Population genetics of the Khorat snail-eating turtle (*Malayemys khoratensis*) in Thailand (#105693)

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Population genetics of the Khorat snail-eating turtle (*Malayemys khoratensis*) in Thailand

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Malayemys khoratensis is an endemic freshwater turtle species in northeastern Thailand and Lao PDR. Even though *M. khoratensis* is relatively common and widespread in Thailand, its population size and trend remain entirely unknown due to the absence of direct studies for estimating its population size. In this study, genetic diversity, population structure and demographic history of *M. khoratensis* in northeastern Thailand were investigated using two mtDNA regions, *Cyt b* and *ND4*. We divided turtle samples from northeastern Thailand according to three basins in this region, Mun, Chi and Northeast Mekong, and tested for isolation-by-basin of its population structure. In our surveys we found 49 *M. khoratensis* in 15 provinces in Thailand and we included 15 sequences from the previous study by *Ihlow et al. (2016)*. We identified 13 unique haplotypes defined by 26 polymorphic sites. The total haplotype diversity of *M. khoratensis* was 0.819. Mekong basin had the highest haplotype number and haplotype diversity followed by Mun basin and Chi basin, respectively. The AMOVA test and the haplotype network did not indicate any population structure among basins. The Bayesian Skyline Plot estimated a stable effective female population size of 130,000 individuals since about 100,000 years ago (the late Pleistocene), followed by a short slight demographic expansion between AD 0 and AD 1000, and a subsequent population decline from AD 1000 to the present. This species might currently be declining, and we thus suggest that it should be protected by law similar to its congeners, *Malayemys subtrijuga* and *Malayemys macrocephala*.

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Abstract

Malayemys khoratensis is an endemic freshwater turtle species in northeastern Thailand and Lao PDR. Even though *M. khoratensis* is relatively common and widespread in Thailand, its population size and trend remain entirely unknown due to the absence of direct studies for estimating its population size. In this study, genetic diversity, population structure and demographic history of *M. khoratensis* in northeastern Thailand were investigated using two mtDNA regions, *Cyt b* and *ND4*. We divided turtle samples from northeastern Thailand according to three basins in this region, Mun, Chi and Northeast Mekong, and tested for isolation-by-basin of its population structure. In our surveys we found 49 *M. khoratensis* in 15 provinces in Thailand and we included 15 sequences from the previous study by *Ihlow et al. (2016)*. We identified 13 unique haplotypes defined by 26 polymorphic sites. The total haplotype diversity of *M. khoratensis* was 0.819. Mekong basin had the highest haplotype number and haplotype diversity followed by Mun basin and Chi basin, respectively. The AMOVA test and the haplotype network did not indicate any population structure among basins. The Bayesian Skyline Plot estimated a stable effective female population size of 130,000 individuals since about 100,000 years ago (the late Pleistocene), followed by a short slight demographic expansion between AD 0 and AD 1000, and a subsequent population decline from AD 1000 to the present. This species might currently be declining, and we thus suggest that it should be protected by law similar to its congeners, *Malayemys subtrijuga* and *Malayemys macrocephala*.

Keywords: Population genetic, *Malayemys khoratensis*, Mitochondrial DNA (mtDNA), Isolation-by-basin, Bayesian Skyline Plot

Introduction

Malayemys is an endemic freshwater turtle genus in Southeast Asia (*Bonin et al., 2006; Brophy 2004,2005; Dawson et al., 2018,2020; Platt et al., 2022*). Three species of *Malayemys* were described in this region: *Malayemys subtrijuga* (*Schlegel & Müller, 1845*) *Malayemys macrocephala* (*Gray, 1859*) and *Malayemys khoratensis* (*Ihlow et al., 2016*). *M. khoratensis* was initially reported to occur in the Khorat (Chi-Mun) Basin of northeastern Thailand (i.e. in Nakhon Ratchasima Province, Udon Thani Province, Nong Bua Lamphu Province and in the Nam Phong River system in Nong Khai Province) and Lao PDR (Vientiane Province, Vientiane Prefecture, Khammouan Province, and Savannakhet Province (*Platt et al., 2022; Ihlow et al., 2016; Sumontha et al., 2016*). *Chaiananporn et al. (in press)* confirmed the distribution range of *M. khoratensis* in northeastern Thailand and reported that in Thailand, *M. khoratensis* distribution is limited to the Chi River Basin and upper Mun River Basin to the Mekong River. It was not found in the lower Mun River to the Mekong River and in eastern Thailand. Thus, *M. khoratensis* is highly endemic to the northeastern Thailand and Lao PDR.

Even though *M. khoratensis* is relatively common and widespread in Thailand, its population size and trend of *M. khoratensis* remains entirely unknown due to the absence of direct studies for estimating population size. Moreover, *M. khoratensis* is threatened by capture and trade for merit releases in Buddhist ceremonies, and turtles and turtle eggs are also consumed by local people or used as medicine (*Dawson et al., 2018,2020; Platt et al., 2022*). However, in Thailand, only *M. subtrijuga* and *M. macrocephala* are protected under Thailand's Wild Animal Conservation and Protection Act, B.E. 2562 (2019), while *M. khoratensis* remains unprotected despite facing similar threats.

Conservation genetics enables the analysis of demographic processes of a species (*Freeland et al., 2011*). Specifically, phylogeography and population genetics of a species help us to understand about population structure, gene flow, connectivity among populations, demographic history and population viability in turtles (*Guinto et al., 2023; Kumar et al., 2024; Mockford et al., 2006; Jordan et al., 2019; McCluskey et al., 2022; Buchanan et al., 2019*). It provides essential tools for the effective planning, conservation, and management of freshwater turtles by offering insights into their genetic health, population structure, and evolutionary history (*Kumar et al., 2024; McCluskey et al., 2022; Schmidt et al., 2018; Escalona et al., 2009; Pineda-Catalan et al., 2012; Buchanan et al., 2019; Bouchard et al., 2019*). Mitochondrial DNA (mtDNA) sequences are important molecular markers used for studying animal genetic diversity and phylogeography because they have a relatively high mutation rate, lack recombination, are haploid, maternally inherited, and have a relatively conserved overall structure (*Freeland et al., 2011*). Moreover, it has been utilized to assess the phylogeography, population structure, genetic diversity, and evolutionary relationships of many freshwater turtle species (*Kumar et al., 2024; Moreno et al., 2022; Schmidt et al., 2018; Pineda-Catalan et al., 2012*).

Here, the genetic diversity, population structure and demographic history of *M. khoratensis* in northeastern Thailand (Isan) were examined using two *mt*DNA regions, *Cyt b* and *ND4*. We divided the turtle samples into three groups according to the northeastern Thailand river basins: Mun, Chi, and Northeast Mekong, which we refer to as the Mekong basin in this study (*Office of the National Water Resources Thailand, 2021*) and we tested for isolation-by-basin hypothesis in its population structure. Understanding

M. khoratensis genetic diversity and population structure can provide important information for effective freshwater turtle conservation and management plans in Thailand.

Materials & Methods

Animal ethics

The study was permitted (DF. 16/2561, DF.27/2562 and DF.1/2566) by the Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand, and the protocol was approved by the Committee on the Ethics of Animal Experiments (permit numbers: ACUC-KKU-24/61, ACUC-KKU-85/62 and ACUC-KKU-84/66) of the Khon Kaen University. All animal handling was in accordance with accepted wildlife husbandry standards of American Society of Ichthyologists and Herpetologists (*American Society of Ichthyologists and Herpetologists 2004*).

Study area, survey, and sampling

We performed multiple field surveys in 2018-2023 in northeastern Thailand during rainy seasons (June to October) and cool dry seasons (November to February) where *Malayemys* turtles are active (*Dawson et al., 2018, 2020*). In total, we conducted 20 surveys across 20 provinces in northeastern Thailand. We traveled to each province and reached out to local fishermen and local markets. We gathered samples directly from fishermen or residents if they had them available, or they informed us when they captured turtles subsequently. All turtles were captured by hand or fish traps from rice fields, ponds, natural wetlands, or irrigation canals, which are their habitats during the rainy season.

The turtles identified as *M. khoratensis* by morphological characteristics, as described by *Ihlow et al. (2016)*, were photographed, had blood samples collected, and

were released afterward. For blood collection, the skin of each turtle was cleaned by using 70% ethyl alcohol before drawing blood. Approximately 0.5 ml of blood from each turtle was collected by drawing blood from the dorsocervical sinus of each living turtle (less than 0.5% of body weight—the range of turtle body weight in this study is between 150 g and 1.5 kg) by using a 23-gauge needle and a 3-ml syringe. Each blood sample was then stored in EDTA K3 disposable vacuum blood collection tube (Zhejiang Gongdong Medical Technology Co., Ltd.) and the blood collection tubes were then stored on ice until returned to the laboratory. Blood samples were then kept in a freezer at -20°C in the Department of Environmental Science, Faculty of Science, Khon Kaen University, Khon Kaen Province, Thailand.

DNA extraction, fragment amplification, and sequencing

Genomic DNA was extracted from of a total of 49 blood samples of *Malayemys khoratensis* using a GF-1 Blood DNA Extraction Kit (Vivantis Technologies Sdn Bhd, Malaysia) according to the manufacturer's protocol. We measured the DNA concentration and purity (the 260 nm:280 nm ratio) of each sample by using Nanodrop spectrophotometer (Thermo Scientific™). Two *mt*DNA genes (*Cyt b* and *ND4*) were amplified separately. The following primers were used for *Cyt b* region (1200 bp): a forward primer, "CytbG" (5'-AACCATCGTTGTAATCAACTAC-3') and a reverse primer, "mt-f-na" (5'- AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG -3'), and for *ND4* region (892 bp): a forward primer, "L-ND4" (5'- GTAGAAGCCCCAATCGCAG -3') and a reverse primer, "H-Leu" (5'-ATTACTTTTACTTGGATTTGCACCA-3') (*Ihlow et al., 2016*). We prepared PCR Master Mix in a total volume of 50 µl containing 1 µl of DNA sample, 1.5 µl of each primer (5 µM), 25 µl KOD One™ PCR master mix-blue (Toyobo,

Japan) and 21 µl distilled water. The cycling conditions were as follows: 5 minutes the initial denaturation (at 95°C for *Cyt b* and at 94°C for *ND4*) followed by 35 cycles with by for 45 seconds (denaturation temperature at 95°C for *Cyt b* and at 94°C for *ND4*), annealing for 30 seconds (annealing temperature at 56°C for *Cyt b* and at 55°C for *ND4*) and extension at 72°C for 60 seconds, with final extension at 72°C for 8 minutes for *Cyt b* and 10 minutes for *ND4*. The final concentration of DNA templates ranged between 20-200 ng per reaction. We checked the PCR products by using 1% agarose gel electrophoresis and then delivered the PCR products to Macrogen (South Korea) or Bio Basic (Canada) for purification and sequencing by utilizing the same primer pairs as those in PCR. The sequences were manually checked and edited using MEGA11 (*Kumar et al., 2018*). All sequences have been submitted to GenBank, with accession numbers provided in Tables S1 in the Supplement.

Sequence analyses

The sequences of *Cyt b* and *ND4* from the 49 *M. khoratensis* samples from this study and 15 samples from *Ihlow et al. (2016)* obtained from GenBank (for accession numbers of these samples see Supplement Table S1) were concatenated and aligned using MUSCLE (*Edgar, 2004*) in MEGA11 (*Tamura et al., 2021*). The final concatenated sequences consisted of 1,459 bp (753 bp for *Cyt b* and 706 bp of *ND4* plus adjacent tRNAs). The sequences of *Cyt b* and *ND4* were further collapsed into haplotypes separately and further analyzed for number of polymorphic sites, number of haplotypes, haplotype diversity and nucleotide diversity by using DnaSP 6.12 (*Rozas et al., 2017*). The haplotype network of concatenated sequences was constructed by median-joining networks (Bandelt et al., 1999) using the program PopART (Population Analysis with

Reticulate Trees) version 1.7.2 (*Leigh & Bryant, 2015*). We analyzed population structure among three basins by utilizing the analysis of molecular variance (AMOVA) in the program PopART version 1.7.2 (*Leigh & Bryant, 2015*).

The Bayesian Skyline Plots (BSP) based on Bayesian Markov Chain Monte Carlo (MCMC) analyses were created using BEAST 2.7.6 (*Bouckaert et al., 2014,2019*). The jModel test 2.1.7 (*Darriba et al., 2012*) were used for the model selection, and HKY was selected as the best fitted substitution model according to the Akaike information criterion (AIC) and Bayesian information criterion (BIC) for BSP calculations. We assumed a neutral mutation rate of 1.75×10^{-8} per site per generation based on a standard molecular clock for the turtle mitochondrial gene (*Formia et al., 2006; Kumar et al., 2024; Naro-Maciel et al., 2014*) which was consistent to 1.2–2.4% pairwise divergence per million years. The analysis was run for 2×10^7 steps with sampling every 10^4 steps using the piecewise-linear Bayesian skyline model and a random starting tree. Results from five replicates were combined using LogCombiner v1.7.5 (*Drummond et al., 2012*). Tracer 1.7.2 was employed to check for chain convergence and to reconstruct Bayesian Skyline Plot (*Rambaut et al., 2018*).

Results

Distribution and genetic diversity of *M. khoratensis*

In our surveys we found 49 *M. khoratensis* in 15 provinces in Thailand (Amnat Charoen, Bueng Kan, Chaiyaphum, Kalasin, Khon Kaen, Mukdahan, Nakhon Phanom, Nakhon Ratchasima, Nong Bua Lam Phu, Nong Khai, Roi Et, Sisaket, Ubon Ratchathani, Udon Thani and Yasothon). We also included the sequences from the previous study by

183 *Ihlow et al. (2016)*, which reported 15 *M. khoratensis* found in three provinces: Nakhon
184 Ratchasima, Nong Bua Lam Phu and Udon Thani. Thus, our turtle sequence samples
185 cover all three basins in Thailand where *M. khoratensis* occurs, 14 turtle samples from
186 Mun basin (Amnat Charoen (partial), Nakhon Ratchasima, Sisaket and Ubon Ratchathani
187 Province), 34 turtle samples from Chi basin (Chaiyaphum, Kalasin, Khon Kaen, Roi Et
188 and Yasothon Province) and 16 samples from northeast Mekong basin (Bueng Kan,
189 Mukdahan, Nakhon Phanom, Nong Bua Lam Phu, Nong Khai, and Udon Thani Province)
190 (Fig. 1 and Table 1).

191 In total, sequences with a length of 1,459 bp of *Cyt b* and *ND4* plus adjacent tRNAs
192 from 64 *M. khoratensis* individuals were analyzed, and 13 unique haplotypes were
193 identified. We observed 26 polymorphic sites accounting for 1.78% of the total sites
194 (Table 1 and Supplement Table S1). The total haplotype diversity of *M. khoratensis* was
195 0.819. There were 9 singleton variable sites and 17 parsimony informative sites. In
196 comparison among basins, Mekong basin had highest haplotype number (8 haplotypes),
197 haplotype diversity (0.858) and nucleotide diversity (0.0047) followed by Mun basin (6
198 haplotypes, haplotype diversity = 0.791, nucleotide diversity = 0.0028) and Chi basin (4
199 haplotypes, haplotype diversity = 0.693, nucleotide diversity = 0.0023) even Chi basin
200 had highest number of samples (34 turtle samples), followed by Mekong (16 samples)
201 and Mun basin (14 samples). AMOVA result did not indicate any population structure
202 among basins (−2.02% of variation, variance components = −0.31, p=0.85). The
203 remaining 102.02% of the genetic variation was found within populations (variance
204 components = 15.66) (Table 2).

The median-joining (MJ) network of 13 haplotypes showed no major branching events among *M. khoratensis*. In addition, the haplotype network showed no population structure among basins (Fig. 2) which reflected the results of AMOVA mentioned above. Four haplotypes were shared haplotypes occurred in more than one individual. Haplotype 1 and 4 were the most common haplotypes (18 individuals each, accounting for 56.3% of turtle samples). Haplotype 1 was found in all basins while haplotype 4 was found only in Chi and Mun basin. Haplotype 3 (6 individuals) and 5 (9 individuals) also occurred in Chi and Mun basin whereas haplotype 8 (4 individuals) and 6 (2 individuals) were found only in Mekong basin. Seven haplotypes occurred only in single individuals and were observed only in Mekong (5 haplotypes: haplotype 2, 6, 7, 9 and 11) and Mun basin (2 haplotypes: haplotype 10 and 12).

The Bayesian Skyline Plot estimated the population size and demographic trend of *M. khoratensis*, indicating a stable effective female population size of 130,000 individuals since about 100,000 years before present (the late Pleistocene), followed by a short slight demographic expansion between AD 0 and AD 1000, and a subsequent population decline from AD 1000 to the present (Fig. 3). The current estimated effective female population size is also about 130,000 individuals.

Discussion

In this study, we utilized mitochondrial DNA from *M. khoratensis* turtles from three basins in northeastern Thailand to investigate their population structure, estimate their population size, and assess their demographic trend. The turtle samples should cover the

entire distribution range of this species in Thailand (*Chaiananporn et al. in press*). Our findings provide valuable genetic insights into *M. khoratensis*, which are important for the future conservation of this species.

The total haplotype diversity of *M. khoratensis* mitochondrial DNA in this study was relatively high (0.819), while the total nucleotide diversity was relatively low (0.0031). This indicates that the differences among haplotypes are only slight. This pattern has been observed in other turtle species and might be explained by the low mitochondrial mutation rate in turtles (*Kumar et al., 2024*). Additionally, it could indicate a rapid population expansion from a small effective population size (*Avise, 2000*).

The haplotype network and AMOVA analysis indicated no significant population structure among the three river basins. However, the haplotype network revealed only one common haplotype (haplotype 1) present in all three basins, while the other haplotypes were found either exclusively in a single basin or shared between the Mun and Chi basins. Notably, 7 out of the 8 haplotypes found in the Mekong basin were unique, in contrast to the Mun basin, which had only 2 unique haplotypes out of 6, and the Chi basin, which had no unique haplotypes. This pattern suggests that the turtles in the Mekong basin are more genetically distinct from those in the Mun and Chi basins. The shared haplotypes between the Chi and Mun basins is possible to be explained that turtles can and do move between these areas because the Chi and Mun basins are geographically close and connected by floodplains while the Mekong basin is separated from the Mun and Chi basins by the mountain range, *i.e.* the Phetchabun Mountains and the Phu Phan Mountains. However, the role of human-mediated transport of turtles was not excluded because *Malayemys* turtles are exploited as food and trade by local Thai

people (*Dawson et al., 2018,2020*). The human-mediated movement may blur the genetic boundaries between populations and complicate efforts to understand natural population structure and dynamics (*González-Porter et al., 2011*).

Claude et al. (2011) reported fossil evidence of *Malayemys* sp. from the Mun River in Nakhon Ratchasima Province. Their findings indicate that the *Malayemys* turtle existed as early as the Middle Pleistocene. The Bayesian Skyline Plot estimated the demographic trend of *M. khoratensis* showing that the effective female population size remained stable since the late Pleistocene (~100,000 years before present) and changed only slightly from AD 0 to the present. This change in the turtle population size might be attributed to human activities in northeastern Thailand. The northeastern Thailand has been human settlements and agricultural sites since at least 1500 BC, as evidenced by several archaeological sites in this region, e.g., Ban Chiang in Udon Thani Province (Mekong basin) (*Higham et al., 2015*), Non Nok Tha in Khon Kaen Province (Chi basin) (*Higham et al., 2014*) and Ban Non Wat archaeological site in Nakhon Ratchasima Province (Mun basin) (*Higham 2011; Peters et al., 2022*). Settlements in Ban Chiang and Ban Non Wat continued until around AD 700 (*Higham et al., 2014,2015; Wohlfarth et al., 2016*). Habitat modification from forest to agricultural areas, such as rice fields and ponds, in this region at that time might have created suitable habitats for *M. khoratensis*, leading to population expansion of this turtle species. However, *M. khoratensis* might have been one of the species exploited by people, as turtle remains were found in several archaeological sites in Southeast Asia (*Bochaton et al., 2023; Higham & Kijngam, 2023; Conrad, 2015*). In the area of present-day northeastern Thailand, the Angkorian Khmer state (AD 802-1431) extended its influence to this region (*Higham, 2014*) which probably enhanced human

population size and intensified agriculture (*Wohlfarth et al., 2012; Kutanan et al., 2014*). *M. khoratensis* may have been increasingly exploited as a food source by local people, leading to a decline in its population. Even today, *M. khoratensis* and its congeners continue to be consumed and exploited by local people in Mainland Southeast Asia (*Platt et al., 2022; Dawson et al., 2018,2020*).

Conclusions

M. khoratensis is currently categorized as Least Concern (LC), and its population trend is unknown according to the IUCN Red List (*Cota, 2018*). In this study, we utilized genetic data to estimate its effective population size, and assess its demographic trend. Our results showed no isolation by river basins in its population structure, and its population trend might currently be declining, similar to its congener *Malayemys subtrijuga* (*Horne et al., 2018*). This decline is likely due to shared threats faced by all three species of *Malayemys*, including exploitation for food consumption, habitat loss, road-kills, and capture and trade for merit releases in Buddhist ceremonies. In addition, this species has also been reported to be under harvest pressure in Lao PDR (*Platt et al., 2022*). As a highly endemic species in Thailand and Lao PDR, we thus suggest that *M. khoratensis* should be included under Thailand's Wildlife Preservation and Protection Act, similar to *M. subtrijuga* and *M. macrocephala*, and its conversation status should be re-evaluated to ensure the long-term survival of this species.

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Figure 1

Map of basins in northeastern Thailand

This figure illustrates three distinct river basins in northeastern Thailand which are the sampling sites for *M. khoratensis*: 1) Mun basin, 2) Chi basin and 3) northeast Mekong basin. The map highlights the province boundaries (dark lines) and major hydrological features of each basin (blue lines). Different colors and labels are used to differentiate between the three basins. An inset map of Thailand is included in the upper corner to show the relative position of the river basins within the country (Office of the National Water Resources Thailand 2021) .

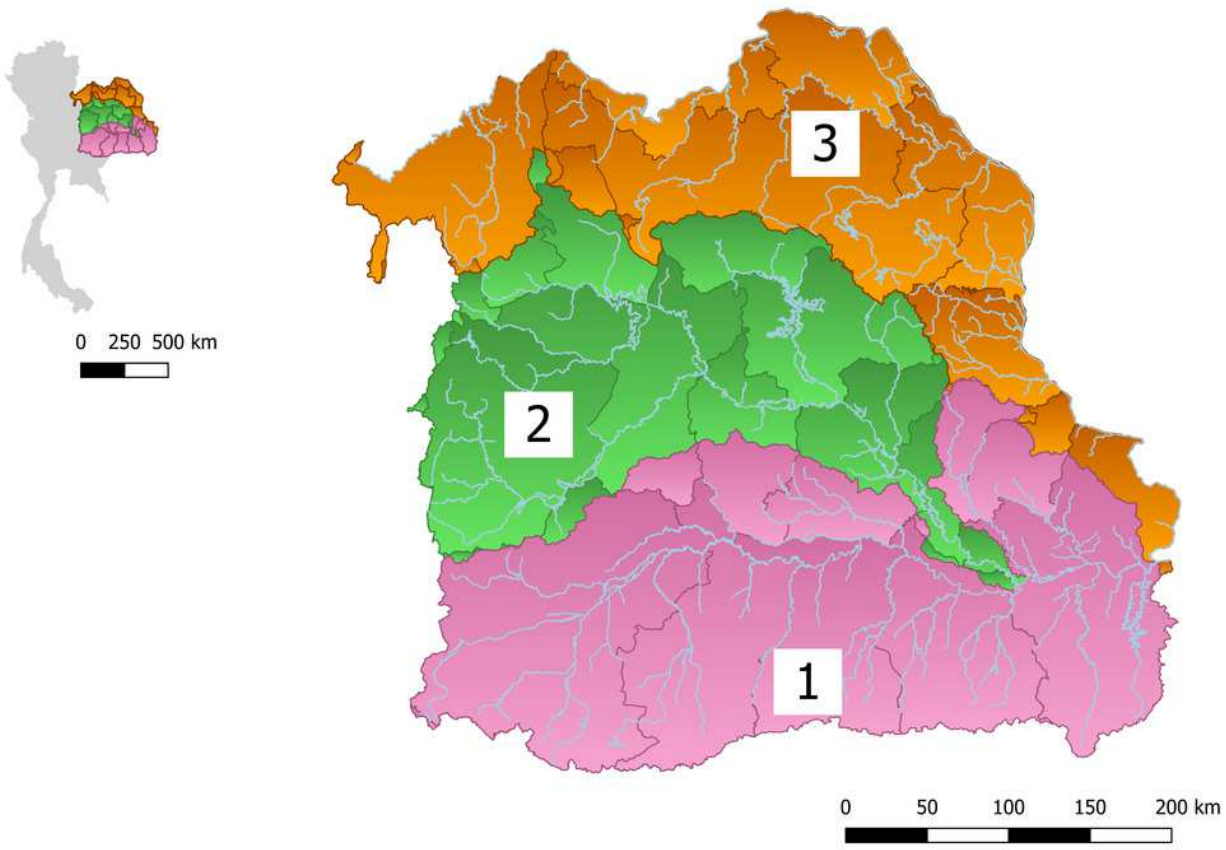

Figure 2

Median-joining haplotype network of *M. khoratensis* mitochondrial DNA

The size of circles presents the relative frequency of each haplotype while short tick lines are the number of mutations between haplotypes. The colors indicate the populations of *M. khoratensis*: Mun basin (purple), Chi basin (red) and northeast Mekong basin (green).

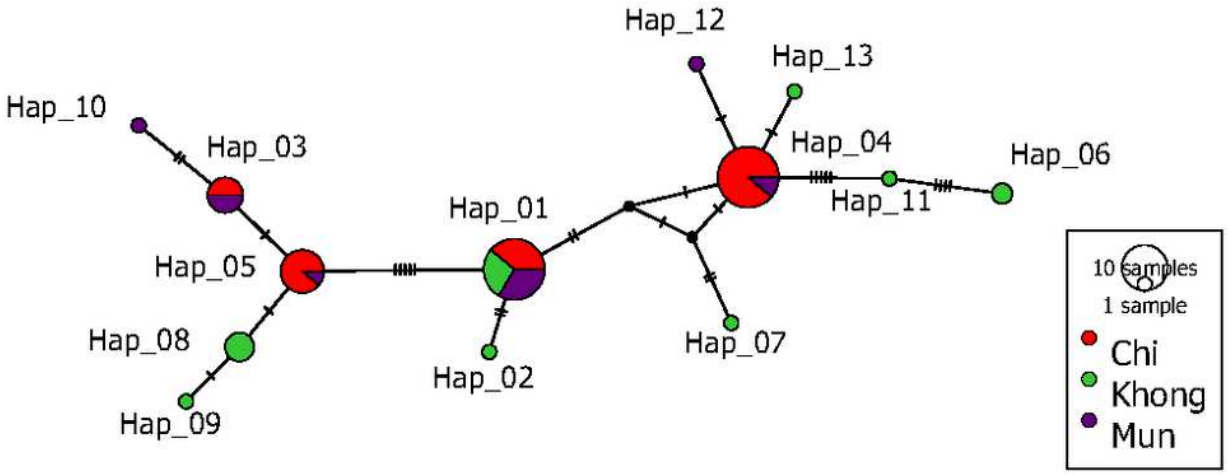

Figure 3

Bayesian skyline plots for the historical demographic trend of *M. khoratensis*

X-axis and Y-axis indicate time before present and effective population size, respectively. The thick solid blue line represents the mean estimate of the effective population size while light blue lines present the 95% highest posterior density intervals.

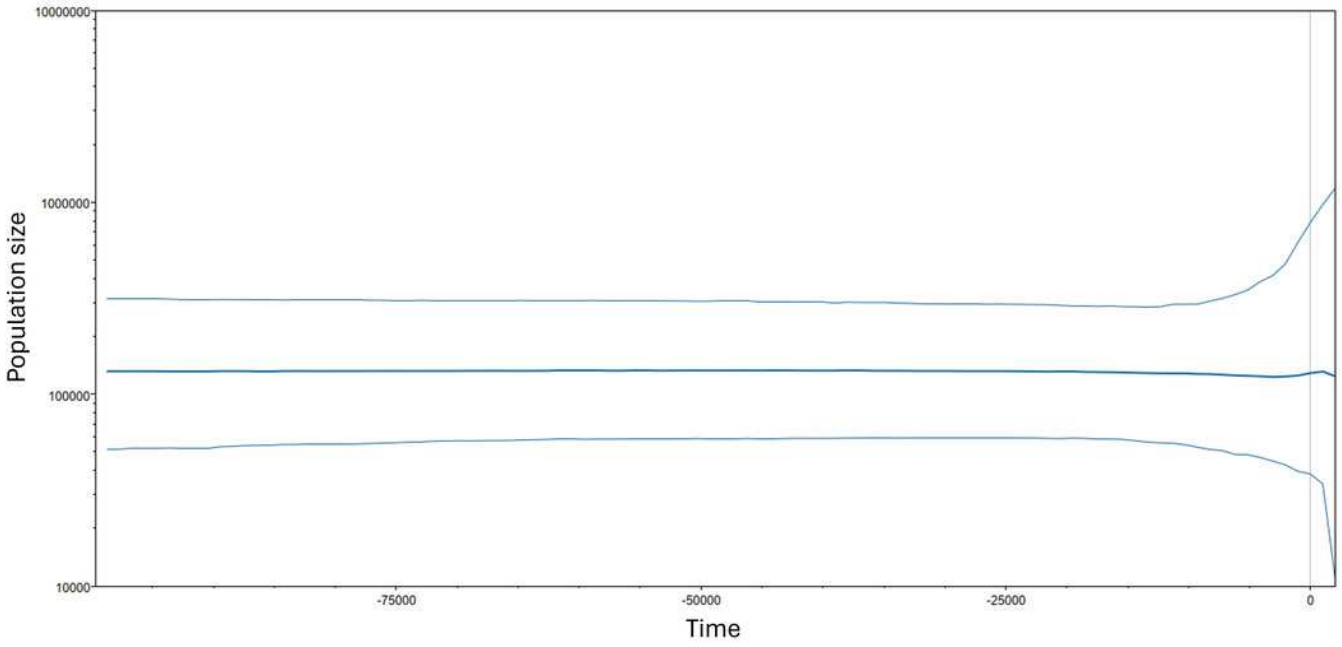

Table 1(on next page)

Sample size (*n*), number of haplotypes (*H*), number of polymorphic sites (*PS*), haplotype diversity (*Hd*) ± SD and nucleotide diversity (<!--[if !msEquation]--> <!--[if !vml]--> <!--[endif]--><!--[endif]-->) ± SD of each group of

Table 1 Sample size (*n*), number of haplotypes (*H*), number of polymorphic sites (*PS*), haplotype diversity (*Hd*) ± SD and nucleotide diversity (π) ± SD of each group of *M. khoratensis* in three river basins.

Group	*n*	*H*	*PS*	*Hd*	π
Chi	34	4	8	0.693 ± 0.051	0.0023 ± 0.0002
Mekong	16	8	22	0.858 ± 0.063	0.0047 ± 0.0007
Mun	14	6	11	0.791 ± 0.09	0.0028 ± 0.0004
All	64	13	26	0.819 ± 0.027	0.0031 ± 0.0003

Table 2(on next page)

Analysis of Molecular Variance (AMOVA) of *M. khoratensis* in three river basins.

Table 2 Analysis of Molecular Variance (AMOVA) of *M. khoratensis* in three river basins.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P- value	Fixation Indices
Among populations	2	11.46	-0.31	-2.02	0.85	-0.02
Within populations	61	951.21	15.66	102.02		
Total	63	966.67	15.35			