RESEARCH NOTE

Genetic diversity and population structure of blue-crested lizard, *Calotes mystaceus* Duméril & Bibron, 1837 (Squamata: Agamidae) in Thailand

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Abstract

The blue-crested lizard, *Calotes mystaceus* Duméril & Bibron, 1837, is listed as a protected wild animal in Thailand. Its population is likely to be dramatically reduced due to massive hunting in several areas in this country. Basic information on its population genetics is therefore needed to facilitate its conservation. Thus, in this study we investigated the mitochondrial cytochrome c oxidase subunit 1 (*CO1*) sequence variation of 238 individual C. *mystaceus* from 42 different geographical localities of the north, west, central, east and northeast regions of Thailand. High genetic diversity and genetic differentiation at the intrapopulation and interpopulation levels was observed. We detected 63 unique haplotypes and 12 common/shared haplotypes. The phylogenetic analysis reveals two major lineages, I and II. These two lineages are separated by mountain ranges, which play an important role as natural barriers blocking gene flow. Our finding reveal at least two cryptic lineages represented in C. *mystaceus* populations in Thailand. However, a comprehensive investigation of the morphology, biology, ecology and genetic diversity of C. *mystaceus* in other regions within its area of distribution is needed.

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Introduction

Members of the lizard genus *Calotes*, including *C. mystaceus*, are listed as protected wild animals in Thailand since 2003. This means that they cannot be hunted for trading, export or possession without permission from the government of Thailand. However, *C. mystaceus* is not listed as protected in other regions of Indo-China, and although illegal, it is still massively hunted in several areas in Thailand, especially in the northeast region, where it is served as local dish. The massive hunting occurs during March and April, which is the lizard's breeding season. Therefore, the population of *C. mystaceus*, especially in hunting areas, is likely to be dramatically reduced. Previous studies performed in other areas of the distributional range of this species show that this taxa contains cryptic

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lineages (Hartmann *et al.* 2013). To date, however, there has been no work done in Thailand in determining whether this is the case, and if so, where these species occur. To facilitate their conservation in the future, intensive information regarding their habitat, biology, ecology, as well as genetic diversity is urgently needed.

There are at least 10 species of genus *Calotes* found in Southeast Asia, namely *Calotes chincollium*, *C. emma*, *C. htunwini*, *C. irawadi*, *C. jerdoni*, *C. kingdonwardi*, *C. maria*, *C. nigrigularis*, *C. versicolor* and *C. mystaceus* (Das 2010). In Thailand, there are three principal species, i.e., *C. versicolor*, *C. emma* (with the ssp. *C. e. emma* and *C. e. alticristatus*) and *C. mystaceus* (Das 2010). *C. mystaceus* is one of the most common and widespread lizards found throughout Thailand, except in the southern region (Chan-ard *et al.* 2015). Moreover, its distribution covers other regions of Indo-China, i.e., northeast India and southern China (Yunan Province), Myanmar, Laos, Cambodia, Vietnam and Malaysia (http://www.reptile-database.org). Its natural habitats include the forest at midaltitudes, as well as gardens in the lowlands and submontane forest at elevations of 180–1500 m above sea level (asl), but it also appears to be able to adapt to encroachment by humans and can be found in tree-rich neighbourhoods and city parks. There are several common names of *C. mystaceus*, according to their distribution region and morph, such as the Indo-Chinese forest lizard, blue forest lizard, blue-crested lizard and white-lipped *Calotes*.

Genetic investigations of the genus *Calotes*, including *C. mystaceus* in Southeast Asia are still very limited. To date, there is only one karyotype study, which showed homology of the karyotype formulae between *C. versicolor* and *C. mystaceus* in Thailand (Patawang *et al.* 2015). A population genetics study performed on *C. versicolor* from China and Vietnam provided evidence for high intrapopulation genetic diversity and high genetic differentiation between populations (Huang *et al.* 2013). For *C. mystaceus*, only one study based on the partial sequence of the mitochondrial cytochrome c oxidase subunit 1 (*CO1*) gene from Cambodia and Vietnam has been published (Hartmann *et al.* 2013). Thus, to obtain information on genetic diversity and population structure to support genetic conservation of this lizard, specimens of *C. mystaceus* were collected from different localities in Thailand using the mitochondrial *CO1* gene as a genetic marker. This maternally inherited marker has proven to be useful in population genetic studies, including the definition of cryptic species (Nazarov *et al.* 2014).

Materials and methods

Two hundred and thirty-eight specimens of *C. mystaceus* were sampled from 42 localities in Thailand (figure 1 and table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The adult *C. mystaceus* were caught using the trap box or fishing pole method (Bennett 1999). Their tails were cut off ∼5 mm from the end, cleaned using 70% alcohol and soaked in 80% alcohol until use. After specimen collection, the lizards were released back into their natural habitat. The total genomic DNA was extracted from their tail samples using E.Z.N.A® Tissue DNA kit (Omega Bio-Tek, Norcross, USA) following the manufacturer's protocol.

A partial region of the mitochondrial *CO1* gene was amplified with the primers and polymerase chain reaction (PCR) condition as reported by Ivanova *et al.* (2006). The amplified band was cut and purified by using E.Z.N.A® Gel Extraction kit (Omega Bio-Tek). The purified PCR product was sequenced directly by using ABI BigDye ver. 3.1 (Warrington, UK) and run on an ABI Prism 377 automated sequencer (Perkin-Elmer Corp., Foster City, USA) and VF1-d was used as a sequencing primer. All new sequences were deposited in GenBank with the accession numbers KX387897–KX388134.

All sequences generated in this study were aligned using the ClustalW program (Larkin *et al.* 2007). The sequences of *C. mystaceus* from Cambodia (CSr(KC016063) and CSr(KC016060)) and Vietnam (VNm(KC016061) and VNm(HM425545)) published by Hartmann *et al.* (2013) were included. Pairwise genetic differentiation between populations with ϕ_{ST} (using genetic distances with the Kimura algorithm) and population structure patterns based on analysis of molecular variance (AMOVA) were conducted using Arlequin ver. 3.5.1.3 (Excoffier and Lischer 2010). Haplotype data was generated using the DnaSp ver. 5 program (Librado and Rozas 2009). A minimum spanning haplotype network was constructed in the Network 5.0.0.0 program based on the median-joining network (Bandelt *et al.* 1999). We performed a suite of analyses to infer the phylogenetic relationship between the different populations. We built a maximum likelihood (ML) tree using the Kimura-2-parameter, and the model was constructed using the MEGA ver. 6.06 program (Tamura *et al.* 2011) with nodal support estimated using 1000 bootstrap resampling. In addition, MrModeltest ver 2.2 program with the corrected Akaike information criterion (AIC) (Nylander 2008) was performed to determine the most appropriate model for molecular evolution utilizable in Bayesian inference. The model selected was GTR+G. BI analysis was performed in MrBayes software package 3.1.2 (Ronquist and Huelsenbeck 2003) using the Markov chain Monte Carlo (MCMC) algorithm. The number of generations used in this analysis was 2,000,000, sampling every 100th generation. To calculate the posterior probabilities from BI analysis, the number of threes sampled at 10,000 after the SD values of the run dipped below 0.01.

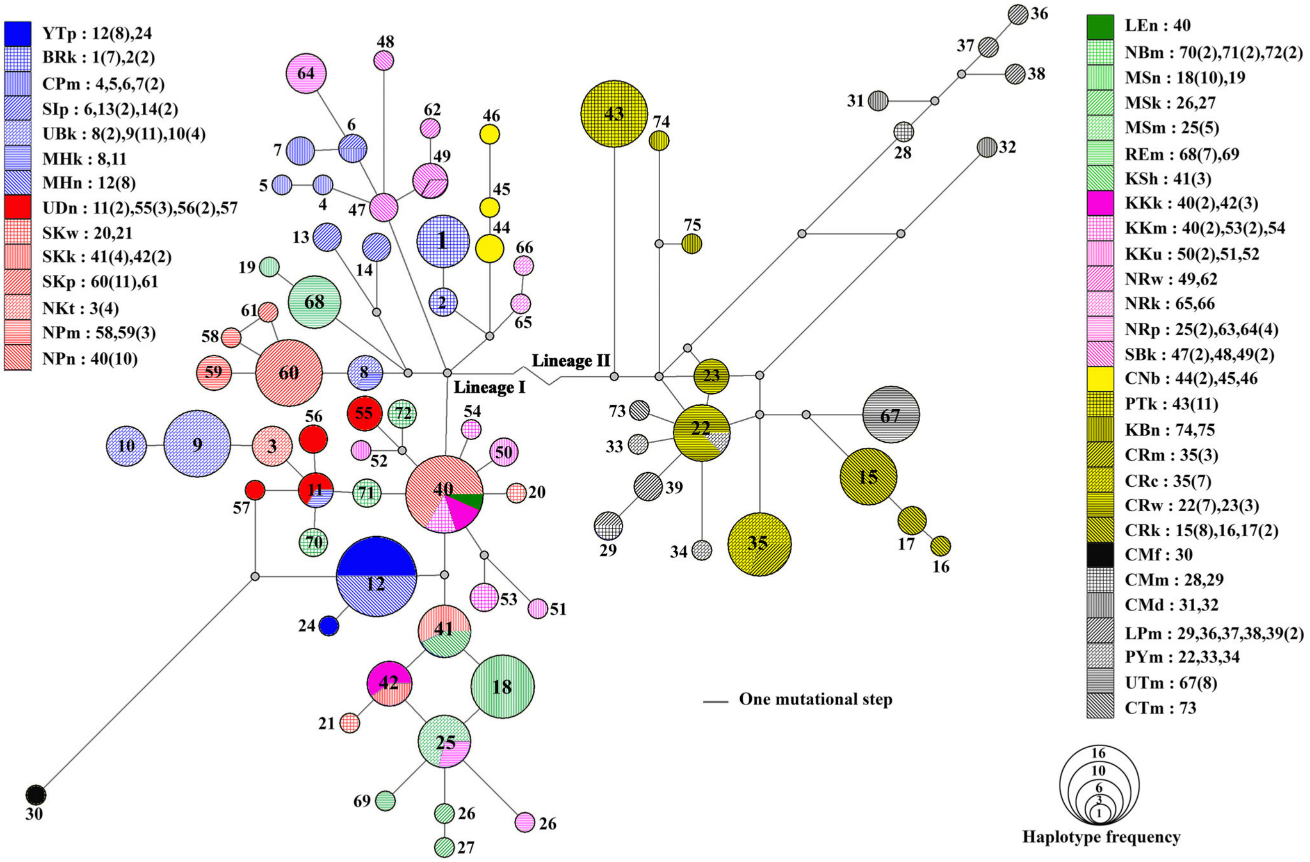

Figure 1. Minimum spanning haplotype network of *C. mysteceus* generated based on partial *CO1* sequence corresponds to their geographical localities separated into 42 different localities in Thailand. The area of the circles represents the proportion of specimen number found in each haplotype. The numbers represent haplotype name in each circle, as well as after the locality code. The number in parenthesis after each haplotype name is number of specimens belong to a particular haplotype, whereas haplotype name without parenthesis represents one specimen.

Results

The 613 bp partial sequence of the mitochondrial *CO1* gene of 238 individuals of *C. mystaceus* was compared within and between populations/localities in Thailand. In total, 96 variable sites were observed. Nucleotide diversity and haplotype diversity within populations are summarized in table 2 in electronic supplementary material. The number of segregation sites (S) and haplotypes (N) within each population ranged between 0–18 and 1–5, respectively. The relatively high genetic variation with a high number of S are from the north, i.e., CMd (13 S), CMm (16 S) and LPm (18 S), as well as NRp (14 S), REm (10 S) and Sip (11 S), and the northeast regions (table 2 in electronic supplementary material). Almost all populations had a unique haplotype (figure 1).

Based on the 96 variable sites, 75 haplotypes were identified and used for a minimum spanning haplotype network construction (figure 1). Of these, there are 63 unique haplotypes. The other 12 haplotypes were shared between at least two localities (figure 1). However, two different lineages (I and II) were defined relating to the ML tree (figure 2). Lineage I included all specimens from the northeast and east, as well as the SBk from central region, whereas all specimens from the north (except CMf), west and central (except SBk) regions belonged to lineage II (figure 1). Moreover, the haplotypes 28, 31, 32, 36, 37 and 38 from CMm, CMd and LPm revealed considerable divergence from the others, ranging between 13 and 17 mutation steps (figure 1). The most distinct haplotype was from CMf (haplotype 30), which shows the greatest divergence from the others at 18 mutation steps, which was not classified into any lineage in this study.

The ML and BI phylogenetic analyses showed similar topologies. Here, only the ML tree was selected to illustrate genetic clustering. At least two well-supported lineages of *C. mystaceus* were demonstrated, namely lineages I and II (figure 2). All specimens from the northeast, CNb from the east and SBk from the central regions, as well as two sequences from Cambodia (CSr) clustered together into lineage I. Lineage II contained all specimens from the northern, CTm and PTk

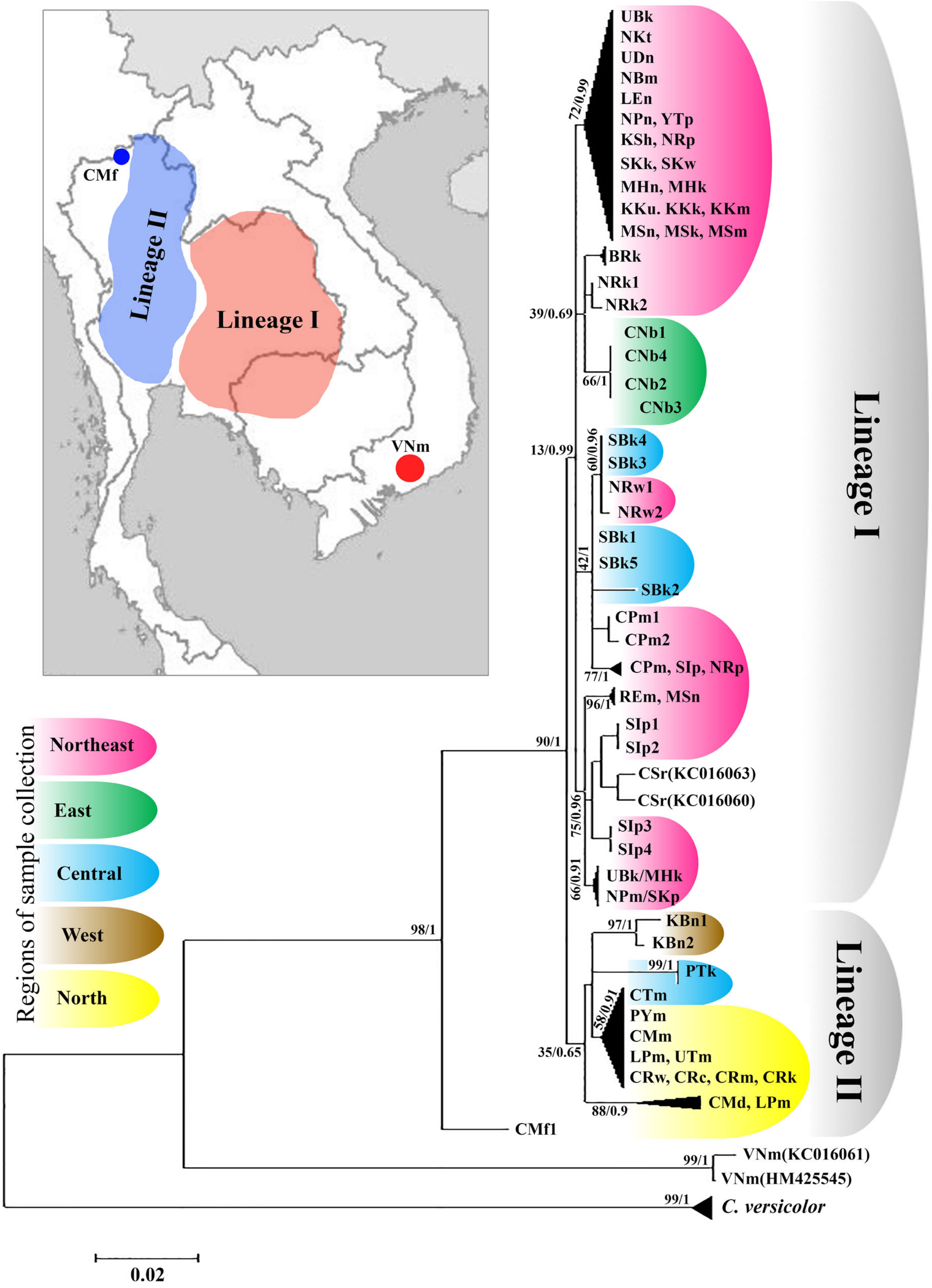

Figure 2. A ML tree was constructed based on 613 bp of *CO1* sequence of *C. mystaceus* from Thailand. The other four sequences are from CSr and VNm that are available in GenBank were also included. Nodal supports are of bootstrap values obtained by ML analyses and Bayesian posterior probability, respectively. The scale bar indicates the expected number of substitutions per site. The sequences of *Calotes versicolor* were used as out-group. The map shows the areas covering two different lineages, where the blue and red circles represent the distinct specimens from CMf and VNm, respectively.

from the central and KBn from the western regions (figure 2). Interestingly, a specimen from CMf identified as a very distinct taxon from the others and could not be included in any lineages of this analysis. Two sequences from Vietnam (VNm) also constituted a distinct clade (figure 2).

Pairwise genetic differentiation (ϕ_{ST}) between populations/localities (only sample sizes >3 were included in this analysis) ranging between 0.000 and 1.000 was observed (table 3 in electronic supplementary material). The population genetic structure of *C. mystaceus* was tested by AMOVA. We found significant genetic substructuring ($F_{CT} = 0.019$, $P < 0.05$) corresponding to the two different lineages generated in the haplotype network and ML tree. The other model of genetic structure based on different regions, provinces or localities in Thailand showed no significant differences, with F_{CT} ranging between 0.007 and 0.148. However, a comparison of populations within each lineage always found significant genetic structuring with F_{SC} ranging between 0.544 and 0.635 ($P < 0.001$), as well as significant genetic structuring for all individuals, with F_{ST} ranging between 0.554 and 0.680 ($P < 0.001$) (table 4 in electronic supplementary material).

Discussion

There is very limited information available on the genetics of *C. mystaceus*. This study is the first to investigate the genetic diversity and population structure of this species in Thailand. We found considerable genetic diversity with high frequencies of unique haplotypes in each population. This finding is similar to that found in China and Vietnam (Huang *et al.* 2013), indicating that the populations of *C. mystaceus* in Thailand have had sufficient time to acquire unique haplotypes. Moreover, the lizards, including *C. mystaceus*, are believed to have limited dispersal abilities (Rutherford and Gregory 2003), which is likely to affect gene flow among populations (Irwin 2002). We also found highly significant genetic differentiation among populations/localities of *C. mystaceus*, even when at a close distance from one another, with no natural barriers, especially between the northeast populations.

We found evidence for the occurrence of two main lineages with AMOVA and different phylogenetic methods. Interestingly, our results show that *C. mystaceus* from Cambodia clustered with the sequences of lineage I together with all populations from northeastern and eastern Thailand, whereas lineage II included the specimens from northern, western and central Thailand. This finding was completely in agreement with the phylogenetic study of *Scolopendra dehaani* from Mainland Southeast Asia, which found that the populations from northeast and east Thailand, as well as Cambodia, represented a monophyletic clade, whereas the populations from northern, western and central Thailand belonged to a different clade (Siriwut *et al.* 2015). The divergence between these two major lineages could be effected by the Phetchabun, Dong Paya Yen and Sankambeng mountain ranges, which might impede gene flow between the Korat Plateau (northeast region) and the northwest and central regions of Thailand. These mountain ranges are also known to limit the gene flow of other organisms, such as the Mekong mud snake, *Enhydris subtaeniata* (Lukoschek *et al.* 2011) and the truncate-snouted burrowing frog, *Glyphoglossus molossus* (Laojumpon *et al.* 2012).

In addition, two *CO1* sequences of *C. mystaceus* from Vietnam were very distinct from the others, which suggests that populations of *C. mystaceus* in other distribution areas in Indo-China may contain more genetic structuring than that determined in this study. More specimens from different dispersal areas should be collected for a more comprehensive study of the genetic diversity and genetic structure of *C. mystaceus* populations throughout the Indo-China region.

The results from this and previous studies suggest that the cryptic lineages of *C. mystaceus* are existed in Mainland Southeast Asia. There have been reports of cryptic lineages/species occurring in amphibian and reptile taxa in this region related to natural barriers (mountain ranges and river systems), for example the Mekong mud snake (Lukoschek *et al.* 2011) and centipede (Siriwut *et al.* 2015). However, the intensive morphological comparisons should be made to define the cryptic species of *C. mystaceus* in Thailand, as well as in other areas of Southeast Asia.

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Abstract

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