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AN EXAMINATION OF CALL AND GENETIC VARIATION IN THREE WIDE-RANGING SOUTHEAST ASIAN ANURAN SPECIES

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***ABSTRACT.* –** We examined common male mating calls and portions of two mitochondrial genes (16S and cytochrome b) of three wide-ranging Southeast Asian anurans to determine whether populations separated by 1,600 km are conspecific. For one species (*Polypedates leucomystax*), calls are significantly different, but genetic distances are relatively low. For the other two species (*Microhyla heymonsi* and *Hylarana erythraea*), calls do not differ significantly, and genetic distances are larger than those observed in *P. leucomystax*. We conclude that the study populations of *P. leucomystax* may represent different species, and that the populations of *M. heymonsi* and *H. erythraea* are likely conspecific. Further sampling of calls and genetics of geographically intermediate *P. leucomystax* populations will help resolve whether or not these populations are conspecific.

***KEY WORDS.* –** Frogs, Thailand, Singapore, *Polypedates*, *Microhyla*, *Rana*.

INTRODUCTION

Taxonomic identification of anurans was traditionally based on morphological variation and little attention was paid to geographic variation until John Moore's studies of *Rana pipiens* (Moore, 1944, 1949). In the 1960's it became clear that mating calls were more biologically meaningful in delimiting anuran species and that typically conservative morphological variation was often a poor indicator of species status (e.g., Ball & Jameson, 1966; Littlejohn & Oldham, 1968). Subsequently, genetic data became commonly used for delimiting species, either alone or in conjunction with morphological or call data (Goldberg, et al., 2004; Lougheed, et al., 2006; Stuart, et al., 2006; Fouquet, et al., 2007). Although different data sets may yield different answers, when examined individually, to the question of whether two individuals are conspecific, collectively they should give a reliable indication of species delimitations.

Morphologically cryptic frog species can be difficult to distinguish based on physical features, but they can often be distinguished based on call differences (Matsui, et al., 1986; Matsui, 1997; Boul, et al., 2007), because calls may contribute to reproductive isolation through female discrimination and choice. However, despite the obvious advantages of using calls and DNA sequences for delimiting frog species, and the use of such data to delimit cryptic neotropical frogs (e.g., Pröhl, et al., 2006; Boul, et al., 2007), few researchers have examined call variation in conjunction with genetic variation in an attempt to discover cryptic Southeast Asian frog species (Narins, et al., 1998; Brown, et al., 2006). Here, we use a combination of call and genetic (16S and cytochrome b) data to examine whether distant populations of three common, widespread Southeast Asian frog species are conspecific (*Polypedates leucomystax*, *Hylarana erythraea*, and *Microhyla heymonsi*).

These three common species were sampled in Singapore and central Thailand, sites 1,600 km apart. All three species range from southern China or northern Thailand to Indonesia (http://globalamphibians.org). The range of *P. leucomystax* extends west to India, but that of *M. heymonsi* and *H. erythraea* extends only as far west as Myanmar (http://globalamphibians.org). The calls of all three species

have been described elsewhere (see Table 1 for a summary of published call descriptions).

Currently, the study populations of these three species are considered conspecific, but *Polypedates leucomystax* is suspected to harbor cryptic diversity (e.g., Narins, et al., 1998). *Polypedates leucomystax* is a medium-sized tree frog, whose snout-vent length (SVL) differs significantly between the two sites: males and females are significantly larger in Thailand (55.24 ± 0.25 mm and 80.94 ± 0.77 mm, respectively) than in Singapore (44.74 ± 0.18 mm and 64.11 ± 0.74 mm; ANOVA P-value < 0.001 for both; Sheridan, 2009). Additionally, individuals in Thailand lack dorsal stripes, while those in Singapore have them (pers. obs.). Snout-vent length of *M. heymonsi* does not differ between sites: males are 19.40 ± 0.08 mm and 19.53 ± 0.11 mm in Singapore and Thailand, respectively (ANOVA $F_{1,341}$ = 0.75, P = 0.39) and females are 24.64 ± 0.39 mm and 24.98 ± 0.24 mm, respectively (ANOVA $F_{1,55}$ = 0.59, P = 0.45, Sheridan, 2009). Similarly, SVL of *H. erythraea* does not differ between the two populations: males are 42.76 ± 0.39 mm and 41.53 ± 0.64 mm in Singapore and Thailand, respectively (ANOVA $F_{1,86}$ = 2.69, P = 0.10) and females are 70.91 ± 1.15 mm and 68.27 ± 1.06 mm, respectively (ANOVA $F_{1,46}$ = 1.64, P = 0.21; Sheridan, 2009). There are no obvious differences in morphology or colour pattern of either *M. heymonsi* or *H. erythraea* between the two study sites. We would thus predict that geographic variation in calls and genetic markers will be higher for *P. leucomystax* than for *H. erythraea* and *M. heymonsi*.

MATERIALS AND METHODS

***Study sites.* –** Observations were conducted in Thailand at Sakaerat Environmental Research Station (14º30'N 101º55'E), and in Singapore at the Singapore Zoo and adjoining Mandai Orchid Garden (1º24'N 103º47'E). These study sites lie approximately 1,600 km apart and the three species are found throughout the intervening area. The amphibian community size is similar (25 species at Sakaerat, 26 species in Singapore), and seven species are common to both sites (Lim & Lim, 2002; Chan-ard, 2003). The study area in Thailand comprised secondary forest and cleared areas, and has a mean annual rainfall of 1,260 mm, with most of the rain falling between April and September (unpubl. meteorological data, Sakaerat Environmental Research Station). The study areas in Singapore were mainly cleared areas adjacent to secondary and primary forest with a mean annual rainfall of 2,345 mm distributed fairly evenly throughout the year (https://inetapps.nus.edu.sg/fas/geog/).

***Comparative analysis of calls.* –** We recorded calls of 7–9 individuals of each species from two populations of each species (total calls analyzed per species: 15–18). Recordings in Thailand were obtained between June and October 2006, and in Singapore between May 2006 and March 2007. Ambient temperature at the time of recording was measured with a mercury thermometer, and recorded individuals were collected and deposited in either the Chulalongkorn University Museum of Natural History (Thailand) or the Raffles Museum of Biodiversity Research (Singapore) (Appendix 3). Recordings were made either with a Sony WM D6C Professional Walkman Cassette Recorder and an Audio-technica condenser microphone or a Marantz PDM 660 digital recorder and a Sennheiser K6 microphone. All analog recordings were digitized using Windows Sound Recorder at 22.0 or 44.1 kHz. Audiospectrograms and oscillograms were quantified with Raven Software 1.2 (Cornell Laboratory of Ornithology, Ithaca, NY, USA).

Because call type differed between species, we analyzed different call characters for each. For all three species, we quantified dominant frequency, call duration, and call rate. For *P. leucomystax*, we also quantified pulse number and pulse rate. For *M. heymonsi* we quantified those five parameters plus number of calls/bout. We tested for differences between the two populations using MANOVA in Statistica v. 6 (StatSoft, Inc. 2001).

***Tissue collection.* –** Tissue samples from Sakaerat, Thailand were collected between April and September 2005 and from Singapore between February and May 2006. Tissues were obtained by extracting liver or calf muscle from a collected specimen or by clipping toes from an adult frog which was then released at point of capture. All tissues were preserved in 95% ethanol. Voucher specimens were deposited in the Natural History Museum at Chulalongkorn University, Bangkok, Thailand, the Raffles Museum of Biodiversity Research, Singapore, and the Natural History Museum and Biodiversity Research Center of the University of Kansas. Other tissue samples from Thailand were obtained via generous loans. Species identifications, voucher numbers, localities (where available), and GenBank accession numbers are listed in Appendix 1. In addition to the 16S and cytochrome b sequences listed in Appendix 1, we downloaded 16S sequences for related species from GenBank (Appendix 2). The few cytochrome b sequences available for each species group are listed in Apprendix 1.

***DNA extraction, amplification, and sequencing.* –** Genomic DNA was extracted from tissue samples using the Qiagen DNeasy Tissue Kit. Approximately 500 base pairs from both the 16S and cytochrome b mitochondrial gene regions were amplified using the following primer sets: 16S: 16Sbi, 16Sar-L (modified from Palumbi et al. 1991); cytb, both designed for this study: tRNAf (GAAAARCTAYYGYTGWWAYTCAACTAC), cytbr (GTCWACTGARAASCCKCCTCAATTCATTG). For the PCR reactions, we amplified samples at 95° C for 3 minutes, followed by 40 cycles of 94°C for 1.5 minutes, 52°C for 1 minute, and 72°C for 1.5 minutes with a further extension at 72°C for 3 minutes. Samples were stored at 4°C until they were purified with Quick-Clean kits (BioLine, London, UK). Samples were then sequenced with the ABI BigDye terminator mix using fluorescent thermal-cycle sequencing followed by electrophoresis in an ABI 3100 automated sequencer (Perkin Elmer) following the manufacturer's instructions.

***Phylogenetic analysis.* –** DNA sequences were obtained from 4–5 individuals of each species from each population (Singapore and Thailand). Each sample was sequenced in both directions and complementary sequences were aligned in Sequencher (Gene Codes Corp., Ann Arbor, MI), followed by multiple sequence alignment in Clustal X (Thompson, et al., 1997) with some manual adjustments in MacClade 4.06 OS X (Maddison & Maddison, 1989). The dataset was subjected to Maximum Parsimony (MP) and Bayesian analyses. For MP, the sequences were analyzed in PAUP* 4.0b (Swofford, 2000) under the MP criteria with all characters weighted equally, indels treated as missing data, and 1,000 independent analyses using random-addition and TBR branch-swapping. Branch support was quantified using bootstrapping as implemented in PAUP* (1,000 replicates analyzed with 100 random-addition runs each). MrModeltest 2.2 (Nylander, 2004) was used to identify a substitution model, nucleotide frequencies, and optimal priors for the gamma parameter. We used the Akaike Information Criteria (AIC) to determine an appropriate model of molecular evolution. The Bayesian analysis was carried out in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) with a Monte Carlo Markov chain (MCMC) length of 1 million generations. We sampled one out of every 100 generations, giving us a total of 10,000 samples. Of these, we discarded the first 2,500 (burn-in), and used the remaining trees to calculate posterior probabilities for each branch. We consider a node well supported if its posterior probability is greater than 95%. We also ran a Bayesian analysis to find the best trees for these data. Trees obtained from Bayesian analyses matched well with those obtained from maximum parsimony analyses (see results of each species for details), and we present a single phylogram for each species with both bootstrap and posterior probability values for each node. Some nodes on the Bayesian tree lack bootstrap values (Fig. 1) because these nodes were either not supported in the maximum parsimony analysis or the support was < 50. We also calculated F_{ST} values between our study populations using DnaSP 4.0 (Rozas et al., 2003).

Our phylograms are not meant to represent the complete phylogeographic history of the species, but rather to examine how the populations are related to one another and to closely related congeners. For *P. leucomystax*, two sequences downloaded from GenBank identified as *P. megacephalus* clustered within our *P. leucomystax* group. The sequences are represented by a single terminal on our tree because they were identical for our markers.

RESULTS

We found the calls of *P. leucomystax* to be significantly different between the two locations, but genetic distances were relatively low. In contrast, the calls of *M. heymonsi* and *H. erythraea* were virtually identical in the two populations but genetic distances were nearly twice as high as those between populations of *P. leucomsytax*. The phylogenetic trees (Figure 1) indicate that individuals sampled for each species form well-supported monophyletic clades with respect to other sampled congeners.

***Polypedates leucomystax.* –** The Singapore population of this species has two types of call. Both are found in the Thai population which also has at least four additional call types (Fig. 2). In our population comparison, we compared the most common call consisting of a single note. MANOVA indicated significant differences in call parameters between the study populations (Wilks' Lambda = 0.32, F = 4.25, df = 5,10, P = 0.02), so we conducted ANOVA on the individual call components. Call duration is longer, pulse number is greater, and call rate is lower in Singapore than in Thailand (ANOVA P-value < 0.02 for each; Table 1). The dominant frequency was lower in Thailand, but the result was not significant (ANOVA $F_{1,14}$ = 4.05, P = 0.06). Pulse rate did not differ between the two populations (ANOVA $F_{1,14}$ = 1.28, P = 0.28). Uncorrected p-distances between the two populations of *P. leucomystax* were 2.95–3.57% and 6.99–7.46% for 16S and cytb, respectively, and ≤1.2% within a population. There were 149 parsimony informative characters in the 16S and cytochrome b data set (CI = 0.65, RI = 0.75, RCI = 0.49; Figure 1). The GTR + I + Γ was the best fit model and F_{ST} between the Sakaerat, Thailand and Singapore clades was 0.75. Two sequences (represented by a single node) downloaded from GenBank labeled as "*P. megacephalus*" cluster with our *P. leucomystax* sequences. We consider this to be a misidentification of the specimens, as other *P. megacephalus* sequences fell outside of our *P. leucomystax* group, and *P. megacephalus* is superficially similar to *P. leucomystax* (Matsui, et al., 1986).

***Microhyla heymonsi.* –** Standard call variables (dominant frequency, call duration, pulse number, pulse rate, call rate, and number of calls/bout) did not significantly differ between the two populations (Wilks' Lambda = 1.31, df = 6,10 P = 0.34; Table 1). Uncorrected p-distances between individuals across study populations of *M. heymonsi* were 4.29–4.56% and 11.90–13.08% for 16S and cytb, respectively, while distances between individuals within each population were less than 1%. There were 144 parsimony informative characters in the combined 16S and cytochrome b data set (consistency index, CI = 0.81, retention index, RI = 0.87, rescaled consistency index, RCI = 0.70; Figure 1). Bootstrap support was high for each population as well as for *heymonsi* relative to the outgroups in our data set. The GTR + Γ model was identified as the best fit for this species with these data, and the resulting phylogram is very similar to our most parsimonious tree. The F_{ST} between the Sakaerat, Thailand and the Singapore clades was 0.62.

***Hylarana erythraea.* –** There was no difference in dominant frequency, call duration, and call rate between the two populations (Wilks' Lambda = 0.54, F = 1.92, df = 4,9, P = 0.19; Table 1). Uncorrected p-distances between the study populations of *H. erythraea* were 5.11% and 10.4% for 16S and cytb, respectively, while distances within each population were 0% (Fig. 1). There were 94 parsimony informative characters for the combined 16S and cytochrome b data set (CI = 0.81, RI = 0.81, RCI = 0.66). As before, GTR + Γ was identified as the best fit model. F_{ST} between the Sakaerat, Thailand and Singapore clades was 0.93.

Table 1. Call features of *Microhyla heymonsi*, *Hylarana erythraea* "chirp" call, and *Polypedates leucomystax* "normal" calls. Values are means ± SE (range). For the Taiwan *M. heymonsi* info, we report what Kuramoto identified as the 3rd note of a 3-note call (by our terminology, this would be the third call in a calling bout). The study by Narins et al. (1998) recorded variables for two different morphs (striped and non-striped) in Kuala Lumpur that they determined to be different species based on allelic polymorphism. *Temperature for these specimens is water, not air temperature. †Values from this study are means ± SD.

Species and Location (n)	Recording Temp (°C)	SVL (mm)	Dominant Frequency (Hz)	Call duration (ms)	Pulse number	Pulse rate (pulse/s)	Call Rate (calls/s)	Source
M. heymonsi Manchou, Taiwan	24.5	20–23	3000	290 ± 20	10.1 ± 0.9	31.2 ± 1.9	N/A	Kuramoto (1987)
M. heymonsi Chiahsien, Taiwan	25.5	20–23	2800	410 ± 30	12.5 ± 0.7	28.3 ± 0.3	N/A	Kuramoto (1987)
M. heymonsi Chiang Mai, Thailand (5)	24–26	N/A	2490.4 ± 66.57	338.82 ± 8.63	13.1 ± 0.30	38.70 ± 0.68	N/A	Garcia-Rutledge and Narins (2001)
M. heymonsi Sakaerat, Thailand (9)	24.5 ± 0.44	19.52 ± 0.11	3292.11 ± 176.69	430.09 ± 20.89	9.63 ± 0.44	22.44 ± 0.28	0.87 ± 0.02	This study
M. heymonsi Sakaerat, Thailand (1)	28	N/A	1700–3000	480	11	23	N/A	Heyer (1971)
M. heymonsi Singapore (9)	26.5 ± 0.23	19.44 ± 0.08	3284.84 ± 82.75	461.99 ± 28.21	10.09 ± 0.53	21.98 ± 0.48	0.93 ± 0.06	This study
H. erythraea Sakaerat, Thailand (8)	24.63 ± 0.71	41.53 ± 0.64	2406.20 ± 126.96	19.90 ± 2.04	N/A	N/A	0.170 ± 0.03	This study
H. erythraea Singapore (7)	25.88 ± 0.59	42.71 ± 0.38	2701.84 ± 191.47	18.79 ± 4.09	N/A	N/A	0.206 ± 0.06	This study
H. erythraea Negros, Philippines	29–30	N/A	N/A	24.7 (16–33)	N/A	N/A	N/A	Alcala et al. (1986)
P. leucomystax Taiwan	25–27	N/A	N/A	153–628	2–5	7.78–11.90	N/A	Kuramoto (1986)
P. leucomystax Ba Be, Vietnam (22)	26.2	N/A	1940	33	2–17	N/A	N/A	Trepanier et al. (1999)
P. leucomystax Pac Ban, Vietnam (2)	26.2	N/A	1950	64	4–16	N/A	N/A	Trepanier et al. (1999)
P. leucomystax Chiang Mai, Thailand (5)	24–26	N/A	N/A	48.22 ± 4.21	5.24 ± 0.51	108.43 ± 3.79	N/A	Garcia-Rutledge and Narins (2001)
P. leucomystax Sakaerat, Thailand (7)	24.17 ± 0.71	58.14 ± 1.52	1197.05 ± 183.50 (516.8–1778.7)	58.47 ± 5.43 (35.7–83.6)	4.18 ± 0.32 (2.7–5)	72.79 ± 4.22 (56.5–83.3)	0.09 ± 0.02 (0.03–0.21)	This study
P. leucomystax Sakaerat-a (2)	N/A	N/A	300–2600	230–380	4–5	N/A	N/A	Heyer (1971)
P. leucomystax Sakaerat-b (3)	N/A	N/A	1700–3100	120–250	2–4	N/A	N/A	Heyer (1971)
P. leucomystax Uthai Thani, Thailand (8)	ca. 26	N/A	660 ± 40	52 ± 15	4.3 ± 1.0	N/A	N/A	Christensen-Dalsgaard et al. (2002)
P. leucomystax KL, Malaysia Non-striped-A (5)	ca. 25	N/A	2336 ± 110.2	24.6 ± 1.4	1.0 ± 0.0	N/A	N/A	Narins et al. (1998)
P. leucomystax KL, Malaysia Striped-A (16)	ca. 25	N/A	2238 ± 43.2	174.3 ± 11.8	13.2 ± 0.8	N/A	N/A	Narins et al. (1998)
P. leucomystax Singapore (9)	26.21 ± 0.41	43.89 ± 0.92	1786.52 ± 214.54 (818.3–2562.5)	101.81 ± 12.21 (46–140.5)	7.97 ± 1.02 (4–12)	78.51 ± 3.03 (63.4–89.1)	0.03 ± 0.01 (0.01–0.07)	This study
P. leucomystax Negros, Philippines (17) †	28–30	41–52	1500–2500	192.7 ± 20.5	16.2 ± 2.3	81.4 ± 10.6	0.1–0.13	Brzoska et al. (1986)
P. leucomystax Kundasang, Sabah, Borneo (3)	21.5*	N/A	2250 (2100–2500)	158 (133–18.5)	11.3 (9–13)	N/A	N/A	Matsui et al. (1986)
P. leucomystax Ranau, Sabah, Borneo (12)	27*	N/A	2390 ± 70 (1900–2700)	125 ± 5 (83–159)	12.3 ± 0.5 (8–16)	N/A	N/A	Matsui et al. (1986)
P. leucomystax Sandakan, Sabah, Borneo (2)	27.5*	N/A	2550 (2400–2700)	144 (143–145)	13.0 (13)	N/A	N/A	Matsui et al. (1986)
P. leucomystax Sarawak, Borneo (3)	22–26	N/A	2623.7 (2398.9–2927.9)	143.5 (130.5–165.7)	13.4 (11.3–15)	94.0 (84.3–107.3)	0.06 (0.06–0.07)	Sanchez-Herriaz et al. (1995)
P. leucomystax Bali (6)	24.5–29	ca. 48	2550.1 (2320.6–2677.7)	218.5 (148–408.3)	16.5 (12.6–23.2)	84.7 (43.8–129.4)	N/A	Marquez and Eekhout (2006)

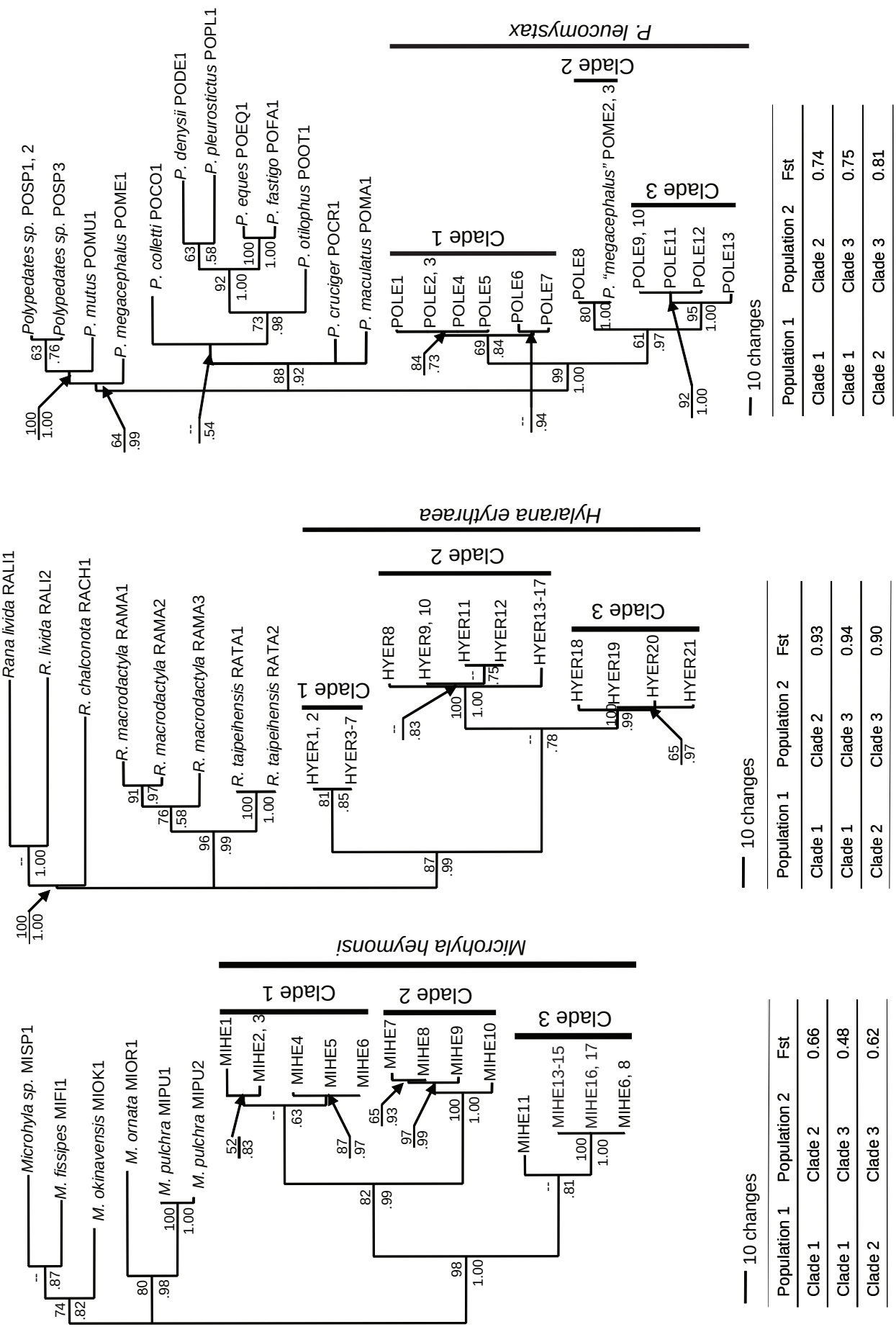

Fig. 1. Phylogenies of *Microhyla heymonsi*, *Hylarana erythraea*, and *Polypedates leucomystax*. Top numbers are bootstrap values from maximum parsimony analyses, and bottom numbers are posterior probabilities from Bayesian analyses.

Table 2. Calls, morphology, and genetics of frogs from Sakaerat, Thailand and Singapore. "Lower" and "greater" indicate values in the Thai population compared to Singapore.

Species	Call duration, pulse number, call rate	Genetic distance (%)		F_{ST}	Taxonomic conclusion by data type	
		16S	Cytb		Call	DNA
M. heymonsi	Same	4.29–4.56	11.90–13.08	0.62	Conspecific	Ambiguous
H. erythraea	Same	5.11	10.4	0.93	Conspecific	Ambiguous
P. leucomystax	Lower, lower, greater	2.95–3.57	6.99–7.46	0.75	Cryptic	Conspecific

DISCUSSION

We found that the calls of *P. leucomystax* were significantly different between the two locations, while the genetic distances were relatively small (Table 2). We found that although our populations are not each other's closest relatives, all sampled populations of *P. leucomystax* formed a well-supported monophyletic clade (Fig. 1). Other studies of *P.*

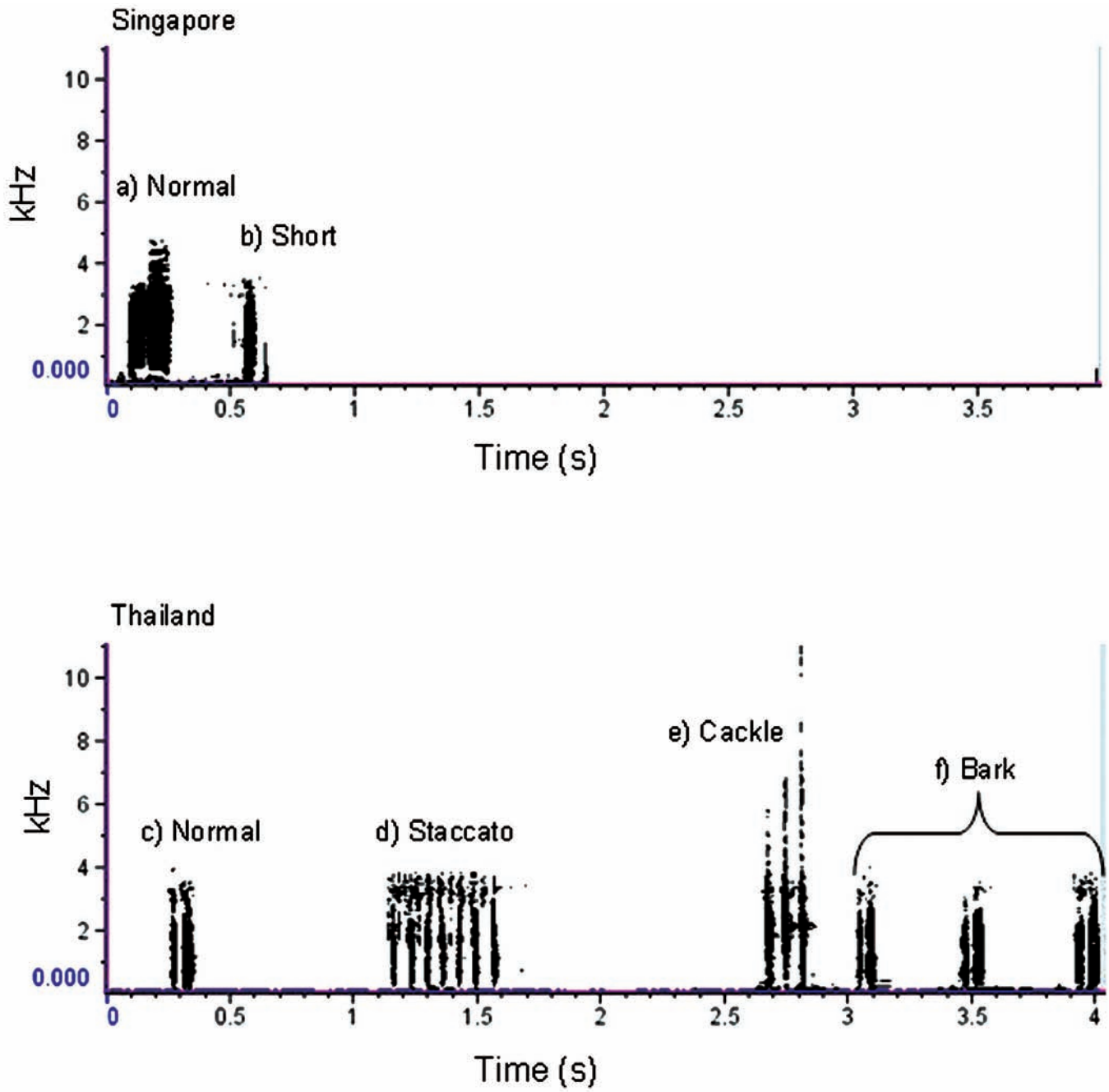

Fig. 2. Spectrograms of *Polypedates leucomystax* calls from Singapore (top) and Thailand (bottom). Calls are from a single individual in each population. For the Thai individual, call elements did not occur consecutively, but were cut and pasted from a 5 minute recording.

leucomystax calls (Table 1) indicate that there may be three or more cryptic species currently under that name across the range of *P. leucomystax*, and our call data support that hypothesis. In other widely distributed species, variation in calls decoupled from genetic divergence is not uncommon. Pröhl et al. (2006) found that differences in calls of *Physalaemus pustulosus* were better explained by geographic distance than by genetic distance, and that females preferred local males to foreign males in phonotaxis experiments. Although we attempted phonotaxis experiments to determine whether the differences in calls between our two populations contribute to prezygotic isolation, the response rate of females was very low (one in 18 trials), so we cannot determine the significance of the observed call variation.

Lougheed et al. (2006) found no geographic structure in the variation of 16S in *Hyla leucophyllata*, but calls varied significantly, similar to what we found in *P. leucomystax*. It is possible that our mtDNA markers do not accurately reflect reproductive isolation between populations, which would explain the observed differences in the call of *P. leucomystax* that was not accompanied by similar differences at our sampled genes. Our goal was to determine whether these two populations were conspecific, and for *P. leucomystax*, our data are unfortunately inconclusive. The calls indicate that the populations have diverged, and adult body size and time to metamorphosis are significantly different between the study populations (Sheridan, 2009). However, the genetic distances are relatively small. These populations may be different species, but we refrain from formally describing them because we were unable to study the geographically intermediate populations. More exhaustive sampling across the range, and phonotaxis experiments would aid in determining whether these two populations of *P. leucomystax* are reproductively isolated and thus represent different species.

For *M. heymonsi* and *H. erythraea*, similar calls indicate that the two populations are conspecific, but the genetic distances are larger than those observed for *P. leucomystax* (Table 2). These relatively large distances may only indicate that the populations have been separated for a long period of time, but long separation does not necessarily lead to speciation (Ferguson, 2002). Other anurans are known to exhibit similar levels of conspecific genetic diversity. For example, Matsui et al. (2005) found distances of over 10% at the cytb marker across populations (but within species) of *M. okinavensis* and *M. fissipes*. Additionally, adults of *M. heymonsi* and *H. erythraea* are not significantly different in body size (Sheridan, 2009) which indicates a lack of morphological divergence between the two populations. Call parameters for *M. heymonsi* have been examined for populations in N. Thailand and Taiwan (Table 1), but no populations intermediate between our study sites, so it is not yet possible to determine how call parameters vary across this species' range, and no data from other populations are available on the call parameters we examined for *H. erythraea*. Given that *M. heymonsi* and *H. erythraea* are probably nearly continuously distributed between our two study sites, the species may be effectively panmictic with minor genetic differentiation by distance. We thus conclude that our study populations of *M. heymonsi* and *H. erythraea* are conspecific. The study of call and genetic variation from isolated samples is unlikely to permit the identification of some species but the combination of these data sets coupled with comprehensive geographic sampling will eventually lead to the full characterization of these morphologically conservative animals.

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Appendix 1. Species, collection location, tissue code, museum code, and GenBank accession number for specimens collected for this study. Museum codes: CU, Chulalongkorn University Natural History Museum; KU, University of Kansas Natural History Museum and Biodiversity Research Center; RM, Raffles Museum of Biodiversity Research.

Species	Locality	Tissue Code	Museum Code	GenBank accession number	
				16S	cytb
Microhyla heymonsi	N/A	MIHE2	N/A	AY458596	AY458596
M. heymonsi	N/A	MIHE3	N/A	NC006406	NC006406
M. heymonsi	Sakaerat, Thailand	MIHE10	KU DSM1136	HM359087	N/A
M. heymonsi	Sakaerat, Thailand	MIHE7	KU DSM1152	HM359088	HQ141539
M. heymonsi	Sakaerat, Thailand	MIHE9	KU DSM1153	HM359089	HQ141545
M. heymonsi	Sakaerat, Thailand	MIHE8	KU DSM1205	HM359090	HQ141542
M. heymonsi	Singapore	MIHE12	RM MIHEJS2	HM359091	HQ141546
M. heymonsi	Singapore	MIHE13	RM MIHEJS3	HM359092	HQ141544
M. heymonsi	Singapore	MIHE14	RM MIHEJS4	HM359093	HQ141541
M. heymonsi	Singapore	MIHE15	RM MIHEJS5	HM359094	HQ141540
M. heymonsi	Singapore	MIHE16	RM MIHEJS6	HM359095	HQ141543
M. heymonsi	Singapore	MIHE17	RM MIHEJS8	HM359096	HQ141538
Polypedates megacephalus	N/A	POME2	N/A	AY458598	AY458598
P. megacephalus	N/A	POME3	N/A	NC006408	NC006408
P. leucomystax	Singapore	POLE2	RM POLEJS2	HM359102	HQ141554
P. leucomystax	Singapore	POLE3	RM POLEJS8	HM359103	HQ141553
P. leucomystax	Singapore	POLE4	RM POLEJS6	HM359104	HQ141549
P. leucomystax	Singapore	POLE5	RM POLEJS1	HM359105	HQ141551
P. leucomystax	Sakaerat, Thailand	POLE10	CU HKV17473	HM359098	HQ141548
P. leucomystax	Sakaerat, Thailand	POLE12	CU HKV17474	HM359100	HQ141556
P. leucomystax	Sakaerat, Thailand	POLE13	CU HKV17475	HM359101	HQ141552
P. leucomystax	Sakaerat, Thailand	POLE11	CU HKV17496	HM359099	HQ141547
P. leucomystax	Sakaerat, Thailand	POLE9	KU DSM1147	HM359097	HQ141555
Rana chalconota	Singapore	RACH1	RM RACHJS2	HM359106	N/A
R. macrodactyla	Sakaerat, Thailand	RAMA1	KU DSM1308	HM359107	N/A
Hylarana erythraea	Sakaerat, Thailand	HYER3	KU DSM1373	HM359111	HQ141557
H. erythraea	Sakaerat, Thailand	HYER4	KU DSM1374	HM359108	HQ141559
H. erythraea	Sakaerat, Thailand	HYER5	CU HKV17482	HM359110	HQ141560
H. erythraea	Sakaerat, Thailand	HYER6	CU HKV17485	HM359109	HQ141558
H. erythraea	Sakaerat, Thailand	HYER7	CU HKV17497	HM359112	HQ141561
H. erythraea	Singapore	HYER13	RM RAERJS2	HM359113	HQ141562
H. erythraea	Singapore	HYER14	RM RAERJS3	HM359114	HQ141566
H. erythraea	Singapore	HYER15	RM RAERJS4	HM359115	HQ141563
H. erythraea	Singapore	HYER16	RM RAERJS5	HM359116	HQ141564
H. erythraea	Singapore	HYER17	RM RAERJS8	HM359117	HQ141565

Appendix 2. 16S sequences downloaded from GenBank.

Species	Tissue Code	GenBank Accession Number
Microhyla sp.	MISP1	AF215371
M. fissipes	MIFI1	AB201186
M. okinavensis	MIOK1	AB201184
M. ornate	MIOR1	AB201188
M. pulchra	MIPU1	AB201191
M. pulchra	MIPU2	AF285205
M. heymonsi	MIHE6	AF215372
M. heymonsi	MIHE5	AF285201
M. heymonsi	MIHE4	AF285200
M. heymonsi	MIHE1	DQ283382
M. heymonsi	MIHE11	AB201190
Polypedates aff. *leucomystax*	POSP1	AF285224
P. aff. *leucomystax*	POSP2	AF285223
P. colettii	POCO1	AF215354
P. cruciger	POCR1	AY141845
P. dennysii	PODE1	AF285219
P. eques	POEQ1	AY141846
P. fastigo	POFA1	AY880518
P. maculates	POMA1	AF215358
P. megacephalus	POME1	AY880519
P. mutus	POMU1	AY880521
P. otilophus	POOT1	AF215356
P. pleurostictus	POPL1	AY880522
P. leucomystax	POLE1	AF215343
P. leucomystax	POLE7	AF026368
P. leucomystax	POLE6	AY141849
P. leucomystax	POLE8	AF285220
P. "leucomystax"	POSP3	DQ283048
Rana livida	RALI1	AF206459
R. livida	RALI2	AB200955
R. macrodactyla	RAMA3	DQ360002
R. macrodactyla	RAMA2	AF206489
R. taipehensis	RATA2	DQ283396
R. taipehensis	RATA1	DQ360005
Hylarana erythraea	HYER1	AF206475
H. erythraea	HYER2	DQ283138
H. erythraea	HYER8	DQ835345
H. erythraea	HYER9	DQ835339
H. erythraea	HYER19	DQ835340
H. erythraea	HYER18	DQ835343
H. erythraea	HYER20	DQ835342
H. erythraea	HYER21	DQ835341
H. erythraea	HYER10	DQ835344
H. erythraea	HYER11	DQ835346
H. erythraea	HYER12	DQ835347

Appendix 3. Details of individuals recorded for this study in Singapore and Thailand. RMBR museum numbers are for Raffles Museum of Biodiversity Research; CU museum numbers are for Chulalongkorn University Museum of Natural History.

Species	Location	Museum Number	Recording temperature (°C)	SVL (mm)	Dominant Frequency (Hz)
Hylarana erythraea	Singapore	RMBR00028	27.2	45	2627
Hylarana erythraea	Singapore	RMBR00036	27.4	42	3521
Hylarana erythraea	Singapore	RMBR00058	N/A	41	2930
Hylarana erythraea	Singapore	RMBR00073	N/A	41	2156
Hylarana erythraea	Singapore	RMBR00074	25.2	41	2406
Hylarana erythraea	Singapore	RMBR00081	24.8	42	2179
Hylarana erythraea	Singapore	RMBR00082	24.8	46	3094
Hylarana erythraea	Thailand	CU 17561	26	46	2627
Hylarana erythraea	Thailand	CU JAS003	27	45	2046
Hylarana erythraea	Thailand	CU JAS004	27	41	2885
Hylarana erythraea	Thailand	CU JAS017	24	46	2821
Hylarana erythraea	Thailand	CU JAS027	25	49	2345
Hylarana erythraea	Thailand	CU JAS028	24	45	1878
Hylarana erythraea	Thailand	CU JAS029	22	39	2438
Hylarana erythraea	Thailand	CU JAS030	22	40	2211
Microhyla heymonsi	Singapore	RMBR00066	N/A	19	3488
Microhyla heymonsi	Singapore	RMBR00069	N/A	19	3316
Microhyla heymonsi	Singapore	RMBR00070	N/A	20	3359
Microhyla heymonsi	Singapore	RMBR00009	25.9	18	1938
Microhyla heymonsi	Singapore	RMBR00011	25.9	17.5	3747
Microhyla heymonsi	Singapore	RMBR00024	26.7	20	3359
Microhyla heymonsi	Singapore	RMBR00029	25.8	21	3445
Microhyla heymonsi	Singapore	RMBR00037	27.0	19	3660
Microhyla heymonsi	Singapore	RMBR00038	27.0	19	3316
Microhyla heymonsi	Thailand	CU 17556	N/A	20	2946
Microhyla heymonsi	Thailand	CU 17557	N/A	19	3557
Microhyla heymonsi	Thailand	CU JAS005	26.0	21	3488
Microhyla heymonsi	Thailand	CU JAS006	26.0	22	3445
Microhyla heymonsi	Thailand	CU JAS007	26.0	23	3018
Microhyla heymonsi	Thailand	CU JAS018	24.0	21	3114
Microhyla heymonsi	Thailand	CU JAS019	23.5	19	3609
Microhyla heymonsi	Thailand	CU JAS022	23.5	20	3277
Microhyla heymonsi	Thailand	CU JAS024	23.5	22	3109
Polypedates leucomystax	Singapore	RMBR00010	25.9	42	818
Polypedates leucomystax	Singapore	RMBR00012	25.9	43	947
Polypedates leucomystax	Singapore	RMBR00003	25.2	41	2191
Polypedates leucomystax	Singapore	RMBR00002	25.2	41	2562
Polypedates leucomystax	Singapore	RMBR00018	25.5	42	1361
Polypedates leucomystax	Singapore	RMBR00019	24.8	45	1478
Polypedates leucomystax	Singapore	RMBR00020	27.8	48	2139
Polypedates leucomystax	Singapore	RMBR00021	27.8	48	2268
Polypedates leucomystax	Singapore	RMBR00022	27.8	45	2311
Polypedates leucomystax	Thailand	CU 17545	24.0	61	1094
Polypedates leucomystax	Thailand	CU 17546	22.0	52	1708
Polypedates leucomystax	Thailand	CU 17562	26.0	57	1382
Polypedates leucomystax	Thailand	CU 17564	26.5	63	517
Polypedates leucomystax	Thailand	CU JAS032	23.0	54	1253
Polypedates leucomystax	Thailand	CU JAS035	23.5	61	1779
Polypedates leucomystax	Thailand	CU JAS038	N/A	59	646