Population Genetic Structure of *Tylototriton panhai* Nishikawa, Khonsue, Pomchote and Matsui, 2013 (Urodela: Salamandridae) from Phu Hin Rong Kla National Park, Thailand

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Abstract

Genetic diversity is one of the criteria commonly used for conservation status assessment. Panhai's crocodile newts, *Tylototriton panhai*, are distributed in Phu Hin Rong Kla National Park (NP), where two major breeding pond sites have been reported: Head Quarter and Thap Boek. Phu Hin Rong Kla NP is a popular tourist destination and is easily accessible from main roads. In this study, genetic diversity and population genetic structure of the *T. panhai* population at Phu Hin Rong Kla NP were examined based on mitochondrial NADH dehydrogenase 2 (ND2) sequences ($n = 28$, 1,035 bp) extracted from tail tissue samples obtained from the Head Quarter ($n = 15$) and Thap Boek areas ($n = 13$). The Head Quarter population revealed relatively higher genetic diversity indices in terms of number of haplotype, haplotype diversity, and nucleotide diversity ($N = 6$, $h = 0.7714$, and $\pi = 0.001785$) compared with the Thap Boek population ($N = 3$, $h = 0.6538$, and $\pi = 0.000743$). Although the results of pairwise genetic distance (p-distance) ranged from 0-0.58%, F_{ST} values showed a significant genetic differentiation ($F_{ST} = 0.23472$, $P < 0.001$) between the Head Quarter and Thap Boek populations. Moreover, the median-joining haplotype network showed eight distinct haplotypes, including seven unique and one shared haplotype. The results of this study suggested that gene flows between breeding ponds, which were 1.4 km apart, were restricted and both populations were valuable as they contributed to the overall genetic diversity and conservation of *T. panhai* at Phu Hin Rong Kla NP.

Keywords: *Genetic diversity, Panhai's crocodile newts, Thailand*

1. Introduction

Genetic diversity is the variation of alleles and genotypes among individuals in a population (Frankham, Ballou, & Briscoe, 2004). Genetic diversity contributes to the ability of populations to adapt to environmental changes (Allentoft & O'Brien, 2010) because genetic diversity governs morphological, physiological, and behavioral variations (Frankham et al., 2004). The loss of genetic diversity may reduce the fitness of populations, followed by population declines, which may ultimately lead to species extinction (Ellegren & Galtier, 2016). Therefore, genetic diversity is one of the major components commonly used for population assessment and conservation of biodiversity.

Habitat loss has long been considered a major factor in population decline and extinction (Arntzen et al., 2017), often through reduced genetic diversity (Ellegren & Galtier, 2016). Amphibians are of top concern since they inhabit both terrestrial and aquatic environments (Bee and Griffiths, 2005). Moreover, amphibians are known for low dispersal ability (Smith & Green, 2005), so they are unlikely to move into new habitats. Amphibians play important parts in ecosystems. Their diets consist mainly of insects, thus, playing important roles in controlling insect populations—particularly disease vectors and agricultural pests. Unfortunately, amphibian populations are decreasing worldwide (Stuart et al., 2004) while there are a few studies concerning genetic diversity and conservation.

Newts of the genus *Tylototriton* are the only group of the urodelan amphibians recorded in Thailand. At present, five nominal species have been reported in Thailand: *T. uyenoi*, *T. panhai*, *T. anguliceps*, *T. verrucosus*, and *T. phukhaensis* (Nishikawa et al., 2013; Le et al., 2015; Pomchote et al., 2020a,b). They are allopatrically distributed at elevations higher than 1,000 meters above mean sea level (AMSL) throughout the northern, northeastern, and western mountain ranges of Thailand (Nishikawa et al., 2013; Le et al., 2015;

Hernandez et al., 2019; Pomchote et al., 2020a,b; Hernandez & Pomchote, 2020a,b,c). *T. verrucosus* and *T. anguliceps* are categorized as least-concerned (LC) species—that is, the species practically has no conservative concern, while the other Thai *Tylototriton* are categorized as data deficiency (DD; IUCN, 2020). Besides, *T. verrucosus* is the only newt species in Thailand that is protected by the Wild Animal Protection Act B.E. 2562 (2019) (Department of National Parks, Wildlife and Plant Conservation, 2021). Although most newts are distributed in protected areas, such as national parks (NP) and wildlife sanctuaries (WS) in Thailand (Nishikawa et al., 2013; Le et al., 2015; Pomchote et al., 2020a,b), many anthropogenic activities such as habitat alteration and modification, deforestation, pesticide usage, and poaching sometimes occur (Hernandez & Pomchote, 2020a,b) and may affect the populations of Thai newts, placing them at risks of extinction.

Panhai's crocodile newts, *T. panhai*, are distributed throughout Loei-Phetchabun ranges: Phu Hin Rong Kla NP, Phu Suan Sai NP, Phu Soi Dao NP, and Phu Luang WS (Nishikawa et al., 2013; Hernandez et al., 2019; Hernandez & Pomchote, 2020a). Phu Hin Rong Kla NP, Phitsanulok Province, is a well-known tourist destination, where large numbers of tourists visit the park each year (Department of National Parks, Wildlife and Plant Conservation, 2019). This park is reported to be the southernmost distribution range of this species and newts have been reported at two major breeding ponds: Head Quarter and Thap Boek (Pomchote et al., 2008; Hernandez et al., 2019; Hernandez and Pomchote, 2020a). Both ponds are easily accessible from the main roads by the tourists. As previously mentioned, amphibians are generally known as poor dispersers (Smith & Green, 2005) and most, including several *Tylototriton* species (Bernardes et al., 2013; Phimmachak et al., 2015; Dowwiangkan et al., 2018), are philopatric to their breeding sites (Beebee, 2005). Thai *Tylototriton* is considered a sensitive species based on its patchy distribution and isolated populations (Wongratana, 1984). For these reasons, distinct population genetic structures between Head Quarter and Thap Boek may exist. Thus, this study aims to assess genetic variation and population genetic structure of *T. panhai* at Phu Hin Rong Kla NP for future management and conservation purposes.

2. Objectives

To study the population's genetic structure and genetic diversity of *T. panhai* populations from Phu Hin Rong Kla NP, Phitsanulok Province, Thailand

3. Materials and Methods

3.1 Sampling localities

Newts were captured at Head Quarter (15 males; Figure 1A) and Thap Boek (12 males and 1 female; Figure 1B) sites at Phu Hin Rong Kla NP (Figure 2). The distance between Head Quarter and Thap Boek is approximately 1.4 km. Field surveys were conducted in May 2019, which was the breeding season of *Tylototriton* spp. (Seglie et al., 2003; Pomchote et al., 2008; Phimmachak et al., 2015). The two breeding ponds were selected based on previous reports (Pomchote et al., 2008; Hernandez et al., 2019; Hernandez and Pomchote, 2020a). The tail tips were removed. Then, the wound was cleaned with an antiseptic liquid. The obtained tissue samples were preserved in 95% ethanol for further molecular analyses (Pomchote et al. 2020a).

Figure 1 Male newt from Head Quarter (A) and Thap Boek sites (B) at Phu Hin Rong Kla NP, Thailand

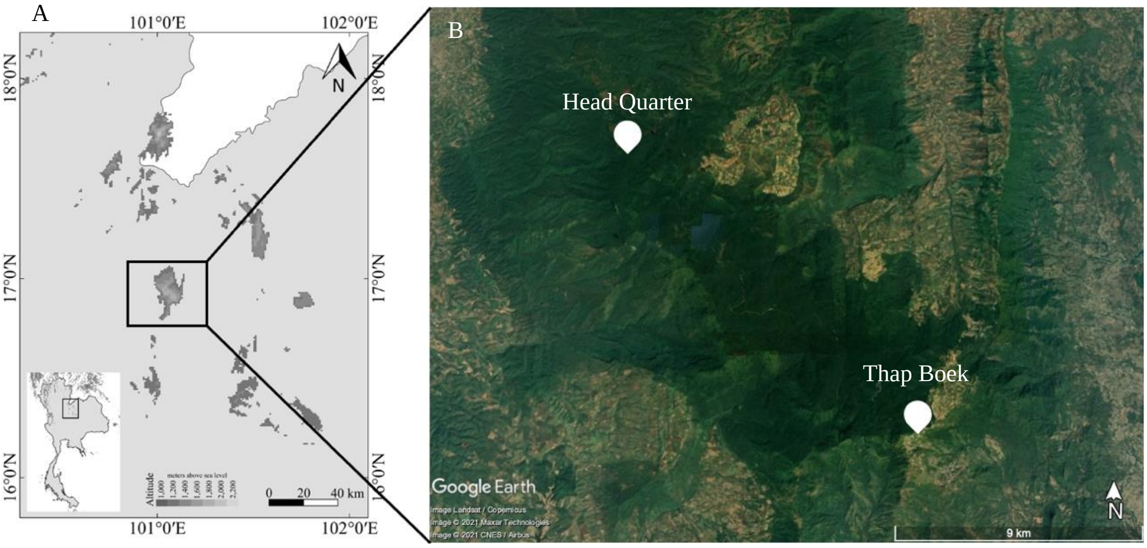

Figure 2 Maps of Phu Hin Rong Kla NP (A) and Head Quarter and Thap Boek sites (B; Google Earth, 2021)

3.2 Molecular analyses

Total DNA was extracted using PureDireX^{TM} genomic isolation kit (Bio-Helix, Taiwan). The mitochondrial NADH dehydrogenase 2 gene (ND2) was amplified by polymerase chain reaction (PCR), using primer SL-1 (5'-ATAGAGGTTCAAACCCTCTC-3') and SL-2 (5'-TTAAAGTGTCTGGGTTGC ATTCA G-3') (Wang et al., 2018). PCR reaction mixture consisted of 15 µl of OnePCR^{TM} Ultra (GeneDirex, Inc., Taiwan), which is a pre-mixed solution of 1.5 µl of 10 µM of each primer, 9 µl of UltraPure^{TM} DNase/RNase-Free distilled water (Invitrogen Inc., USA), and 3 µl of DNA template (Khantasup, 2021). PCR conditions included an initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min 30 sec (Wang et al., 2018). Then, PCR products were checked by the Gel electrophoresis technique to confirm sizes and concentrations (Khantasup, 2021). PCR products were purified and sequenced by Bioneer Inc in South Korea.

3.3 Data analyses

All sequences were manually checked and trimmed. Alignments were conducted by Muscle algorithms in MEGA version 7.0 (Kumar, Stecher, & Tamura, 2016). Genetic diversity indices, including the number of haplotypes (N), haplotype diversity (h), nucleotide diversity (π), genetic differentiation (F_{ST}), and pairwise genetic distance (p-distance) were calculated by Arlequin version 3.5 (Excoffier & Lischer, 2010). The median-joining haplotype network was constructed by Population Analysis with Reticulate Trees (PopART; Bandelt et al., 1999) to examine the relationship of *T. panhai* populations in Phu Hin Rong Kla NP.

4. Results and Discussion

ND2 gene sequences revealed a total of 6 haplotypes ($N = 6$) and 3 haplotypes ($N = 3$) at Head Quarter (n = 15, 1,035 bp) and Thap Boek (n = 13, 1,035 bp), respectively. Genetic diversity indices of the Head Quarter population ($N = 6$, $h = 0.7714$, and $\pi = 0.001785$) were relatively higher than those of the Thap Boek population ($N = 3$, $h = 0.6538$, and $\pi = 0.000743$) (Table 1). Since Thap Boek is a tourist destination and easily accessible by cars, human activities may cause negative impacts on the habitats, including ecosystem and resource degradation. The surrounding areas may receive irreparable damage from tourism (Belsoy et al., 2012), and subsequent population decline (Arntzen et al., 2017). The resulted decrease in the effective population size could result in decreased genetic diversity in a population (Frankham et al., 2004). On the other hand, the effective population size may be affected by breeding pond size as previously reported in the California tiger salamander, *Ambystoma Californians*, which showed a positive correlation between effective population size and breeding pond area (Wang et al., 2011). In this study, while the pond at Thap Boek was a small permanent pond, the breeding pond at Head Quarter was part of a long and slow-moving stream and was comparatively larger in size, comparing the results of this study to previous reports (Hernandez et al., 2019; Hernandez and Pomchote, 2020a). The pond at Head Quarter may serve as a more suitable habitat, supporting a larger population size. Thus, Thap Boek's population size was possibly smaller compared with that of Head Quarter and resulted in the relatively lower observed genetic diversity. Nonetheless, unequal sample sizes in this study may have affected the genetic diversity indices as well (Frankham et al., 2004).

The numbers of haplotypes, haplotype diversity, and nucleotide diversity obtained from the present study were rather similar to those previously reported in the tiger salamander, *A. tigrinum*, ($N = 9$, $h = 0.84$, and $\pi = 0.009$) (Bos et al., 2008), which was also categorized as LC species. However, *A. tigrinum* comprised a large number of subpopulations and locations and larger population size and had no indications of anthropogenic population decline (IUCN, 2020). Thus, those factors must be considered to determine the conservation status of the *T. panhai*. The results of this study revealed a relatively high haplotype diversity while the nucleotide diversity was relatively low. The results suggested that the small populations were undergoing rapid population growth (Grant and Bowen, 1998), the period of which was sufficient for genetic differentiation but not for the accumulation of nucleotide differences (Lowe et al., 2009).

The results of p-distances (0-0.58%) and F_{ST} values showed a significant genetic differentiation ($P < 0.001$) between the Head Quarter and Thap Boek populations (Table 1). Likewise, the median-joining haplotype network revealed eight haplotypes, including seven unique haplotypes (PH-1-PH-5, PH-7-PH-8) and only one shared haplotype (PH-6) (Figure 3). Some newts and salamanders, including some *Tylototriton* sp., are philopatric to their breeding ponds (Beebee, 2005; Bernardes et al., 2013; Phimmachak et al., 2015; Dowwiangkan et al., 2018). This behavior may restrict gene flows between the populations (Mitton, 2013). For this reason, *T. panhai* may also be philopatric to their breeding ponds and restricted gene flows may have resulted in the observed population genetic structure between Head Quarter and Thap Boek. Furthermore, these two populations may have shared the same gene pool in the past as was evident in the single shared haplotype.

Table 1 Genetic diversity indices of *T. panhai* populations at Phu Hin Rong Kla NP based on ND2 gene.

	Head Quarter	**Thap Boek**
Coordinate	16°59'N 100°59'E	16°53'N 101°05'E
Sample size (n)	15	13
Number of haplotype (N)	6	3
Haplotype diversity ($h \pm$ S.D.)	0.7714 ± 0.0979	0.6538 ± 0.0985
Nucleotide diversity ($\pi \pm$ S.D.)	0.001785 ± 0.001215	0.000743 ± 0.000652
Genetic differentiation (F_{ST})	0.23472 ($P < 0.001$)	
Pairwise genetic distance (p-distance)	0-0.58%	

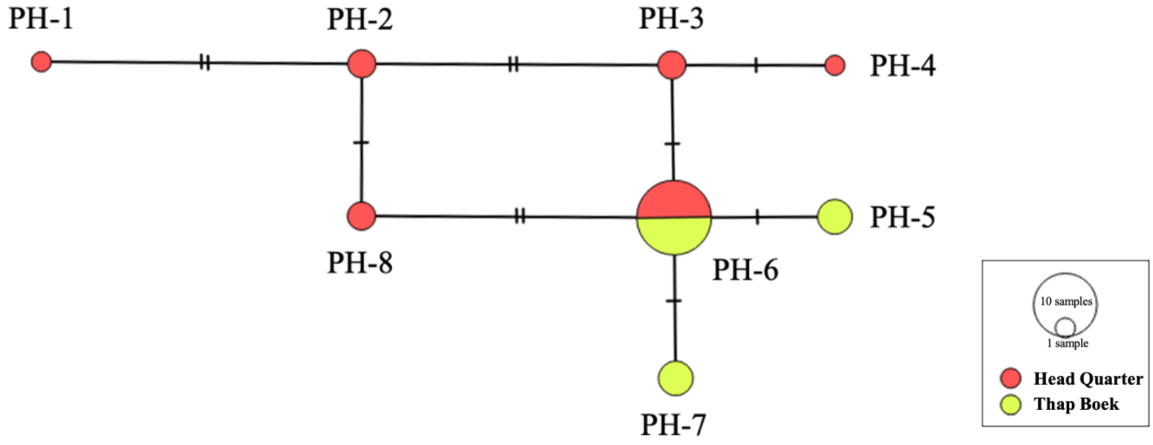

Figure 3 Median-joining haplotype network of *T. panhai* populations in Phu Hin Rong Kla NP

5. Conclusion

T. panhai population distributed at Head Quarter revealed a relatively higher genetic variation compared with the Thap Boek population. Therefore, anthropogenic effects that may have resulted in population decline and loss of genetic variations should be evaluated. Although the genetic diversity indices of *T. panhai* at Phu Hin Rong Kla NP were similar to other salamander species categorized as LC species, there is still a lack of long-term population monitoring data in *T. panhai*, which is necessary for the determination of conservation status. Moreover, a significant genetic differentiation between Head Quarter and Thap Boek populations was observed, which suggested that each population should be considered genetically unique and both populations are important for the maintenance of the overall genetic diversity. It is particularly essential to ensure physiological adaptations to environmental changes, survival of species, and preservation of biodiversity at Phu Hin Rong Kla NP.

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