Agr. Nat. Resour. 54 (2020) 1–8

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Molecular identification of the morphologically cryptic Asian common treefrogs (Anura: Rhacophoridae, *Polypedates leucomystax* complex) in Thailand

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Article Info

Article history:
Received 31 Marcg 2018
Revised 14 February 2019
Accepted 21 Februay 2019
Available online 28 February 2020

Keywords:
Coconut milk,
Mixing order,
Palm sugar,
Physical properties

Abstract

New amphibian species in Southeast Asia have been continuously reported. However, the diversity of amphibians in this region is underestimated due to the prevalence of cryptic species. Recent molecular studies revealed that many amphibian species in Southeast Asia including *Polypedates leucomystax* comprised cryptic species. In this study, we reveal the cryptic diversity within *P. leucomystax* complex in Thailand. The *16S rRNA* gene (588 to 621 bp) was analyzed from 218 specimens in 54 provinces of Thailand. Then, a different region of *16S* (376 to 379 bp) was analyzed using 19 selected sequences from the first analyses and six tadpole from GenBank. Based on phylogenetic analyses, the first tree showed *P. leucomystax* complex was a monophyletic group. Our specimen collection fell within four clades; A-clade (*P. leucomystax*), B-clade (*Polypedates* sp.), C-clade (*P. megacephalus*) and E-clade (*P. discantus*). The most intraspecific p-distances were less than or equal to 0.03 while the interspecific p-distance were greater than 0.03. Moreover, the second phylogenetic analysis showed that six tadpole sequences were clustered in the clades of *P.* cf. *mutus*1, *P. braueri* and *Polypedates* sp. We found *P. megacephalus* and *P. leucomystax* were the dominant species in Indochinese and Sundaic subregions, respectively. Furthermore, the B-clade could be considered as a new clade because their genetics differred from the others. Our results concluded that there was cryptic diversity within Asian common treefrogs in Thailand. Molecular identification using *16S* marker in our study provided reliable results to identify this cryptic species and will be useful for further studies in population genetics and biogeography.

Introduction

Southeast Asia is one of the biodiversity hotspots in the world due to its geographical complexity and historical climate patterns. Thus, it contributes to the diversity of ecosystems and organisms including amphibians (Bain and Hurley, 2011; Hertwig et al., 2012). Although new amphibian species in this region have been continuously reported (Köhler et al., 2005; Hasan et al., 2014), their diversity remains underestimated due to the prevalence of cryptic species (sibling species or species complexes) (Stuart et al., 2006; Hasan et al., 2014). "Cryptic species" refers to more than one distinct species that were classified as the same species due to morphological similarity (Bickford et al., 2007; Trontelj and Fišer, 2009). This is a common pattern in the widely distributed amphibian species in Southeast Asia (Stuart et al., 2006; Brown et al., 2010).

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https://doi.org/10.34044/j.anres.2020.54.1.01

The Asian common treefrog (*Polypedates leucomystax*) is one of the widespread species in Southeast Asia. Its distribution ranges from Nepal through continental China, Taiwan and Southeast Asia including Thailand (Matsui et al., 1986; Diesmos et al., 2004; Li et al., 2012). Recent molecular studies revealed there were cryptic species within *P. leucomystax*, and recognized them as *P. leucomystax* complex, which was difficult to identify and distinguish from *P. leucomystax* (Kuraishi et al., 2013; Rujirawan et al., 2013).

Previously, two subspecies of *P. leucomystax* in Thailand had been recognized; *P. l. leucomystax* and *P. l. sexvirgatus*. *Polypedates leucomystax leucomystax* was distributed throughout Thailand while *P. l. sexvirgatus* was distributed in Yala Province in southern Thailand (Taylor, 1962). However; the molecular studies concluded that the geographic distribution of the valid *P. leucomystax* should be restricted to the Sundaic subregion, which covered the type locality in Java, Indonesia and the southern area of the Isthmus of Kra in Thailand (the geographic distribution of *P. l. sexvirgatus*), whereas *P. l. leucomystax* was reidentified into *P. megacephalus*, distributed in the Indochinese subregion covering its type locality in Taiwan and the northern area of the Isthmus of Kra in Thailand (Kuraishi et al., 2013; Buddhachat and Suwannapoom, 2018).

At present, there are 24 species of *Polypedates* spp. in the world (Frost, 2019). In Thailand there are seven species; *P. colletti*, *P. macrotis*, *P. leucomystax*, *P. megacephalus*, *P. mutus*, *P. discantus* and *P. impresus* (Taylor, 1962; Chan-ard, 2003; Rujirawan et al., 2013; Buddhachat and Suwannapoom, 2018). The *P. colletti* and *P. macrotis* can be distinguished from *P. leucomystax* based on external morphology. The other five species are recognized as *P. leucomystax* complex (Kuraishi et al., 2013).

In the previous extensive molecular studies of *P. leucomystax* complex (Brown et al., 2010; Sheridan et al., 2010; Blair et al., 2013; Kuraishi et al., 2013; Rujirawan et al., 2013; Buddhachat and Suwannapoom, 2018), most of the sample collections did not cover their distribution areas in Thailand. To address this problem, we collected comprehensive samples of *P. leucomystax* complex to more thoroughly cover their broad distribution. The mitochondrial *16S ribosomal RNA* gene (*16S*) was used as a molecular marker since it has been proposed as a marker for species identification in amphibians. The *16S* is conserved, easy for design as a universal primer, and it also provides a higher amplification success rate in PCR product when compared to the other mitochondrial genes (*cytochrome c oxidase subunit I* gene; *COI*) (Vences et al., 2005; Fouquet et al., 2007). In addition, there were *16S* sequences of *Polypedates* spp. rather than *COI* sequences available on GenBank.

The purpose of this study was to use phylogenetic analyses and genetic differences of *16S* sequences to identify the morphologically cryptic diversity within Asian common treefrogs (*P. leucomystax* complex) in Thailand.

Materials and Methods

Specimen collection

All specimens were collected from 48 provinces in Thailand (Fig. 1) during November 2013 to April 2017. The specimens were preliminarily identified by external morphology following the descriptions of Taylor (1962) and Inger (1966) as *P. leucomystax* complex. Tissue samples were preserved in 95% EtOH for DNA extraction.

Ethics Statements

The animals in all experimental procedures were approved by the Animal Experiment Committee, Kasetsart U. with the number ACKU61-SCI-013.

DNA extraction and PCR amplification

Total DNA were extracted by GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). The quality and concentration of DNA were investigated by 1.2% agarose gel electrophoresis and spectrophotometry, respectively. The *16S* primers were designed (5'GAAGAGGCAAGTCGTAACATGG3'

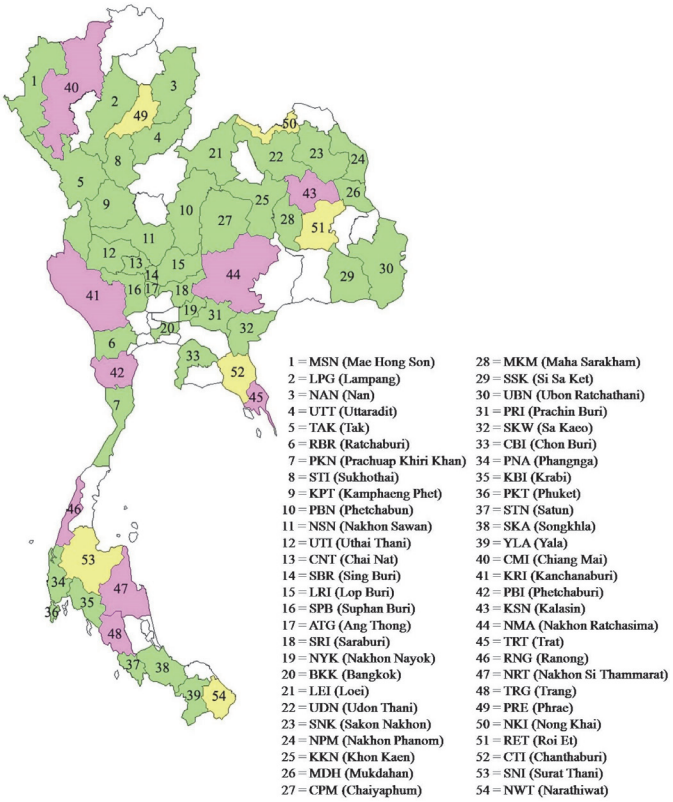

Fig. 1 The total 54 provinces comprise 39 provinces from our sample collection (green: no.1–39), nine provinces from both our sample collection and Genbank (pink: no.40–48), and six provinces (yellow: no.49–54) from Genbank.

and 5'CTTTATTGGGACCTTTGCGGTG3'). The PCR reaction contained 5µl 10x Buffer, 5µl 25mM $MgCl_2$, 5µl 2mM dNTPs, 1µl 10µM of each primer, 0.25U 5U/µl *Taq* DNA Polymerase, 50–100ng DNA template and total volume was adjusted to 50µl with ultrapure water. The PCR conditions were run as follows: preheat 3.00 min at 92°C, 35 cycles of 1.00 min at 92°C, 1.00 min at 54–60°C, 1.30 min at 72°C, and followed by 7.00 min at 72°C.

Sequencing and phylogenetic analyses

The cleaned up PCR products obtained using GeneJET Gel Extraction Kit (Thermo Scientific, USA) were sent to Macrogen Inc. (Korea) for sequencing. The 175 sequences of *16S* obtained were edited manually using BioEdit v7.2.5 (Hall, 1999) and submitted to GenBank (Table S1). Multiple alignments were performed by CLUSTALW 1.83.XP (Thompson et al. 1994). The best fit model of nucleotide substitution and the genetic differences (p-distances) were calculated with MEGA 6.06 (Tamura et al., 2013).

The phylogenetic analyses were performed based on maximum likelihood (ML) and Bayesian inference (BI) methods. The ML was simulated with the TN93+G model and 1,000 bootstraps using MEGA 6.06. The MrBayes v3.2.6 (Ronquist et al. 2012) was used for BI and simulated with the HKY+G model. The parameters were set as the default priors and run for 10,000,000 generations. The trees were sampling in every 100 generations and the first 25% of sampled trees were discarded as burn-in.

The first analysis is using 218 sequences of *16S* (588 to 621 bp) (Table S1) that represent the sequences from 54 provinces in Thailand (Fig. 1). The 218 sequences comprised our 175 sequences plus 43 sequences available on GenBank including *P. megacephalus* (type locality; TL, from Hong Kong, China), *P. leucomystax* (TL: Java, Indonesia), *P.* cf. *mutus*1, *P.* cf. *mutus*2 and the *Polypedates* sp. "Malay clade" (Kuraishi et al., 2013), which was inferred as *P. discantus* (Rujirawan et al., 2013).

Later, we analyzed a different region of *16S* (376 to 379 bp) from 27 sequences that comprised: 19 selected sequences that represent each group in the first tree, along with the six tadpole sequences available on Genbank (the specimens were reported as collected from Thailand and identified as *Polypedates* sp., *P. mutus* and *P. braueri*), and two sequences of *P. braueri* (TL: Taiwan) from GenBank (Table S2). For all analyses, the sequences of *Rhacophorus bipunctatus* and *P. colletti* were used as outgroups. The data of 218 sequences in the first analysis (Table S1) and 27 sequences in the second analysis (Table S2) are available on https://drive.google.com/file/d/1XhRtBFTCRzwzkY1CTWTmigux3m8GbvjH/view?usp=sharing

Results

Phylogenetic analyses

In the first analysis, the 218 sequences of *16S* (588 to 621 bp) comprised 46 gaps, 189 parsimony informative sites, 48 singletons and 85 haplotypes. The phylogenetic trees (Fig. 2) exhibited identical topology and six clades with high statistical support (61, 0.81). Our specimen collection fell within four clades: A-clade (*P. leucomystax* from the southern provinces), B-clade (*Polypedates* sp. from Maehongson and Tak), C-clade (*P. megacephalus* from northern, northeastern, eastern, western and central provinces) and E-clade (*P. discantus* from Satun). For the D-clade (*P*. cf. *mutus*2) and F-clade (*P.* cf. *mutus*1), there were none of our specimen collections included. The first phylogenetic tree showed that *P.* cf. *mutus*1 separated first, followed by *P. discantus*. The other four clades (*P. leucomystax*, *Polypedates* sp., *P. megacephalus* and *P.* cf. *mutus*2) were closely related species with high statistical support (99, 1.00).

The tree topology in the second analysis (Fig. 3) was similar to those of the first analysis with high statistical support (88, 1.00). Four of the tadpole sequences on GenBank (the specimens from Petchabun and Phitsanulok: GbKR828029PBN, GbKR828031PBN, GbKR828032PBN and GbKR828030PLK) were clustered within our *Polypedates* sp. clade, while the sequence of *P. mutus* from Prachuabkhirikhan (GbKR828028PKN), and *P. braueri* from Phitsanulok (GbKR827990PLK) were clustered within *P.* cf. *mutus*1 and *P. braueri* clades, respectively.

Genetic differences

The genetic differences (p-distances) of *16S* (588 to 621 bp) from the 218 sequences is shown in Table 1. Most intraspecific p-distances were less than or equal to 0.03 (≤3%), whereas the interspecific p-distances were above 0.03 (>3%). However, it was remarkable that approximately 33.67% of intraspecific p-distances within *P. leucomystax* were relatively higher than 0.03.

Discussion

Phylogenetic relationships and genetic differences

The first phylogenetic tree showed *P. leucomystax* complex was a monophyletic group, as in previous studies (Kuraishi et al., 2013; Pan et al., 2013; Buddhachat and Suwannapoom, 2018). When the *Polypedates* sp. clade was excluded, the relationships of the other three species (*P. leucomystax*, *P. megacephalus* and *P.* cf. *mutus*2) were similar to those in the study of Kuraishi et al. (2013). Therefore, our results confirmed that *P. leucomystax*, *P. megacephalus* and *P.* cf. *mutus*2 were more closely related than *P.* cf. *mutus*1 and *P. discantus* (Kuraishi et al., 2013).

Most of the intraspecific p-distances in this study were less than or equal to 0.03 (≤3%), while the interspecific p-distances were greater than 0.03 (>3%) (Table 1). These are credible values that occur within amphibian species, which should not be over 3% for the *16S* gene (Fouquet et al., 2007). However, the intraspecific p-distances within the clade of *P. leucomystax* were relatively high. This might be due to their polymorphism in the large ancestral population, or could be caused by an introgressive hybridization with different lineages, as reported in some other amphibian species (Vences et al., 2004; Langone et al., 2016).

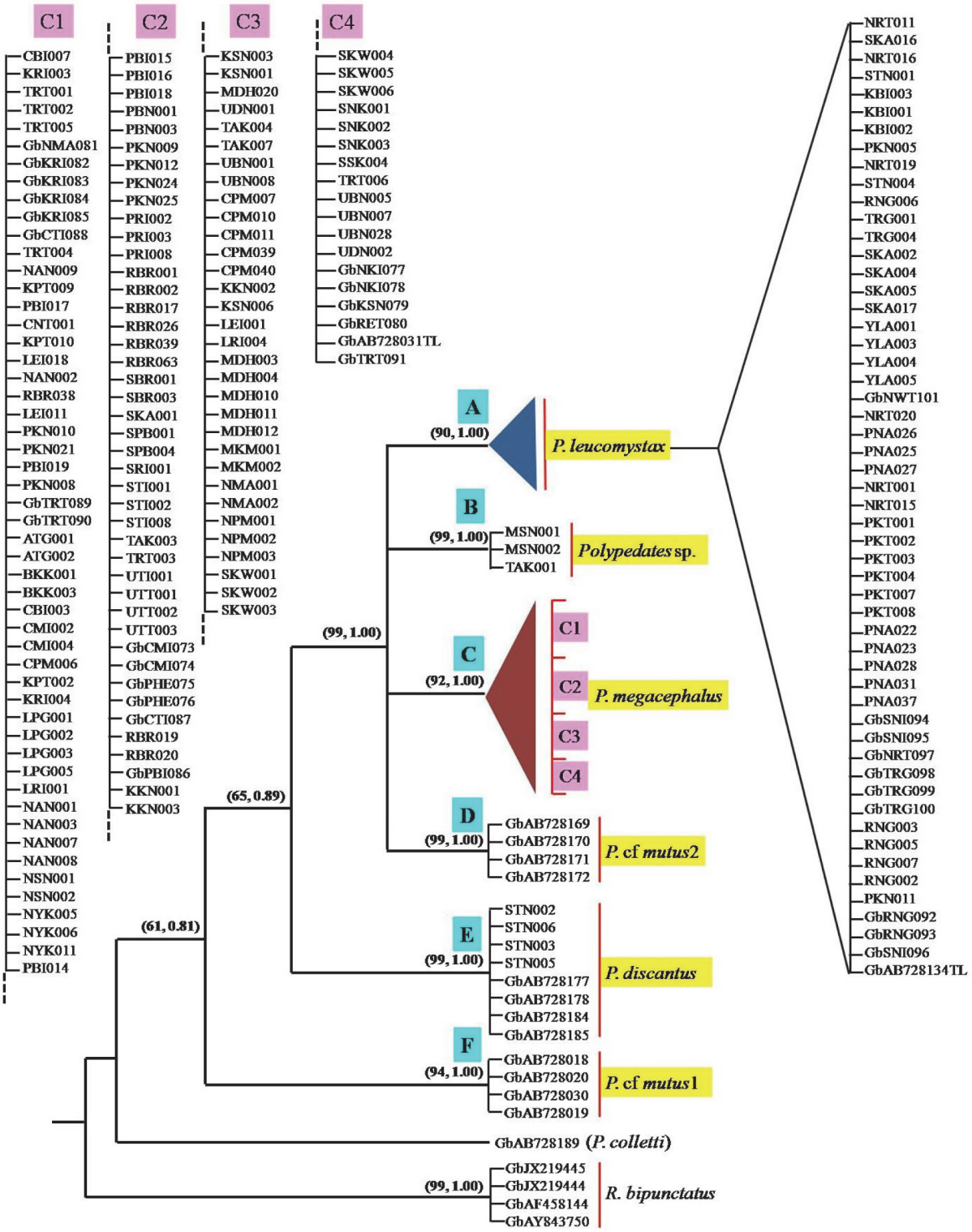

Fig. 2 Phylogenetic tree of 223 specimens (outgroups included) using 588 to 614 bp of *16S* sequences based on ML and BI. The numbers at the nodes were the percentage of bootstrap in ML and the Bayesian posterior probability, respectively.

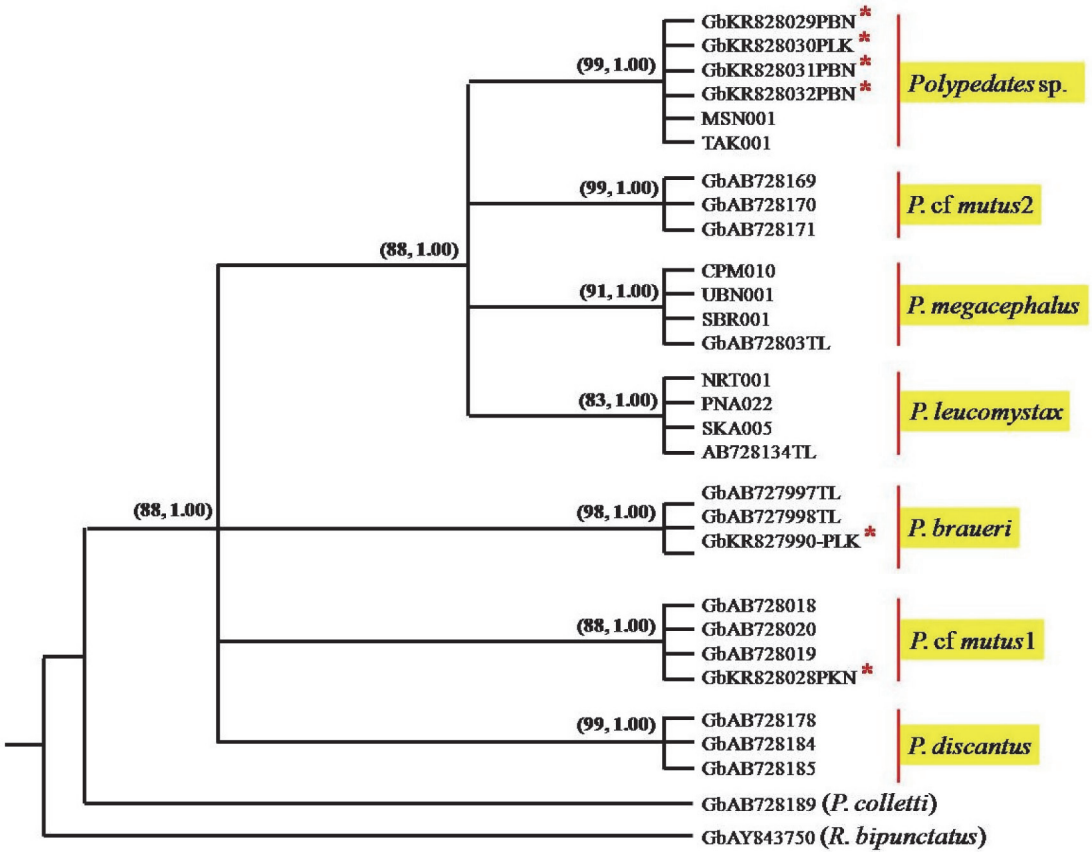

Fig. 3 Phylogenetic tree of 29 specimens (outgroup included) using 376 to 379 bp of *16S* sequences based on ML and BI. The numbers at the nodes were the percentage of bootstrap in ML and the Bayesian posterior probability, respectively. The specimens with asterisk were tadpole specimens from GenBank that were collected in Thailand. The specimens that were identified as *Polypedates* sp. (GbKR828029PBN, GbKR828031PBN, GbKR828032PBN and GbKR828030PLK) were clustered with our *Polypedates* sp. clade. The specimens that were identified as *P. mutus* (GbKR828028PKN) and *P. braueri* (GbKR827990PLK) were clustered within *P.* cf. *mutus*1 clade and *P. braueri* clade, respectively.

Table 1 The p-distances within and between species of *P. leucomystax* complex calculated from 218 sequences (588 to 621 bp), and classified into three ranges; less than or equal to 0.03, more than 0.03 to 0.10 and more than 0.10. The percentages in the blanket were the pairwise frequencies in each range, the percentages were not shown if they were 100%

Species	P. megacephalus *n* = 145	P. leucomystax *n* = 54	*Polypedates* sp. *n* = 3	P. discantus *n* = 8	*P.* cf. *mutus* 1 *n* = 4	*P.* cf. *mutus* 2 *n* = 4
P. megacephalus	≤ 0.03 (99.95%) > 0.03 (0.05%)					
P. leucomystax	> 0.03–0.10 (85.43%) > 0.10 (14.57%)	≤ 0.03 (66.33%) > 0.03–0.10 (13.67%) > 0.10 (20.00%)				
Polypedates sp.	> 0.03–0.10	> 0.03–0.10 (83.64%) > 0.10 (16.63%)	0.00			
P. discantus	> 0.10	> 0.10	> 0.10	0.00–0.01		
P. cf. *mutus* 1	> 0.10	> 0.10	> 0.10	> 0.10	0.00–0.01	
P. cf. *mutus* 2	> 0.03–0.10	> 0.03–0.10 (77.73%) > 0.10 (22.27%)	> 0.03–0.10	> 0.10	> 0.10	0.00–0.01

Considering the localities in our specimen collection, *P. megacephalus* was sympatric with *Polypedates* sp. in the north, while *P. leucomystax* was sympatric with *P. discantus* in the south. However, from our data and that of others, the values of p-distance between morphologically cryptic species might be low or high between sympatric species just as with allopatric species, and it was not dependent on the geographic distribution (Kuraishi et al., 2013; Rujirawan et al., 2013).

Cryptic diversity of P. leucomystax complex in Thailand

According to our results and the previous studies (Kuraishi et al., 2013; Rujirawan et al., 2013; Grosjean et al., 2015; Buddhachat and Suwannapoom, 2018), there are seven species of the *P. leucomystax* complex in Thailand; *P. leucomystax*, *P. megacephalus*, *P. mutus*, *P. braueri*, *P. discantus*, *P. impresus* and *Polypedates* sp.

In our comprehensive sampling, we have supported the finding that *P. megacephalus* and *P. leucomystax* are the dominant species in the Indochinese and Sundaic subregions, respectively as in previous study (Kuraishi et al., 2013). The correctly identified species were concordant with most of the sampling localities as in previous studies (Kuraishi et al., 2013; Buddhachat and Suwannapoom, 2018). However three specimens (PKN005, PKN011 and SKA001) were clustered into a clade which was discordant to their geographic distribution. The PKN005 and PKN011 from the north were established in A-clade (*P. leucomystax*), while SKA001 from the south was established in C-clade (*P. megacephalus*). In addition, the p-distances of these three specimens were consistent with the other members of each clade. It might result from introgression, which maintained the genetic integrity in mtDNA of closely related taxa, or the mtDNA in some individuals of these two species may have recently diverged and maintained the trace of a common ancestor, due to the incomplete lineage sorting as found in many amphibian species (Vences et al., 2005; Fouquet et al., 2007; Chen et al., 2009; Gvozdik et al., 2010; Kuraishi et al., 2013). To solve this problem, using mitochondrial and nuclear DNA as multilocus markers has been suggested for further study (Liu et. al, 2010). On the other hand, many amphibian species had shown turnover distribution across the Indochinese and Sundaic subregions, especially in the boundary area of Isthmus of Kra (Inger, 1966; Hughes et al., 2003) In addition, the treefrogs in *P. leucomystax* complex is considered as human-mediated species, because their dispersal may be impacted by human activities, such as agricultural transportation (Kuraishi et al., 2009; Brown et al., 2010; Blair et al., 2013). Therefore, some individuals or a small established population may appear outside their normal geographic distribution.

For *P. mutus* and *P. braueri*, there were no specimens in our collection. *Polypedates mutus* (TL: N'Chang Yang, Myanmar) has been distinguished by considering the lack of vocal sacs in males (Smith, 1940). However, this species has been reported in Thailand with photographs and their geographic distribution was noted, but no mention was ever made about the vocal sac in males (Chan-ard, 2003; Chuaynkern and Chuaynkern, 2012). Based on both vocal sac diagnosis and molecular study, Kuraishi et al. (2013) recognized *P. mutus* as *P.* cf. *mutus*1 and *P.* cf. *mutus*2 with substantial genetic differences. Later, Grosjean et al. (2015) studied DNA barcodes in tadpoles and reported them as the tadpoles of *P. mutus* and *P. braueri* from Thailand (the latter species had never previously been reported in Thailand). In our second analysis, the results supported the finding that both *P. mutus* (clustered within *P.* cf. *mutus*1 clade) and *P. braueri* were found in Thailand. According to the geographic distribution of *P. mutus*, *P. braueri* and *P. megacephalus*, they were sympatric in the Indochinese subregion (Kuraishi et al., 2013; Pan et al., 2013). Therefore, these three sympatric species may co-occur in northern Thailand. To indicate the current status of *P. mutus* and *P. braueri*, more specimen collections of either adults or tadpoles will be required.

Originally, *P. discantus* was recognized as *P. leucomystax* "Morph B" (non-striped pattern at dorsum) by Narins et al. (1998). Later, Kuraishi et al. (2013) mentioned that *P. leucomystax* "Morph B" was *Polypedates* sp. "Malay clade". Eventually, Rujirawan et al. (2013) recognized this species as a new species of *P. leucomystax* complex, the *P. discantus* (TL: Songkhla Province, Thailand) by using the integrated taxonomy (molecular data, morphology and bioacoustics). In our first phylogenetic tree, we found our four collected specimens from Satun Province were clustered within the *P. discantus* clade. Moreover, they showed substantial genetic difference from the sympatric *P. leucomystax* (12 to 30% p-distance).

In a recent study of *P. leucomystax* complex in Thailand using phylogenetic and spanning network analyses of *COI* gene by Buddhachat and Suwannapoom (2018), they reported 12 specimens of *P. impresus* (TL: Yunnan, China) which were collected from Nan Province, northern Thailand. In addition, they found a new clade of *Polypedates* sp. ($n = 40$) in which the specimens were collected from Phetchaburi, Kanchanaburi and Mae Hong Son provinces.

In our study as well, we found a new clade of *Polypedates* sp. ($n = 3$) in which the specimens were collected from Tak and Mae Hong Son provinces. Our *Polypedates* sp. clade (B-clade) was established with high statistical support (99, 1.00). The interspecific p-distances between B-clade *Polypedates* sp. and the other species were higher than 3% of *16S*, even in the case of *P. megacephalus,* which was a sympatric species (6 to 8%). Moreover; in the second analysis, our *Polypedates* sp. were clustered with four of the tadpole sequences of *Polypedates* sp. from Petchabun and Phitsanulok provinces. However, we could not compare our *Polypedates* sp. sequences with *Polypedates* sp. reported by Buddhachat and Suwannapoom (2018) due to the difference of DNA markers.

From our comprehensive samples, we found cryptic diversity within Asian common treefrogs (*P. leucomystax* complex) in Thailand. Although the integrated taxonomy has been suggested for identification, there are limitations in practice because they are geographically widespread species (Hasan et al., 2014). We used the phylogenetic analyses and genetic differences in *16S*, it provided reliable results in species identification of *P. leucomystax* complex, as in previous molecular studies of morphologically cryptic amphibians (Lehtinen et al., 2007; Hasan et al., 2012; Stock et al., 2012; Crawford et al., 2013; Guarnizo et al., 2015; Dufresnes et al., 2016).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We would like to thank Assoc. Prof Virayuth Lauhachinda for his excellent advice. We also convey my appreciation to Apiwat Suttiwisaet, Apisak Sukprasert, Thavin Bodharamik, Kittipong Lerdrungroj, Poramad Trivalairat, Wachiryah Thong-Asa and Jeerayot Saiyasuk for assistance in laboratory and field work. I am grateful to Patchara Danaisawadi for reviewing and providing the useful comments in this manuscript. We are indebted to all local people concerned for the specimen collections. Thanks to Valerie Suwanseree for English editing. Lastly, We thank the Department of Zoology, Kasetsart U. and Biology Division, Rajamangala University of Technology, Krungthep, for permitting us to use laboratory equipment and facilities.

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