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# Phylogenetic relationships and genetic diversity of the *Polypedates leucomystax* complex in Thailand

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Controversy in the taxonomic evaluation of the Asian tree frog *Polypedates leucomystax* complex presents the challenging task of gaining insight into its biogeographical distribution and diversification. Here, we describe the dispersion and genetic relationship of these species in Thailand where we connect the population of the *P. leucomystax* complex of the Sunda Islands to the mainland population based on the mitochondrial cytochrome c oxidase subunit I (*COI*) gene, derived from 266 samples. Our maternal genealogy implies that there are four well-supported lineages in Thailand, consisting of Northern A (clade A: *Polypedates* sp.), Nan (clade B: *P. cf. impresus*), Southern (clade C: *P. cf. leucomystax*) and Northern B (clade D: *P. cf. megacephalus*), with Bayesian posterior probability >0.9. Phylogeny and haplotype networks indicate that clades A, B and D are sympatric. In contrast, clade C (*P. cf. leucomystax*) and clade D (*P. cf. megacephalus*) are genetically divergent due to the geographical barrier of the Isthmus of Kra, resulting in allopatric distribution. Climatic conditions, in particular rainfall, that differ on each side of the Isthmus of Kra may play an important role in limiting the immigration of both clades. For the within-populations of either clades C or D, there was no significant correlation between geographic and genetic distance by the isolation-by-distance test, indicating intraspecific gene flow of each clade. Population expansion occurred in clade C, whereas clade D showed a constant population. Taken together, the *P. leucomystax* complex in Southeast Asia may be diversified by climatic oscillation, leading to allopatric and/or sympatric speciation.

1 **Phylogenetic relationships and genetic diversity of the *Polypedates leucomystax* complex in**  
2 **Thailand**

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22 **ABSTRACT**

23 Controversy in the taxonomic evaluation of the Asian tree frog *Polypedates leucomystax*  
24 complex presents the challenging task of gaining insight into its biogeographical distribution and  
25 diversification. Here, we describe the dispersion and genetic relationship of these species in  
26 Thailand where we connect the population of the *P. leucomystax* complex of the Sunda Islands to  
27 the mainland population based on the mitochondrial cytochrome c oxidase subunit I (*COI*) gene,  
28 derived from 266 samples. Our maternal genealogy implies that there are four well-supported  
29 lineages in Thailand, consisting of Northern A (clade A: *Polypedates* sp.), Nan (clade B: *P. cf.*  
30 *impresus*), Southern (clade C: *P. cf. leucomystax*) and Northern B (clade D: *P. cf.*  
31 *megacephalus*), with Bayesian posterior probability >0.9. Phylogeny and haplotype networks  
32 indicate that clades A, B and D are sympatric. In contrast, clade C (*P. cf. leucomystax*) and clade  
33 D (*P. cf. megacephalus*) are genetically divergent due to the geographical barrier of the Isthmus  
34 of Kra, resulting in allopatric distribution. Climatic conditions, in particular rainfall, that differ  
35 on each side of the Isthmus of Kra may play an important role in limiting the immigration of  
36 both clades. For the within-populations of either clades C or D, there was no significant  
37 correlation between geographic and genetic distance by the isolation-by-distance test, indicating  
38 intraspecific gene flow of each clade. Population expansion occurred in clade C, whereas clade D  
39 showed a constant population. Taken together, the *P. leucomystax* complex in Southeast Asia  
40 may be diversified by climatic oscillation, leading to allopatric and/or sympatric speciation.

41

42 **Keywords** Amphibian, Allopatric, Demographic expansion, Evolution

43

## 44 INTRODUCTION

45 Southeast Asia is a hotspot of substantial genetic diversity of amphibians. Recent molecular  
46 phylogenetic analyses have disclosed many anuran lineages that contain cryptic species.  
47 Historically, complex changes in the region's geology and climate (e.g., Pleistocene climatic  
48 oscillations) altered the topology and environmental conditions, resulting in an initial  
49 fragmentation of habitat. These mechanisms generated high species richness in the current period  
50 (Hall, 1998; Woodruff, 2010). Of interest to our research were the numerous frog species in  
51 Southeast Asia whose taxonomy is still controversial, such as *Microhyla fissipes* (Yuan et al.,  
52 2016), *Staurois tuberilinguis* (Matsui et al., 2007), *Microhyla ornata* (Matsui et al., 2005) and  
53 *Polypedates leucomystax* (Kuraishi et al., 2013; Rujirawan, Stuart & Aowphol, 2013). The  
54 clarification of ambiguous species is essential to better understand their speciation and  
55 diversification and their biogeography for conservation purposes.

56 The Asian tree frog, the *Polypedates leucomystax* (Gravenhorst, 1829) complex, is an  
57 Asian Rhacophoridae frog. These species are widely distributed in Southeast Asia, South China  
58 and India. In addition, this species has phenotypic plasticity and high adaptation to local  
59 environment, leading to its existence in diverse habitats such as forests and even buildings. These  
60 high levels of phenotypic plasticity present a great challenge for classification. Phylogenetic and  
61 taxonomic relationships of the *P. leucomystax* complex throughout Southeast Asia exhibit  
62 adaptive radiation (Kuraishi et al., 2013; Pan et al., 2013; Rujirawan, Stuart & Aowphol, 2013).  
63 At least six valid species, including *P. braueri*, *P. leucomystax*, *P. macrotis*, *P. megacephalus*, *P.*  
64 *mutus* and *P. impresus*, have been delimited from the *P. leucomystax* complex based on their  
65 morphology, advertisement calls and molecular data (Matsui, Seto & Utsunomiya, 1986; Brown

66 et al., 2010; Kuraishi et al., 2011; Kuraishi et al., 2013; Pan et al., 2013). Five species, *P.*  
67 *leucomystax*, *P. mutus*, *P. macrotis*, *P. colletti* and *P. megacephalus*, can be found in Thailand  
68 (Taylor, 1962; Heyer, 1971; Frost, 2013; Kuraishi et al., 2013; Pan et al., 2013; Rujirawan,  
69 Stuart & Aowphol, 2013). A study by Brown et al. (2010) indicated that much of the genetic  
70 divergence of the *P. leucomystax* complex was discovered in mainland rather than in insular  
71 populations distributed throughout thousands of islands of the Malay Archipelago, presumably  
72 resulting from range expansion mediated by transportation of agricultural products. Recently a  
73 new species, *P. discantus*, belonging to the *P. leucomystax* species complex from southern  
74 Thailand was discovered using data on morphological characteristics, advertisement calls and  
75 molecular evidence, which were dominantly dissimilar to those of *P. leucomystax* and *P.*  
76 *megacephalus* (Rujirawan, Stuart & Aowphol, 2013). Several studies have confirmed the  
77 presence of highly cryptic species of the *Polypedates leucomystax* complex (Matsui, Seto &  
78 Utsunomiya, 1986; Kuraishi et al., 2011; Blair et al., 2013; Kuraishi et al., 2013; Pan et al.,  
79 2013). We believe that the *P. leucomystax* complex in Thailand remains a highly cryptic species,  
80 as is the case elsewhere.

81         Understanding the phylogenetic relationships among species can give insight into how  
82 lineages diverged and how new species arose. The process of speciation can be organized based  
83 on the geographic overlap of emerging species during divergence. In this study, we investigated  
84 the genetic variation, phylogenetic relationships and other relevant factors that limit the dispersal  
85 of *P. leucomystax* complex in Thailand. The present results illustrate the range of distribution of  
86 *P. leucomystax* and *P. megacephalus*, which is influenced by climatic conditions, and the  
87 possible existence of *P. impresus* in sympatry with *P. megacephalus* in the north of Thailand.

88

## 89 MATERIALS AND METHODS

### 90 Sample collection, DNA extraction and sequencing

91 In this study, a total of 266 adult *Polypedates leucomystax* complex individuals were collected  
92 from 15 different localities in Thailand (Table 1). All samples were dissected to obtain the liver,  
93 which was then stored in absolute ethanol. Collecting and euthestication was approved by  
94 Center For Animal Research Naresuan University under project number NU-AE591028.  
95 Genomic DNA was extracted from liver tissue using a DNA extraction kit (RBC Bioscience,  
96 Singapore) and kept at  $-20^{\circ}\text{C}$  for further use. Individual DNA was used as a template for PCR  
97 amplification of the mitochondrial COI gene using Taq DNA polymerase in a total volume of 25  
98  $\mu\text{L}$  under the following condition: an initial denaturation at  $94^{\circ}\text{C}$  for 5 min, followed by 35–40  
99 cycles at  $94^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 1 min, and a final extension step at  $72^{\circ}\text{C}$  for  
100 7 min. PCR products were visualized on 1.5% agarose gel under UV light. The expected size of a  
101 partial mitochondrial COI gene sequence was 688 bp. Subsequently, all PCR products were  
102 purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and then sequenced  
103 (Macrogen, South Korea).

104

### 105 Phylogeny

106 Phylogenetic reconstructions were executed using Bayesian inference (BI) and maximum  
107 likelihood (ML) independently. The best-fit model of DNA sequence evolution for this locus was  
108 identified with the Akaike information criterion (AIC) implemented in MrModeltest v2.3  
109 (Nylander, 2004). The GTR+I+G model was selected as the best model and used in the following

110 analysis. A Bayesian tree was generated using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003).  
111 For BI analysis, two independent searches with random starting trees were run for 5 million  
112 generations while sampling over 1,000 generations and compared using four Markov chain  
113 Monte Carlo chains (temp = 0.2). Convergence was assessed by plotting the log-likelihood  
114 scores in Tracer v1.5 (Rambaut et al., 2013), and the first 25% of the generations from each run  
115 were discarded before building a consensus tree. Maximum likelihood analysis was performed  
116 using RAxML 7.0.4 (Stamatakis, Hoover & Rougemont, 2008). The same model of nucleotide  
117 substitution used for BI analysis was employed for the ML tree search with 1,000 bootstrap  
118 replicates. COI gene sequences of the *Polypedates leucomystax* complex were retrieved from  
119 GenBank, as follows: *P. impresus*: KP996822 (China), KP996846 (China), KP087862-70  
120 (Laos); *P. leucomystax*: KR087871-2 (Thailand); *P. megacephalus*: KR087879, KR087881  
121 (Thailand).

122

### 123 **Population genetics and structure**

124 A total of 266 sequences of mitochondrial *COI* were aligned using ClustalW (implemented in  
125 MEGA 6.0 with default parameters). The number of polymorphic sites, the parsimony-  
126 informative sites, singleton sites, the number of haplotypes, haplotype diversity ( $H_d$ ), and  
127 nucleotide diversity for each clade were calculated using DnaSP 5.0 (Librado & Rozas, 2009).  
128 Genetic distances among taxa were calculated using the *p*-distance model in MEGA 6.0 (Tamura  
129 et al., 2011). Furthermore, we detected a boundary line in the genetic landscape between *P.*  
130 *megacephalus* and *P. leucomystax* using Barrier 2.2 (Manni, Guérard & Heyer, 2004). The  
131 minimum spanning network was constructed using PopART (Population Analysis with



132 Reticulate Trees) population genetics software to define the relationships among haplotypes and  
133 the distribution of haplotypes in each locality (Bandelt, Forster & Röhl, 1999). To evaluate the  
134 effect of geographic distance on the genetic divergence among populations of *P. megacephalus*  
135 and among populations of *P. leucomystax*, a Mantel test with 1,000 permutations was carried out  
136 using GenAEx 6.5 (Peakall & Smouse, 2012).

137

### 138 **Demographic history**

139 To investigate the demographic history of *P. megacephalus* and *P. leucomystax* populations in  
140 Thailand, multiple approaches were explored. Neutrality tests of Tajima's *D* and Fu's *F<sub>s</sub>* for the  
141 two species were calculated using DnaSP 5.0. A significantly positive value indicates a process  
142 of subdivision or recent population bottleneck, whereas a population expansion shows a  
143 significantly negative value. Pairwise mismatch distribution was used to assume a constant  
144 population size using DnaSP 5.0. Multiple mismatch distribution implies stability of the  
145 population, while unimodal mismatch distribution reflects an expanding population. In addition  
146 to these methods, the raggedness index ( $r_g$ ) of the observed distribution was calculated using  
147 DnaSP 5.0. A small  $r_g$  indicates a demographic expansion.

148

## 149 **RESULTS**

### 150 **Sequence characteristics**

151 A total of 266 samples of the *P. leucomystax* complex yielded 688 bp fragments of the  
152 mitochondrial *COI* gene. All new sequences in this study were deposited in the GenBank  
153 database (xxxx-xxxx) [available upon manuscript acceptance]. After multiple alignment of all  
154 *COI* sequences, the sequences were trimmed to the same length, given as 437 bp before  
155 downstream analysis. We observed 82 polymorphic sites, which are 82 parsimony-informative  
156 sites without a singleton site, acquiring 15 haplotypes (Table 2). Overall nucleotide and  
157 haplotype diversity were 0.0664 and 0.9000, respectively (Table 2).

158

### 159 **Phylogenetic analyses and haplotype distribution**

160 Based on 266 mitochondrial *COI* sequences of the *P. leucomystax* complex, matrilineal  
161 genealogy was analyzed through a Bayesian analysis model with MrModeltest v2.3. Our results  
162 indicated that the *P. leucomystax* complex in Thailand could consist of four clades, including:  
163 clade A (Northern A), *Polypedates* sp.; clade B (Nan), *P. cf. impresus*; clade C (Southern), *P. cf.*  
164 *leucomystax*; and clade D (Northern B), *P. cf. megacephalus* (Fig. 1). With respect to  
165 phylogenetic interference, *Polypedates* sp. was treated as a sister group of *P. cf. impresus*, which  
166 was found in Nan. *Polypedates* sp., however, can be seen in genetic samples obtained from  
167 Kanchanaburi (KCB), Mae Hong Son (MHS) and Phetchaburi (PCB) provinces, and shared a  
168 habitat with clade D, which was recognized as *P. cf. megacephalus*; its distribution range was in  
169 the far north of the Isthmus of Kra as Chiang Mai (CM), MHS, KCB, PCB, Saraburi (SRB), Loei  
170 (LPR), Nakhon Ratchasima (NRS) and Prachuap Khiri Khan (PKK), whereas the dispersal areas  
171 of the clade C population as *P. cf. leucomystax*, including Chumphon (CP), Nakhon Si  
172 Thammarat (NST), Phuket (PK) and Ranong (RN), were south of the Isthmus of Kra (Fig. 2). To

173 determine a barrier for immigration between *P. megacephalus* and *P. leucomystax* based on the  
174 dataset of genetic distance (Kimura's two-parameter model), Barrier 2.2 was ed. Likely, the  
175 Isthmus of Kra could be a significant area to restrict their immigration.

176 The minimum spanning network among the mitochondrial haplotypes was also  
177 constructed as a result of four groups having similar results of phylogenetic inference (Fig. 3).  
178 The haplotypes of each group showed unique features, and each group had a different number of  
179 haplotypes. *P. megacephalus* exhibited the highest number of haplotypes at seven ( $H_d = 0.746$ ),  
180 followed by *P. leucomystax* with five haplotypes ( $H_d = 0.7526$ ), as shown in Table 2.  
181 Haplotypes A and B, seen in *Polypedates* sp., and haplotype C found in *P. cf. impresus* were  
182 unique haplotypes. Haplotypes D–I were noted in *P. cf. megacephalus*, while populations of  
183 northern, western and upper southern Thailand (KCB, PCB and PKK, respectively) shared  
184 haplotype J. Besides, we noted that the haplotypes G and F of *P. cf. megacephalus* in NRS had  
185 relatively high divergence from the congeners. *P. cf. leucomystax* had high haplotype diversity,  
186 and contained three unique haplotypes (M, N and O) and two shared haplotypes (K and L).

187 In addition, there was no significant isolation-by-distance effect among populations of *P.*  
188 *cf. megacephalus* and *P. cf. leucomystax* based on analyses of Mantel tests between the genetic  
189 distance of the mitochondrial *COI* gene sequence and the geographical distance (Fig. 4).

190

### 191 **Demographic history**

192 When we defined a significant barrier around the Isthmus of Kra leading to the genetic  
193 divergence between *P. megacephalus* and *P. leucomystax*, neutrality tests (Tajima's *D* and Fu's

194  $F_s$ ) of both species were not significantly positive, whereas  $F_u$ 's  $F_s$  of *P. leucomystax* was  
195 significantly positive (Table 3). Furthermore, the mismatch distribution was tested as a result of  
196 left-skewed multimodal mismatch distribution for *P. megacephalus* with moderate  $r_g$  (0.2031)  
197 but unimodal mismatch distribution for *P. leucomystax* with low  $r_g$  (0.0569) (Fig. 5). Overall,  
198 these results suggested a constant population size of *P. megacephalus* and population expansion  
199 of *P. leucomystax*.

200

## 201 **DISCUSSION**

202 The taxonomy of the Asian tree frog of the *Polypedates leucomystax* complex, one of the most  
203 notoriously challenging, is contentious owing to the species' widespread distribution and their  
204 similar morphology. To solve the taxonomic status of the *P. leucomystax* complex in Thailand,  
205 *COI* mitochondrial gene sequences of these species were analyzed. Unfortunately, several  
206 studies on the genealogy of the *P. leucomystax* complex used mitochondrial 12S rRNA, tRNA  
207 valine, and the 16S rRNA gene (Brown et al., 2010; Kuraishi et al., 2013; Pan et al., 2013). We,  
208 therefore, could not exploit these sequences for our analyses. Our matrilineal genealogy implied  
209 four well-supported lineages, consisting of *P. cf. megacephalus* (Northern B clade), *P. cf.*  
210 *leucomystax* (Southern clade), *P. cf. impresus* (found only in Nan) and *Polypedates* sp. (Northern  
211 A clade). That *Polypedates* sp. occurs in the same geographic areas as *P. cf. megacephalus* was  
212 considered as sympatric distribution; it, however, is a sister lineage with *P. cf. impresus*.  
213 Nevertheless, to clarify the taxonomy of *Polypedates* spp., morphological observations, call  
214 advertisement and ecological habitat would need to be investigated in future work. Recently, Pan  
215 et al. (2013) validated *P. impresus* molecularly, based on approximately 2 kb of mitochondrial

216 12S rRNA, tRNA valine and the 16S rRNA gene, as a valid species found in southern China and  
217 northern Laos. This is the first report to verify the existence of *P. impresus* in Nan, Thailand,  
218 which is located at the border with northern Laos, indicating its dispersal range. The main  
219 characteristics of *P. impresus* originally described by Yang (2008) are: the top of the head  
220 obviously concave, upper lip margin white, dorsal body light brown, and with no zebra-like  
221 stripes. The putative *P. impresus* individuals in our study are consistent with these  
222 characteristics.

223 Bayesian inference strongly supported that the northern and southern clades were  
224 considered as *P. megacephalus* and *P. leucomystax*, respectively, indicating geographically  
225 distinct species. Furthermore, Monmonier's algorithm suggested that the Isthmus of Kra seemed  
226 to be a significant region separating them due to a large genetic divergence between the  
227 populations north and south of the isthmus. This result was in accordance with a previous study  
228 by Kuraishi et al. (2013). In addition, we noted that the majority of the populations of the *P.*  
229 *leucomystax* complex in Thailand are *P. megacephalus* and *P. leucomystax*. They are widespread  
230 species, with the former having a dispersal range from the south of China (Guangxi and Yunnan)  
231 to the north of Thailand, Laos and Vietnam (Kuraishi et al., 2013; Pan et al., 2013), and the latter  
232 having a distribution range from the south of Thailand to the Malay Archipelago (Brown et al.,  
233 2010; Kuraishi et al., 2013). Considering that the Isthmus of Kra shapes the spatial distribution  
234 of the genetic lineage between the northern and southern clades, it seems remarkable that it lacks  
235 substantial topological features, e.g. a mountain range or river, which usually block migration  
236 routes of animals. Yet the Isthmus of Kra serves as a geographic barrier between the two clades,  
237 with *P. leucomystax* absent from areas north of the isthmus and, likewise, *P. megacephalus* not  
238 distributed south of the isthmus. Moreover, the matrilineal haplotype network was strong

239 evidence of the suppression of migration of the northern and southern clades. In contrast, a  
240 previous study revealed the range expansion of insular populations of *P. leucomystax* with small  
241 genetic distance among islands of the Malay Archipelago, as they can migrate from one island to  
242 another mediated by anthropogenic effects, in particular transportation of agricultural products  
243 (Brown et al., 2010). We therefore postulated that climatic conditions might be the key barrier  
244 for restricting the distribution range of the modern populations, because the Isthmus of Kra is a  
245 climatic joint between a drier climate to the north and a more humid climate to the south of the  
246 isthmus. Data on rainfall in Thailand by the Thai Meteorological Department indicates a  
247 difference in the amount of rainfall between areas to the north and south of the isthmus (Fig. 6).  
248 Todd et al. (2011) observed a shift in reproductive timing in ten amphibian species at a wetland  
249 in South Carolina, USA, over 30 years as a result of climate change. Furthermore, the variation  
250 of breeding season length of the *P. leucomystax* complex of Thailand (putative *P. megacephalus*;  
251 6 months a year for breeding) and Singapore (putative *P. leucomystax*; every month) led to  
252 adaptation to shortened breeding season length by increasing clutch size (Sheridan, 2009). It is  
253 clear that climate can influence the alteration in phenotypes of frogs (Sheridan, 2009; Todd et al.,  
254 2011). The different climates of the northern and southern clades' habitats likely led to the  
255 alteration of biological features such as behavior, reproductive timing or specific niche,  
256 contributing to the restricted distribution of both species. However, an initial cladogenesis of the  
257 *P. leucomystax* complex in Indochina, which includes the two species, was caused by climatic  
258 oscillations during the Miocene and the subsequent Plio–Pleistocene, resulting in increased  
259 aridity and a monsoonal weather system, sea fluctuation and habitat fragmentation which in turn  
260 promoted species diversification (Blair et al., 2013). Kuraishi et al. (2013) inferred that the most  
261 recent divergence time between *P. megacephalus* and *P. leucomystax* was in the late Pliocene or

262 early Pleistocene (1.4–4.0 MYBP). The speciation process was caused by a short disjunction of  
263 the Malay Peninsula and Sunda Islands by the South China Sea in the early Pliocene (about 5  
264 MYBP) when the sea level rose (Hall, 1998), resulting in genetic divergence into the two valid  
265 species.

266         Within the population of *P. megacephalus*, maternal genealogy demonstrated that the  
267 genetic samples from Nakhon Ratchasima province (NRS) seemed to be a naturally occurring  
268 divergence because of the emergence of endemic haplotypes; however, it was a low-supported  
269 lineage, with 0.7 Bayesian posterior probability (BPP). When we considered the topography of  
270 this region, the population of NRS as clade D1 is partitioned from the other populations within  
271 clade D by the two mountain ranges, Dong Phaya Yen and Sankamphaeng. This may be a  
272 possible barrier to gene flow among the modern populations of *P. megacephalus* between  
273 western and eastern Thailand. Unfortunately, only a limited number of populations from the east  
274 of Thailand were investigated in this study. Further work for validating if Dong Phaya Yen and  
275 Sankamphaeng are a great barrier for gene flow in *P. megacephalus* is required. According to the  
276 demographic history, the population of *P. megacephalus* in Thailand was a stable population but  
277 the population of *P. leucomystax* in southern Thailand showed a relatively similar unimodal  
278 distribution with small raggedness index, possibly indicating a population expansion. This result  
279 was similar to that observed in the population of *P. leucomystax* in the northern Philippines  
280 (Brown et al., 2010). This scenario implied a genetically homogenous population, especially in  
281 the population of Phuket Island which shared a haplotype with NST. We believe that these  
282 events might be mediated by human activities like agricultural transportation (Brown et al.,  
283 2010). Although the population of *P. leucomystax* expanded, it was limited to localities south of  
284 the Isthmus of Kra.

285

286 **CONCLUSIONS**

287 Our matrilineal genealogy of the *Polypedates leucomystax* complex in Thailand suggested four  
288 lineages, i.e. Nan (putative *P. impresus*), Northern B (putative *P. megacephalus*), Southern  
289 (putative *P. leucomystax*) and Northern A (*Polypedates* sp.) clades. We noted that the  
290 populations of the Northern B clade, Nan and *Polypedates* sp. are in sympatry while their  
291 distributions are allopatric to the southern clade (*P. leucomystax*), separated by the Isthmus of  
292 Kra. Climatic conditions may be a major contributor to limited migration of the current  
293 populations of both clades but climatic oscillation in the Pliocene and Pleistocene is a highly  
294 possible scenario that drove a speciation mechanism for diversification of the *P. leucomystax*  
295 complex in Southeast Asia and China, the divergence of the southern and northern clades in  
296 Thailand included.

297

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302

303 **Author Contributions**



304 K.B. designed the experiments, analyzed the statistical data and wrote the manuscript. C.S.  
305 conducted the experiments on *P. leucomystax* complex samples. Both authors read and approved  
306 the final manuscript.

307

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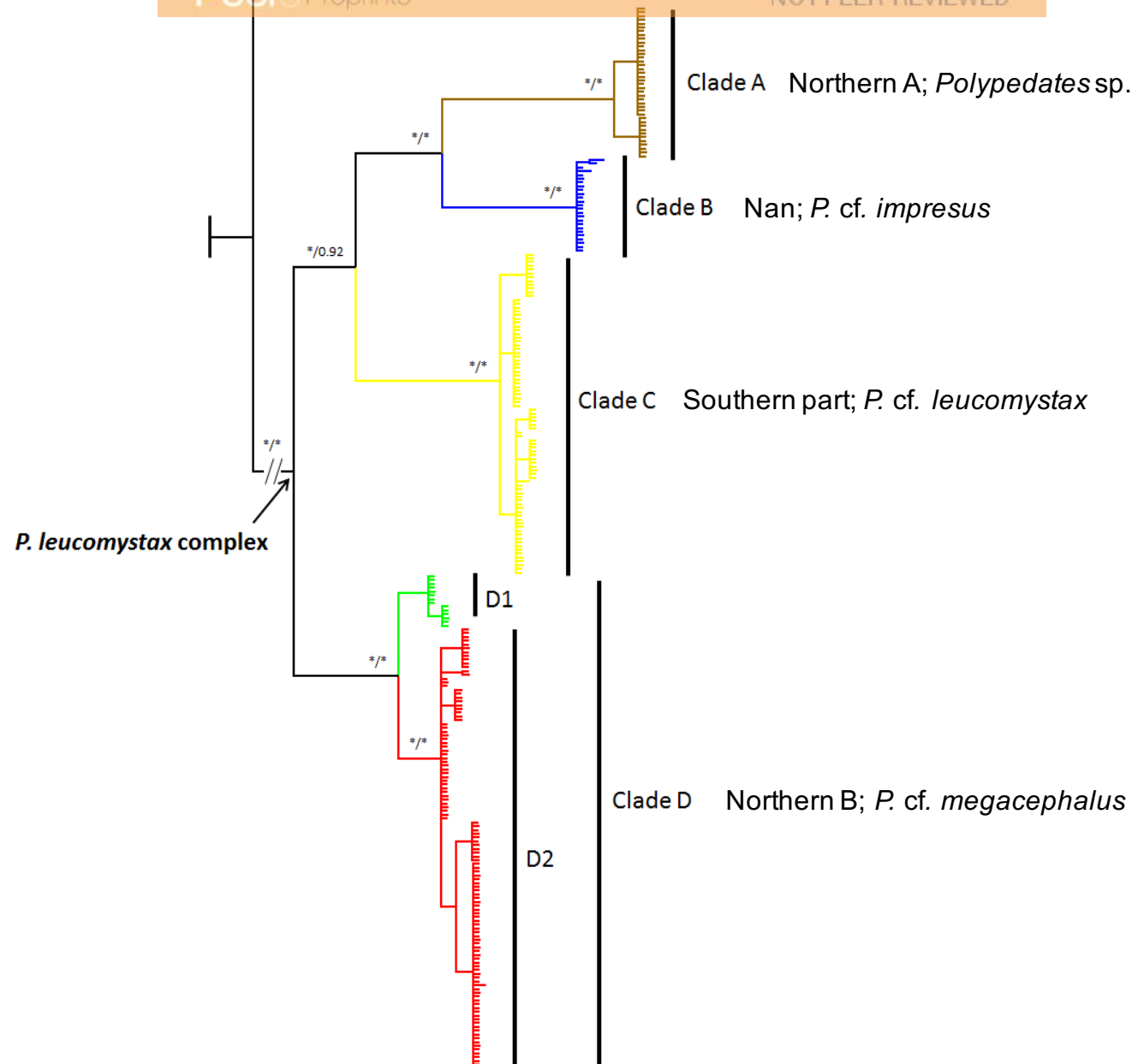
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**Figure 1**(on next page)

Phylogeographic relationships of the *Polypedates leucomystax* complex among populations in Thailand

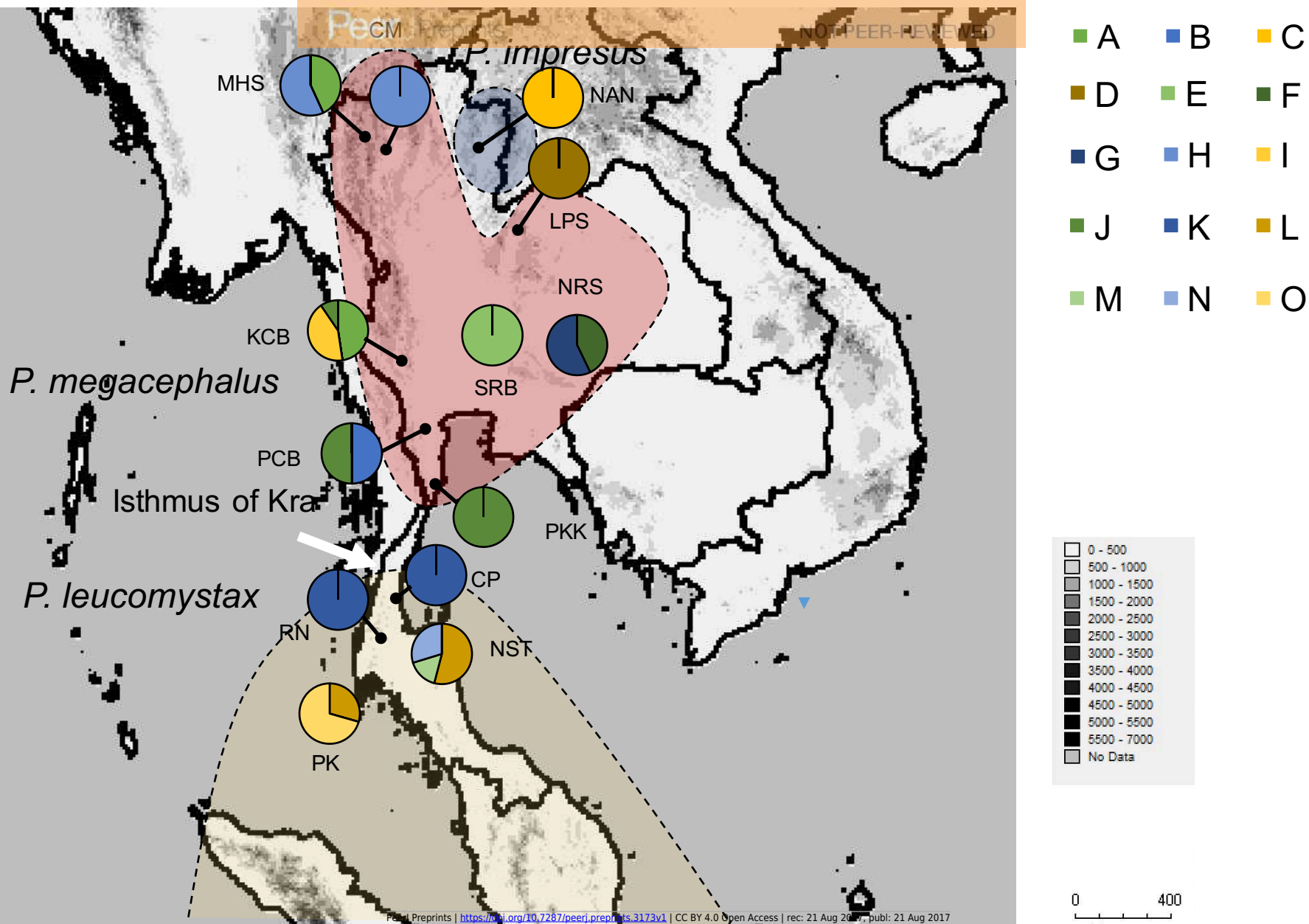
It consists of clade A as *Polypedates* sp., clade B as *P. cf. impresus*, clade C as *P. cf. leucomystax*, and clade D as *P. cf. megacephalus*, as well as outgroups (KR087858, KP996762 = *P. braueri*) inferred from Bayesian analysis of mitochondrial *COI* gene sequences. Bayesian posterior probability values are expressed above internodes. The asterisks above branches represent bootstrap support for Bayesian posterior probabilities and maximum likelihood (>95%). Scale bar represents 0.5 nucleotide substitutions per site.



**Figure 2** (on next page)

Haplotype distribution of the *Polypedates leucomystax* complex throughout Thailand.

The abbreviations for each locality are given in Table 1. Different colors represent the different haplotypes.

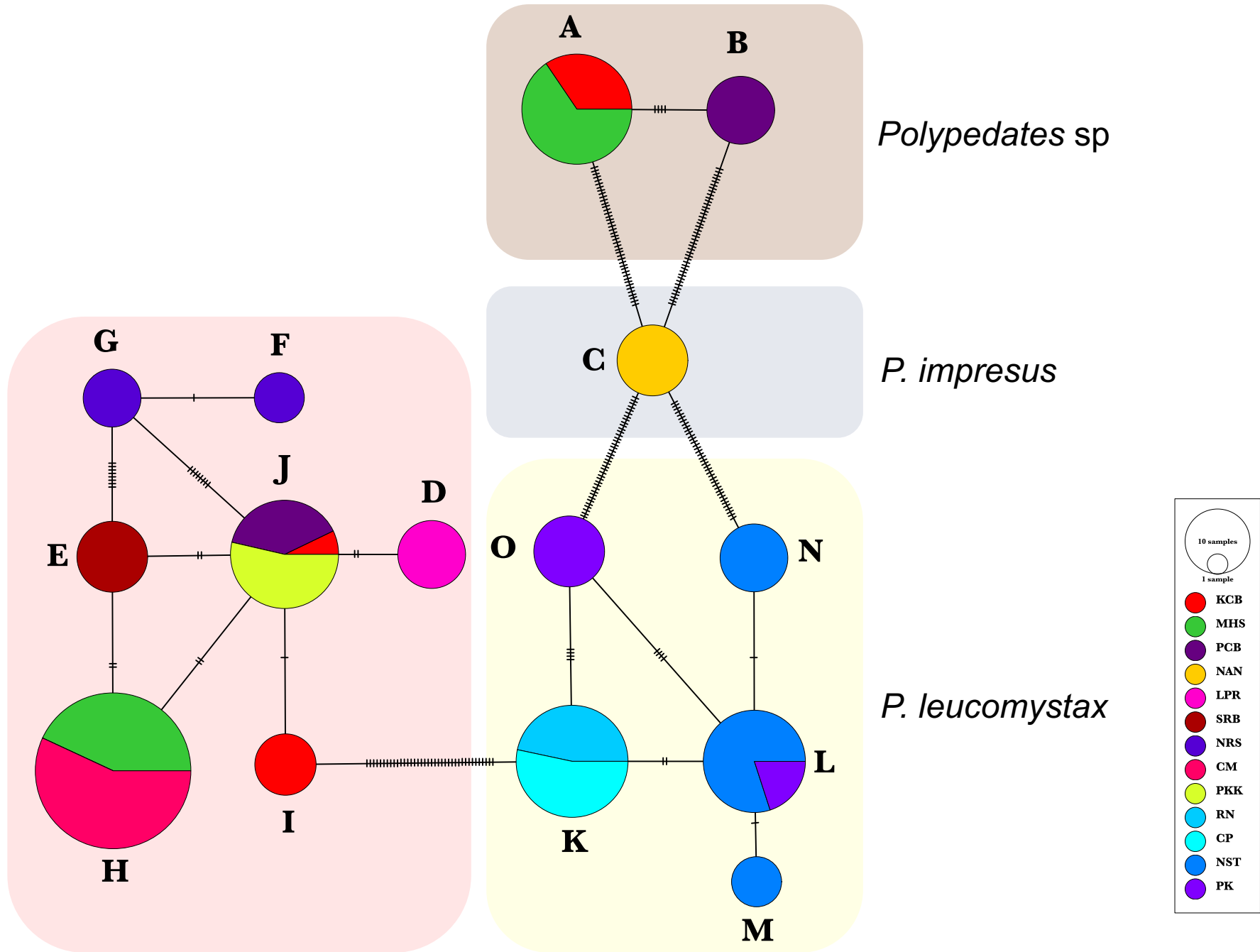




**Figure 3**(on next page)

The minimum spanning network of all haplotypes found in the *Polypedates leucomystax* complex in Thailand.

The mutation points between haplotypes are expressed by hatch marks. The different colors indicate the localities where the samples were collected.

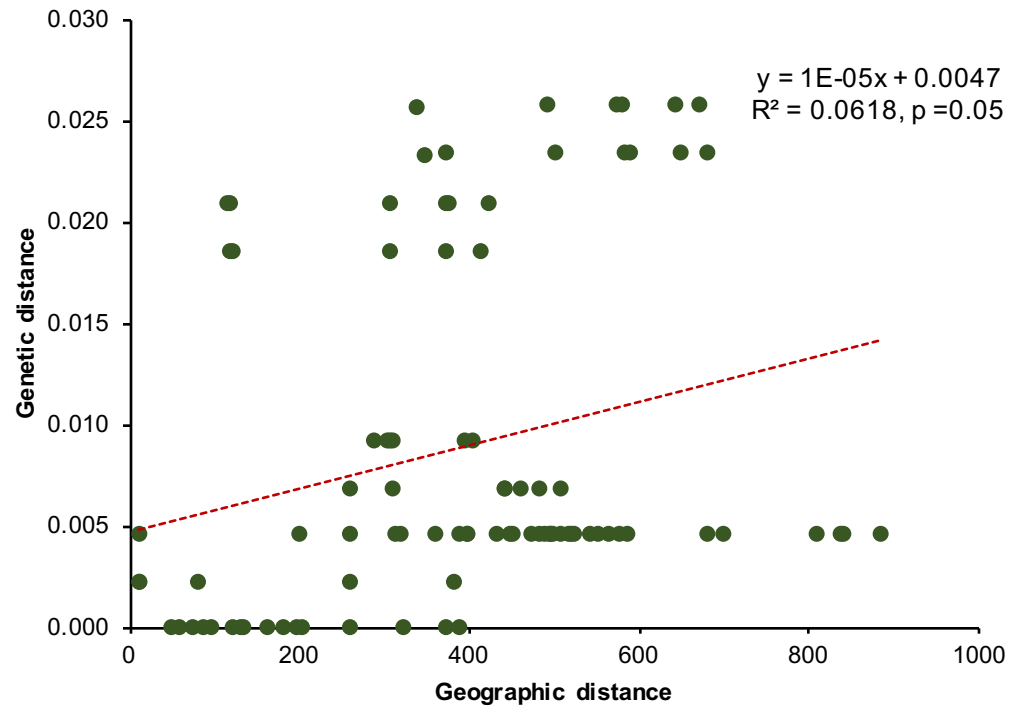


*P. megacephalus*

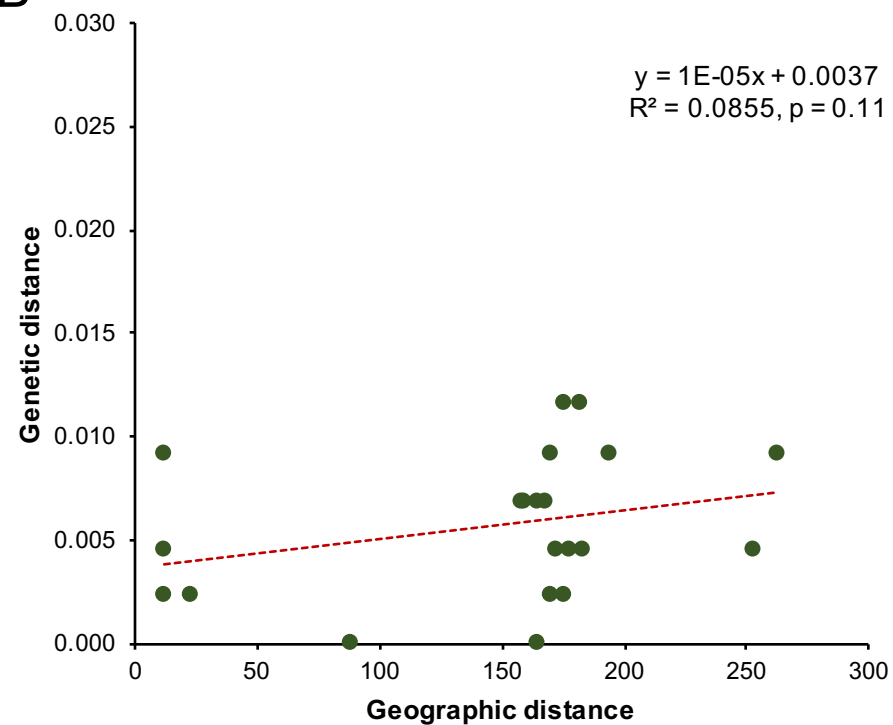
**Figure 4**(on next page)

The correlation of genetic distance and linear geographic distance (km) for *Polypedates megacephalus* (A) and *P. leucomystax* (B).

A



B

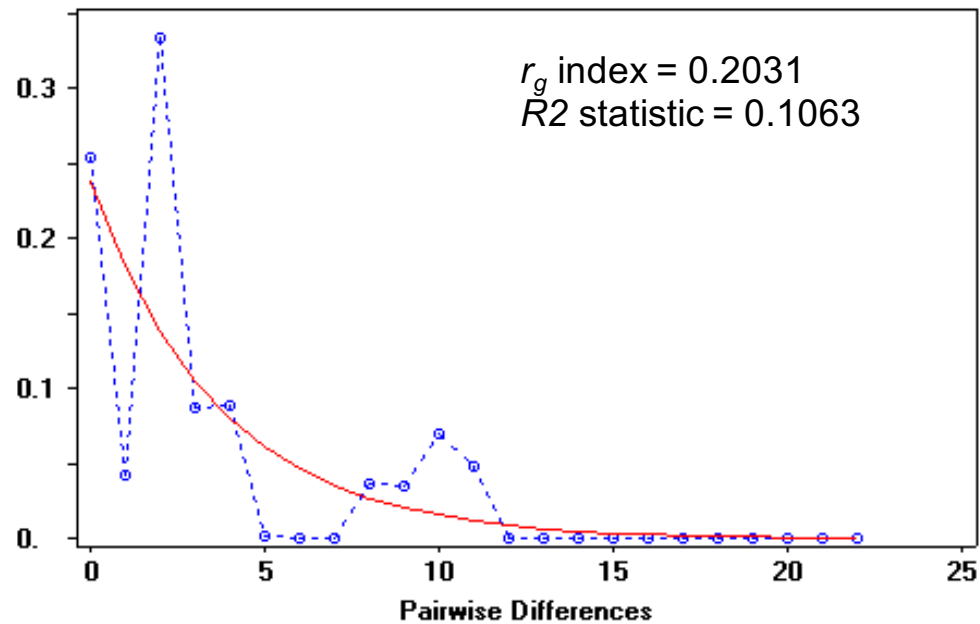


**Figure 5** (on next page)

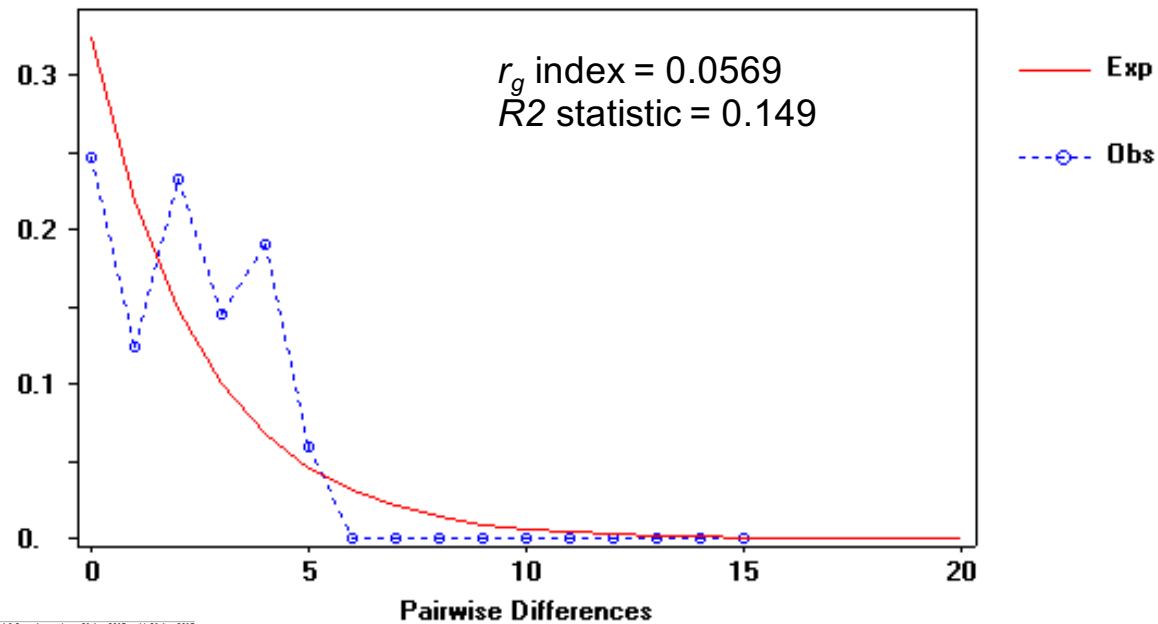
Mismatch distribution of the mitochondrial *COI* gene in *Polypedates megacephalus* (A) and *P. leucomystax* (B).

The raggedness ( $r_g$ ) index is calculated to evaluate the population expansion of each species. Ramos-Onsins and Rozas's  $R_2$  statistic represents the population growth.

A



B

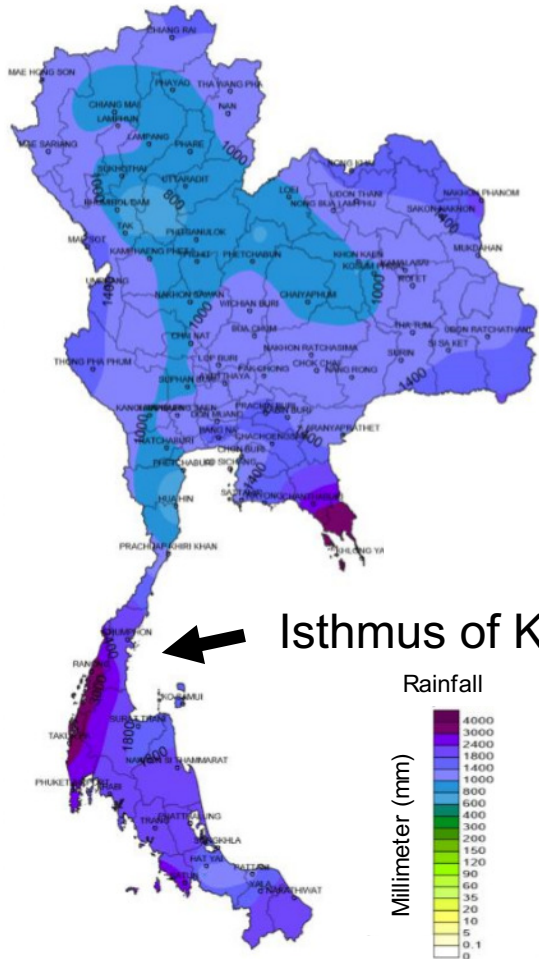


**Figure 6**(on next page)

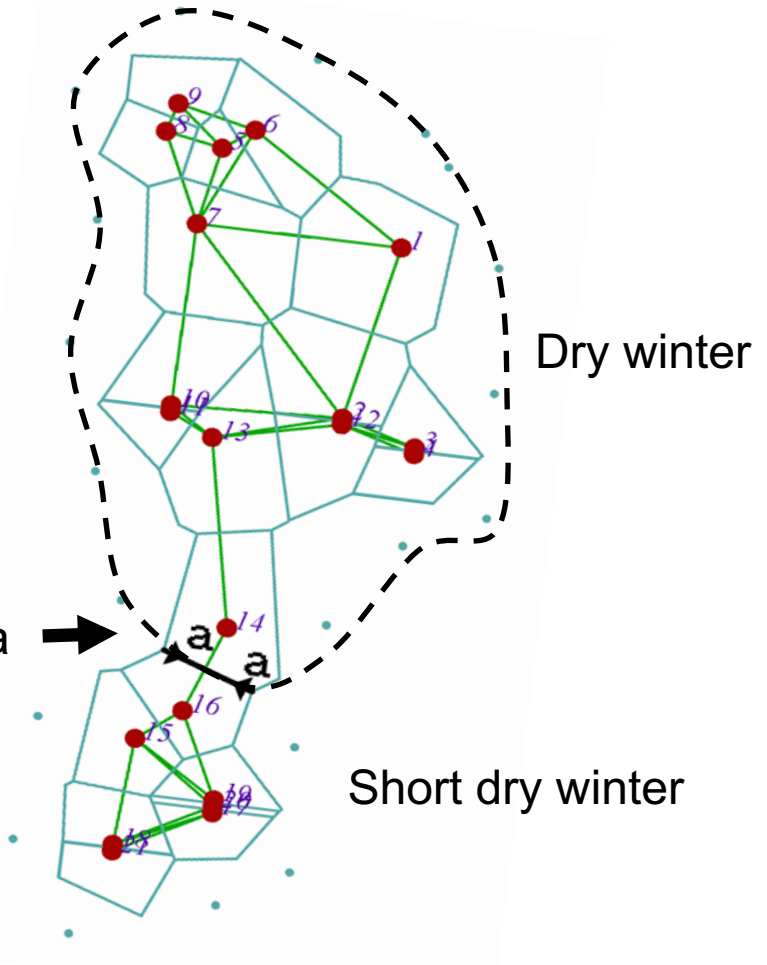
The relationship between climatic condition and genetic differentiation across *Polypedates megacephalus* and *Polypedates leucomystax*.

(A) annual rainfall (in mm) in Thailand for 2015 (Image credit: Thai Meteorological Department); and (B) a significant barrier to partition the distribution of the Northern B (*P. megacephalus*) and Southern (*P. leucomystax*) clades, by Barrier version 2.2.

A



B





**Table 1** (on next page)

Localities of sample collection for *Polypedates leucomystax* complex in Thailand.

1 **Table 1** Localities of sample collection for *Polypedates leucomystax* complex in Thailand.

Locality	Abbreviation	Number	Altitude (m above sea level)	Longitude	Latitude
Nan province	NAN	12	665	18.980974	101.182594
Kanchanaburi province	KCB	20	917	14.69329	98.40535
Loei province: Phu Ruea	LPR	11	939	17.48193	101.34982
Nakhon Ratchasima province	NRS	14	865	14.49336	101.87364
Chiang Mai province: Mae Wang	CM	7	678	18.657305	98.681831
Chiang Mai province: Doi Saket	CM	13	402	18.98777	99.11455
Chiang Mai province: Omkoi	CM	13	460	17.47137	98.45785
Mae Hong Son province	MHS	44	396	19.24797	97.99542
Saraburi province	SRB	12	105	14.70993	100.81819
Phetchaburi province	PCB	22	329	14.70993	100.81819
Prachuap Khiri Khan province	PKK	15	23	11.43678	99.56011
Ranong province	RN	14	18	9.6052	98.4669
Nakhon Si Thammarat province	NST	37	97	8.76902	99.80349
Phuket province: Thalang	PK	17	31	7.96804	98.33589
Chumphon province	CP	15	103	10.110278	99.082778

2

**Table 2** (on next page)

Summary of the *P. leucomystax* complex in Thailand

major lineages clades, putative scientific name, number of individuals ( $N$ ), number of mtDNA haplotypes ( $n$ ), number of polymorphic sites ( $P$ ), parsimony-informative sites ( $PI$ ) and singleton sites ( $S$ ), haplotype diversity ( $H_d$ ) and nucleotide diversity ( $\pi$ ).

1 **Table 2** Summary of the *P. leucomystax* complex in Thailand: major lineages clades,  
 2 putative scientific name, number of individuals ( $N$ ), number of mtDNA haplotypes ( $n$ ),  
 3 number of polymorphic sites (P), parsimony-informative sites (PI) and singleton sites (S),  
 4 haplotype diversity ( $H_d$ ) and nucleotide diversity ( $\pi$ ).

Clade	Scientific name	$N$	$n$	$\pi$	$H_d$	P	S	PI
A	<i>Polypedates</i> sp.	40	2	0.0037	0.4089	4	0	4
B	<i>P. impresus</i>	12	1	0	0	0	0	0
C	<i>P. megacephalus</i>	131	7	0.0048	0.746	15	1	14
D	<i>P. leucomystax</i>	83	5	0.0073	0.7526	7	0	7
Total		266	15	0.0664	0.9	82	0	82

5

**Table 3** (on next page)

Summary of statistics used to compute the demographic history of populations of *P. megacephalus* and *P. leucomystax*.

1

2 **Table 3** Summary of statistics used to compute the demographic history of populations of  
3 *P. megacephalus* and *P. leucomystax*.

Species	Tajima's <i>D</i>		Fu's <i>F<sub>s</sub></i>	
	<i>D</i>	<i>P</i> value	<i>F<sub>s</sub></i>	<i>P</i> value
<i>P. megacephalus</i>	0.439	>0.1	3.213	0.045
<i>P. leucomystax</i>	1.176	>0.1	3.031	0.071

4

5