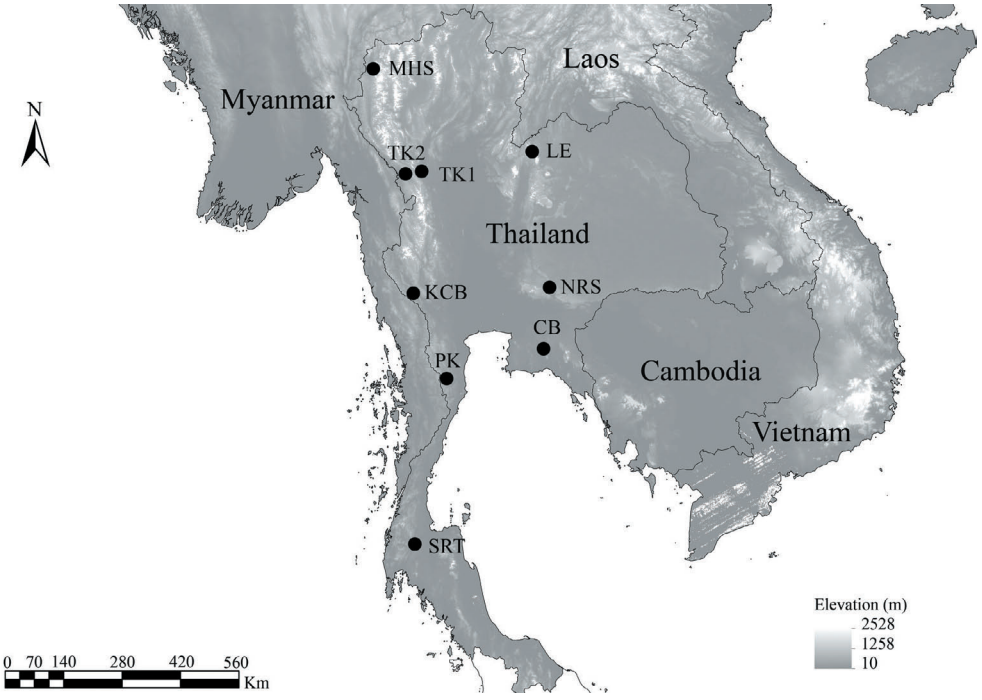

Figure 1 Map of Thailand showing sampling localities of *Chiromantis hansenae* populations incorporated into this study: Mae Hong Son (MHS); Tak1 (TK1); Tak2 (TK2); Loei (LE); Kanchanaburi (KCB); Nakhon Ratchasima (NRS); Chonburi and Chanthaburi (CB); Prachuap Khiri Khan (PK); and Surat Thani (SRT).

reaction (PCR) using previously published primers and protocols (16Sc, 16Sd; Moriarty and Cannetella, 2004). Additionally, a fragment of the nuclear DNA proopiomelanocortin (POMC) gene was amplified via PCR using published primers and protocols (POMC-1, POMC-7; Kuraishi *et al*., 2013). PCR amplifications were carried out in 25 µl volumes containing: 1 µl of DNA template, 10 nM dNTP, 10 µM of each primer, 50 nM $MgCl_2$, 10× PCR buffer, and 5U *Taq* DNA polymerase. Annealing temperatures for 16S and POMC were 52°C and 55°C, respectively. PCR products were purified using the Qiagen PCR Purification Kit (Qiagen, Inc.). Sequencing reactions were performed by Macrogen Inc. (Seoul, Korea) using an ABI 3730 automatic sequencer. Novel sequence data was deposited in GenBank (Accession numbers for 16S: KJ546807–KJ546839; POMC: KJ546672–KJ546806).

2.3 Phylogenetic analyses Initial alignments were produced in Geneious v5.6.3 (Biomatter, Ltd.), with subsequent manual adjustments made visually. Fifteen base pairs of ambiguous alignments were excluded from analyses, and gaps from the alignment were treated as missing data. To assess phylogenetic congruence between the mitochondrial and nuclear data, we inferred the phylogeny for each subset independently using Bayesian analyses. Following the observation of several instances of strongly supported incongruence between datasets, we conducted separate phylogenetic analyses for each dataset.

Partitioned Bayesian analyses were conducted in MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003). We treated the 16S dataset as a single data partition, but partitioned POMC by codon position. The Akaike information criterion (AIC), as implemented in jModelTest v2.1.4 (Darriba *et al*., 2012), was used to select the best model of nucleotide substitution for each partition (16S = GTR + Γ; POMC position 1 = HKY + Γ, positions 2, 3 = GTR + Γ). A rate-multiplier model was used to allow substitution rates to vary among subsets, and default priors were used for all substitution parameters. We ran four independent Markov chain Monte Carlo (MCMC) analyses, each with four Metropolis-coupled chains, and an incremental heating temperature of 0.02 for 16S and POMC, respectively. All analyses were run for 10 million generations, with parameters and topologies sampled every 1000 generations. To assess chain stationarity, all sampled parameter values and log-likelihood scores from the cold Markov chain were plotted against generation time and compared among independent runs using Tracer v1.4 (Rambaut and Drummond, 2007). We conservatively discarded the first 25% of samples as burn-in.

2.4 Geographic and Population structure Due to the low intraspecific genetic diversity observed for the nuclear sequence data, only mitochondrial data (16S) was used for population structure analyses. To assess general population genetic diversity among and within sampled populations, we calculated haplotype diversity (*h*; Nei, 1987), nucleotide diversity (π; Nei and Tajima, 1981), the numbers of haplotypes (N_h), and the numbers of unique haplotypes (N_u) using DnaSP v5.10.01 (Librado and Rozas, 2009). Uncorrected pairwise sequence divergences (%, *p*-distances) were calculated in MEGA v5.2 (Tamura *et al*., 2011). Analyses of molecular variation (AMOVAs) were conducted using Arlequin v3.5.1.3 (Excoffier *et al*., 2005) with 1000 permutations to estimate the amount of genetic variation explained within sampled populations, between populations and between geographically separated groups of populations. Pairwise F_{ST} values were calculated to explore genetic differentiation between paired populations. Analyses of isolation by distance (IBD) were conducted to investigate correlations between F_{ST} / (1-F_{ST}) and logarithms of inter-population geographical distance, using IBDWS v3.23 (Jensen *et al*., 2005). To determine haplotype relationships and better visualize population genetic structure among populations, we constructed a median joining network using Network v4.6.0 (Fluxus Technology Ltd.). A phylogenetic network in the program SplitsTree v4.10 (Huson and Bryant, 2006) using the Neighbor-Net algorithm (Bryant and Moulton, 2004). To assess the support for inferred splits in the phylogenetic network, a bootstrap analysis was conducted with 1000 pseudoreplicates.

2.5 Demographic history To assess the demographic histories of the sampled populations for evidence of recent changes in effective population sizes, we calculated mismatch distributions in DnaSP. Each population mismatch distribution was assessed for ragged and/or multimodal distributions, which can show signs of structured versus smooth or unimodal populations. Unimodal distributions may be indicative of recent population expansion or sudden panmixia (Harpending *et al*., 1998). We estimated Fu's Fs (Fu, 1997) and Tajima's D (Tajima, 1989) to test for neutrality of populations and further assess the data for evidence of recent population expansion. Finally, we estimated a population-specific raggedness index (*r*) as a test of the expected distribution of population expansion in Arlequin.

3. Results

3.1 Genetic Diversity The final dataset consisted of 871 bp of mitochondrial (16S), and 562 bp of nuclear (POMC), data from 135 individuals of *Chiromatis hansenae*. Observed variable and parsimony informative sites for the two sampled genes were: 106/99 (16S); 43/29 (POMC). The numbers of observed haplotypes among sampled genes were quite similar, with 57 and 54 haplotypes identified for 16S and POMC, respectively. Overall, mitochondrial haplotype diversity (*h*) was 0.975 ± 0.004, and ranged from 0.667 ± 0.314 (PK) to 1.000 ± 0.500 (LE) and 1.000 ± 0.177 (TK2). There were 54 unique mitochondrial haplotypes among nine populations. The overall mitochondrial nucleotide diversity (π) was 0.03561 ± 0.00101, and ranged from 0.00078 ± 0.00037 (PK) to 0.00796 ± 0.00192 (TK2). Finally, the average frequency distributions of nucleotides for the mitochondrial dataset were A = 34.5%, C = 22.7%, G = 17.5% and T = 25.2%. The haplotype distribution among the nine localities is presented in Table 1.

3.2 Phylogenetic analyses Bayesian phylogenetic analyses (BI) of the 16S dataset support the monophyly of sampled populations of *Chiromantis hansenae* (Figure 2). Additionally, analyses of 16S data recover two genetically divergent clades within Thailand with strong support (Figure 2). Clade A (posterior probability [PP]: 1.0) consisted of three groups of populations from PK (A1), KCB and TK2 (A2a), and MHS (A2b) distributed across the northwest and western regions of Thailand (Figure 1). Clade B (PP: 0.97) consisted of two groups of populations from TK1 (B1a), SRT (B1b) and LE, NRS and CB (B2) distributed across the northeast, eastern and southern regions of Thailand (Figure 1). The PP of the nuclear POMC dataset (Figure 3) showed several, well-supported inconsistencies with the 16S dataset (Figures 2–3).

The median-joining network of mitochondrial haplotypes resulted in observed intraspecific genetic structure (Figure 4). To better visualize haplotype structure across Thailand, we divided the network into two haplogroups consistent with the topology of the 16S gene tree (Figure 2). Haplogroup1 consists of haplotypes from MHS, TK2, KCB, and PK. Haplogroup2 consists of haplotypes from LE, NRS, CB and TK1 and SRT. The networks show a large number of unique haplotypes within individual sampling localities, and displayed a low degree of sharing among localities, with the exception of Haplotype H16 shared by two populations from NRS and LE, and the Haplotype H39 and H40 shared by two populations from NRS and CB. Phylogenetic networks of the 16S dataset produced by splitstree provide consistent support for seven well-supported genetic clusters (bootstrap support [BS] > 70%; Figure 5). Comparatively, analyses of the sequenced portion of POMC recovered three genetic clusters (Figure 6).

3.3 Population genetic structure Pairwise genetic divergences (%) among populations are presented in Table 2. The results revealed high mean genetic divergences (4.4%–6.3%) between the two major clades identified in phylogenetic analyses. Interestingly, genetic divergences between sampled localities from the supported clade of populations LE, NRS, CB, TK1 and SRT (0.0–3.4%) were observed to be lower than those between populations recovered in the clade of PK, KCB, TK2 and MHS (1.3%–5.0%). The AMOVA analysis (Table 3) supported similar amounts of significant genetic variation explained among and within populations (49.25% and 45.81%, respectively; $P < 0.01$). Most pairwise F_{ST} values were observed to be significant ($P < 0.01$; Table 2). F_{ST} values inferred for the CB, MHS, and TK1 populations with other populations showed significant differentiation including low gene flow among these populations. Correlation of $F_{ST} / (1\text{-}F_{ST})$ and the logarithms of inter-population geographical distance (Figure 7) showed negative values of *r* and not significant in Mantel test analysis ($r = –0.118$, $p = 0.675$). Therefore, the genetic differentiation among populations did not relate with geographic distance among populations.

3.4 Population demographic history Calculated mismatch distributions of all populations were multimodal (Figure 8), and both sum of squared deviations (SSD) and Harpending's raggedness indices (*r*) were not significant (SSD = 0.011, $p > 0.01$; $r = 0.004$, $p > 0.01$; Table 4). Tajima's D tests and Fu's Fs tests were negative and not significant ($p > 0.01$; Table 4) which support the results of mismatch distribution analyses. Populations at demographic equilibrium or decline should provide a multimodal distribution of pairwise differences (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). Therefore, these results are in line with the possible inference of stable population dynamics among Thailand populations of *C. hansenae*.

4. Discussion

4.1 Phylogenetic relationships among populations The phylogenetic analyses of the mitochondrial dataset recovered multiple, genetically divergent, endemic

Table 2 Uncorrected pairwise sequence divergences (%) for the mitochondrial 16S rRNA dataset below diagonal, showing inter-population and intra-population genetic diversity for *Chiromantis hansenae* across Thailand. Percentages on the diagonal represent intra-population genetic diversity. Mean pairwise genetic divergences shown in parentheses for reference. Pairwise F_{ST} values among and within sampling localities of *C. hansenae* across Thailand shown above diagonal, based on the mitochondrial (16S) dataset. Significant F_{ST} values (*p*-values < 0.05) are bolded for emphasis.

	LE	NRS	CB	TK1	SRT	MHS	KCB	TK2	PK
LE	0.4 (0.4)	0.00955	**0.50014**	**0.93872**	**0.90261**	**0.9576**	0.95304	0.90918	0.97248
NRS	0.0–0.7 (0.5)	0.1–0.9 (0.6)	**0.31539**	**0.89767**	**0.88699**	**0.94353**	**0.9418**	**0.9312**	**0.92706**
CB	0.2–1.1 (0.7)	0.0–1.2 (0.6)	0.0–0.9 (0.3)	**0.91498**	**0.90063**	**0.95309**	**0.94796**	**0.9378**	**0.94308**
TK1	2.4–2.7 (2.5)	2.3–2.9 (2.5)	2.3–3.2 (2.7)	0.0–0.5 (0.1)	**0.88216**	**0.97000**	**0.96779**	**0.96617**	**0.97706**
SRT	2.6–2.9 (2.7)	2.6–3.2 (2.8)	2.6–3.4 (2.9)	1.5–2.0 (1.7)	0.0–0.7 (0.3)	**0.9558**	**0.95437**	**0.94905**	**0.95767**
MHS	4.5–5.0 (4.7)	4.6–5.5 (5.0)	4.8–5.9 (5.2)	5.4–5.9 (5.6)	4.8–5.4 (5.0)	0.0–0.5 (0.2)	**0.94316**	**0.9427**	**0.95767**
KCB	5.1–5.7 (5.4)	4.9–5.9 (5.5)	5.0–5.7 (5.4)	5.6–6.3 (5.9)	5.2–6.0 (5.5)	3.5–-4.4 (3.8)	0.0–0.7 (0.2)	**0.82583**	**0.92838**
TK2	5.7–6.0 (5.9)	5.7–6.3 (5.9)	5.5–6.1 (5.8)	5.9–6.1 (6.0)	5.9–6.2 (6.0)	3.8–4.5 (4.2)	1.3–2.1 (1.8)	0.4–1.0 (0.6)	**0.94877**
PK	4.4–4.5 (4.5)	4.4–5.1 (4.7)	4.6–5.2 (4.9)	5.4–5.5 (5.5)	4.8–5.6 (5.5)	4.0–4.3 (4.1)	4.1–4.5 (4.2)	4.8–5.0 (4.9)	0.0 (0.0)

Table 3 Results of Analysis of Molecular Variance (AMOVA) of genetic differences in mtDNA sequences of sampled populations of *Chiromantis hansenae*.

Source of variation	*df*	Variance component	Percent (%) variation	Fixation index	*p*-value
Among groups	1	10.60915	49.25	0.90277	0.00000 ± 0.00000
Among populations within groups	7	9.8679	45.81	0.95066	0.00000 ± 0.00000
Within populations	128	1.06285	4.93	0.49253	0.00391 ± 0.00185

Table 4 Results of statistical tests of neutrality and mismatch distributions of sampled populations of *Chiromantis hansenae* based on the mitochondrial 16S rRNA dataset. Significant values (*p*-values < 0.05) are bolded for emphasis.

Lacalities	Tajima's D	*p*	Fu's Fs	*p*	SSD	*p*	*r*	*p*
LE	-	-	-	-	-	-	-	-
NRS	-0.841	0.221	**-8.617**	0.000	0.007	0.600	0.0262	0.800
CB	-1.290	0.066	**-6.405**	0.000	0.000	1.000	0.0330	0.770
TK1	-0.580	0.311	-0.775	0.299	0.015	0.280	0.1000	0.420
SRT	-0.525	0.317	-0.934	0.305	**0.131**	0.010	0.0480	0.950
MHS	0.567	0.725	-2.443	0.630	0.004	0.520	0.0680	0.480
KCB	-0.005	0.535	-0.362	0.439	0.141	0.610	0.0560	0.700
TK2	-0.154	0.587	-0.568	0.187	0.065	0.790	0.2220	0.760
PK	-	-	-	-	-	-	-	-
All	-0.315	0.530	-2.111	0.000	0.011	0.116	0.004	0.178

Thailand populations of *C. hansenae*. One well-supported clade (Clade A) includes sampled populations from northwestern and western regions of Thailand, with a second clade (Clade B) made up of populations from northeastern, eastern and southern regions of Thailand as well as a single population (TK1) from western regions of the country. In contrast, although phylogenetic analyses of the nuclear dataset were roughly consistent with analyses of 16S, given the low levels of genetic variation within the gene, several well-supported relationships were not concordant with the mtDNA gene tree (Figures 2–3).

Buckley *et al*. (2006), among others, have reviewed the relatively common observations of discordant mitochondrial and nuclear gene trees in the literature. There are several evolutionary processes that have been proposed as responsible for this conflict among gene trees, including, but not limited to, incomplete lineage sorting, genetic polymorphism, hybridization,

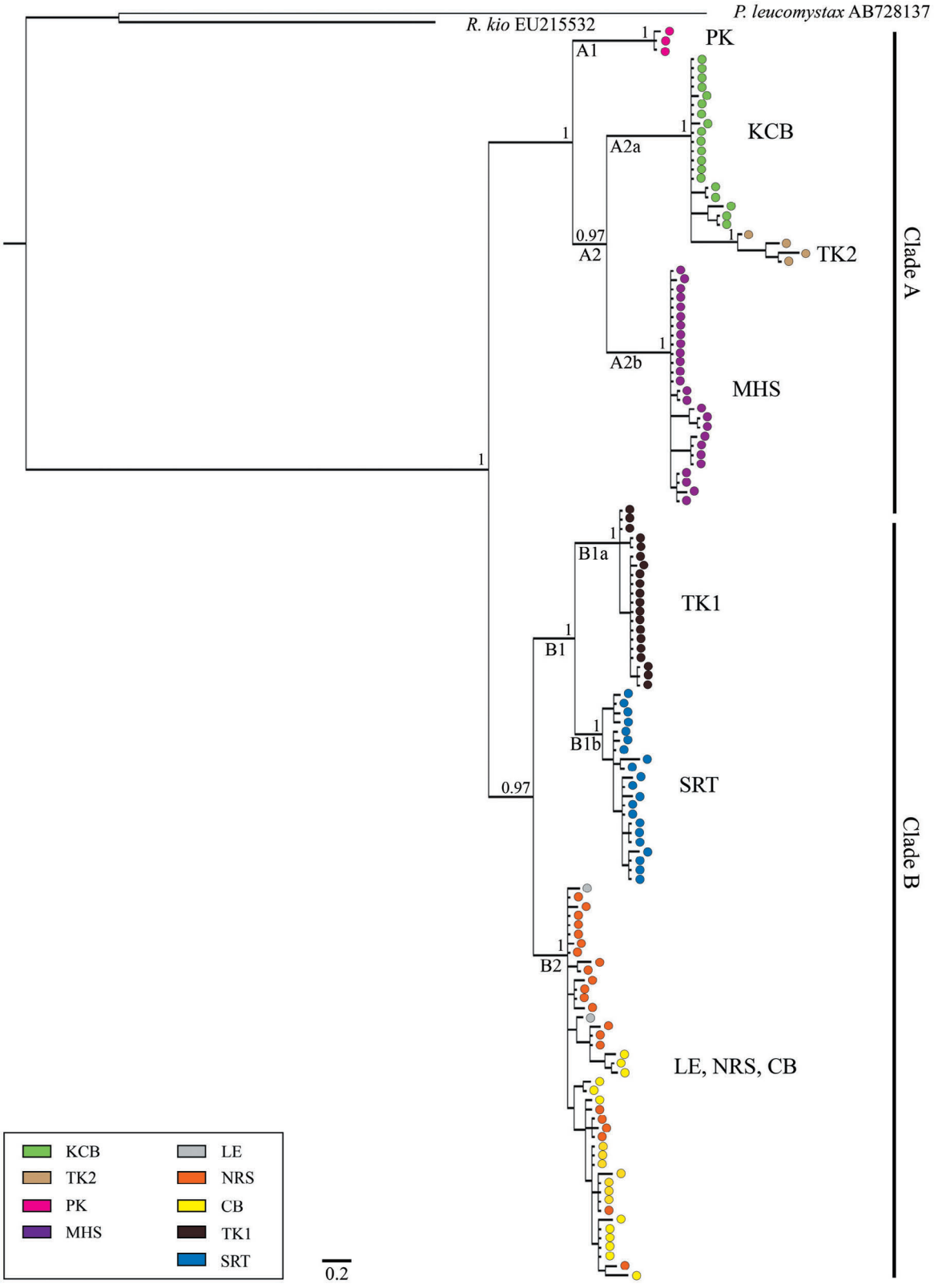

Figure 2 Hypothesized relationships of among sampled populations of *Chiromantis hansenae*, illustrated by the maximum clade credibility tree resulting from Bayesian analyses of the mitochondrial 16S rRNA dataset. Bayesian posterior probabilities are shown above branches, with alphanumeric labels shown below branches referencing focal clades discussed in the Results and Discussion sections. Nodes supported by > 0.95 Bayesian PP were considered highly supported. Terminals are labeled with colors corresponding to sampling localities shown in Figure 1.

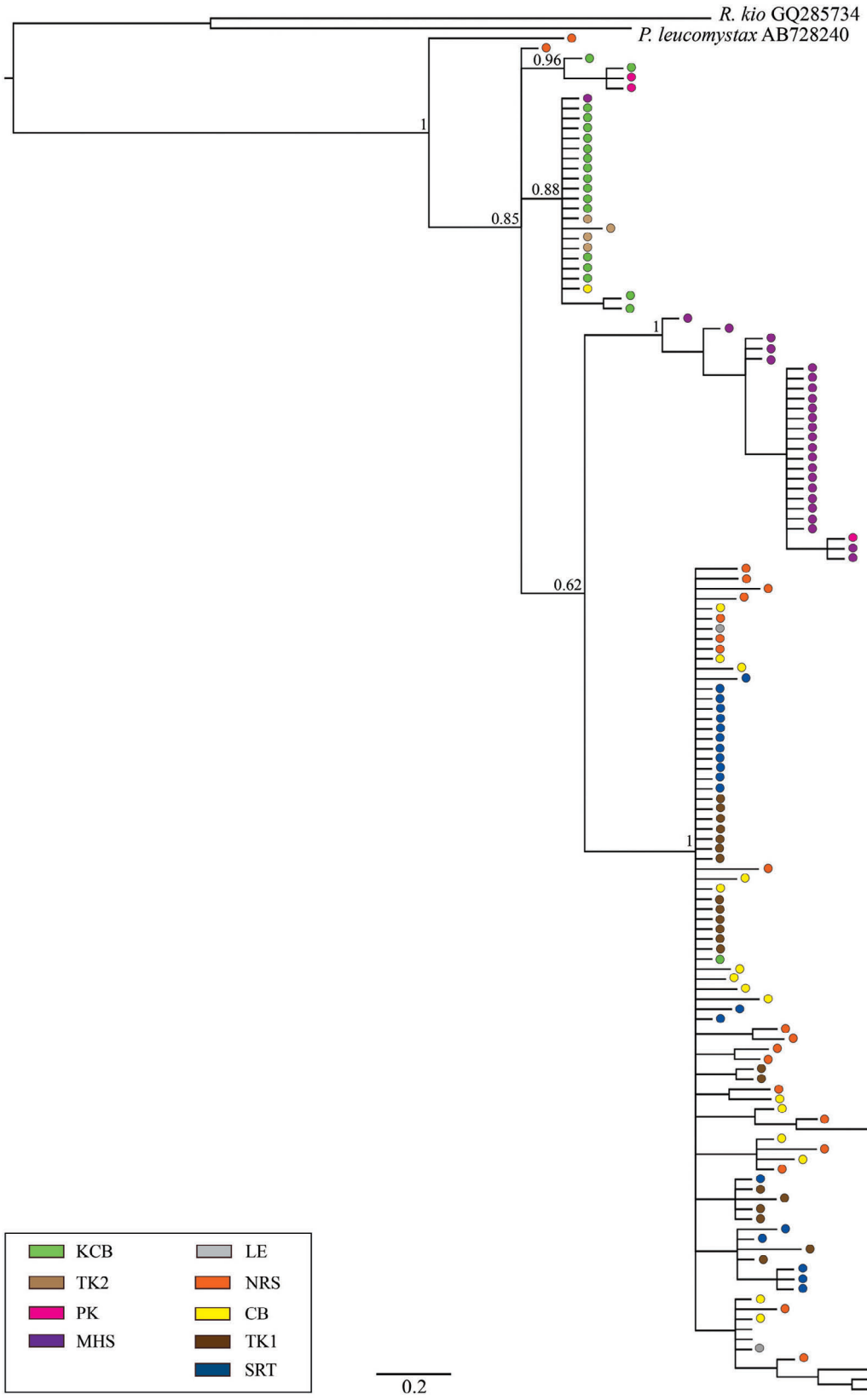

Figure 3 Hypothesized relationships of among sampled populations of *Chiromantis hansenae*, illustrated by the maximum clade credibility tree resulting from Bayesian analyses of the nuclear POMC dataset. Bayesian posterior probabilities are above the branches. Nodes supported by > 0.95 Bayesian PP were considered highly supported. Terminals are labeled with colors corresponding to sampling localities shown in Figure 1.

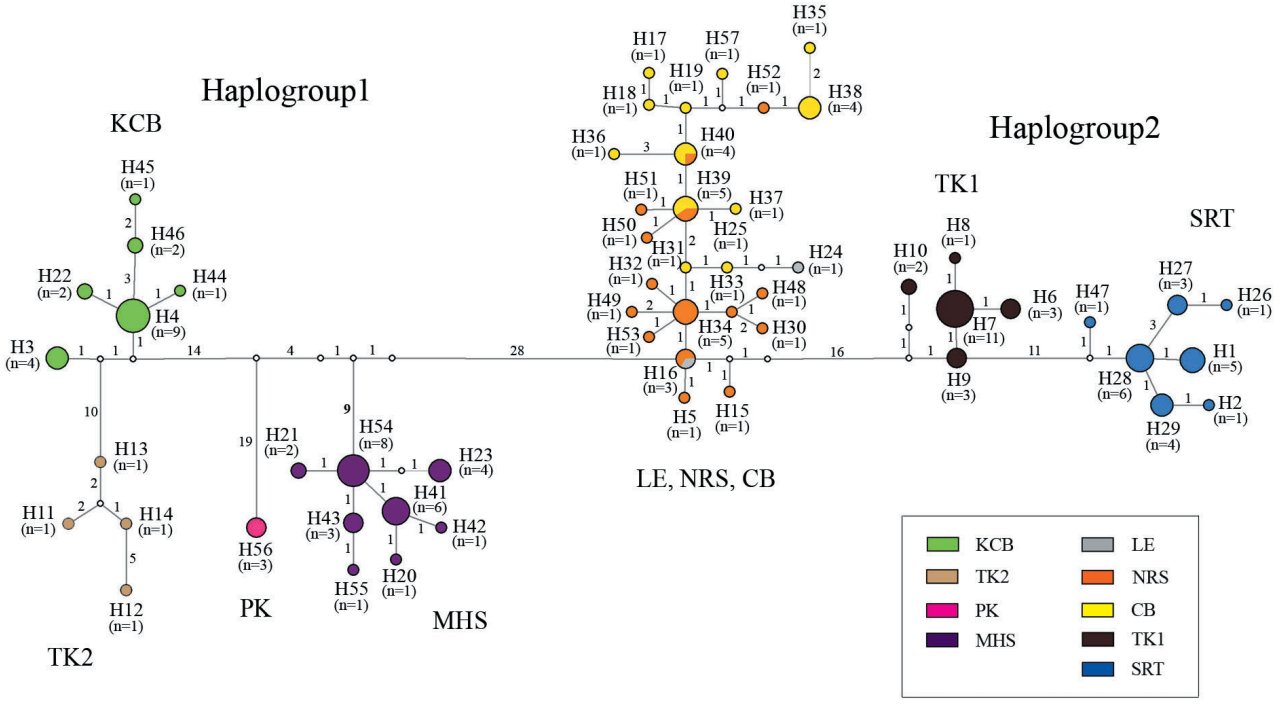

Figure 4 Median joining 16S haplotype network (MJN) depicting hierarchical relationships among haplotypes represented by sampled populations of *Chiromantis hansenae*. Each circle represents a haplotype and the size of circle is scaled to the number of individuals sharing that haplotype. White circles represent median vectors, and branch numbers represent the estimated number of mutational steps.

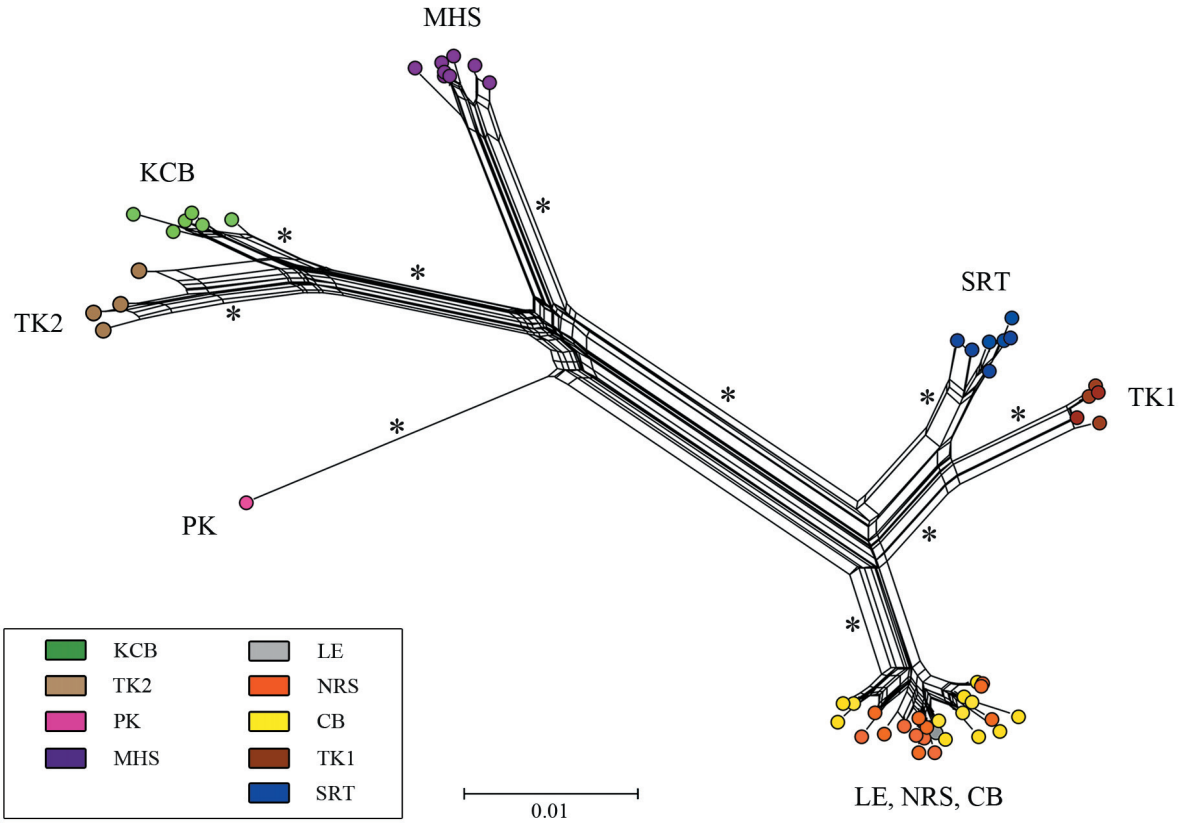

Figure 5 SplitsTree network (Huson and Bryant, 2006) with bootstrap support values for the mitochondrial (16S) dataset. Colors correspond to the same colors on the phylogenies and in the other figures. Asterisks represent bootstrap support values greater than 70%.

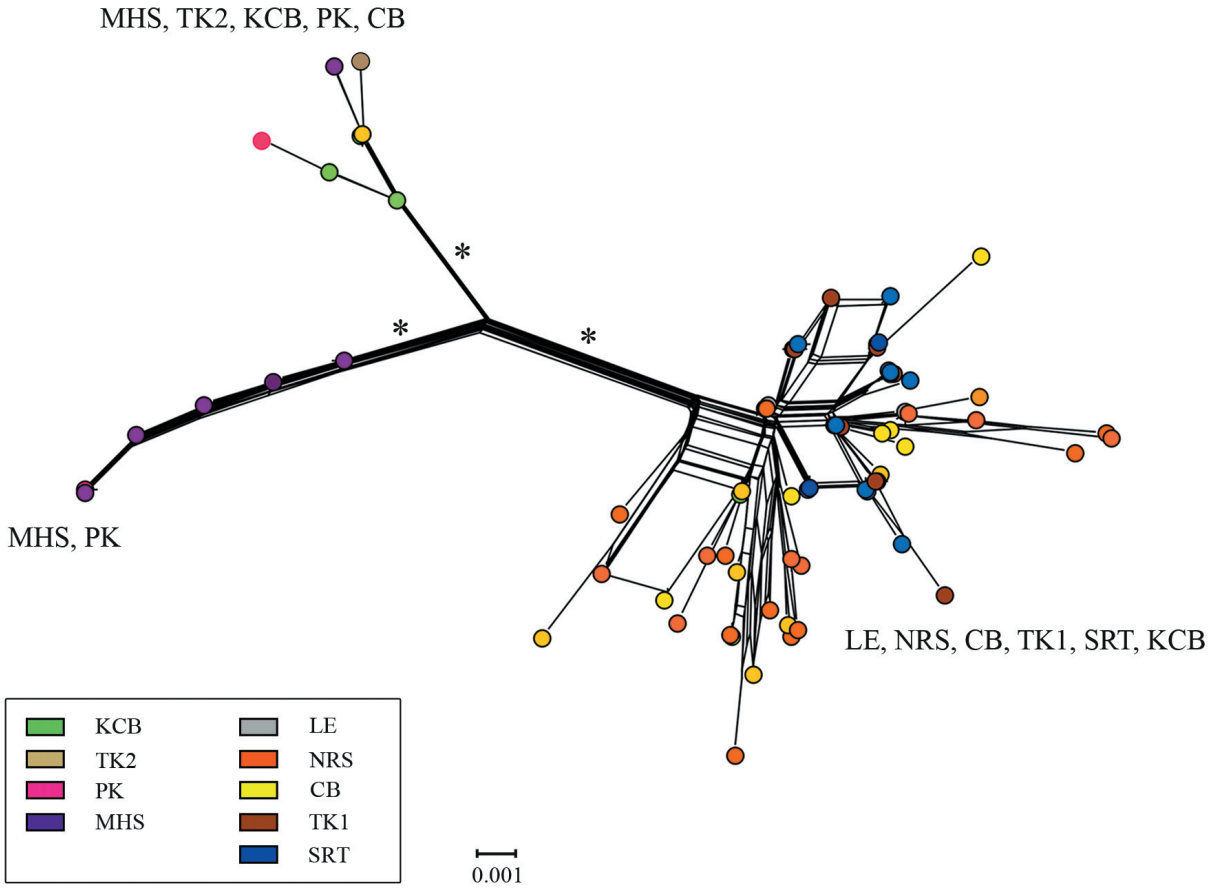

Figure 6 SplitsTree network (Huson and Bryant, 2006) with bootstrap support values for the nuclear (POMC) dataset. Colors correspond to the same colors on the phylogenies and in the other figures. Asterisks represent bootstrap support values greater than 70%.

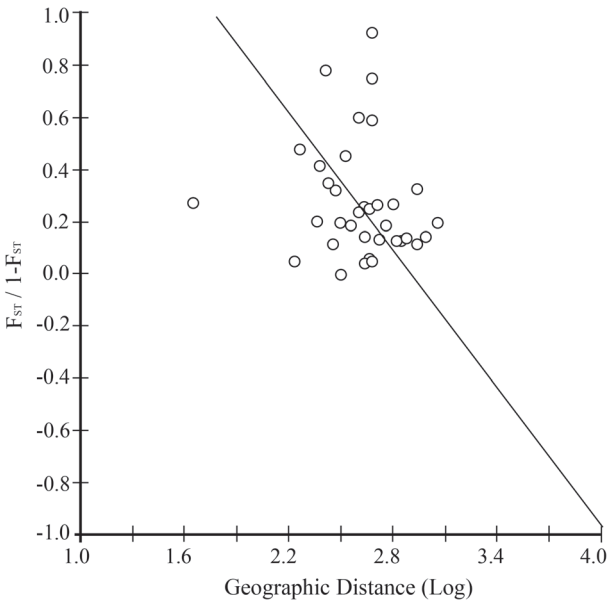

Figure 7 Isolation by distance plot of *Chiromantis hansenae* populations showing relationship of pairwise genetic distance $F_{ST}/ (1-F_{ST})$ and logarithms of geographic distance between populations. A linear regression line based on data points overlays the scatter plot for reference.

and introgression (Buckley *et al*., 2006; Gompert *et al*., 2008; Bossu and Near, 2009; Leaché *et al*., 2009; Siler *et al*., 2010). Although currently it remains difficult to determine definitively which process, or processes, might be responsible for observed mito-nuclear gene tree discordance, the rapid development of genomic approaches to collecting datasets of many, unlinked loci will likely provide new insights into these patterns. For the focal species of *Chiromantis hansenae*, additional data would be needed before any robust hypotheses could be made. However, given the low observed genetic diversity within the POMC dataset (Figure 3), and the shallow mtDNA divergences between many sampled populations in Thailand (Figure 2), we suspect that the gene tree conflicts are likely the result of incomplete lineage sorting in the nuclear marker.

4.2 Genetic structure of *Chiromantis hansenae* The populations of *C. hansenae* in Thailand revealed strong genetic structure based on the mtDNA dataset. The haplotype network displayed unique haplotypes in each population and did not reveal widespread haplotypes

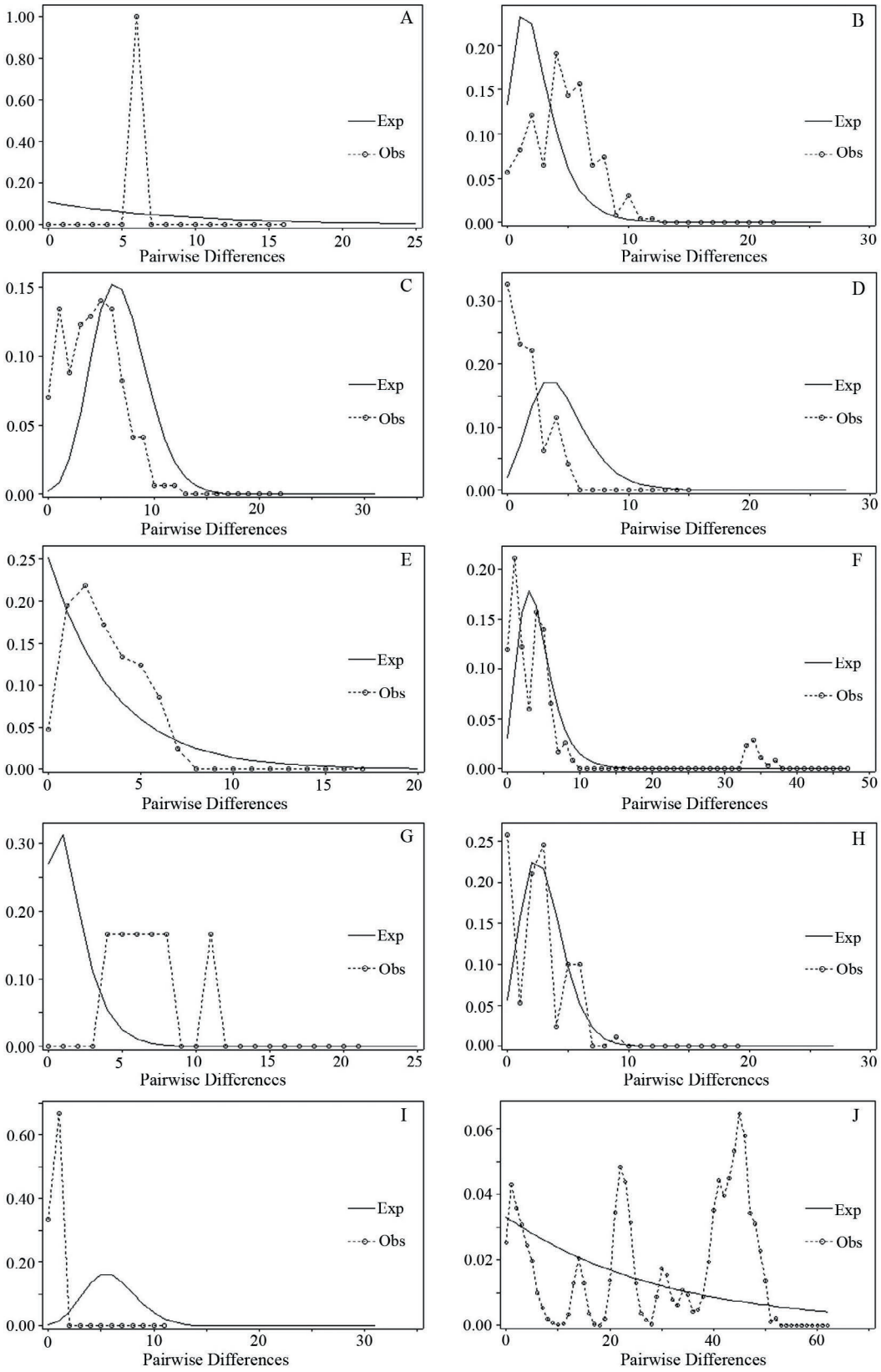

Figure 8 Observed frequencies of pairwise nucleotide differences among mitochondrial sequences (dashed lines) and expected frequencies under a model of sudden population expansion (solid lines) (Rogers and Harpending, 1992). Mismatch distributions depict frequencies of pairwise differences for: (A) LE, (B) NRS, (C) CB, (D) TK1, (E) SRT, (F) MHS, (G) TK2, (H) KCB, (I) PK and (J) all samples of *Chiromantis hansenae*.

with a few exceptions in populations LE, NRS, and CB populations (Figure 4). With sequence divergences (16S) among populations of *C. hansenae* inferred to be relatively high (Table 2), and AMOVA analyses supporting high amounts of genetic variation explained among geographic groups of populations and among populations within these groups (Table 3), the results suggest the possible presence of geographic barriers to dispersal and gene flow across Thailand.

Early studies on the home range sizes of amphibians suggested that they were limited to short-distances (less than 0.5 km; Zug, 1993). Additionally, Smith and Green (2005) reported amphibians as having limited dispersal abilities; with 44% of amphibian species they reviewed being capable of natural movement in excess of 400 m. Not only might low dispersal abilities potentially increase or maintain genetic differentiation among populations (e.g. Garcia-Paris *et al*., 2000; Cabe *et al*., 2007; Martinez-Solano *et al*., 2007), but also, geographic barriers such as mountain ranges may naturally obstruct gene flow in certain amphibian taxa (Hagemann and Pröhl, 2007; Zhang *et al*., 2010). Based on the geographic topology of Thailand, the northwestern and western regions of the country contain many continental steep mountain ranges that lay in north-south orientations with the Tenasserim and Thanon Thong Chai mountain ranges that border Thailand and Myanmar. The average elevations of these mountain ranges are higher on the Myanmar side, with many mountain peaks reaching 1 000 m asl, while on the Thai side the highest summits remain around 800 m asl (Gupta, 2005). The northeastern and eastern regions of Thailand have lower elevations, with plateaus and isolated mountain chains (Inger, 1999). *Chiromantis hansenae* is documented to occur in lowland (Sheridan and Ocock, 2008) to mid-elevation forest (~900 m asl; Stuart *et al*., 2004). Future studies on the microhabitat preferences or ecological requirements of this species may eventually reveal these as mechanisms limiting the dispersal ability of *Chiromantis* populations across Thailand and Southeast Asia.

Within the SRT population in southern Thailand, phylogenetic analyses revealed populations to be most closely related to the northeastern and eastern populations sampled in this study. Although the SRT population currently is located more than 500 km from eastern Thailand, and is separated from other populations by the Gulf of Thailand, historically, these regions experienced cyclical connections during the Pleistocene. During glacial maxima, both regions were connected as sea levels dropped by more than 100 m (the depth of the Gulf of Thailand), providing a potential land bridge connection between these geographically distant populations (Hall, 1998; Voris, 2000; Sathiamurthy and Voris, 2006). This historical land positive connection might help explain some of the observed phylogenetic results, a possibility that has been supported by studies of allozyme variation in *Rana nigrovittata* across Thailand (Matsui *et al*., 2001). Moreover, this is congruent with the study of phylogenetic relationships among *Hoplobatrachus rugulosus* in the country (Pansook *et al*., 2012).

Populations from PK and SRT, which are located on the Thai-Malay Peninsula (~400 km apart), are geographically more proximate to each other than the population from SRT is to sampled populations in northeastern and eastern Thailand. However, the results of phylogenetic analyses do not support the monophyly of populations from PK and SRT. This result highlights the need to better understand the impact historical land connections across the Isthmus of Kra may have had on vertebrate diversification in Thailand, as populations PK and SRT are located to the north and south of the Isthmus of Kra, respectively. Not only is the Isthmus of Kra recognized as an ecotone between the Indochinese and Sundaic subregions (Hughes *et al*., 2003; Woodruff, 2003b; Woodruff and Turner, 2009), but also, this region represents a major faunal turnover zone across which species dispersal may be limited (for review, see Inger and Voris, 2001; Hughes *et al*., 2003; Woodruff and Turner, 2009). Future studies across this transition zone may support the region as a promoter of genetic divergence between northern and southern regions of Thailand.

Interestingly, two populations from Tak Province were recovered as genetically divergent, despite being geographically proximate to each other (roughly 45 kilometers apart). Our results support a close relationship between the TK1 population and populations from northeastern, eastern and southern Thailand, whereas we find support for TK2 being closely related to populations from northwestern and western Thailand. Furthermore, we observe high genetic divergence between these two populations (5.9%–6.1%), which further can be visualized in inferred haplotype networks (Figure 4). Preliminary data on external morphology does not readily distinguish these two lineages from each other (Yodthong and Aowphol, unpublished data; however, current sampling available for the TK2 population is limited. Additionally, analyses of mating call variation have yet to be performed between these two populations, and such data might reveal differences between these genetically divergent lineages. Biogeographically, the TK1 population was

sampled from regions within the Sam Ngao District and Mueang District in Tak Province, at elevations of 160 and 209 m asl, respectively. The TK2 population was sampled from Mae Sod District in Tak Province, at an elevation of 315 m asl. Additionally, population-level sampling across Thailand may reveal that these two divergent populations are separated by part of the Thanon Thong Chai Mountain Range, which may present a moderate to high elevational barrier to dispersal for this species (~1 000 m asl).

Before broad scale conclusions can be reached about the influence of historical biogeographic processes on vertebrate diversification in Thailand, it is clear that studies are needed on many other vertebrate taxa recognized to span this biogeographic barrier (i.e., amphibians, reptiles, birds, mammals). Furthermore, future studies should focus on patterns of historical and modern gene flow between eastern continental and southern peninsula populations, investigating the impact of historical land bridge connections across the Gulf of Thailand on species diversification on the Sunda Shelf.

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Appendix A Sample and sequence used in this study.

Museum No.	Locality	Haplotype No. of 16S	Accession No.	
			partial 16S	POMC
ZMKU AM 00613	Nakhon Ratchasima	H53	KJ546836	KJ546672
ZMKU AM 00614	Nakhon Ratchasima	H52	KJ546835	KJ546673
ZMKU AM 00615	Nakhon Ratchasima	H34		KJ546674
ZMKU AM 00616	Nakhon Ratchasima	H34		KJ546675
ZMKU AM 00617	Nakhon Ratchasima	H39		KJ546676
ZMKU AM 00618	Nakhon Ratchasima	H34		KJ546677
ZMKU AM 00619	Chonburi	H57	KC357638[a]	KJ546678
ZMKU AM 00632	Chanthaburi	H18	KJ546821	KJ546679
ZMKU AM 00633	Chanthaburi	H35	KC357639[a]	KJ546680
ZMKU AM 00634	Chanthaburi	H37	KC357641[a]	KJ546681
ZMKU AM 00635	Chanthaburi	H19	KJ546822	KJ546682
ZMKU AM 00636	Chanthaburi	H17	KJ546820	KJ546683
ZMKU AM 00667	Mae Hong Son	H20	KJ546823	KJ546684
ZMKU AM 00668	Mae Hong Son	H42	KC357654[a]	KJ546685
ZMKU AM 00669	Mae Hong Son	H23		KJ546686
ZMKU AM 00670	Mae Hong Son	H41	KC357653[a]	KJ546687
ZMKU AM 00671	Mae Hong Son	H43	KC357655[a]	KJ546688
ZMKU AM 00672	Mae Hong Son	H43	KC357656[a]	KJ546689
ZMKU AM 00673	Mae Hong Son	H23		KJ546690
ZMKU AM 00678	Mae Hong Son	H54	KJ546837	KJ546691
ZMKU AM 00679	Mae Hong Son	H54		KJ546692
ZMKU AM 00680	Mae Hong Son	H54		KJ546693
ZMKU AM 00681	Mae Hong Son	H23	KJ546826	KJ546694
ZMKU AM 00682	Mae Hong Son	H21	KJ546824	KJ546695
ZMKU AM 00683	Mae Hong Son	H21		KJ546696
ZMKU AM 00687	Nakhon Ratchasima	H15	KJ546818	KJ546697
ZMKU AM 00688	Nakhon Ratchasima	H34		KJ546698
ZMKU AM 00689	Nakhon Ratchasima	H51	KJ546834	KJ546699
ZMKU AM 00690	Loei	H24	KJ546828	KJ546700
ZMKU AM 00707	Chonburi	H38	KC357642[a]	KJ546701
ZMKU AM 00710	Chonburi	H38	KC357644[a]	KJ546702
ZMKU AM 00711	Chonburi	H38	KC357643[a]	KJ546703
ZMKU AM 00712	Chanthaburi	H39	KC357647[a]	KJ546704
ZMKU AM 00718	Chonburi	H38	KC357645[a]	KJ546705
ZMKU AM 00722	Nakhon Ratchasima	H49	KJ546832	KJ546706
ZMKU AM 00723	Nakhon Ratchasima	H48	KJ546831	KJ546707
ZMKU AM 00728	Nakhon Ratchasima	H40		KJ546708
ZMKU AM 00729	Nakhon Ratchasima	H50	KJ546833	KJ546709
ZMKU AM 00733	Nakhon Ratchasima	H39		KJ546710
ZMKU AM 00746	Nakhon Ratchasima	H16	KJ546819	KJ546711
ZMKU AM 00781	Surat Thani	H26	KC357625[a]	KJ546712
ZMKU AM 00782	Surat Thani	H28	KC357628[a]	KJ546713
ZMKU AM 00783	Surat Thani	H1	KC357627[a]	KJ546714
ZMKU AM 00784	Surat Thani	H27	KC357626[a]	KJ546715
ZMKU AM 00785	Surat Thani	H28	KC357630[a]	KJ546716
ZMKU AM 00786	Surat Thani	H29	KC357629[a]	KJ546717
ZMKU AM 00799	Kanchanaburi	H4	KC357661[a]	KJ546718
ZMKU AM 00800	Kanchanaburi	H4	KC357662[a]	KJ546719
ZMKU AM 00802	Kanchanaburi	H3	KC357658[a]	KJ546720
ZMKU AM 00803	Kanchanaburi	H4	KC357664[a]	KJ546721
ZMKU AM 00805	Kanchanaburi	H44	KC357660[a]	KJ546722
ZMKU AM 00806	Kanchanaburi	H3	KC357659[a]	KJ546723
ZMKU AM 00817	Kanchanaburi	H3		KJ546724
ZMKU AM 00818	Kanchanaburi	H4	KC357665[a]	KJ546725
ZMKU AM 00819	Kanchanaburi	H46	KC357668[a]	KJ546726
ZMKU AM 00822	Kanchanaburi	H4		KJ546727
ZMKU AM 00823	Kanchanaburi	H4		KJ546728

(Continued Appendix A)

Museum No.	Locality	Haplotype No. of 16S	Accession No.	
			partial 16S	POMC
ZMKU AM 00825	Kanchanaburi	H22	KJ546825	KJ546729
ZMKU AM 00828	Kanchanaburi	H4	KC357663[a]	KJ546730
ZMKU AM 00829	Kanchanaburi	H4	KJ546829	KJ546731
ZMKU AM 00831	Kanchanaburi	H3	KC357657[a]	KJ546732
ZMKU AM 00832	Kanchanaburi	H46	KC357669[a]	KJ546733
ZMKU AM 00846	Kanchanaburi	H22		KJ546734
ZMKU AM 00870	Chonburi	H40	KC357650[a]	KJ546735
ZMKU AM 00871	Chonburi	H25		KJ546736
ZMKU AM 00872	Chonburi	H31	KC357632[a]	KJ546737
ZMKU AM 00873	Chonburi	H40		KJ546738
ZMKU AM 00874	Chonburi	H36	KC357640[a]	KJ546739
ZMKU AM 00875	Chonburi	H40	KC357652[a]	KJ546740
ZMKU AM 00876	Chonburi	H39	KC357648[a]	KJ546741
ZMKU AM 00877	Chonburi	H39	KC357649[a]	KJ546742
ZMKU AM 00878	Prachuap Khiri Khan	H56	KJ546839	KJ546743
ZMKU AM 00879	Prachuap Khiri Khan	H56		KJ546744
ZMKU AM 00880	Prachuap Khiri Khan	H56		KJ546745
ZMKU AM 00887	Mae Hong Son	H43		KJ546746
ZMKU AM 00888	Mae Hong Son	H54		KJ546747
ZMKU AM 00891	Mae Hong Son	H54		KJ546748
ZMKU AM 00892	Mae Hong Son	H54		KJ546749
ZMKU AM 00895	Mae Hong Son	H23		KJ546750
ZMKU AM 00896	Mae Hong Son	H55	KJ546838	KJ546751
ZMKU AM 00899	Mae Hong Son	H54		KJ546752
ZMKU AM 00900	Mae Hong Son	H41		KJ546753
ZMKU AM 00902	Mae Hong Son	H41		KJ546754
ZMKU AM 00903	Mae Hong Son	H54		KJ546755
ZMKU AM 00939	Nakhon Ratchasima	H16		KJ546756
ZMKU AM 00942	Surat Thani	H28		KJ546757
ZMKU AM 00943	Surat Thani	H28		KJ546758
ZMKU AM 00944	Surat Thani	H1		KJ546759
ZMKU AM 00945	Surat Thani	H27		KJ546760
ZMKU AM 00946	Surat Thani	H1		KJ546761
ZMKU AM 00947	Surat Thani	H47	KJ546830	KJ546762
ZMKU AM 00948	Surat Thani	H1	KJ546827	KJ546763
ZMKU AM 00949	Surat Thani	H1	KJ546807	KJ546764
ZMKU AM 00950	Surat Thani	H2		KJ546765
ZMKU AM 00951	Surat Thani	H28		KJ546766
ZMKU AM 00952	Surat Thani	H29		KJ546767
ZMKU AM 00953	Surat Thani	H29		KJ546768
ZMKU AM 00954	Surat Thani	H28		KJ546769
ZMKU AM 00955	Surat Thani	H27		KJ546770
ZMKU AM 00956	Surat Thani	H29		KJ546771
ZMKU AM 01112	Nakhon Ratchasima	H5	KJ546808	KJ546772
ZMKU AM 01113	Tak 1	H9	KJ546812	KJ546773
ZMKU AM 01114	Tak 1	H8	KJ546811	KJ546774
ZMKU AM 01115	Tak 2	H14	KJ546817	KJ546775
ZMKU AM 01116	Tak 2	H12	KJ546815	KJ546776
ZMKU AM 01117	Tak 2	H13	KJ546816	KJ546777
ZMKU AM 01118	Tak 2	H11	KJ546814	KJ546778
ZMKU AM 01119	Tak 1	H9		KJ546779
ZMKU AM 01120	Tak 1	H9		KJ546780
ZMKU AM 01121	Tak 1	H6	KJ546809	KJ546781
ZMKU AM 01122	Tak 1	H7		KJ546782
ZMKU AM 01123	Tak 1	H7		KJ546783
ZMKU AM 01124	Tak 1	H6		KJ546784
ZMKU AM 01125	Tak 1	H6		KJ546785

(Continued Appendix A)

Museum No.	Locality	Haplotype No. of 16S	Accession No.	
			partial 16S	POMC
ZMKU AM 01126	Tak 1	H7		KJ546786
ZMKU AM 01127	Tak 1	H7		KJ546787
ZMKU AM 01128	Tak 1	H7		KJ546788
ZMKU AM 01129	Tak 1	H7		KJ546789
ZMKU AM 01130	Tak 1	H10	KJ546813	KJ546790
ZMKU AM 01131	Tak 1	H7	KJ546810	KJ546791
ZMKU AM 01132	Tak 1	H7		KJ546792
ZMKU AM 01133	Tak 1	H10		KJ546793
ZMKU AM 01134	Tak 1	H7		KJ546794
ZMKU AM 01135	Tak 1	H7		KJ546795
ZMKU AM 01136	Tak 1	H7		KJ546796
ZMKU AM 00968	Loei	H16	KC357636[a]	KJ546797
ZMKU AM 00971	Nakhon Ratchasima	H30	KC357631[a]	KJ546798
ZMKU AM 00972	Nakhon Ratchasima	H34	KC357637[a]	KJ546799
ZMKU AM 00975	Nakhon Ratchasima	H33	KC357634[a]	KJ546800
ZMKU AM 00977	Nakhon Ratchasima	H32	KC357633[a]	KJ546801
ZMKU AM 00985	Kanchanaburi	H45	KC357667[a]	KJ546802
ZMKU AM 00986	Kanchanaburi	H4	KC357666[a]	KJ546803
ZMKU AM 00987	Mae Hong Son	H41		KJ546804
ZMKU AM 00988	Mae Hong Son	H41		KJ546805
ZMKU AM 00989	Mae Hong Son	H41		KJ546806

[a] Aowphol *et al.* (2013)