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.

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2 **Thailand**

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22 **ABSTRACT**

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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

INTRODUCTION

44

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

MATERIALS AND METHODS

89

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 enthuestication 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 ℃ 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 μL under the following condition: an initial denaturation at 94 ℃ for 5 min, followed by 35–40

99 cycles at 94 ℃ for 30 s, 50 ℃ for 30 s and 72 ℃ for 1 min, and a final extension step at 72 ℃ 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 GenAlEx 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 *Fs* 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 *Fs*) of both species were not significantly positive, whereas Fu's *Fs* 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

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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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.

P. braueri

Clade A   Northern A; *Polypedates* sp.

Clade B   Nan; *P.* cf. *impresus*

Clade C   Southern part; *P.* cf. *leucomystax*

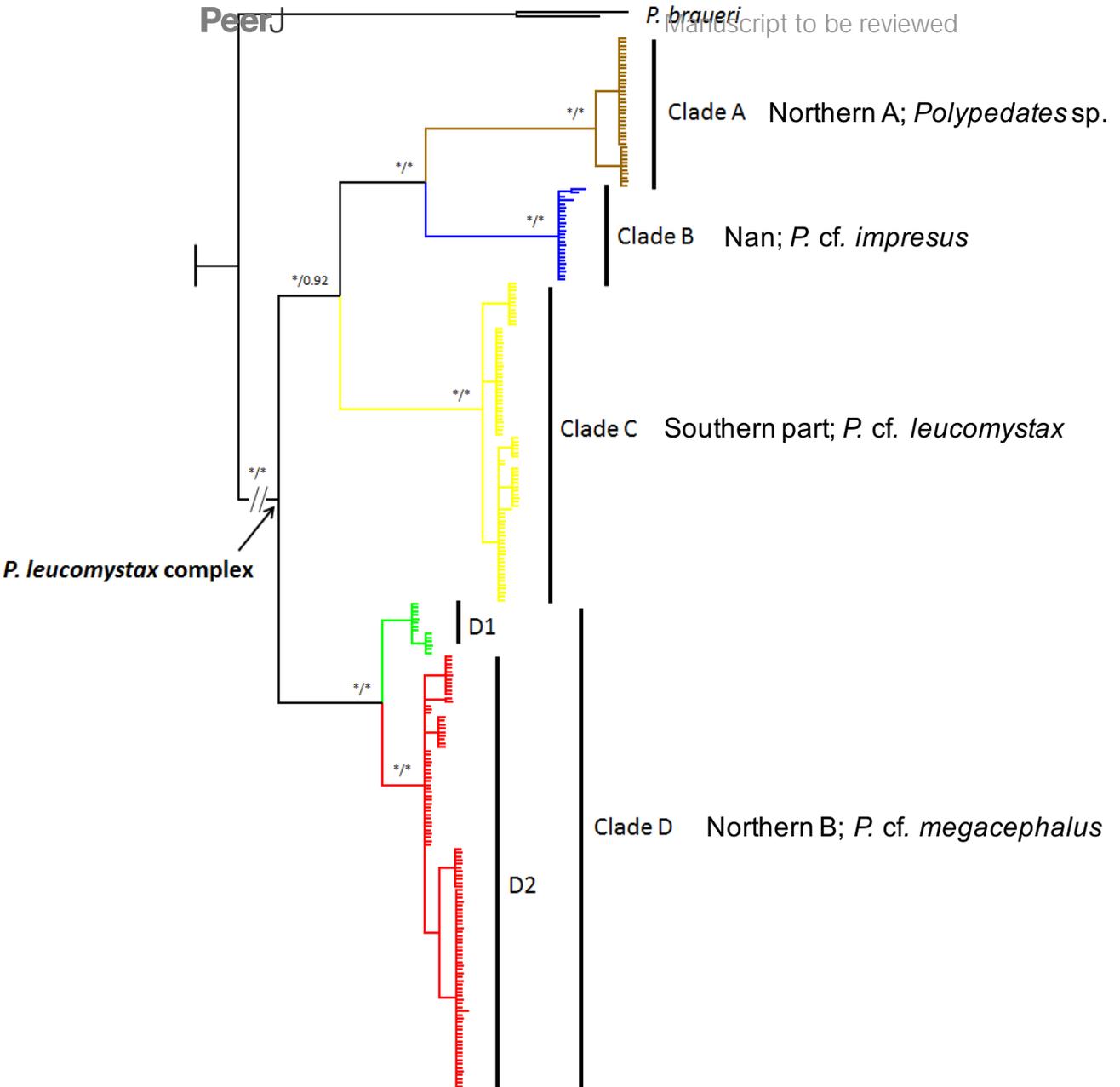
P. leucomystax complex

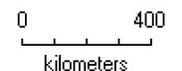
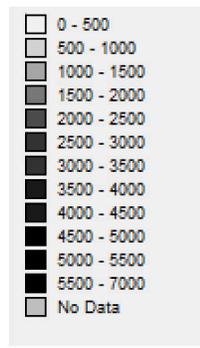
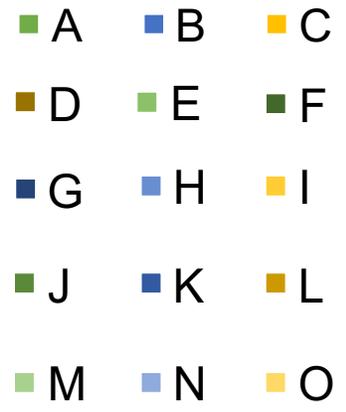
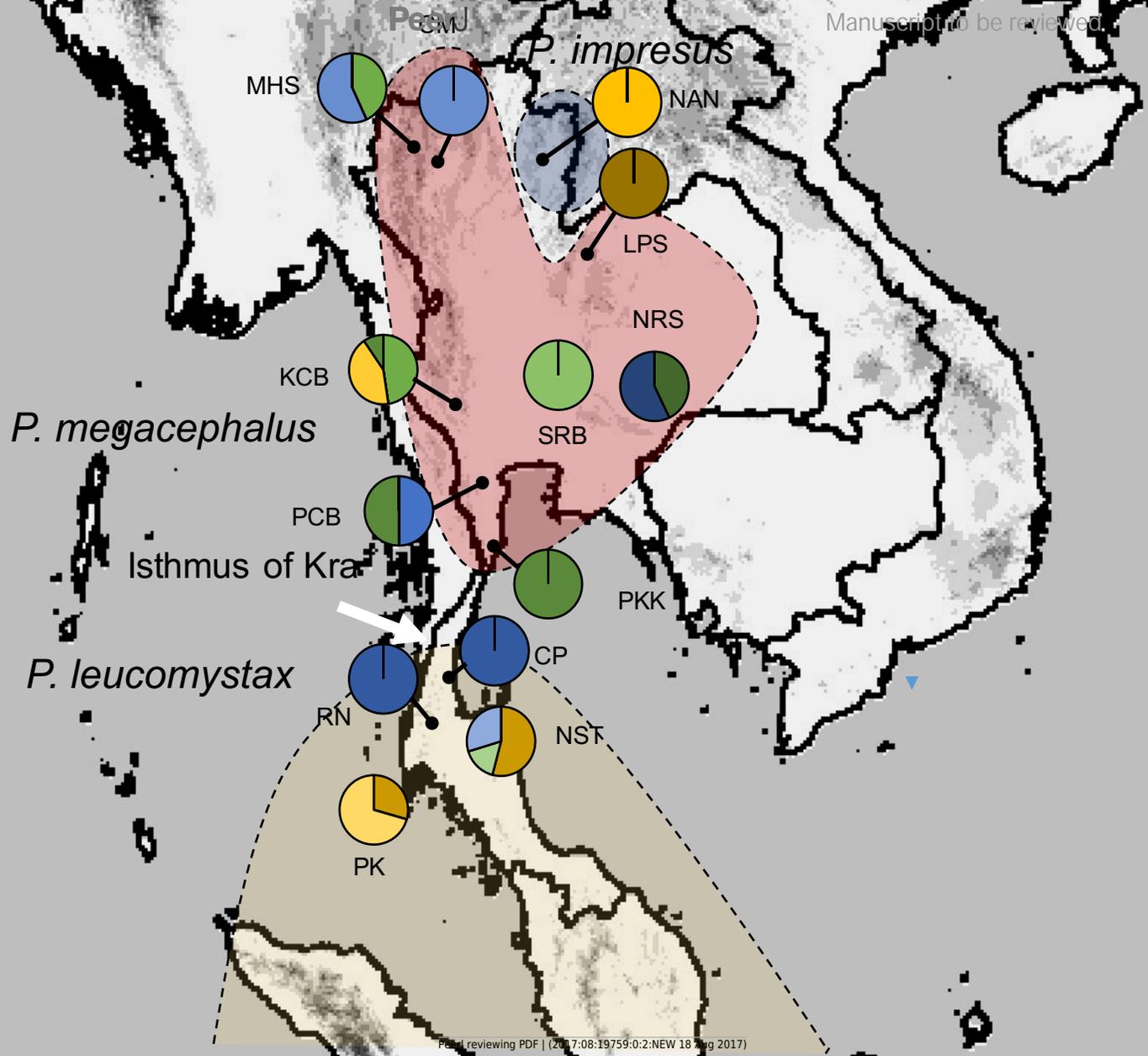
D1

Clade D   Northern B; *P.* cf. *megacephalus*

D2

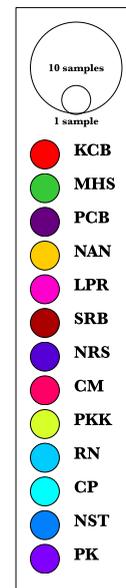
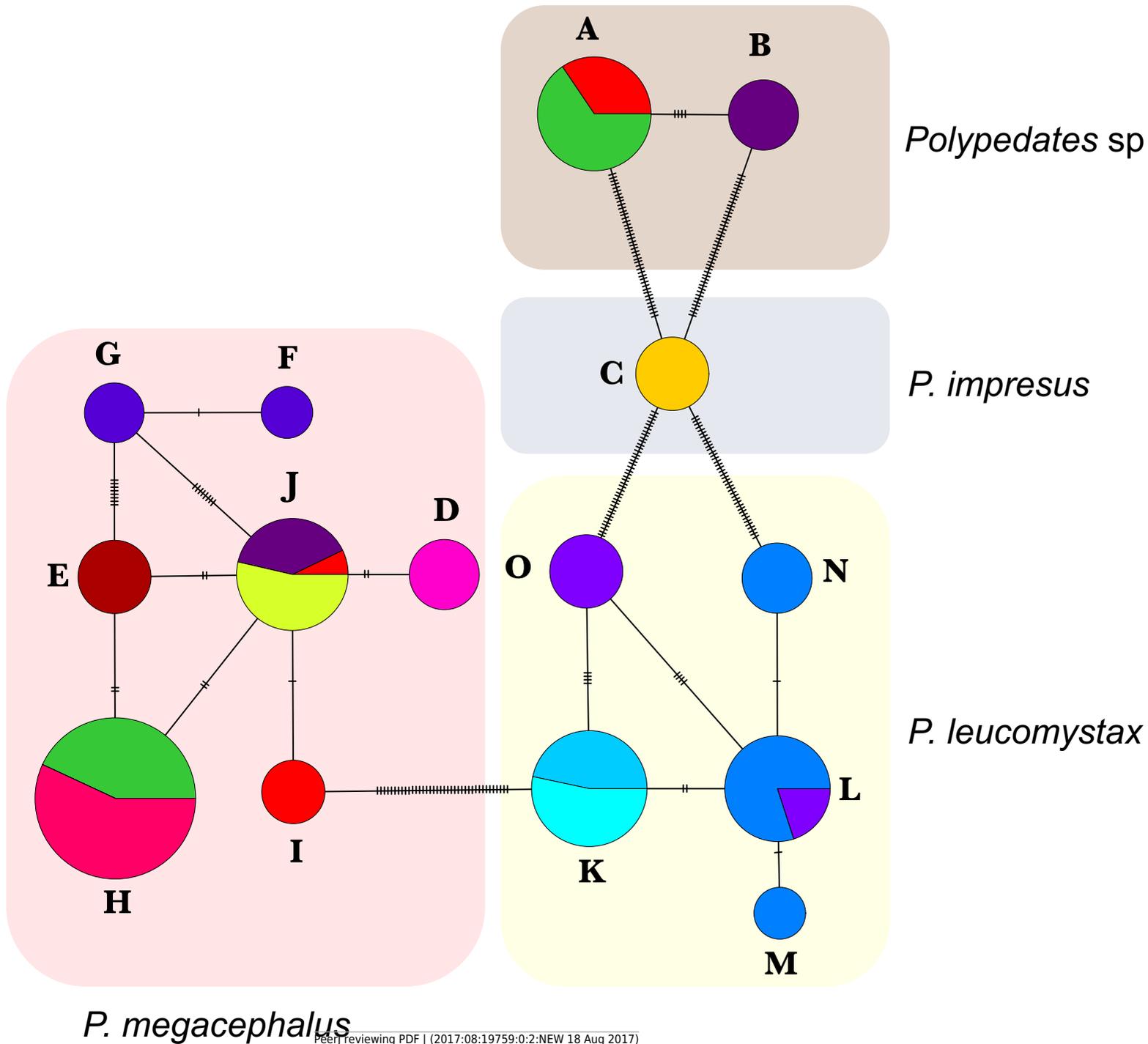
/
/
*/0.92
/
/
/
/
/
/

0.5

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

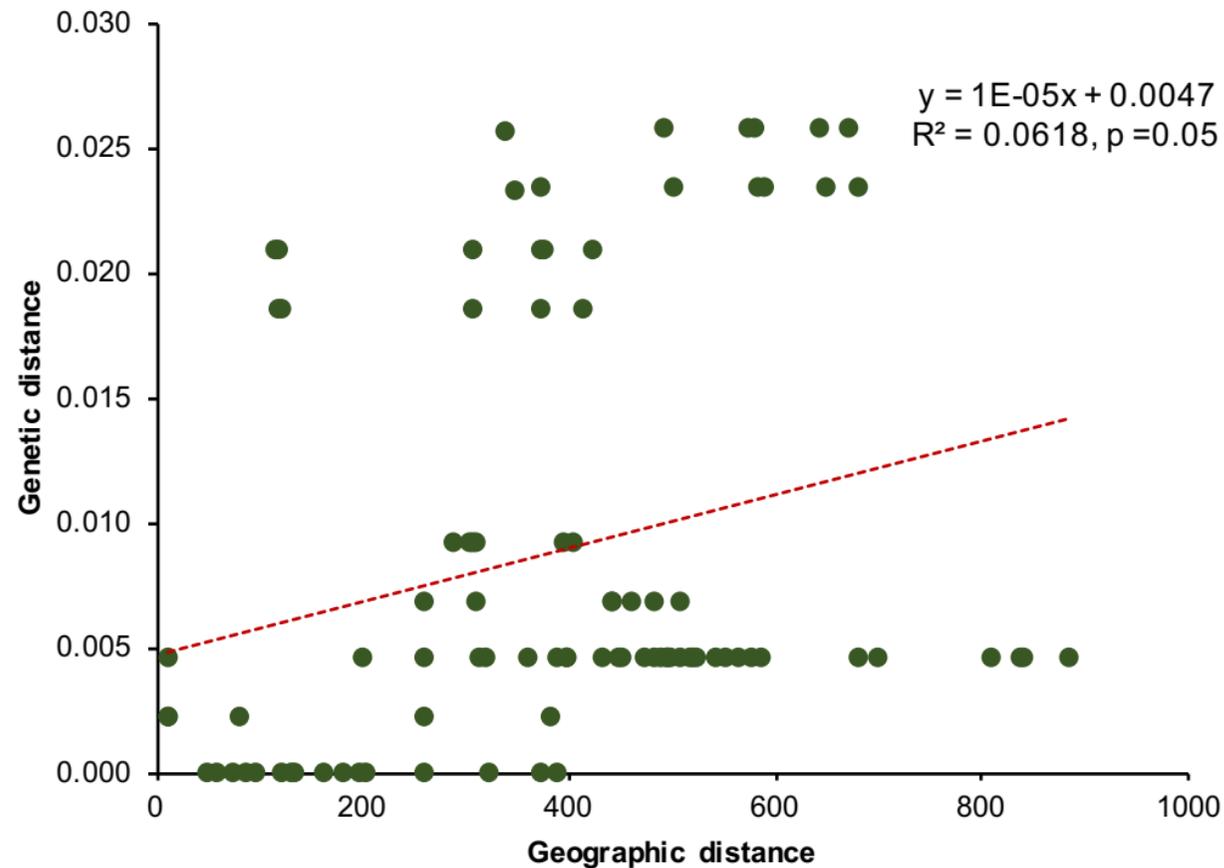
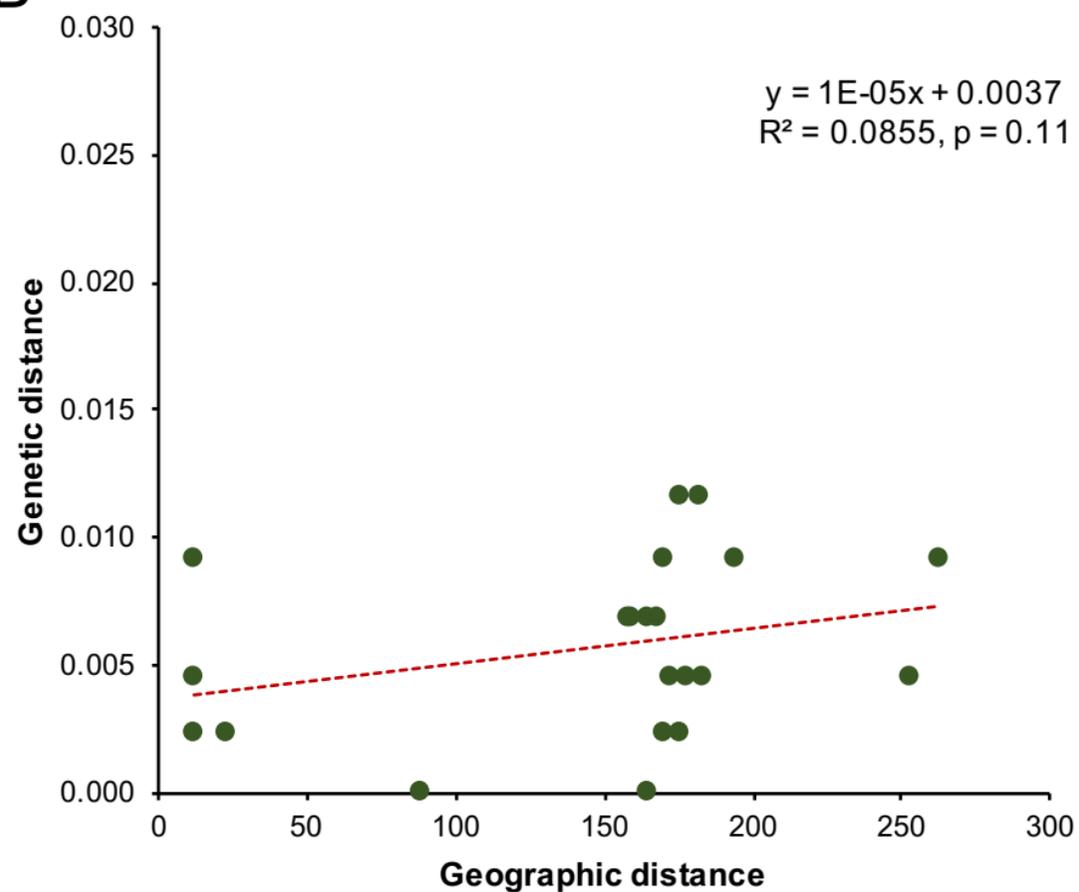
The abbreviations for each locality are given in Table 1. Different colors represent the different haplotypes.

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.

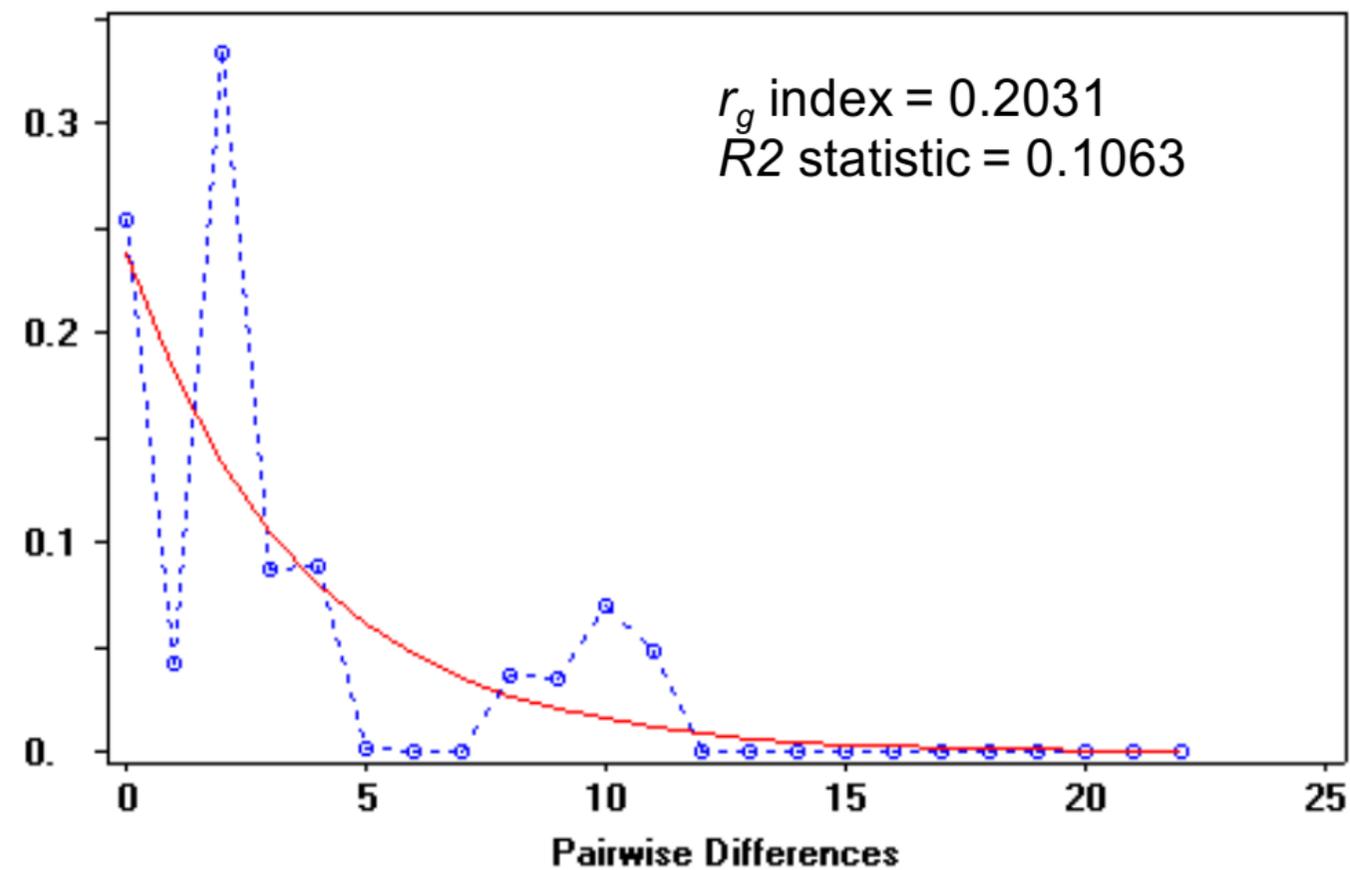
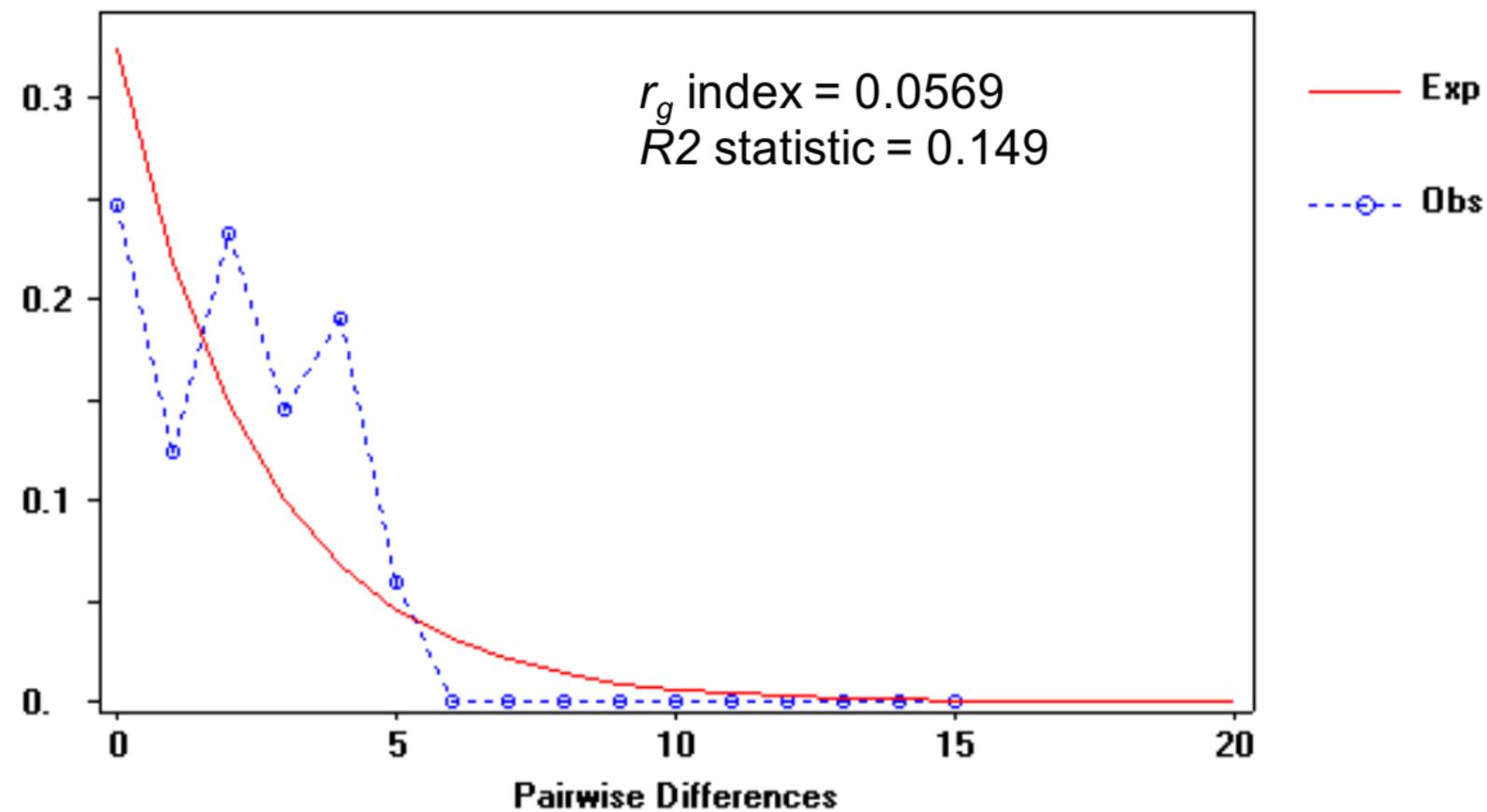
Polypedates sp

P. impresus

P. leucomystax

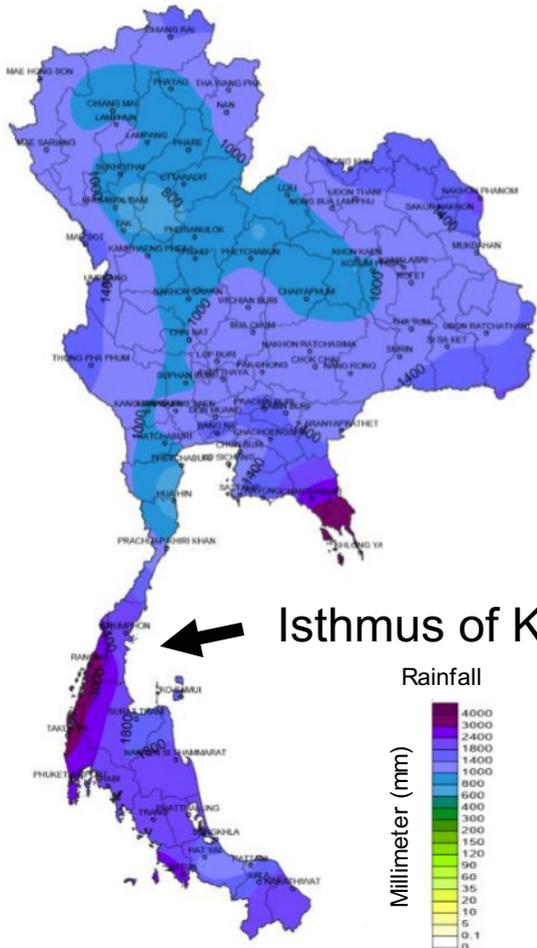
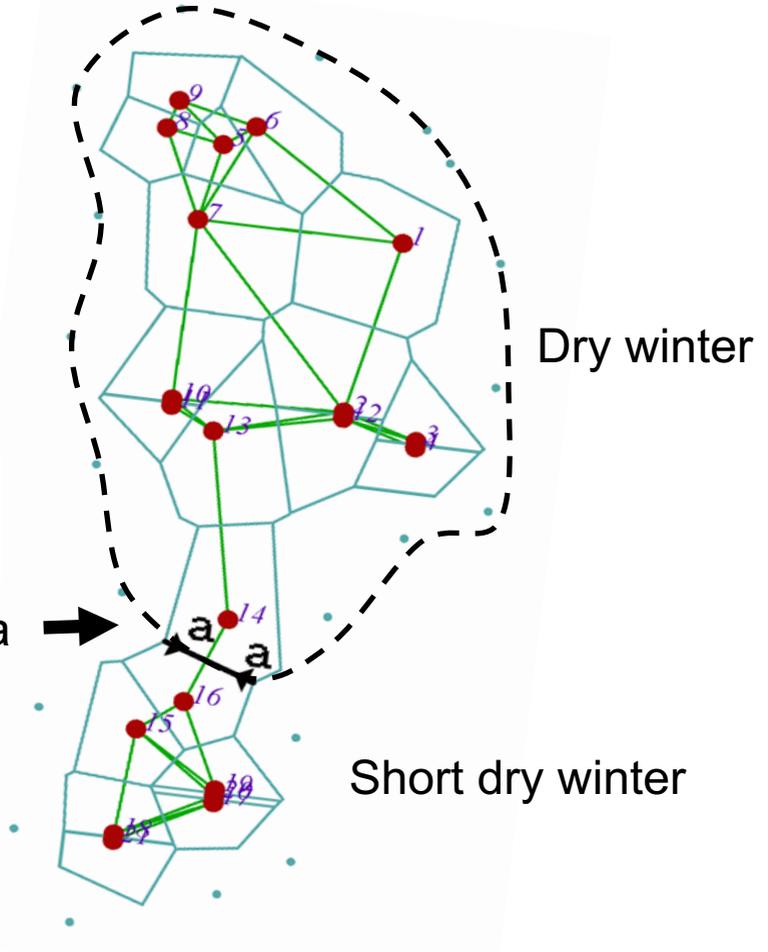
P. megacephalus

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

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 *R2* statistic represents the population growth.

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

Isthmus of Kra

Dry winter

Short dry winter

Rainfall

Millimeter (mm)

| 4000 |
| 3000 |
| 2400 |
| 1800 |
| 1400 |
| 1000 |
| 800 |
| 600 |
| 400 |
| 300 |
| 200 |
| 150 |
| 120 |
| 90 |
| 60 |
| 35 |
| 20 |
| 10 |
| 5 |
| 0.1 |
| 0 |

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

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 (π).

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 (π).

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

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

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

Species	Tajima's D		Fu's Fs	
	D	P value	Fs	P value
P. megacephalus	0.439	>0.1	3.213	0.045
P. leucomystax	1.176	>0.1	3.031	0.071