

Thai limestone karsts: an impending biodiversity crisis

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Abstract

Due to the high level of endemic species that they support and the high threats they are facing, such as unsustainable limestone quarrying, hunting and urbanization, limestone karsts are recognized as biodiversity hotspots needing urgent protection.

The first aim of our study was to investigate the mammal rodent diversity of Thai limestone karsts. Our second aim was to examine the diversity at a finer scale than the species level (intraspecific biodiversity) using phylogeographic approaches. Therefore, we studied the phylogeographic structure of a threatened rodent, endemic to limestone karsts of Thailand, the Murinae *Leopoldamys neilli*. We sampled 76 limestone karsts in whole Thailand and live-trapped 444 rodents including 115 *Leopoldamys neilli*.

Our study revealed an important rodent diversity in Thai limestone karsts. Besides endemic rodent species, karsts also host typical forest species to which they provide forest refugia in deforested regions. At the intraspecific level, our study revealed an unexpected high level of genetic differentiation within the rodent species *L. neilli*. As each limestone area of Thailand is characterized by a particular genetic lineage of *L. neilli*, the destruction of these karsts would lead to the disappearance of unique intraspecific strains not found elsewhere. Our results highlight the importance of protecting limestone habitats to preserve not only their interspecific but also intraspecific rodent diversity that is highly threatened as more than 20% of limestone karsts in Thailand have already been quarried. Management plans of limestone, a non-renewable resource, should urgently take into account this high biological importance of karsts.

Keywords: Thailand; rodent diversity; limestone karsts; conservation; *Leopoldamys neilli*

1. Introduction

Limestone karsts are sedimentary rock outcrops consisting of calcium carbonate created millions of years ago by calcium-secreting marine organisms, and subsequently lifted above sea level by tectonic movement (Clements *et al.*, 2006). Within Southeast Asia, karsts cover an area of 460,000 km² - about 10% of the total land area of this region (Day & Urich, 2000). Southeast Asian limestone karsts are highly threatened by unsustainable limestone quarrying, hunting and urbanization (Vermeulen & Whitten, 1999; Clements *et al.*, 2006). Limestone is an extremely important raw material and is used in Thailand in 14 major industries as crushed rock for building and construction, cement and lime among others (ESCAP, 2001). More than 122 limestone mines are operating in Thailand and more than 20% of limestone karsts in Thailand have already been quarried (World Bank, 2004).

Southeast Asian limestone karsts support very high level of endemic species of plants, vertebrates and invertebrates (Vermeulen & Whitten, 1999; Schilthuizen *et al.*, 2005;

Clements *et al.*, 2006; 2008) and are recognized as biodiversity hotspots of high biological importance (Vermeulen & Whitten, 1999; Clements *et al.*, 2006). Thai limestone karsts host several endemic animals, among which are two rodent species, *Leopoldamys neilli* and *Niviventer hinpoon*, one bird species, *Napothera crispifrons calcicola*, at least seven fish species and dozens of snails species (World Bank, 2004). However, limestone karsts remain poorly studied despite their great scientific interest (Sodhi *et al.*, 2010) and they urgently need an increase of biological knowledge to better preserve their huge biodiversity.

The first aim of this study was to develop a mammal rodent diversity inventory of Thai limestone karsts on the basis of morphological as well as genetics methodologies.

Our second aim was to examine the rodent diversity at a finer scale than the species level (intraspecific biodiversity) using phylogeographic approaches. Indeed, in contrast to other regions in the world, the Southeast Asian region is poorly studied on this point of view, as only 3.3% of the phylogeographic studies published between 1987 and 2006 concern taxa from this region (Beheregaray, 2008). Phylogeographic approaches therefore appear highly important to better understand the level of limestone karsts biodiversity, not only on the interspecific but also intraspecific level. Therefore, we studied the phylogeographic structure of a threatened rodent, endemic to limestone karsts of Thailand, the Murinae *Leopoldamys neilli* based on molecular markers. *L. neilli*, a large and long-tailed rat with grayish-brown fur and a white belly, was discovered in 1973 in Saraburi province, central Thailand (Lekagul & McNeely, 1988). The species has also been recorded in a few locations in north and southwest Thailand (Lekagul & McNeely 1988; Waengsothorn *et al.*, 2007). *L. neilli* was previously classified as “Endangered” on the IUCN Red List, but is now listed as “Data deficient” since very little information is available about its biology and ecology (IUCN, 2010).

2. Material & Methods

2.1 Mammal rodent diversity inventory

A total of 444 mammal rodents were collected (live-trapped) in limestone karsts from 76 localities in 15 provinces of Thailand (Figure 1). All the collected animals were released after sampling of a small piece of ear skin. The skin samples were stored in 96% ethanol. Forty-five samples (Table 1) were selected among the 444 specimens in order to maximize the number of species and geographic locations and were used in phylogenetic analyses. Field identifications were made based on morphological criteria according to Lekagul & McNeely (1988) and Corbet & Hill (1992).

Table 1. Samples used in the biodiversity inventory

Code	Species	Locality of origin (Figure 1)	Province
LK012	<i>Niviventer fulvescens</i>	9	Loei
LK021	<i>Leopoldamys edwardsi</i>	9	Loei
LK034	<i>Leopoldamys neilli</i>	10	Loei
LK047	<i>Maxomys</i> sp. 2	10	Loei
LK054	<i>Rattus tanezumi</i>	3	Nan
LK059	<i>Leopoldamys neilli</i>	1	Nan

Table 1. Samples used in the biodiversity inventory (cont.)

Code	Species	Locality of origin (Figure 1)	Province
LK062	<i>Maxomys surifer</i>	2	Nan
LK072	<i>Maxomys</i> sp. 1 (lineage M1 in Pagès et al., 2010)	5	Phrae
LK073	<i>Rattus tanezumi</i>	6	Lampang
LK074	<i>Leopoldamys edwardsi</i>	8	Lampang
LK083	<i>Maxomys surifer</i>	7	Lampang
LK089	<i>Maxomys</i> sp. 2	17	Saraburi
LK090	<i>Leopoldamys neilli</i>	17	Saraburi
LK101	<i>Niviventer hinpoon</i>	18	Saraburi
LK109	<i>Niviventer hinpoon</i>	18	Saraburi
LK112	<i>Rattus tanezumi</i>	16	Nakhon Ratchasima
LK122	<i>Niviventer hinpoon</i>	19	Lopburi
LK141	<i>Rattus tanezumi</i>	20	Kanchanaburi
LK142	<i>Leopoldamys neilli</i>	21	Kanchanaburi
LK202	<i>Maxomys</i> sp. 3	27	Krabi
LK207	<i>Leopoldamys sabanus</i>	27	Krabi
LK215	<i>Rattus tiomanicus</i>	28	Krabi
LK222	<i>Rattus tiomanicus</i>	28	Krabi
LK254	<i>Leopoldamys sabanus</i>	29	Krabi
LK261	<i>Rattus tanezumi</i>	30	Krabi
LK262	<i>Leopoldamys sabanus</i>	25	Nakhon Si Thammarat
LK264	<i>Maxomys</i> sp. 3	25	Nakhon Si Thammarat
LK280	<i>Maxomys</i> sp. 3	24	Nakhon Si Thammarat
LK290	<i>Rattus tanezumi</i>	23	Nakhon Si Thammarat
LK297	<i>Leopoldamys sabanus</i>	26	Nakhon Si Thammarat
LK303	<i>Rattus tanezumi</i>	22	Surat Thani
LK304	<i>Leopoldamys sabanus</i>	22	Surat Thani
LK310	<i>Niviventer fulvescens</i>	11	Chaiyaphum
LK311	<i>Maxomys</i> sp. 2	11	Chaiyaphum
LK317	<i>Niviventer fulvescens</i>	11	Chaiyaphum
LK319	<i>Rattus tanezumi</i>	11	Chaiyaphum
LK364	<i>Maxomys</i> sp. 2	9	Loei
LK377	<i>Niviventer fulvescens</i>	9	Loei
LK387	<i>Leopoldamys edwardsi</i>	13	Petchabun
LK396	<i>Leopoldamys edwardsi</i>	4	Phrae
LK409	<i>Rattus andamanensis</i>	1	Nan
LK414	<i>Rattus andamanensis</i>	1	Nan
LK419	<i>Maxomys</i> sp. 2	12	Petchabun
LK425	<i>Rattus tanezumi</i>	15	Petchabun
LK430	<i>Leopoldamys edwardsi</i>	14	Petchabun

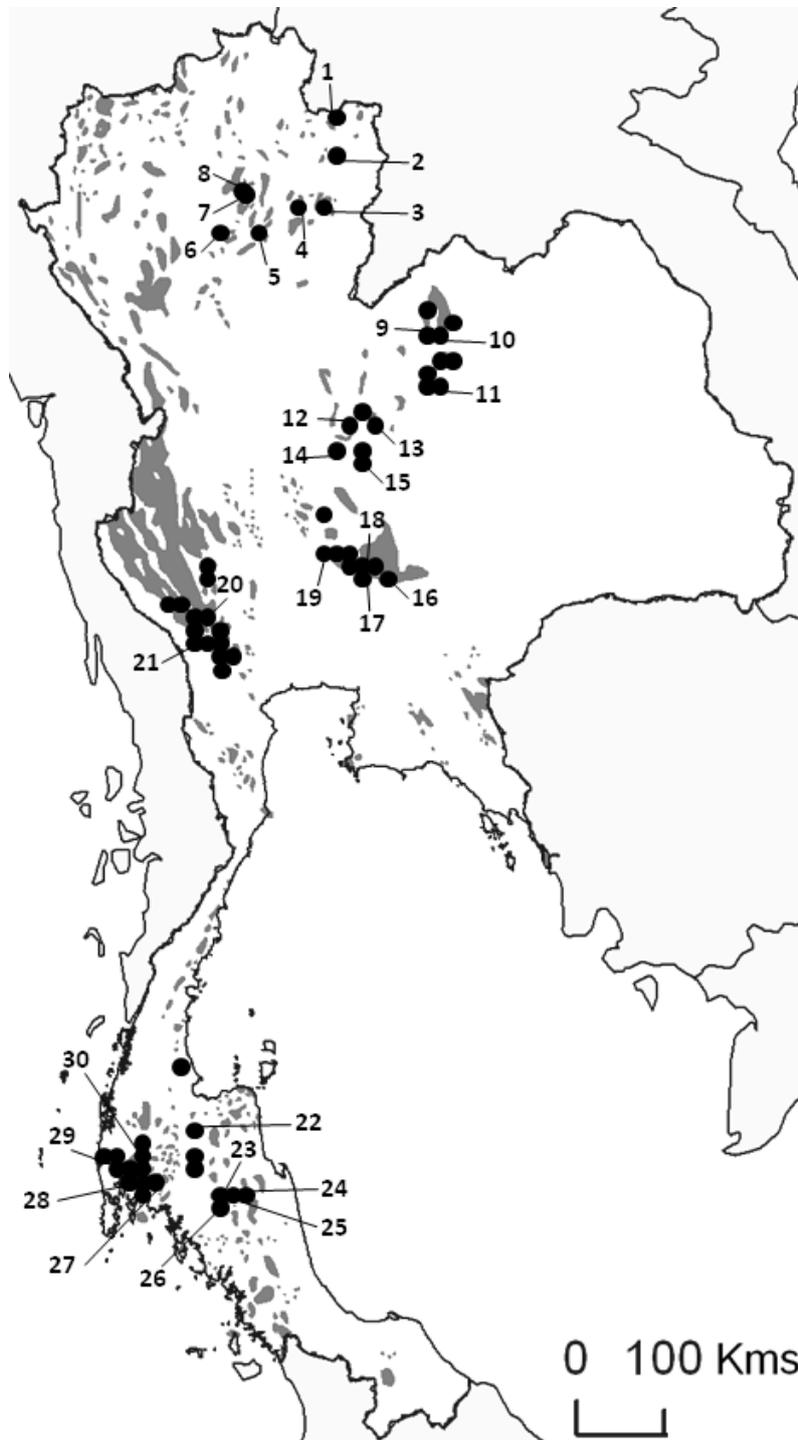


Figure 1: Sampling locations of this study. Shaded areas represent the distribution of limestone karsts in Thailand. Black dots represent the 76 limestone karsts sampled in this study. Sampling localities of the 45 Rattini specimens included in our phylogeny and biodiversity inventory are numbered (see Table 1).

Genomic DNA was extracted from skin samples using the DNeasy Tissue Kit (Qiagen Inc., Valencia, California) following the manufacturer’s instructions.

One mitochondrial marker, the cytochrome *b* gene (*cytb*) (1000 bp), was amplified for the 45 samples using primers H6 (5’-TCTCCATTTCTGGTTTACAAGAC-3’) and L7 (5’-ACCAATGACATGAAAAATCATCGTT-3’). A phylogenetic tree was reconstructed using the Neighbor-Joining method with 100 bootstraps implemented in MEGA v4.1 (Tamura *et al.*, 2007). To allow accurate species identification and confirm our field identification based on morphological criteria, several *cytb* sequences belonging to voucher specimens from Pagès *et al.* (2010) and available on GenBank were added to our dataset (*Leopoldamys edwardsi*: R4370, *Leopoldamys neilli*: R4485, *Leopoldamys sabanus*: R3033, *Rattus andamanensis*: R2953, *Rattus tiomanicus*: R1833, *Rattus tanezumi*: R4424, *Maxomys surifer*: R4223-R3464, *Maxomys* sp.: R3118-R3116, *Niviventer fulvescens*: R3427-R4525). Two sequences of *Micromys* were used as outgroup.

2.2 Phylogeography of *L. neilli*

A total of 115 *Leopoldamys neilli* collected (live-trapped) in limestone karsts from 20 localities in 7 provinces of Thailand (Loei, Nan, Phrae, Saraburi, Nakhon Ratchasima, Lopburi, Kanchanaburi provinces) has been analyzed (Figure 2). Field specimen identifications and locality information are given in Latinne *et al.* (2011).

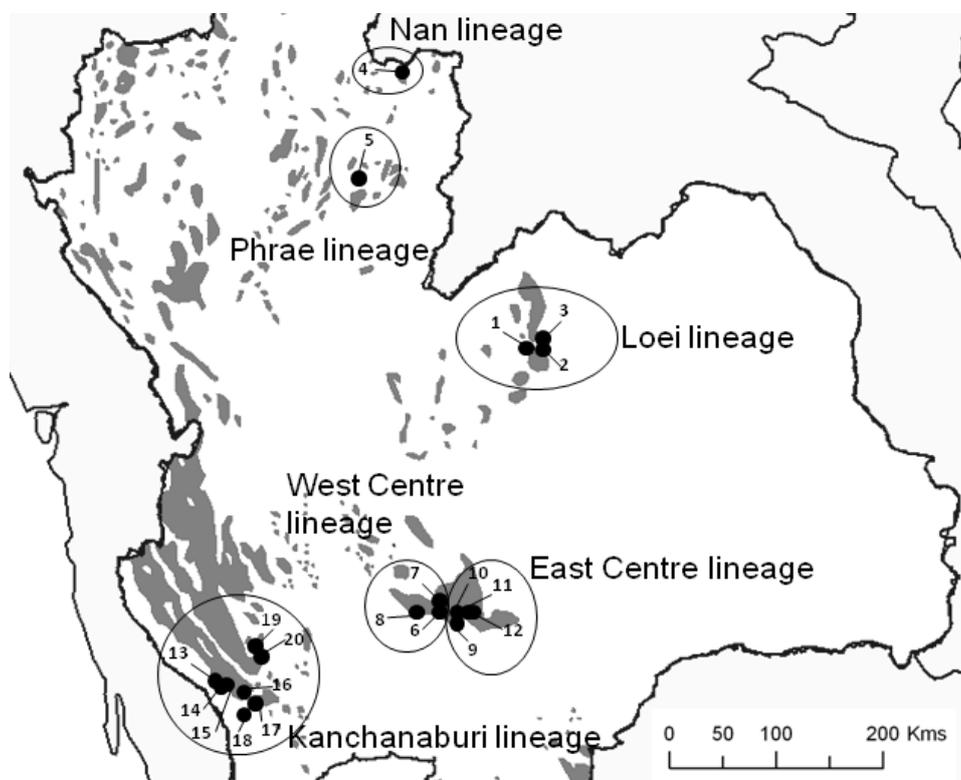


Figure 2 (modified from Latinne *et al.*, 2011): Sampling localities of the 115 *Leopoldamys neilli* analyzed in this study and geographic distribution of the different genetic lineages observed within *L. neilli* in Thailand. Shaded areas represent the distribution of limestone karsts in Thailand.

Two mitochondrial markers, the cytochrome *b* gene (*cytb*) (900 bp) and the cytochrome *c* oxidase subunit I gene (*COI*) (713 bp), were amplified for all the *L. neilli* samples. Moreover, a nuclear locus, the β -fibrinogen intron 7 (*bfibr*) (745 bp), was targeted for a subset of 65 samples representative of the main mitochondrial clades found, using primers designed by Seddon *et al.* (2001). DNA extraction and amplification are detailed in Latinne *et al.* (2011).

Phylogenetic trees were reconstructed for the phylogeographic purposes using two probabilistic approaches: the maximum likelihood method (ML) and Bayesian inferences (BI) as described in Latinne *et al.* (2011). Analyses were run on the three combined genes dataset (*cytb*/*COI*/*bfibr*). A median joining network was performed with NETWORK v4.5.1.6. (Bandelt *et al.*, 1999) to explore relationships between haplotypes of the combined dataset (*cytb*/*COI*/*bfibr*).

Haplotype (*h*) and nucleotide (π) diversities of the main lineages corresponding to the studied karstic regions were estimated for each locus using Arlequin 3.11 (Excoffier *et al.*, 2005). An Analysis of Molecular Variance (AMOVA) performed on the combined dataset (*cytb*/*COI*/*bfibr*) in Arlequin 3.11 was used to assess the distribution of genetic variation among populations. The genetic groups were defined as detailed in Latinne *et al.* (2011). The genetic differentiation between lineages was assessed using population pairwise *F*_{st} and *N*_{st} calculated in DNASP v5 (Librado & Rozas, 2009).

3. Results

3.1 Mammal rodent diversity inventory

According to our phylogeny (Figure 3), four genera belonging to the Rattini tribe were encountered in our sampling sites: *Leopoldamys*, *Rattus*, *Maxomys* and *Niviventer*. Three *Leopoldamys* species (*L. edwardsi*, *L. neilli*, *L. sabanus*), three *Rattus* species (*R. andamanensis*, *R. tiomanicus*, *R. tanezumi*), four *Maxomys* species (*M. surifer*, *M. sp. 1*, *M. sp. 2*, *M. sp. 3*) and two *Niviventer* species (*N. fulvescens*, *N. hinpoon*) were identified in our dataset.

3.2 Phylogeography of *L. neilli*

The phylogenetic tree (Figure 4) and median joining network (data not shown) of the three combined genes gave similar results and indicated that the 36 haplotypes clustered into six geographically well-structured lineages: Loei lineage (samples from Loei province), Phrae lineage (samples from Phrae province), Nan lineage (samples from Nan province), West Centre lineage (samples from Lopburi province and from the western part of Saraburi province), East Centre lineage (samples from Nakhon Ratchasima province and from the eastern part of Saraburi province) and Kanchanaburi lineage (samples from Kanchanaburi province) (Figure 2). The monophyly of these lineages is very well supported with BS: 100% - BP: 1.0.

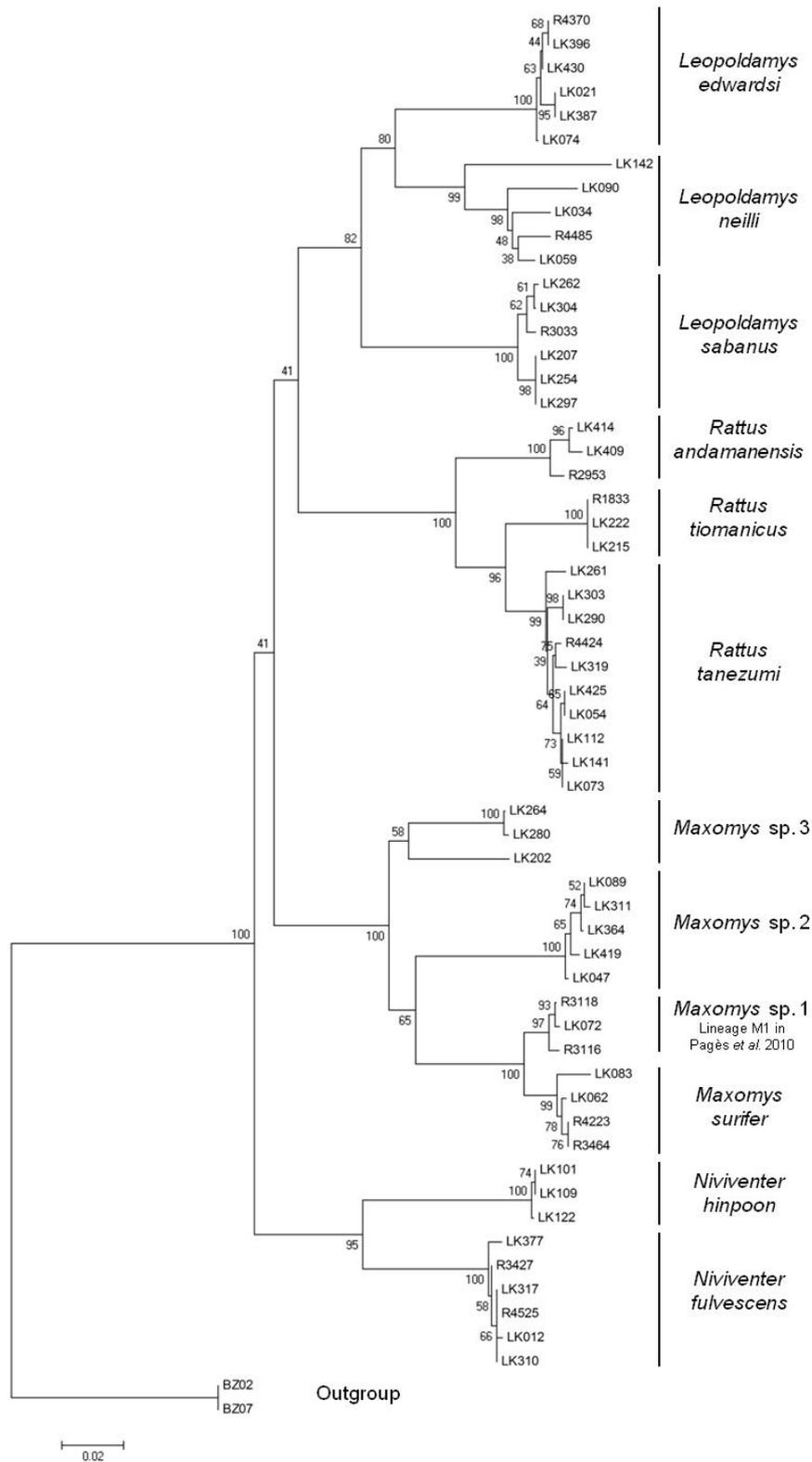


Figure 3: Neighbor-Joining phylogenetic tree depicting relationships among Rattini species of Thai limestone karsts. Bootstrap supports (100 replicates) of the nodes are indicated above the branches.

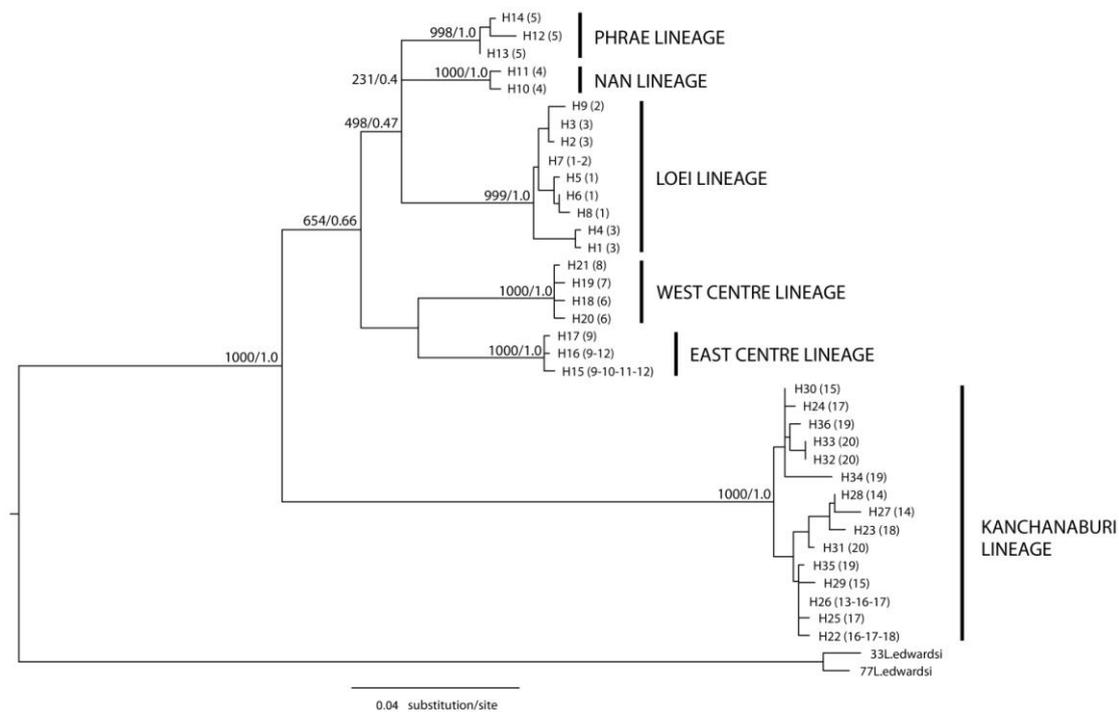


Figure 4: Maximum likelihood tree summarizing the phylogenetic relationships among the 36 haplotypes of the studied populations based on the three combined genes (GTR + G model). The same topology was obtained with the Bayesian analyses. Bootstrap support (1000 replicates)/posterior probabilities are indicated above the branches. The geographic distribution of each haplotype has been included in parentheses (see Figure 1 for numbers and localities correspondences).

Haplotype and nucleotide diversities were calculated for the six main lineages for the three independent markers (cytb, COI, and b fibr). The results indicate that all the lineages show a very low genetic diversity for all the markers (between 0 and 0.81 for haplotype diversity and between 0 and 0.58% for nucleotide diversity).

The AMOVA performed on the three combined genes dataset showed that 94% of variation is explained by differences among geographical groups, whereas only 2% and 4% of this variation are explained by differences among populations within groups and within populations, respectively.

Fst and Nst values calculated on the three combined genes dataset are very high among lineages (>0.9), evidencing low gene flow among them.

4. Discussion

4.1 Interspecific rodent diversity of Thai limestone karsts

Our study revealed an important rodent diversity in Thai limestone karsts. The two species of Thai rodent endemic to limestone karsts, *Leopoldamys neilli* and *Niviventer hinpoon*, have been identified in our sampling sites and share narrow ecological niche. Our study confirms the presence of *L. neilli* in Saraburi, Kanchanaburi and Loei provinces (IUCN,

2010) but extends its geographical range to the northern (Nan and Phrae provinces) part of Thailand. The distribution range of *N. hinpoon* appears to be more restricted and limited to karst area of central Thailand (Saraburi and Lopburi provinces). The high endemism of *N. hinpoon* to limestone karsts of this region makes this species highly vulnerable and threatened by habitat destruction.

Besides endemic species, Thai limestone karsts also host typical rodent forest species. *Leopoldamys edwardsi*, *Leopoldamys sabanus*, *Rattus andamanensis*, *Maxomys surifer* and *Niviventer fulvescens* are all living in wild forest ecosystems (Lekagul & McNeely, 1988). Since Thailand has lost about 30% of its forest cover between 1950 and 2000 (World Bank, 2004), limestone karsts provide important forest refugia for these species in deforested and highly developed regions. *Rattus tiomanicus* and *R. tanezumi* are highly adaptable species living in a large variety of habitats from domestic and agricultural to natural environments. It is interesting to note that their generalist ecology allow them to live also in limestone karsts. Our study also evidenced the presence in the studied areas of two additional *Maxomys* species (*Maxomys* sp. 2 and *Maxomys* sp. 3) not found in the study of Pagès *et al.* (2010). The *Maxomys* sp. 3 lineage includes specimens from southern Thailand (Krabi and Nakhon Si Thammarat provinces). This region represents the northern limit of the geographical range of two *Maxomys* species: *M. rajah* and *M. whiteheadi* (Wilson & Reeder, 2005). So the *Maxomys* sp. 3 lineage could be one of these species. The *Maxomys* sp. 2 lineage includes specimens from central and northeastern Thailand (Saraburi, Petchabun, Loei and Chaiyaphum provinces) where *M. surifer* is the only *Maxomys* species reported. Further investigations using independent data (morphology, nuclear genes) will help to determine if these two lineages correspond to cryptic species endemic to the limestone karsts or if they belong to species already described in Thailand but not analysed in previous genetic studies.

4.2 Intraspecific rodent diversity of Thai limestone karsts

Our study evidenced a high geographic structure of the genetic diversity for *L. neilli*. Indeed, six highly differentiated genetic lineages were observed. Each of them corresponds to particular regions of Thailand (Loei, Nan, Phrae, Saraburi-Nakhon Ratchasima, Saraburi-Lopburi, Kanchanaburi provinces) and gene flows between them are extremely low. These results are surprising as some populations are geographically very close to each other (Figure 2). Such fine-scale population differentiation at the intraspecific level is very rare among rodents and small mammals.

These results suggest a severe population fragmentation correlated to the scattered distribution of the karst habitat of this species; the lowland areas between limestone karsts thus act as biogeographical barriers and prevent gene flow between lineages. These results also attest the high endemism of *L. neilli* to limestone karsts and its high ecological specialization.

4.3 Implication for conservation and management of limestone karsts

More than 20% of limestone karsts in Thailand have already been quarried for cement, lime and hard core, and many have completely disappeared from the landscape (World Bank, 2004). The cement production of Thailand has increased by about 10% every year in the early

2000's but is slightly decreasing since 2007 (ESCAP, 2001; Shi, 2008). An average of 37 Mt of cement is produced every year in Thailand (Shi, 2008). As a result of these activities, some endemic species became dramatically endangered. Management plans of limestone karsts should urgently take into account the high biological importance of karsts in order to protect their great biodiversity.

Our phylogenetic analyses proved the importance of limestone karsts in the conservation of Southeast Asian biodiversity as they host endemic species (*L. neilli* and *N. hinpoon*) but also ubiquitous (*R. tiomanicus* and *R. tanezumii*) and forest (*L. edwardsi*, *L. sabanus*, *R. andamanensis*, *M. surifer* and *N. fulvescens*) species. Our study also evidenced the existence of highly differentiated genetic lineages within *L. neilli* corresponding to different limestone regions spread all over Thailand. As the six *L. neilli* lineages that we described exchange very few migrants and are geographically and genetically isolated, they should be considered as distinct Management Units (MU) (Moritz, 1994; Avise, 2000), and may require separate management and conservation plans. This information is extremely important for the conservation of *L. neilli*. Indeed, as the Thai limestone areas are characterized by particular endemic genetic lineages of this species and as they are presently highly threatened by quarrying, hunting, and urbanization (Vermeulen and Whitten, 1999; Clements *et al.*, 2006), it is very likely that each karst destruction will lead to the disappearance of unique intraspecific *L. neilli* lineages not found elsewhere in Southeast Asia. Large undisturbed limestone karsts areas should be preserved in each karst region of Thailand in order to protect the whole genetic diversity of *L. neilli*. We strongly suggest following the recommendations of Vermeulen & Whitten (1999) for limestone quarrying management and to avoid the quarrying of isolated limestone hills and to locate quarries in the largest limestone areas and let a considerable part of it untouched.

5. Conclusion

From the interspecific point of view, our molecular study revealed a high number of Rattini species living in Thai limestone karsts, confirming the importance of limestone karsts as forest refugia for rodents in deforested and developed regions. At the intraspecific level, our study revealed an unexpected high level of genetic differentiation within the species *L. neilli*.

These results highlight the importance of protecting limestone habitats to preserve not only their interspecific but also intraspecific biodiversity that is highly threatened. Management plans of limestone, a non-renewable resource, should urgently take into account this high biological importance of karsts.

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