

**Exploring the patterns and mechanisms determining
geographic variation of tropical Asian warty newts**

TRAN Van Dung

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To my late wife (Thuy Nguyen),

Who always believed in me

CONTENTS

	Page
Contents	
Chapter 1	General introduction 1
Chapter 2	Demographic and ecological niche dynamics of the Vietnam warty newt, <i>Paramesotriton deloustali</i> : Historical climate influences 15
Chapter 3	Ecological niche variation drives morphological differentiation in tropical stream-dwelling newts from Indochina 61
Chapter 4	Population genetics provides insights into the impact of climate change on the genetic structure and distribution of Asian warty newts 106
Chapter 5	Clay model reveals the difference in day and night predation rates on Vietnam warty newt (Caudata: Salamandridae) 139
Chapter 6	Variation in ventral coloration pattern and aposematism in tropical warty newts 157
Chapter 7	General discussion 177
Summary	189
Acknowledgements	191

CHAPTER 1

General Introduction



One of the central questions in Ecology, Biogeography, and Conservation Biology is “What factors determine the geographic variation of organisms?”. Geographic variation refers to differences in the typical characteristics of more than one existing populations (Endler, 1977; Gould & Johnston, 1972; Šunje, 2022). This may reflect genetic distinction, phenotypic adaptability, ecological niche differentiation or a combination thereof (Peñalver-Alcázar, 2019). Studying the geographic variation can provide insight to the process of evolution, speciation, and adaptation of species to environmental changes (Olvido & Mousseau, 2012). The information is also important for developing sustainable management strategies for common species and effective conservation strategies for rare species. Two components of studying geographic variation are patterns (distribution) and processes (mechanisms) at different temporal and spatial scales (Huston, 1999; Mayr, 1956; Peñalver-Alcázar, 2019). Generally, the geographical distribution of organisms can be directly observed, while the processes are often hidden and challenging to reveal due to possibly multiple driving mechanisms (Frank, 2009). Understanding the patterns and processes of geographic variation is important to advance knowledge in biodiversity and natural systems through ecological and evolutionary theories (Ashton, 2001; Pickett et al., 2010).

During the great exploration of the 19th century by biogeographers, ecologists and naturalists, they documented many patterns of geographic variation and how they are related to the environment. For example, Bergmann (1847) stated an increase in body size of mammals with latitude/altitude explored (Bergmann’s rule). Joel Asaph Allen developed an biogeographical rule that demonstrated that species exhibit longer appendages in warmer environments (Allen, 1877). Another rule in evolutionary biology is Foster’s rule, or the island rule, (Foster, 1964), which states that small organisms are larger on islands compared to those on mainland (gigantism), while large species are smaller on islands than on the mainland (dwarfism). Rapoport’s rule indicates that species increase in distribution size with latitude (Stevens, 1989). Steven’s rules, an extension of Rapoport’s rule, stated that species will increase their elevational range with high elevation (Stevens, 1992). Along with that, scientists have focused extensively on studying the rules of geographic variation. Peñalver-Alcázar (2019) demonstrated that nearly 1500 papers related to these rules were published in the Scopus database from 1948 to 2018. Among these, Bergmann’s rule emerged as the hottest topic, with 644 articles (Peñalver Alcázar, 2019).

Geographic variation may arise from genetic and/or non-genetic factors in heterogeneous environmental conditions (Blanquart et al., 2013; Kaltz & Shykoff, 1998; Kirkpatrick & Barton, 1997). For genetic factor, population genetic analytical approaches provide a major advantage to understanding the local adaptation of species, and geographical variable selection (Tiffin & Ross-Ibarra, 2014). Regarding non-genetic factors, phenotypic characteristics may change in response to environmental variability. The response of organisms via phenotypic diversification, including aposematic variation, may drive the

local distribution of species (Ojala et al., 2007). For example, terrestrial plethodontid salamanders have longer limbs in comparison to their aquatic counterparts (Edgington & Taylor, 2019). Additionally, the interaction between the genotype and the environment (G x E) may also cause geographic variation for species. This occurs when populations comprise individuals exhibiting different reaction norms (Pigliucci, 2005).

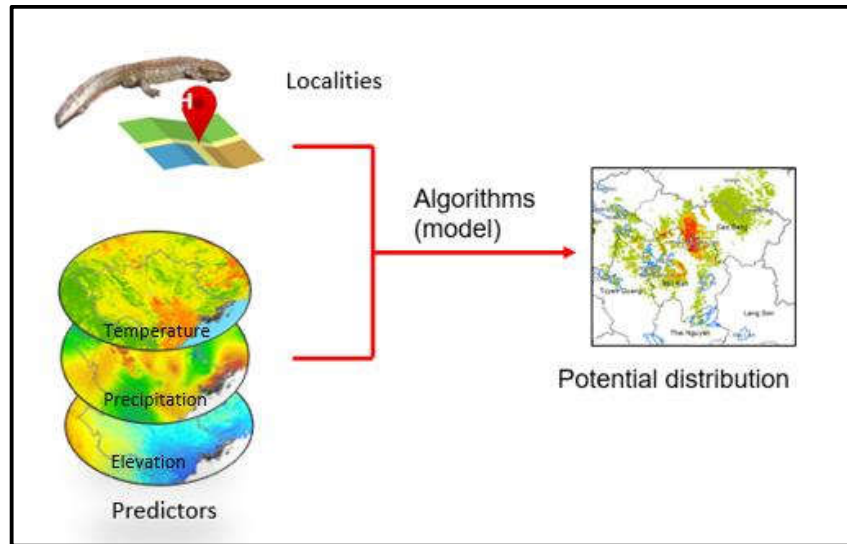


Figure 1-1. Ecological Niche Modeling

In order to study patterns and processes of geographic variation, ecological niche models (ENMs) can be employed to answer questions regarding where organisms are distributed, and which factors are driving their distributions. ENMs – also known as species distribution models (SDMs), habitat suitable models (HSMs), are computational powerful approach to predict the spatial distribution of species based on their known occurrences and environmental variables (Figure 1-1; Guisan & Thuiller, 2005). ENMs employs a variety of algorithms, for example, Maximum Entropy (MaxEnt; Phillips et al., 2006), Random Forest (RF; Breiman, 2001), Generalized Linear Models (GLM; Nelder & Wedderburn, 1972), Artificial Neural Networks (ANN; Lek & Guégan, 1999), or ensemble species distribution modeling (eSDM) by averaging the outputs from multiple species distribution models using various algorithms (Araújo & New, 2007). Recently, the approach has also been widely applied to address many questions in biogeography. Researchers can use ENMs to quantify the environmental niche of species (Broennimann et al., 2012; Warren et al., 2008), support taxonomic classification (Raxworthy et al., 2007; Zhang et al., 2014), increase conservation efforts (van Schingen et al., 2014), assess risk and impact of invasive species (Chapman et al., 2017; Mainali et al., 2015; Srivastava et al., 2019). Especially, many studies have applied ENMs to assess the effects of climate change on distribution of many species' groups, for instance, Amphibians (Abreu-Jardim et al., 2021; Ashrafzadeh et al., 2019; Zhang et al.,

2020), Reptiles (Ngo et al., 2022; Tran et al., 2023b), Birds (Chen et al., 2022; Velásquez-Tibatá et al., 2013), Mammals (Trinh et al., 2022; Vu et al., 2020); plants (Banerjee et al., 2019; Cong et al., 2024; Ovalle-Rivera et al., 2015; Zhang et al., 2014).

Differentiation in morphology of organisms also can be influenced by genetic differentiation (Relethford, 2004; Wright, 1943), local adaptation (Williams, 2018), or the combination of both genetic differentiation and local adaptation (Colombelli-Négrel, 2016). Morphological traits of salamanders and newts are often shaped by a range of environmental and ecological factors (Edgington & Taylor, 2019; Nishikawa et al., 2007; Tominaga et al., 2005). For instance, Edgington and Taylor (2019) demonstrated that limbs of terrestrial plethodontid salamanders are mainly longer than those of aquatic salamanders due to the difference in their living environments. Investigating the underlying causes of morphological variation is crucial for gaining insights into the evolutionary histories of species and understanding their impact on geographic and ecological patterns. To date, no studies have assessed the morphological variation of Asian warty newts at both intra- and interspecies levels.

Urodeles can be an ideal example to explore the geographic variation through time and across space due to their deep evolutionary histories, low dispersal abilities, and high environmental constraints (they need cool climate and high humidity) (Vieites et al., 2009). Many researchers have used salamanders and newts as focal species to reveal geographic variation, such as Iberian newt (*Lissotriton boscai*; Peñalver-Alcázar, 2019) and Alpine salamander (*Salamandra atra prenzensis*; Šunje, 2022). Additionally, several studies have provided information on the variation in salamanders and newts using ENMs and genetic variation (e.g., Rissler & Apodaca, 2007; van Riemsdijk et al., 2017). However, effects of biotic factors were not considered in these models. Other studies showed the relationships between the distribution and aposematic variation in salamanders (Mochida, 2009), but the population genetic divergence and the interaction among factors in ecological niches were ignored. Furthermore, most of the studies have been conducted in temperate zones, while there are very few such studies on tropical urodeles, although more than half of them occur in tropical ecosystems (Wiens et al., 2007). Thus, the main factors affecting the distribution pattern and evolution processes of newts, especially in the tropics, are still poorly understood.

The Asian warty newt, belonging to the genus *Paramesotriton* in the family Salamandridae, is exclusively distributed in China and northern Vietnam (Sparreboom, 2014). The species have rough skin, and a prominent vertebral ridge, and lateral ridge along each side of the back. The parietal ridges on the skull are well-defined (Sparreboom, 2014). At present, 15 species within this genus have been identified (Frost, 2024). According to molecular studies, *Paramesotriton* is identified as a monophyletic group, positioned as the sister taxon to *Cynops* (Pyron & Wiens, 2011; Weisrock et al., 2006) or *Laotriton* (Gu et al.,

2012). The preferred habitat of the species is cool montane streams within closed-canopy evergreen forests (Sparreboom, 2014). In northern Vietnam, two species *Paramesotriton deloustali* and *P. guangxiensis* (Sparreboom, 2014) occur that are southernmost species in the genus and expected to suffer from severe environmental condition, because they favor cool habitat. The Vietnam warty newt (*P. deloustali*) was initially discovered in Tam Dao National Park (Vietnam) and has subsequently been recorded in more than ten regions across northern Vietnam (Nguyen et al., 2009; Sparreboom, 2014). The Guangxi warty newt (*P. guangxiensis*) was initially described by Huang et al. (1983) based on a holotype collected from Guangxi Zhuang Autonomous Region, China. Then, the newt was found in Cao Bang Province, northern Vietnam (Nguyen et al., 2009). These two species could serve as suitable models for this study because they exhibit allopatric distribution in northern Vietnam. Their occurrence ranges are wide but fragmented in high mountains, resembling “sky islands”, especially *P. deloustali*. Additionally, the species display a high level of phenotypic diversification, as shown by considerable red ventral coloration (Figure 1-2).

Newts are well-known to possess aposematic coloration because they have toxin inside their bodies. The aposematic coloration pertains to the noticeable and distinct color patterns or markings displayed by certain animals, often characterized by red, yellow, or white patterns on a black background (Cott, 1940). Understanding the evolutionary origins of aposematic coloration is considered one of the most complex and challenging tasks (Endler & Mappes, 2004; Mappes et al., 2005; Briolat et al., 2019). Species frequently employ aposematism as a strategy to signal their unpalatability and use warning signals to communicate these defenses to their natural predators (Mappes et al., 2005). Newts serve as excellent model species for gaining insights into the respective roles of adaptation and plasticity in shaping the variation of aposematic patterns (Wells, 2007). Asian warty newts often possess a red or orange ventral coloration with a black line network (Figure 1-2), and display aposematic coloration as a defensive behavior (Tran & Nishikawa, 2021; Wells, 2007). However, our understanding of the aposematic coloration of the newts and their relationships with their geographic distribution remains limited.



Figure 1-2. The variation in ventral coloration of Asian warty newts in northern Vietnam

What are the underlying causes of the geographic variation of warty newts in the tropics, and what is the relationship between these species and their environment? In order to address the questions, I explored the patterns and mechanisms underlying the geographic variation of the Asian warty newts, focusing on *P. deloustali*, and *P. guangxiensis*, in northern Vietnam by examining the ecological, genetic, and phenotypic data at both inter- and intraspecific levels. Specifically, I revealed the patterns of geographic variation by examining the morphological, ecological niche and genetics differentiation, then uncovered the relationship among them. I also assessed the influence of genotype and environment on the variation (Figure 1-3). The study aims to provide new, and comprehensive knowledge on the geographic variation of tropical stream-dwelling newts from Indochina. The research will advance our understanding of the biogeography, ecology, and evolution of organisms, as well as provide updated information to safeguard biodiversity in the face of future drastic climate change.

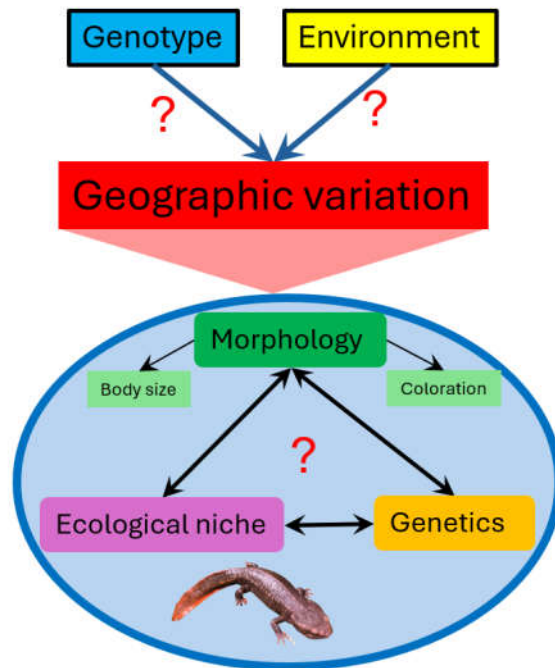


Figure 1-3. The main objectives of the study are to reveal the patterns and processes of geographic variation in Asian warty newts from northern Vietnam

The dissertation includes seven chapters:

Chapter 1: General introduction.

Chapter 2: I integrated mitochondrial DNA (mtDNA) sequence data with environmental data in ENM to examine the historical demographic and distribution patterns of *P. deloustali* at both the species and population levels. Here, I demonstrated how Quaternary climate played an important role in driving the genetic diversification, demographic structure, and paleodistribution of the species.

Chapter 3: I examined morphological and ecological variation within two related *Paramesotriton* species (*P. deloustali* and *P. guangxiensis*) to assess both intra- and interspecific variation and identify the key factors shaping their morphological characteristics. Then, I explored the correlation between morphological differentiation and environmental niche overlap to examine whether local adaptation could account for the observed morphological differences.

Chapter 4: I employed genome-wide nuDNA SNP data to reveal the genetic structure and gene flow among populations of Asian warty newts (*P. deloustali* and *P. guangxiensis*). Then, I incorporated genetic structure into ENM to project changes in suitable habitat for each population under climate change scenarios in 2050 and 2090. Furthermore, I evaluated the dynamics of genetic clusters in relation to climatic variables over time.

Chapter 5: I applied the clay model method to assess predator pressure on the nocturnal Vietnam warty newt (*Paramesotriton deloustali*) in Tam Dao NP, northern Vietnam. Then, I compared the proportions of attacks on newt clay models between the day and night. Furthermore, I also used camera traps to confirm the specific predators of *P. deloustali* in nature.

Chapter 6: I examined the aposematic ventral coloration of adult warty newts across diverse populations in different regions of northern Vietnam, with the goal of revealing interpopulation variation. Additionally, I conducted clay model experiments to test two hypotheses concerning the interaction between predators and the aposematic signals of the warty newts.

Chapter 7: General discussion.

The papers were published from the dissertation:

Chapter 2:

Tran VD*, Vu TT, Fukutani K, & Nishikawa K. (2023). Demographic and ecological niche dynamics of the Vietnam warty newt, *Paramesotriton deloustali*: Historical climate influences. *Plos One*, 18(8), e0290044. DOI: <https://doi.org/10.1371/journal.pone.0290044>

Chapter 3:

Tran VD*, Phan VD, Lau AK, & Nishikawa K. (2024). Ecological niche variation drives morphological differentiation in tropical stream-dwelling newts from Indochina. *Journal of Zoology*. DOI: <http://doi.org/10.1111/jzo.13169>

Chapter 4:

Tran VD*, Suzuki T, Fukuyama I, & Nishikawa K. Population genetics provides insights into the impact of climate change on the genetic structure and distribution of Asian warty newts. (*in preparation*).

Chapter 5:

Tran VD* & Nishikawa K. (2023). Clay model reveals the difference in day and night predation rates on Vietnam warty newt (Caudata: Salamandridae). *Current Herpetology*, 42(2), 124-135. DOI: <https://doi.org/10.5358/hsj.42.124>

Chapter 6:

Tran VD* & Nishikawa K. (2024). Variation in ventral coloration pattern and aposematism in tropical warty newts. *Zoological Science*. 41(3). DOI: <https://doi.org/10.2108/zs230109>

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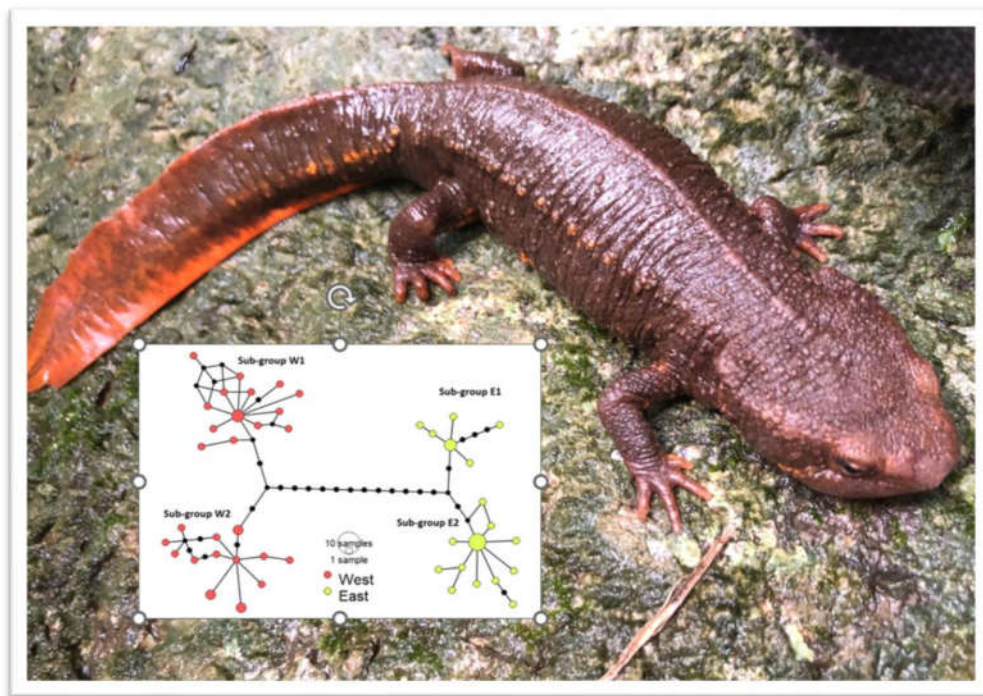
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CHAPTER 2

Demographic and ecological niche dynamics of the Vietnam warty newt, *Paramesotriton deloustali*: historical climate influences



2-1. Introduction

The factors underlying the distribution patterns and divergence of organisms are a central topic in biogeography (Wiens & Donoghue, 2004), and understanding them is crucial to developing sustainable management and conservation strategies (Willi et al., 2022; Woodruff, 2001). The geographic distribution of species is controlled by combination of abiotic and biotic factors over species' evolutionary histories (Soberon & Peterson, 2005). As the distribution of a species is affected by numerous processes with complex patterns across time and space, understanding its driving factors remains a major challenge (Elith & Leathwick, 2009). The recent integration of phylogeography with species distribution modeling (SDM) provides a robust approach to exploring population dynamics and historical distribution processes, revealing the drivers underlying the current ranges of species (Hickerson et al., 2010; Kozak et al., 2008; Pahad et al., 2020; Raxworthy et al., 2007; van Riemsdijk et al., 2017). Phylogeography can be used to illustrate the spatial genetic diversity and structure of species as well as to estimate demographic dynamics (Avise, 2000; Hickerson et al., 2010; Pahad et al., 2020), while SDM provides insights into the relationships of species with environmental space, ecological interactions among species, and the process of speciation (Guisan & Thuiller, 2005; Raxworthy et al., 2007; Rissler & Apodaca, 2007).

Northern Vietnam is located in Southeast Asia, a global biodiversity hotspot (Lohman et al., 2011; Myers et al., 2000). The geology and environment of northern Vietnam are highly complex due to its location at the convergence of the tropical and subtropical zones and biotic interactions among three biogeographic units: Indochina, south China, and coastal Indochina (Sterling et al., 2006). This area is dominated by the Hoang Lien Son Mountain Range (the southeasternmost extension of the Himalaya Range), karst formations, and the Red River (Sterling et al., 2006). The climate is highly seasonal, with considerable variation in temperature and rainfall. In addition, the area is affected by cold and arid air delivered by the northeast monsoons from the Tibetan Plateau (Sterling et al., 2006). During the Pleistocene, cooler and drier climatic events greatly impacted northern Vietnam, despite the absence of glaciers (Bird et al., 2005; Tamburini et al., 2003). The majority of lowland areas were likely covered by wetlands and grasslands rather than tropical rainforests during the last glacial maximum (LGM) (Bird et al., 2005; Wurster et al., 2010). Multiple studies have demonstrated that the genetic divergence, population demographics, and current ranges of a variety of organisms have been significantly influenced by climatic oscillations during the Quaternary period (Byrne, 2008; Goczał et al., 2020; Hampe & Petit, 2005; Hewitt, 2000, 2004, 2011; Turchetto-Zolet et al., 2013). However, species may exhibit diverse responses to ensure their survival through significant climate changes (Hewitt, 2000).

Urodeles are excellent examples for exploring the demographic dynamics and

historical ranges of organisms over time and across space due to their long evolutionary histories, low dispersal abilities, and strong environmental constraints (Vieites et al., 2009). Historical climate significantly shapes the geographic distributions and genetic diversification of salamanders and newts (Luo et al., 2021, 2022; Rissler & Apodaca, 2007; Vaissi, 2021a; van Riemsdijk et al., 2017; Yuan et al., 2022). The Vietnam warty newt (*Paramesotriton deloustali*, Caudata: Salamandridae) provides an ideal model for assessing the effect of Quaternary climate on the demographic and range dynamics of organisms. This species occurs widely in northern Vietnam (IUCN SSC Amphibian Specialist Group, 2017; Nguyen et al., 2009) and southern China (Zhang et al., 2018), with a wide elevational range of 200–1900 m asl (IUCN SSC Amphibian Specialist Group, 2017; Sparreboom, 2014). Currently, *P. deloustali* is classified as “Least Concern” in the International Union for Conservation of Nature (IUCN) Red List (IUCN SSC Amphibian Specialist Group, 2017). Similar to other warty newts, *P. deloustali* prefers cool montane streams in closed-canopy evergreen forest habitats and possesses limited physiological tolerance to extremely hot and dry environments (Sparreboom, 2014). However, the relationships between genetic structure patterns and dynamic environmental characteristics of this species have not yet been studied.

The dynamics of various species during the glacial period in Indochina have been elucidated in previous studies, including the Ornate chorus frog, *Microhyla fissipes* (Yuan et al., 2016); Giant spiny frog, *Quasipaa spinosa* (Luo et al., 2021); forest-dwelling murine rodents of the genus *Leopoldamys* (Latinne et al., 2015); and Fishing cat, *Prionailurus viverrinus* (Petersen et al., 2022). Despite interest in clarifying the responses of warty newts to past climate variation, a shortage of research in this area persists. Here, I integrated mitochondrial DNA (mtDNA) sequence data with environmental data in SDMs to reveal the historical demographic and distribution patterns of *P. deloustali*, the southernmost species of genus *Paramesotriton*, at both the species and population levels. I highlighted the role of the Quaternary climate in driving the genetic diversification, demographic structure, and paleodistribution of the species. Specifically, I assessed the genetic structure of *P. deloustali*, examined how its population demography is influenced by evolutionary processes, and evaluated the effects of climatic variation over space and time on shaping the current genetic structure.

2-2. Materials and methods

Population genetics

DNA sampling

In total, 67 muscle samples were taken from the tails of live newts collected at 12 locations during field surveys (Figure 2-1). The newts were released back into their natural habitat after tissue collection. No animals were euthanized or subjected to anesthesia or

analgesia. The tissues were preserved in 99% ethanol in a freezer at Vietnam National University of Forestry, Vietnam. The procedures for collecting specimens and conducting animal operations followed the guidelines for animal experimentation of Kyoto University, Japan (Nos. 20-A-7 and 22-A-2). I extracted total DNA from muscle using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Then, the tissues were sequenced for the partial NADH dehydrogenase subunit 2 region (ND2) of mtDNA. The methods used for polymerase chain reaction (PCR), purification of PCR products, and sequencing have previously been described in Nishikawa et al. (2011, 2013). I sequenced the amplified fragments using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) at Kyoto University, Japan. A portion of each sample was sent to 1st Base (Malaysia) for sequencing. Then, I assembled the sequences using ChromasPro v.1.34 software (Technelysium Pty Ltd., South Brisbane, Queensland, Australia), and aligned the 1,011 bp ND2 sequences using MAFFT v7.222 (default settings followed a previous study (Kato & Standley, 2013)). Then, the sequences were submitted to the DNA Data Bank of Japan (DDBJ; accession numbers are from LC758635 to LC758702). The ND2 gene has been widely used for genetic studies of newts in the genus *Paramesotriton* (Gu et al., 2012; Luo et al., 2022; Weisrock et al., 2006; Wu et al., 2009, 2010; Yuan et al., 2016, 2022; Zhang et al., 2008). In addition, I obtained sequences of *P. deloustali* and *P. guangxiensis* from GenBank (<https://www.ncbi.nlm.nih.gov/>), including accession numbers FJ744600 (Wu et al., 2009), DQ517802, DQ517804 (Weisrock et al., 2006), ON794062, ON794063, ON794071, and ON794072 (Luo et al., 2022). The sequences listed under accession numbers ON794085 (*P. longliensis*), ON794083 (*P. caudopunctatus*), ON794119 (*P. chinensis*) (Yuan et al., 2022), and KU375031 (*Pachytriton inexpectatus*; <https://www.ncbi.nlm.nih.gov/nuccore/KU375031>) were used as the outgroup.

Phylogenetic and phylogeographic analyses

I used the maximum likelihood (ML) and Bayesian inference (BI) methods to construct phylogenetic trees. To select the best substitution model, I used Kakusan4 (Tanabe, 2011) to calculate Akaike information criterion (AIC; Akaike, 1974) values for the ML method and Bayesian information criterion (Schwarz, 1978) values for the BI method. Then, I employed RAXML 8.2 software (Stamatakis, 2014) to produce an ML tree with 1,000 replicates. Nodes were considered to have adequate support when their bootstrap support (BS) values were greater than 70% (Huelsenbeck & Hillis, 1993). I used MrBayes 3.2.6 software (Ronquist et al., 2012) with two parallel runs of four Markov chain Monte Carlo (MCMC) methods for 10 million generations to generate the BI tree. Tracer v.1.7 (Rambaut et al., 2018) was employed to check the parameter estimates and convergence of BI analysis. Next, I discarded the first 10% of the generations as burn-in. Bayesian posterior

probability (BPP) $\geq 95\%$ was the criterion for adequate statistical support of a node. I constructed haplotype networks for all ND2 sequences of *P. deloustali* to support the phylogenetic lineages and their geographic structure using the median joining network method in PopART software (Leigh & Bryant, 2015).

I estimated the divergence times of groups using mtDNA data under a Bayesian molecular clock framework in BEAST v.2.6 (Bouckaert et al., 2019). I added two secondary calibration points obtained from previous studies. The first was selected as the node of divergence between the *P. caudopunctatus* group and *P. chinensis* group of the genus *Paramesotriton*, which has been dated to 23.8 million years ago (Mya; 95% highest posterior density [HPD]: 18.0–29.9 Ma; (Zhang et al., 2008)) as a normal prior (mean 23.8; sigma 3.4; offset 0.0). The second calibration point was placed at the split between *P. deloustali* and *P. guangxiensis* at 4.96 Mya (95% HPD: 3.55–6.43 Ma; [Yuan et al., 2022]) as a normal prior (mean 4.96, sigma 0.9, offset 0.0). I ran the analysis for 20 million generations, with sampling every 100,000 generations using the HKY85_Gamma function in Kakusan4 (Tanabe, 2011) with the uncorrelated lognormal relaxed clock model. Then, I used Tracer v.1.7 (Rambaut et al., 2018) to assess the stationarity and ensure an effective sample size (ESS) greater than 200 for all estimated parameters. Finally, I employed TreeAnnotator v. 2.6 (Bouckaert et al., 2019) to generate a maximum group credibility consensus tree with mean node heights, discarding the first 10% as burn-in.

To assess the hypothesis of diversification among groups of *P. deloustali* in northern Vietnam, I conducted historical biogeography analysis using parsimony-based statistical dispersal–vicariance analysis (S-DIVA; Yu et al., 2010) in RASP v.3.2 (Yu et al., 2015). Based on the natural landscape of northern Vietnam, along with the phylogenetic tree (BI method), haplotype network, and occurrence locations of *P. deloustali*, I designated the following four distribution regions: E1, the northeastern area including mountainous area of Tuyen Quang and Bac Kan provinces; E2, the southeastern area including the Tam Dao Mountains in Vinh Phuc, Tuyen Quang, and Thai Nguyen provinces; W1, the northwestern area in Lao Cai and Ha Giang provinces on the right side of the Hoang Lien Son Mountain Range; and W2, the southwestern area covering Yen Bai and Son La provinces, along with the southern area of the Hoang Lien Son Mountain Range.

Demographic analysis

To reveal the genetic diversity of the overall population and each major group of *P. deloustali*, I calculated haplotype diversity (H_d) and nucleotide diversity (π) using DnaSP v.6 (Rozas et al., 2017). To assess the demographic history of each group, I performed mismatch distribution analysis by displaying the frequency distribution of pairwise differences (Fu, 1997) between two models: demographic expansion (Rogers & Harpending,

1992) and spatial expansion (Excoffier, 2004). The goodness-of-fit of the simulated distribution was evaluated based on expected distributions obtained from a population expansion model. This evaluation was conducted by computing the sum of the square deviation (SSD) with 10,000 replicates. To estimate potential expansion, I conducted neutrality tests for each group using Fu's F_s index (Fu, 1997) with 10,000 simulations. Significant negative values in these tests indicate that a demographic expansion likely occurred in the past. In this study, I implemented mismatch distribution and neutrality tests in Arlequin ver. 3.5 software (Excoffier & Lischer, 2010).

To infer the dynamics of effective population size over time for each group, I conducted Bayesian skyline plot (BSP) analysis (Drummond et al., 2005) in BEAST v.2.6 (Bouckaert et al., 2019). I used Kakusan4 (Tanabe, 2011) to select the best model (HKY model) for site model input in BEAST. An evolutionary rate of 0.80% divergence per million years for the mitochondrial DNA of Salamandridae (Tan & Wake, 1995) was used to construct a strict clock model. Then I ran MCMC analysis with 20 million generations, sampling every 100,000 generations and discarding 10% as burn-in. The parameters were evaluated for convergence using Tracer v.1.7 (Rambaut et al., 2018), with an ESS greater than 200. Then, the historical demographic events were depicted as a BSP using Tracer v.1.7 (Rambaut et al., 2018).

Species distribution modeling

Occurrence data

Presence records of the Vietnam warty newt were gathered from my field surveys conducted from 2018 to 2022 across the entire known geographic range of the species in northern Vietnam. In addition, presence records for this newt were obtained from published documents (Nguyen, 2015; Pham et al., 2022) and from the Global Biodiversity Information Facility (GBIF; <https://www.gbif.org/species/2431843>). In total, I gathered 146 presence locations of the Vietnam warty newt. To prevent spatial autocorrelation of the occurrence data, I thinned out presence points located within 1 km of each other by randomly selecting one record and deleting the others; this process was conducted with 10 replicates using the "spThin" package (Aiello-Lammens et al., 2015). I used one km as the criterion for thinning presence locations because warty newts have relatively small home ranges. For example, the mean home range of the Hong Kong newt (*Paramesotriton hongkongensis*) was estimated to be only around 0.04 ha (Lau et al., 2017). This thinning process reduced the presence locations of Vietnam warty newt to 55 points, which were employed as input data for species distribution modeling (Figure 2-1).

Environmental data

Recent climatic data at the highest available resolution (30 arcsecs) were downloaded from the WorldClim database (<http://www.worldclim.com/>; Fick & Hijmans, 2017). The dataset contains 11 variables of temperature and eight layers of precipitation. To project the paleo-distribution of the Vietnam warty newt, I gathered historical climate datasets representing the mid-Holocene (MH), LGM, and last interglacial (LIG) from the WorldClim database. Climatic data for the MH (~6 thousand years ago [Kya]) and LGM (~21 Kya) were obtained from global climate models of MIROC-ESM (Sueyoshi et al., 2013). The paleo-climate during LIG (~140–120 Kya) was reconstructed in Otto-Bliesner et al. (2006). To prevent autocorrelation among climatic variables, I calculated Pearson's correlation index (r) using ENMTools version 1.4.4 (Warren et al., 2010) and retained only one variable from each highly correlated pair ($|r| > 0.80$). Ultimately, seven predictors were selected for inclusion in the final models: annual mean temperature (Bio1), mean diurnal range (Bio2), temperature seasonality (Bio4), temperature annual range (Bio7), annual precipitation (Bio12), precipitation in the driest month (Bio14), and precipitation seasonality (Bio15).

Ensemble species distribution modeling

The availability of numerous techniques for species distribution modeling, which might show considerable differences in their results, causing difficulty for researchers selecting the most appropriate methodology for their needs (Guisan et al., 2017; Thuiller et al., 2009). The technique of ensemble species distribution modeling (eSDM) is widely employed for predicting species distributions and functions by averaging the outputs from multiple species distribution models using various algorithms (Abrahms et al., 2019; Araújo & New, 2007; Guisan et al., 2017; Hao et al., 2019; Robinson et al., 2017; Vaissi, 2021b). BIOMOD (Thuiller, 2003; Thuiller et al., 2009, 2016) is a highly regarded ensemble package that is widely used for species distribution modeling. In addition, this model is open access, and its usage is expected to persist (Hao et al., 2019). Here, I applied the BIOMOD2 package (Thuiller et al., 2016, 2023) version 4.2–2 of the R programming language (R Core Team, 2023) to create ensemble models for *P. deloustali* with the default settings following the recommendations of Guisan et al. (2017). Nine algorithms were run for my models, as suggested by Vaissi (2021b): generalized boosted models (GBM, n.trees = 1000, 3-fold cross-validation); random forest (RF, n.trees = 1000); generalized linear models (GLM, type = 'quadratic,' interaction.level = 1, stepwise procedure using AIC); generalized additive models (GAM, algo = 'GAM_mgcv'); multivariate adaptive regression splines (MARS, simple with no interaction); surface range envelope (SRE, quant = 0.025); flexible discriminant analysis (FDA); classification tree analysis (CTA, CV.tree = 50, 5 Fold Cross-Validation); and maximum entropy (MaxEnt.Phillips, https://biodiversityinformatics.amnh.org/open_source/maxent/).

Absence records were not available for the newt, and presence–absence data were required for most of the models (except SRE and MaxEnt). Therefore, pseudo-absence records were randomly created using the `BIOMOD_FormatingData` function of the BIOMOD2 package (Thuiller et al., 2016) with a minimum geographic distance of one km between pseudo-absence points (Vaissi, 2021b; Arenas-Castro et al., 2018) to avoid autocorrelation and cover the distinct environmental conditions across the modeled area (Wisz & Guisan, 2009). I set the number of pseudo-absence points equal to three times the number of presence points and conducted ten repetitions following the advice of the BIOMOD2 team (Thuiller et al., 2023). For each individual model, I allocated 80% of the data to model calibration and the other 20% to performance assessment of the predictions with four replicates to prevent an effect from splitting the total records (Guisan et al., 2017; Vaissi, 2021b).

I applied Cohen’s kappa (KAPPA), the area under the receiver operating characteristic (ROC) curve (AUC), and true skill statistic (TSS) to evaluate the performance of each model. KAPPA and TSS values vary from -1 to $+1$, with a value of $+1$ indicating perfect performance of the model and values between 0.6 and 0.9 representing excellent model performance (Allouche et al., 2006). AUC ranges from 0 to $+1$; AUC scores below 0.6 are considered to indicate poor models, scores between 0.6 and 0.9 indicate moderate models, and scores above 0.9 are classified as excellent models (Peterson et al., 2011). The mean values of important environmental predictors were calculated and plotted using the BIOMOD2 package version 4.2–2 (Thuiller et al., 2023). In this study, I employed three eSDMs, including a model of the entire species range, as well as West and East group models.

Ecological niche comparison

To compare the differentiation between lineages occupying different ecological niches, I used two widely used statistical indices: Schoener’s D index (Schoener, 1968) and Hellinger’s I index (Warren et al., 2008) for identity and background testing. The identity test (also called the equivalency test) is used to determine whether the SDMs of two groups are equivalent or non-equivalent, whereas the background test (also called the similarity test) is generally used to test for niche conservatism or divergence between two groups (Warren et al., 2008). The observed niche overlap values between niches of the two groups are compared to the niche overlap values of a null distribution with 100 pseudo-replicates using a one-side test for the identity test (alpha level of 0.05) and a two-sided test for the background test (alpha level of 0.05). For the identity test, if the observed niche overlap value fell within the bottom of 5% of the null distribution, I rejected the hypothesis of niche equivalence. For the background test, observed niche overlap values above the 95%

confidence interval of the null distribution suggest that the groups may have similar ecological requirements, which is consistent with the concept of niche conservatism. On the other hand, if observed overlap values are lower than the values expected due to chance, the species may have different ecological requirements, referred to as niche divergence (Warren et al., 2008). All of these ecological niche tests were performed using the ENMTools package version 1.0 (Warren et al., 2021) of the R programming language (R Core Team, 2023) version 4.2.2.

2-3. Results

Phylogenetic reconstruction and divergence times

Phylogenetic reconstruction based on mtDNA ND2 (1,011 bp) revealed two species of warty newts in northern Vietnam (*P. deloustali* and *P. guangxiensis*). The BI and ML trees are generally identical; therefore, I show only the BI tree in Figure 2-2. Within *P. deloustali*, the BI tree strongly supported two major groups, designated East and West, although the monophyly of the East group was not significantly supported by the ML tree (Figure 2-2). The East group was distributed across the eastern region of northern Vietnam, on the right banks of the Lo and Gam Rivers (Figure 2-1). This group could be divided into two subgroups: E1 at locations 5 and 7, and E2 at locations 3 and 4 (Figure 2-2). The West group occurred in northwestern Vietnam, on the left sides of the Lo and Gam Rivers. This group also included two subgroups: W1 and W2 (Figures 2-1 and 2-2). Interestingly, the W1 subgroup covered an area running across the Red River in Lao Cai and Ha Giang Provinces at sampling locations 8, 9, and 10. The W2 group encompassed individuals from locations 11 and 12 in Yen Bai Province (Figure 2-1).

My analysis confirmed the presence of the Guangxi warty newt (*P. guangxiensis*) in northern Vietnam at sampling location 6 in Cao Bang Province, in addition to two other populations in Ningming and Banna, China. In particular, my mitochondrial DNA ND2 analysis revealed that the samples from locations 1 and 2 in Quang Ninh Province were *P. guangxiensis*, while previous studies have reported this population as *P. deloustali* (Nguyen et al., 2009; Figure 2-2).

Divergence dates between the two main groups of *P. deloustali* (East and West) were estimated to be in the Early Pleistocene, approximately 1.92 Mya, with 95% confidence intervals of HPD ranging from 1.25 to 2.75 Mya. The E1 and E2 groups within the East group diverged from a common ancestor at around 0.88 Mya (HPD: 0.45–1.36 Mya). Within the West group, the split between subgroups W1 and W2 occurred at roughly 0.80 Mya (HPD: 0.41–1.21 Mya) (Figure 2-3).

Phylogeography

The numbers of haplotypes in the East and West groups of *P. deloustali* were 18 and 27, obtained from 25 and 32 sequences, respectively (Table 2-1). The haplotype network indicates that the East group was separated from the West group by 13 mutation steps. The subgroups were separated by only four mutation steps (Figure 2-4).

S-DIVA performed for ancestral area reconstruction indicated that the two main groups diverged due to a vicariance event between the East and West groups (Figure 2-2 and Figure 2-5).

Historical demography

Mismatch distribution analysis revealed multiple peaks for the model of overall population, and two peaks (bimodal) for the models of both the West and East groups (Figure 2-6), providing evidence that two distinct populations were presented within each group (Bremer et al., 2005; Jenkins et al., 2018). The SSD values for both demographic and spatial expansion were rejected in the analysis of the overall population ($p < 0.05$; Figure 2-6A). However, they did not support rejecting demographic and spatial expansion for the West and East groups ($p > 0.05$; Figures 2-6B, C). Based on analysis of the sequences of each subgroup, my results indicate a unimodal mismatch distribution for each of the four subgroups (Figure 2-7), indicating sudden population expansion events (Fukutani et al., 2022; Jenkins et al., 2018). Based on SSD values, I did not reject the possibility of demographic and spatial expansion for any of the groups ($p > 0.05$; Figure 2-7).

My analysis of genetic diversity revealed high haplotype diversity (H_d) values for the overall population as well as the West and East groups (0.985, 0.940, and 0.988, respectively). However, the nucleotide diversity (π) values were comparatively low, measuring 0.012, 0.004, and 0.005 for the overall population, West group, and East group, respectively (Table 2-1). Furthermore, the genetic and nucleotide diversity of the West group were higher than corresponding values for the East group. The neutrality test produced significantly negative F_u 's F_s values for all tests (overall population, West, and East group). These results support the proposed pattern of historical demographic expansion for *P. deloustali* and each group within this species.

Historical demographic estimation using BSPs of the overall population and two main groups of *P. deloustali* revealed that a population expansion began around 0.15 Mya. In particular, the effective population size of the West group was estimated to increase sharply from ca. 0.11–0.07 Mya, and then to stabilize from this period to the present (Figure 2-8). For the East group, I estimated a steadily increasing effective population size from approximately 0.15–0.04 Mya, which then became stable until the present (Figure 2-8).

Ecological niche models

Ecological niche models of the entire range of *P. deloustali* as well as each separate group showed high values of KAPPA, TSS, and AUC, ranging from 0.649 to 1 (Table 2-2). This indicates that my models performed better than random prediction. RF was the best-fitting model, whereas SRE showed the worst fitting performance (Table 2-2). The most important variable of the model for the entire range species was annual precipitation (Bio12) in five model algorithms (CTA, GBM, MAXENT, RF, SRE), while mean diurnal range (Bio2) made the largest contribution in three models (GAM, GLM, and MARS). For the FDA algorithm, temperature seasonality (Bio4) was the most important predictor (Figure 2-9). The potential distribution of *P. deloustali* was predicted to cover a wide and fragmented area, including the East and West regions of northern Vietnam, as well as a small area of southern China (Figure 2-12A). For the West group, annual precipitation (Bio12) was the strongest predictor in all model algorithms (Figure 2-10). The predicted suitable area for the group covered two main areas of Yen Bai, Lao Cai, and Ha Giang Provinces and the western part of northern Vietnam (Figure 2-12B). For the East group, mean diurnal range (Bio2) was predicted as the variable making the largest contribution in all model algorithms, followed by annual precipitation (Bio12; Figure 2-11). The estimated range of the East group was mainly continuous, concentrated in the Vinh Phuc, Thai Nguyen, Tuyen Quang, and Bac Kan Provinces in the eastern part of northern Vietnam (Figure 2-12C).

Paleodistribution models

My projected historical distributions of the entire species and major groups of *P. deloustali* showed different trends from the LIG (~120 Kya) to the present. For models of the entire species range, the predicted distributions during the MH (~6 Kya) and the LIG represented a much larger area than the current potential distribution, covering most of northern Vietnam (Figure 2-12D, J). However, the high-suitability area in the paleodistribution model for the LGM (~21 Kya) was predicted to be smaller than other models, concentrated in the Hoang Lien Son Mountain Range with an upward shift in elevation, while low-suitability areas covered most of northern Vietnam (Figure 2-12G). The paleodistribution of the West group was predicted to be a small area in the Hoang Lien Son Mountain Range during the LIG and LGM periods (Figure 2-12K, H). During the MH period, the distribution of the West group expanded to cover a larger area, stretching from the east to the west in northern Vietnam (Figure 2-12E). In the historical distribution models of the East group, a large area ranging from central northern Vietnam to the coast of northern Vietnam and southern China was suitable during the LIG (Figure 2-12L). The range of this group narrowed and shifted to the west during the LGM (Figure 2-12I), and covered a significantly larger area during the MH, which was focused on the northeast provinces of

Vietnam and southern China (Figure 2-12F).

Niche overlap among major groups

Identity testing between the East and West groups of *P. deloustali* showed that the values of Hellinger's I and Schoener's D were significant ($p < 0.05$), and the true overlap value was smaller than the overlap of the null distribution (Figure 2-13), supporting rejection of the null hypothesis in the equivalent test.

For the background tests, the null hypothesis was not rejected for the direction of the East group versus the background of the West group ($p > 0.05$ for Hellinger's-based I; $p > 0.05$ for Schoener's D). However, for the direction of the West group versus the background of the East group, the null hypothesis was rejected ($p < 0.05$ for Hellinger's-based I; $p < 0.05$ for Schoener's D), and the true niche overlap value was lower than the value for the null distribution (Figure 2-14), indicating niche divergence.

2-4. Discussion

Several studies have attempted to reveal the evolutionary history and geographical distribution of species in the genus *Paramesotriton* based on phylogenetic analysis (Gu et al., 2012; Lu et al., 2004; Luo et al., 2021; Weisrock et al., 2006; Yang et al., 2017; Yuan et al., 2014; Zhang et al., 2008) or integration of phylogenetic analysis with ecological niche modeling (Luo et al., 2022; Yuan et al., 2022). However, these studies have primarily focused on genus or species groups. The present study is the first to investigate the influence of historical processes on shaping the genetic structure and distribution within one species, *P. deloustali*, at both the species and population levels.

The boundary of species

Paramesotriton deloustali has been observed in several locations in northern Vietnam, including the provinces of Vinh Phuc, Bac Kan, Ha Giang, Yen Bai, Tuyen Quang, Thai Nguyen, Lao Cai, and Quang Ninh (Frost, 2024; IUCN SSC Amphibian Specialist Group, 2017; Nguyen et al., 2009), as well as Yunnan, China (Zhang et al., 2018). My analysis using the BI method strongly supported two major groups (West and East) of *P. deloustali* in northern Vietnam. However, the East group was supported with lower confidence based on the ML method (Figure 2-2). I considered the East and West populations as two major geographic groups of this newt species. The same contrast (highly significant in BI but nonsignificant in ML) was found in previous phylogenetic studies of tailed amphibians, including the Shangcheng stout salamander *Pachyhynobius*

shangchengensis (Zhao et al., 2013), Black salamander *Aneides flavipunctatus* (Reilly et al., 2013), and other organisms (Choi et al., 2020; Suzuki et al., 2014). I generally present the results obtained from BI in the following discussion.

The West group occurs in northwestern Vietnam, covering sampling locations in Yen Bai, Lao Cai, and Ha Giang provinces. By contrast, the East group is distributed across the eastern region of northern Vietnam, encompassing Thai Nguyen, Vinh Phuc, Tuyen Quang and Bac Kan provinces (Figure 2-1). These results are largely consistent with previous reports, except for the locations in Quang Ninh Province, Vietnam. Nguyen et al. (2009) identified the newt population in Quang Ninh as *P. deloustali*. However, my analysis of ND2 mitochondrial DNA revealed that this population was nested within *P. guangxiensis* (Figure 2-2). Furthermore, the analysis of morphological characteristics revealed a significant difference between the newts in Quang Ninh Province and other newt populations (Dung Tran 2023, unpublished data). I also highly recommended the use of nuclear DNA analysis in confirming the taxonomy classification of newts in the Quang Ninh province.

Recently, *P. deloustali* has been listed as “Least Concern” on the IUCN Red List due to its widespread distribution (IUCN SSC Amphibian Specialist Group, 2017). However, my findings suggest that the population in Quang Ninh may not be *P. deloustali*, which would greatly reduce the geographic distribution of this species. The distribution map on the IUCN’s official website currently shows a broad range for this species, including areas of Quang Ninh Province (IUCN SSC Amphibian Specialist Group, 2017). I highly recommend revision of the current distribution map to accurately represent the range of this species, which will provide useful data for conservationists and wildlife managers undertaking conservation activities (e.g., conducting field surveys, long-term monitoring, assessing threats, establishing and managing protected areas) (Kass et al., 2021). *Paramesotriton deloustali* is rarely found in lowlands, and most of my observed populations are associated with cool streams in mountainous areas (Dung Tran, personal observations). Increasing areas of agricultural lands, as well as water pollution and flooding in lowland areas, have degraded its habitat (IUCN SSC Amphibian Specialist Group, 2017; Sparreboom, 2014), likely contributing to the current isolation of populations in high mountain regions. Based on my analysis of genetic structure and ecological niche, I strongly recommend prioritizing conservation efforts for the species in mountainous areas located in Vinh Phuc, Thai Nguyen Province (Tam Dao National Park), Tuyen Quang Province (Ba Be National Park), Yen Bai, and Lao Cai Province. These regions have been identified as having high suitability for the species, but they also exhibit significant isolation. Thus, focusing on conservation initiatives in these areas would be crucial for the long-term preservation of the *P. deloustali*. My models were assembled from nine different species distribution model algorithms but considered the influence of climatic predictors only at a large scale, providing insights related to macroecology, biogeography, and conservation biology (Hortal et al., 2014; Isaac et al.,

2020). Future studies using finer-scale models with more presence locations, specific vegetation layers, and human footprint variables should be conducted to increase the accuracy of model results for practical conservation purposes. Furthermore, my genetic data and eSDM results indicate that the populations of *P. deloustali* exhibit low nucleotide diversity and are generally isolated in fragmented areas. This isolation may reduce the effective population size of the species. Thus, conservation efforts for *P. deloustali* should prioritize the preservation of all populations.

Diversification and ecological niche differentiation

The presence of two major groups of *P. deloustali* was strongly supported based on the BI tree, haplotype network, and eSDM analysis. Ecological niche comparison showed significant differences between the ecological niches of the West and East groups. As expected, the identity test revealed that ecological niches were non-equivalent, demonstrating a lack of capacity for ecological exchange between the populations. Background tests showed significance in the direction of the West group against the background of the East group, but not the opposite (Figure 2-14). According to Warren et al. (2008), background test results with significance in one direction based on the null hypothesis indicate that the groups may be more similar than expected. Therefore, I considered that these two groups show evidence of ecological niche divergence, indicating that their ecological niches are less similar than would be expected under the null model. Despite both groups inhabiting cool montane streams (Dung Tran, personal observation; (Sparreboom, 2014)), their niches showed evidence of divergence. This was likely due to the niches of these species having different environmental conditions in their respective allopatric ranges, despite their similar niche preferences (Yuan et al., 2022). In addition, the higher haplotype and nucleotide diversity of the West group may allow it to adapt better to climatic and elevational variation than the East group. My ecological tests showed a difference in niches of *P. deloustali* at the population level, supporting a west-east distribution pattern. This finding is consistent with the hypothesis of west-east evolutionary divergence within the genus *Paramesotriton* (Luo et al., 2021, 2022; Yuan et al., 2022) as well as other amphibians and reptiles in southern China (Guo et al., 2020; Yan et al., 2021; Zhou et al., 2017).

The time of divergence between two major groups of *P. deloustali* was estimated at around 1.92 Mya (HPD: 1.25–2.75 Mya), in the early Pleistocene epoch and Quaternary period (Figure 2-3), consistent with the estimate of 1.86 Mya (HPD: 0.92–3.06 Mya) reported by Lou et al. (2022). The combined effects of geology and climate change likely triggered the diversification of several species of *Paramesotriton* (Luo et al., 2021, 2022). Northern Vietnam is characterized by abundant karst formations dating to the mid-Devonian

through early Triassic periods and the Hoang Lien Son Mountain Range, which uplifted around 65 Mya (Sterling et al., 2006). Dramatic increases in the strength of monsoons and droughts in Indochina and southern China have occurred several times (Clift et al., 2008). The increase event during the late Pliocene and Pleistocene epochs (Clift et al., 2008) was consistent with the divergence date of the two major groups of *P. deloustali* (Figure 2-3). Therefore, historical climate change likely played a more significant role in promoting the divergence of the East and West groups than geological processes.

Yuan et al. (2022) indicated that several valleys among the mountains served as major dispersal obstacles, driving the diversification of warty newts in south China. For *P. deloustali*, wide valleys in the basins of the Lo River and Gam River on the upper side (Tuyen Quang Province) and the Red River on the lower side (Phu Tho and Vinh Phuc Provinces) of the range are potential dispersal barriers for the East and West groups during periods of historical climate change. These barriers prevented newt populations from spreading throughout the lowland valleys and isolated them in cool streams among high mountains (Figure 2-15). The results of S-DIVA analysis based on biogeographical history strongly supported that the current distributions of the East and West groups were shaped by vicariance events (Figure 2-2).

The Red River in northern Vietnam serves as a major boundary in the transition zone between subtropical and tropical climates (Peel et al., 2007). This river has also been considered a natural barrier for many organisms (Bain & Hurley, 2011; Rawson et al., 2011; Yuan et al., 2016; Zhang et al., 2010; Zhang et al., 2010). However, my genetic analysis of *P. deloustali* showed that the West group occurred on both sides along the upper part of the river at locations 8, 9, and 10 in Ha Giang and Lao Cai provinces (Figure 2-1). The northeast region of this area is shielded by a large domal structure with an elevation greater than 2000 m a.s.l., known as the Song Chay Massif (Figure 2-10), which formed during the Proterozoic era between 2500 and 538 Mya (Roger et al., 2000). It is likely that this massif dampened the effect of monsoons in the area during the Pleistocene period, thereby preventing divergence of the newt population. In addition, the valleys formed by the Red River and Chay River are relatively narrow in that area (Figure 2-1), possibly supporting migration between the two sides of the river by aquatic amphibians such as *P. deloustali*.

Historical demographic and distribution dynamics

Mismatch distribution analysis and neutrality testing provided evidence of historical demographic and spatial expansions of *P. deloustali*. In addition, the BSP illustrated that the species began its effective population size expansion around 0.15–0.11 Mya (Figure 2-8), aligning with the period of the LIG (0.14–0.12 Mya; Otto-Bliesner et al., 2006). My eSDM analysis predicted expansion of the paleodistribution of *P. deloustali* during the LIG (Figure

2-13). Despite the absence of glaciers, southern China and Indochina experienced relatively low temperatures throughout the LIG (Bird et al., 2005; Li et al., 2004; Tamburini et al., 2003). This cooler climate likely promoted expansion of both the population and geographic range of *P. deloustali*, a cold-adapted newt (Sparreboom, 2014). However, the predicted distribution of the West group showed contraction, whereas the range of East group expanded during the LIG (Figure 2-12K, L). In northern Vietnam, western areas have numerous high mountain ranges, particularly the Hoang Lien Son Mountain Range, that are absent from the east area (Sterling et al., 2006). High mountains can serve as barriers that hinder the movement of cold air masses (Dong et al., 2022; Rahbek et al., 2019). Therefore, the presence of high mountainous topography likely reduced the influence of cold air during the early stages of the LIG period, decelerating expansion of the West group. This delay in expansion was supported by the results of the BSP: demographic expansion of the West group began at approximately 0.11 Mya, whereas the East group's expansion commenced around 0.15 Mya (Figure 2-8B, C).

My demographic reconstruction results also revealed that *P. deloustali* maintained stable populations from approximately 0.7–0.4 Mya to the present (Figure 2-8). No signs of bottleneck events were found in the estimation of effective population size; however, the eSDM results indicate a reduction in the suitable range during the LGM in models for the overall population as well as the East and West groups (Figure 2-12). The contrasting results of demographic and species distribution modeling suggest a high population density of this species at the LGM (Niwa et al., 2022). Interestingly, suitable habitat areas tended to narrow down to high mountain ranges, such as the Hoang Lien Son Mountain Range (Figure 2-13G). During the LGM, the temperature in Indochina might have decreased by 6–7°C, and precipitation was approximately 30–50% lower than today (Kershaw et al., 2001). The area of tropical rain forests probably decreased, being replaced by wetlands and grasslands in low-lying areas (Bird et al., 2005; Kershaw et al., 2001; Wurster et al., 2010) but was maintained in mountainous areas (Hope et al., 2004; Walker & Xiangjun, 1988). As a species requiring tropical montane forest for survival, *P. deloustali* was likely forced to relocate to high-elevation regions where rainforest persisted during the LGM. Refugia, which are areas with stable climates since the LGM, provide consistent habitats for endemic fauna (Sandel et al., 2011). Maintenance of suitable habitat and the stable effective population size of *P. deloustali* during the LGM indicate that mountainous areas (e.g., Hoang Lien Son Mountain Range) in the western portion of northern Vietnam were potential refugia over that period of climate change. The Hoang Lien Son Mountain Range is considered to be an important site for biodiversity conservation due to its high level of endemism (Ohler et al., 2000; Tapley et al., 2017). Information on historical refugia can improve my understanding of ecological resilience and migration patterns in response to changing climates, providing insights into how populations may respond to future climate changes (Wielstra et al., 2010).

I found that the suitable distribution of *P. deloustali* increased significantly and expanded to lowland areas during the MH (~6 Kya) for all models of the overall population and the East and West groups (Figure 2-12). Previous studies have indicated the recovery of lowland rainforest in this region after the LGM (Hunt & Rabett, 2014; Li et al., 2006). Furthermore, Li et al. (2006) presented evidence based on pollen deposition that northern Vietnam likely experienced a cool climate during the period of 6.5–5.2 Kya, which would promote the expansion of populations inhabiting high mountains into lowland areas. This possibility is consistent with the hypothesis of elevation shift: cold-adapted taxa occupying high-elevation regions expand into lower-elevation regions during periods of cool climate (Galbreath et al., 2009). The same expansion trend has been found in several studies of tropical or subtropical amphibians, including the Santa barbara treefrogs *Bokermannohyla alvarengai* and *Bokermannohyla oxente* (de Oliveira et al., 2021).

In this study, I documented the demographic and distribution dynamics of a montane warty newt driven by historical climate changes. Changes in the historical climate promoted the divergence of two major populations of the newt, and significantly impacted its distributions during the LIG, LGM, and MH, as well as at present. In addition, my eSDM model revealed that both temperature and precipitation variables (mean diurnal range [Bio2] and annual precipitation [Bio12]) play important roles in shaping the distribution of *P. deloustali*. Therefore, I predict that this species could be considerably impacted by the future effects of climate change.

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Table 2-1. Genetic diversity analysis and neutrality tests for each major group of *P. deloustali* based on mitochondrial DNA ND2 (Note: N-number of individuals; Nh-number of haplotypes; Hd-haplotype diversity; π -nucleotide diversity; SD-standard deviation. Asterisk indicates significant *p*-value (<0.01)).

Population	N	Nh	Hd $\pm$ SD	$\pi \pm$ SD	Fu's Fs	p-value
Overall	57	45	0.985 $\pm$ 0.008	0.01285 $\pm$ 0.00033	-23.3626*	<0.001
East	25	18	0.940 $\pm$ 0.037	0.00411 $\pm$ 0.00051	-10.5066*	<0.001
West	32	27	0.988 $\pm$ 0.012	0.00585 $\pm$ 0.00032	-20.5022*	<0.001

Table 2-2. The value of Cohen's kappa (KAPPA), ROC curve (AUC), and True skill statistic (TSS) of Generalized Boosted Models (GBM), Random Forest (RF), Generalized Linear Models (GLM), Generalized Additive Models (GAM), Multivariate Adaptive Regression splines (MARS); Surface Range Envelops (SRE); Flexible Discriminant Analysis (FDA), Classification Tree Analysis (CTA) and Maximum Entropy (MaxEnt) models projecting for the Vietnam wary newt (*Paramesotriton deloustali*) for entire range species, East group and West group.

Model	GBM	RF	GLM	GAM	MARS	SRE	FDA	CTA	MaxEnt
KAPPA	0.899±0.018	0.973±0.015	0.924±0.052	0.928±0.054	0.888±0.027	0.687±0.026	0.850±0.026	0.816±0.067	0.865±0.063
Entire species									
AUC	0.992±0.002	0.999±0.001	0.989±0.009	0.984±0.012	0.987±0.004	0.824±0.014	0.979±0.004	0.955±0.024	0.910±0.042
TSS	0.923±0.012	0.980±0.013	0.953±0.038	0.957±0.038	0.922±0.018	0.649±0.029	0.876±0.021	0.883±0.050	0.821±0.083
KAPPA	0.922±0.014	0.985±0.016	0.917±0.077	0.911±0.099	0.926±0.044	0.800±0.032	0.845±0.042	0.790±0.108	0.859±0.023
East group									
AUC	0.995±0.002	1.000±0.001	0.988±0.015	0.984±0.018	0.993±0.005	0.883±0.020	0.980±0.008	0.964±0.025	0.922±0.039
TSS	0.959±0.015	0.995±0.006	0.967±0.036	0.971±0.034	0.957±0.039	0.765±0.040	0.889±0.028	0.921±0.046	0.835±0.057
KAPPA	0.919±0.036	0.995±0.010	0.972±0.056	0.856±0.125	0.978±0.030	0.808±0.047	0.895±0.059	0.841±0.117	0.936±0.069
West group									
AUC	0.996±0.003	1±0.000	0.994±0.014	0.975±0.022	0.998±0.003	0.870±0.024	0.991±0.007	0.972±0.024	0.960±0.053
TSS	0.966±0.017	0.998±0.003	0.987±0.028	0.950±0.045	0.992±0.012	0.741±0.049	0.952±0.023	0.939±0.049	0.921±0.106

Figure legends

Figure 2-1. Geographic distribution of samples of warty newts in northern Vietnam. The map was created in ArcMap version 10.2 (ERSI). Blue lines present main rivers. The administrative boundary dataset was downloaded from the GADM (https://gadm.org/download_country_v3.html). The elevation map was extracted from Terrain Elevation Data 2010 (GMTED2010), USGS EROS (<http://eros.usgs.gov/#>).

Figure 2-2. The phylogenetic relationships of warty newts in northern Vietnam based on mitochondrial DNA ND2 sequences. Bootstrap supports (Maximum-Likelihood - ML)/posterior probabilities (Bayesian Inference - BI) were provided for only major nodes. Asterisks indicate adequate statistical support ($>70\%$ for ML method, and $> 0.95\%$ for BI method). Pink arrows indicate groups diverging through vicariance/ dispersal (S-DIVA, [Yu et al., 2010]) performed in RASP 4.3 (Yu et al., 2015).

Figure 2-3. Time-calibrated topology of main populations of newts in northern Vietnam based on mitochondrial DNA ND2. C1 and C2 represent the calibration priors. Blue bar nodes indicate 95% confidence intervals of the highest posterior density (HPD).

Figure 2-4. Haplotype network for *P. deloustali* based on the mitochondrial DNA ND2 region. Circle diameter presents the number of individual sharing haplotypes. The color of circles indicates the region of the samples (East and West population). The black dots show the number of mutational steps between individuals.

Figure 2-5. Reconstructed ancestral area of *Paramesotriton deloustali* using Statistical Dispersal-Vicariance Analysis (S-DIVA, Yu et al 2010) performed in RASP 4.3 (Yu et al 2015).

Figure 2-6. Mismatch distribution analyses based on the mitochondrial DNA ND2 (1011 bp) of *Paramesotriton deloustali*, overall population (A), West group (B), and East group (C). Red and blue lines indicate the demographic expansion, and spatial expansion models, respectively. The sums of squared deviations (SSD) and *p*-value were presented for each group.

Figure 2-7. Mismatch distribution of four sub-groups of *Paramesotriton deloustali*.

Figure 2-8. Bayesian Skyline Plots (BSP) presenting demographic variation over time based on mitochondrial DNA ND2 of *Paramesotriton deloustali*: overall population (A) West group (B), East group (C). Dark blue solid lines: median estimates, and light blue area: 95% confidence intervals of highest posterior densities.

Figure 2-9. The contribution of environmental variables in Generalized Boosted Models (GBM), Random Forest (RF), Generalized Linear Models (GLM), Generalized Additive Models (GAM), Multivariate Adaptive Regression splines (MARS); Surface Range Envelops (SRE); Flexible Discriminant Analysis (FDA), Classification Tree Analysis

(CTA) and Maximum Entropy (MaxEnt) models projecting for the Vietnam warty newt (*Paramesotriton deloustali*) for entire species range model.

Figure 2-10. The contribution of environmental variables in Generalized Boosted Models (GBM), Random Forest (RF), Generalized Linear Models (GLM), Generalized Additive Models (GAM), Multivariate Adaptive Regression splines (MARS); Surface Range Envelops (SRE); Flexible Discriminant Analysis (FDA), Classification Tree Analysis (CTA) and Maximum Entropy (MaxEnt) models projecting for the Vietnam warty newt (*Paramesotriton deloustali*) of the West group model.

Figure 2-11. The contribution of environmental variables in Generalized Boosted Models (GBM), Random Forest (RF), Generalized Linear Models (GLM), Generalized Additive Models (GAM), Multivariate Adaptive Regression splines (MARS); Surface Range Envelops (SRE); Flexible Discriminant Analysis (FDA), Classification Tree Analysis (CTA) and Maximum Entropy (MaxEnt) models projecting for the Vietnam warty newt (*Paramesotriton deloustali*) of the East group model.

Figure 2-12. Predicted current distribution and projected historical ranges of overall population, West and East groups of *Paramesotriton deloustali* to the past: Mid Holocene (MH, ~6 Kya), Last Glacial Maximum (LGM, ~21 Kya), Last interglacial (LIG, ~120 Kya). The violet polygon in (G) shows the location of Hoang Lien Son Mountain Range.

Figure 2-13. Niche identity test between ecological niche models for groups East and West of *Paramesotriton deloustali*. Asterisk indicates statistical significance ($p < 0.05$). Dot line presents the true niche overlap values, red column indicates the niche overlap values of the null distribution (100 replicates).

Figure 2-14. Niche background tests between ecological niche models for groups East and West of *Paramesotriton deloustali*. Asterisk indicates statistical significance ($p < 0.05$). Dot line presents the true niche overlap values, red column indicates the niche overlap values of the null distribution (100 replicates).

Figure 2-15. Illustration on the divergence of two main groups of *P. deloustali* in northern Vietnam under the effects of historical climate change. The administrative boundary dataset was downloaded from the GADM (https://gadm.org/download_country_v3.html). The elevation map was extracted from Terrain Elevation Data 2010 (GMTED2010), USGS EROS (<http://eros.usgs.gov/#>).

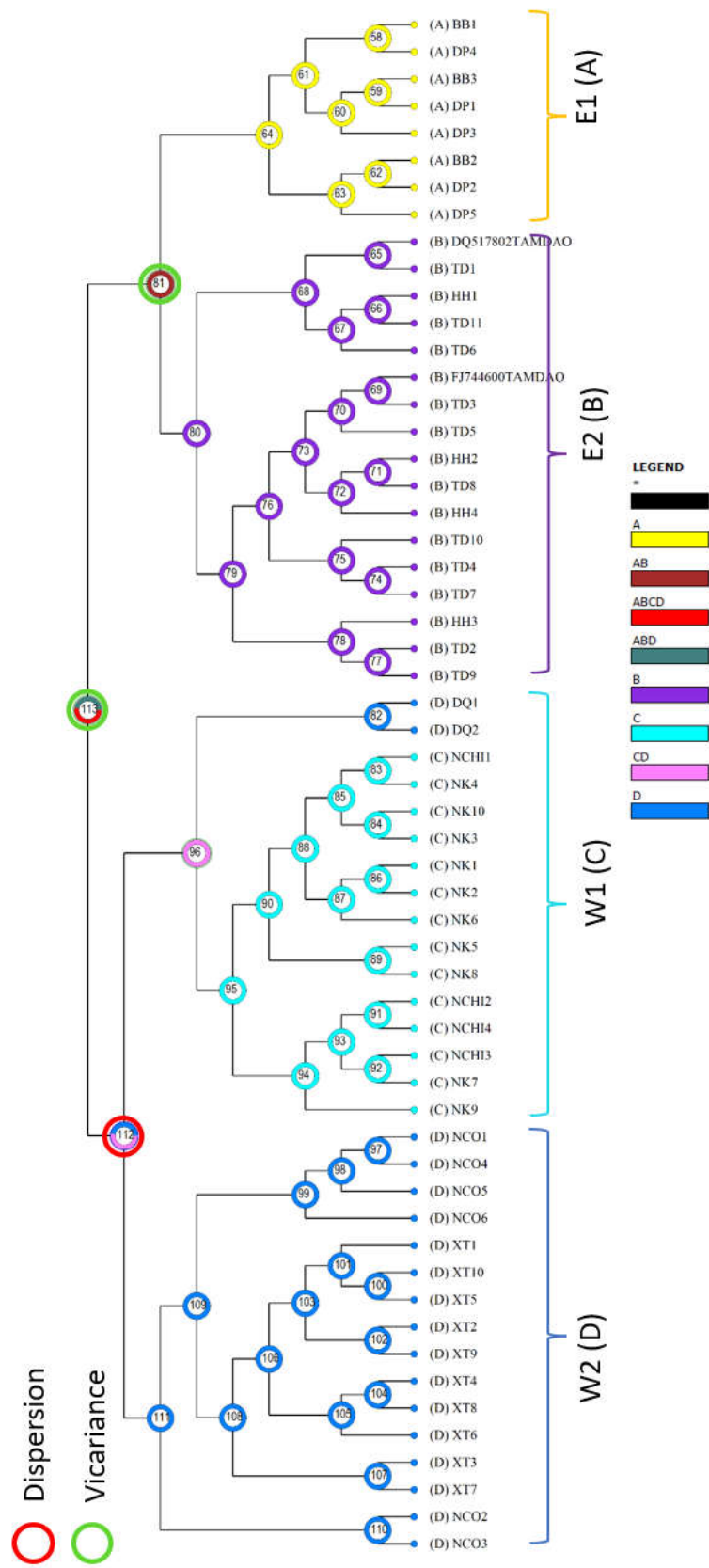
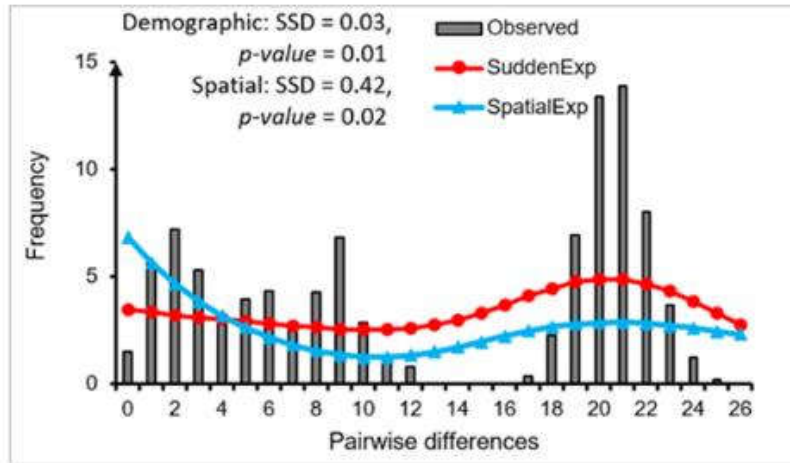
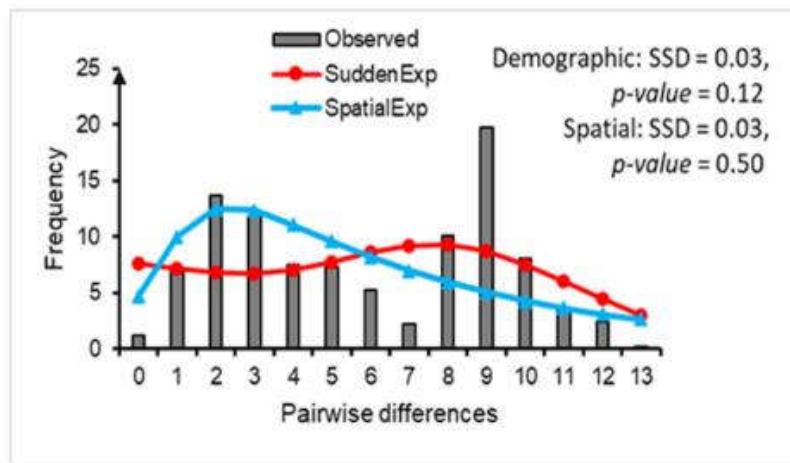


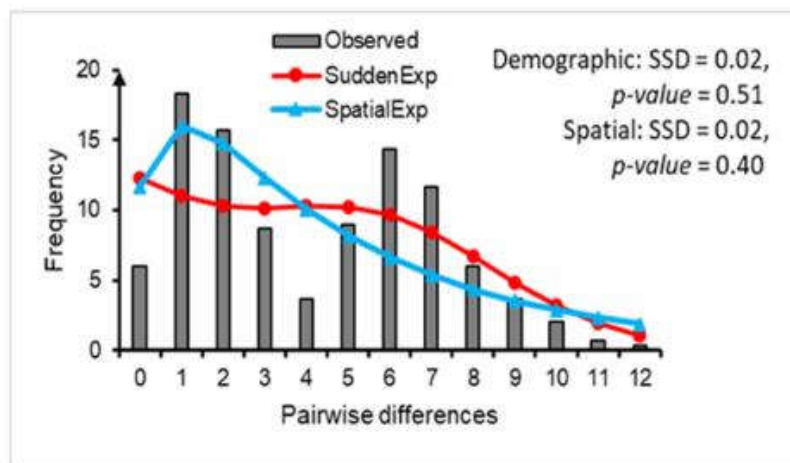
Figure 2-5



(A)



(B)



(C)

Figure 2-6

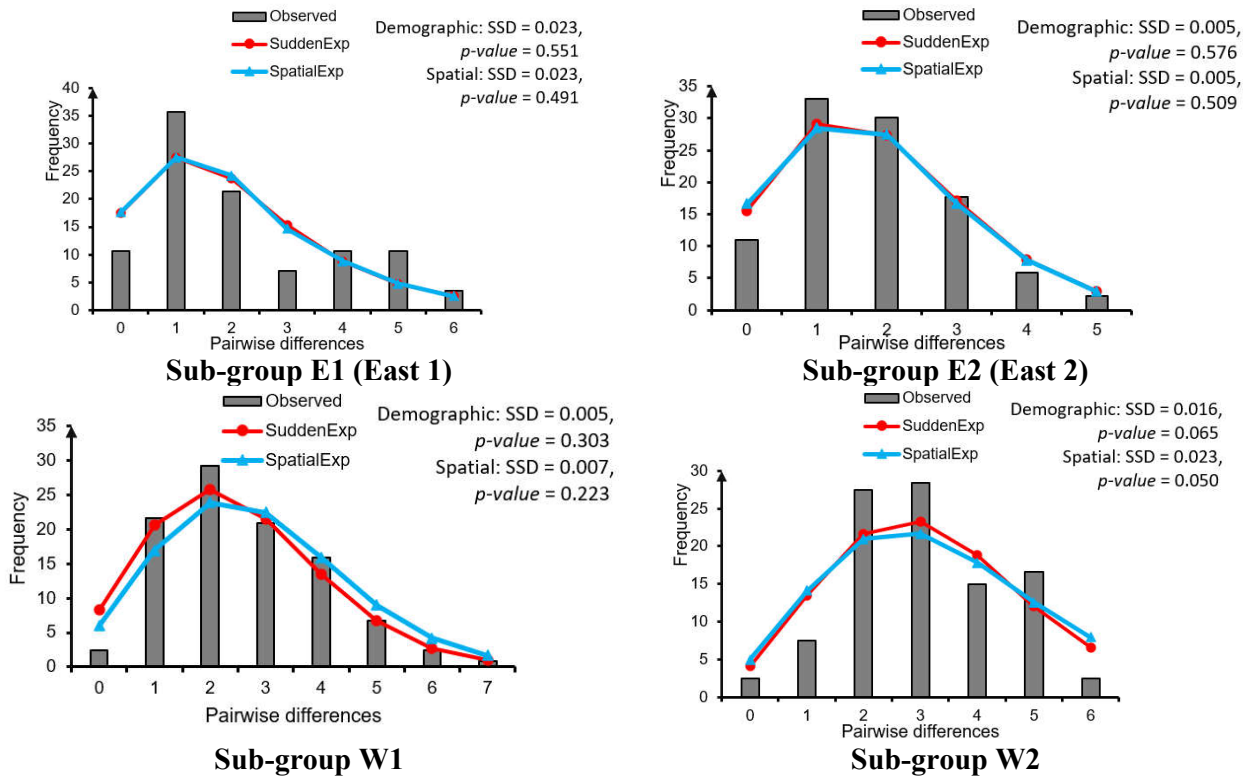


Figure 2-7

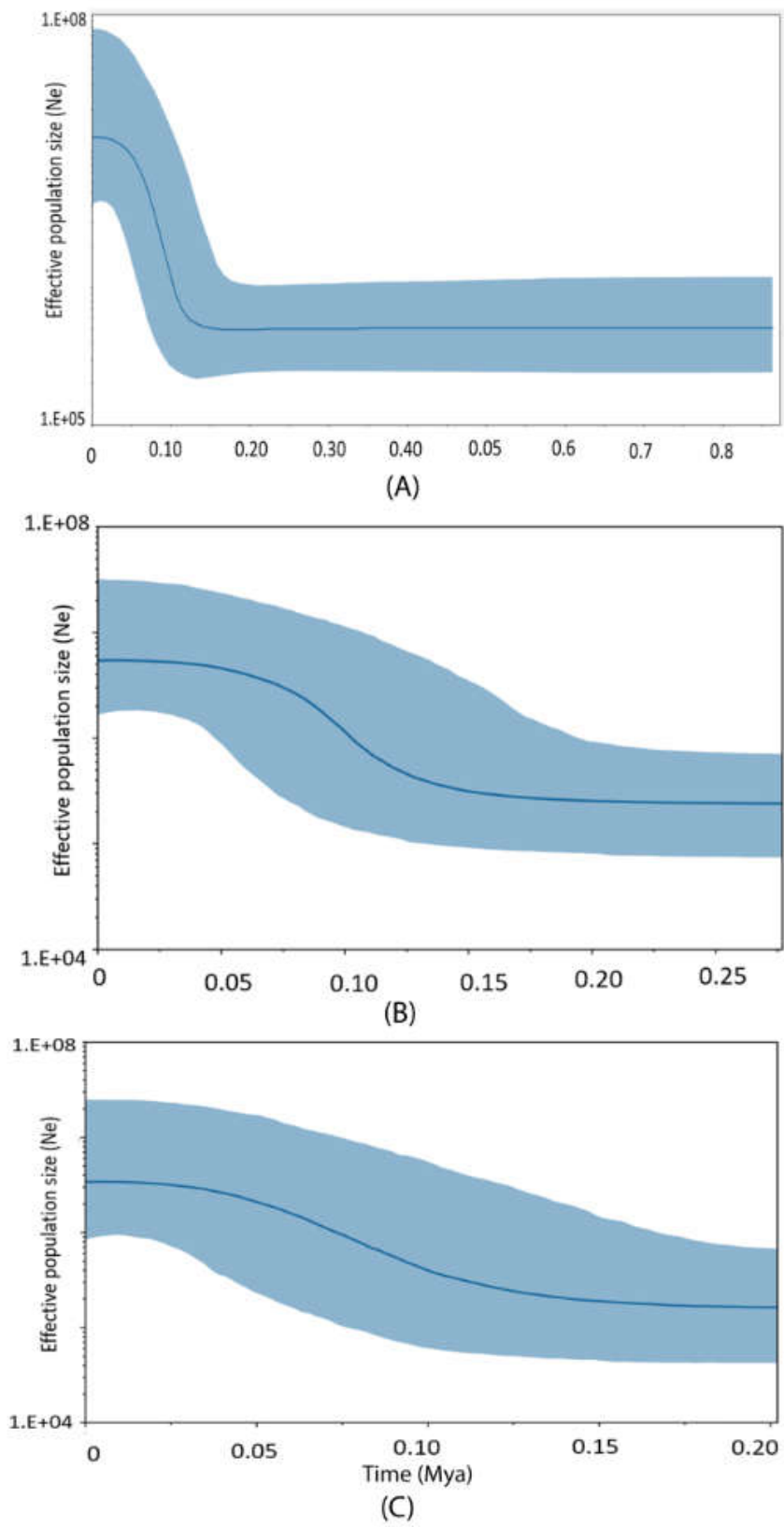


Figure 2-8

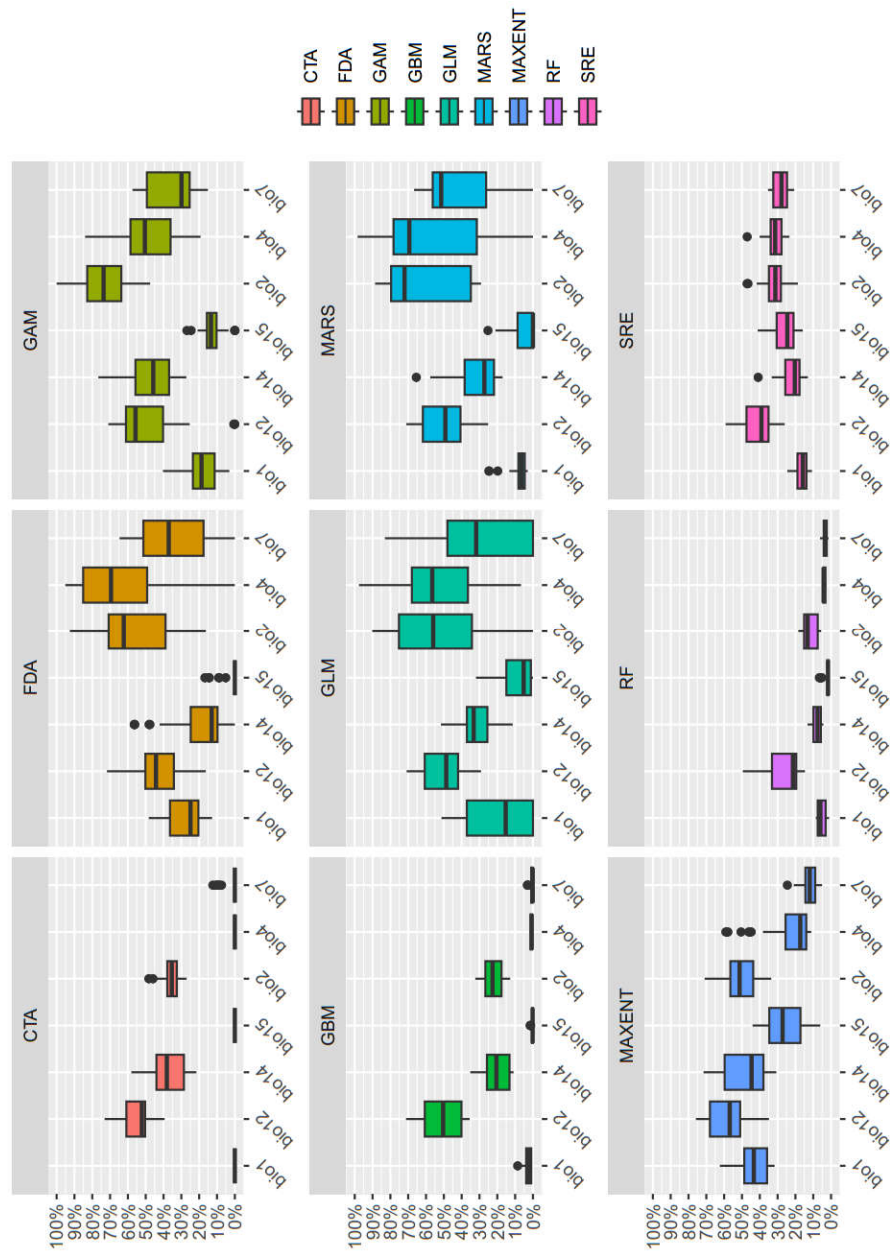


Figure 2-9

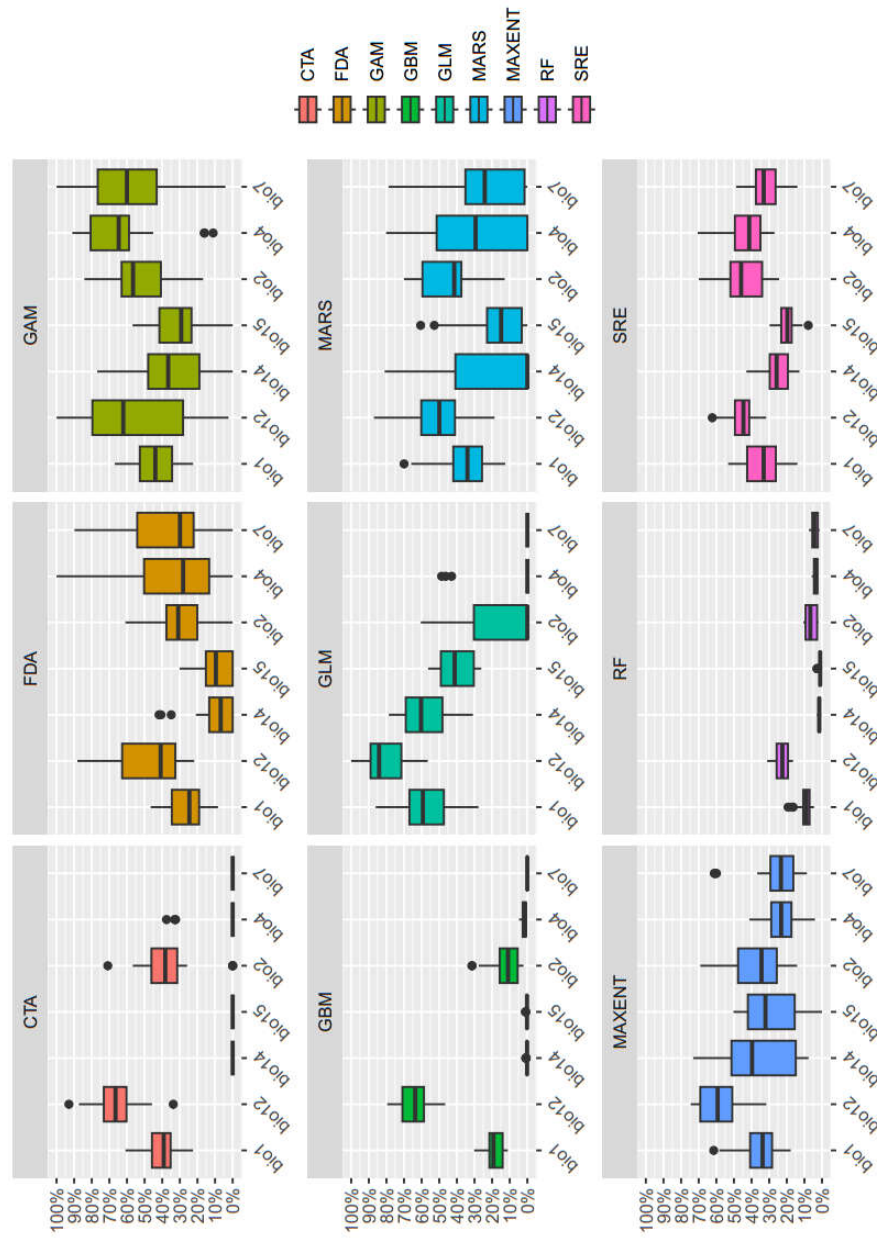


Figure 2-10

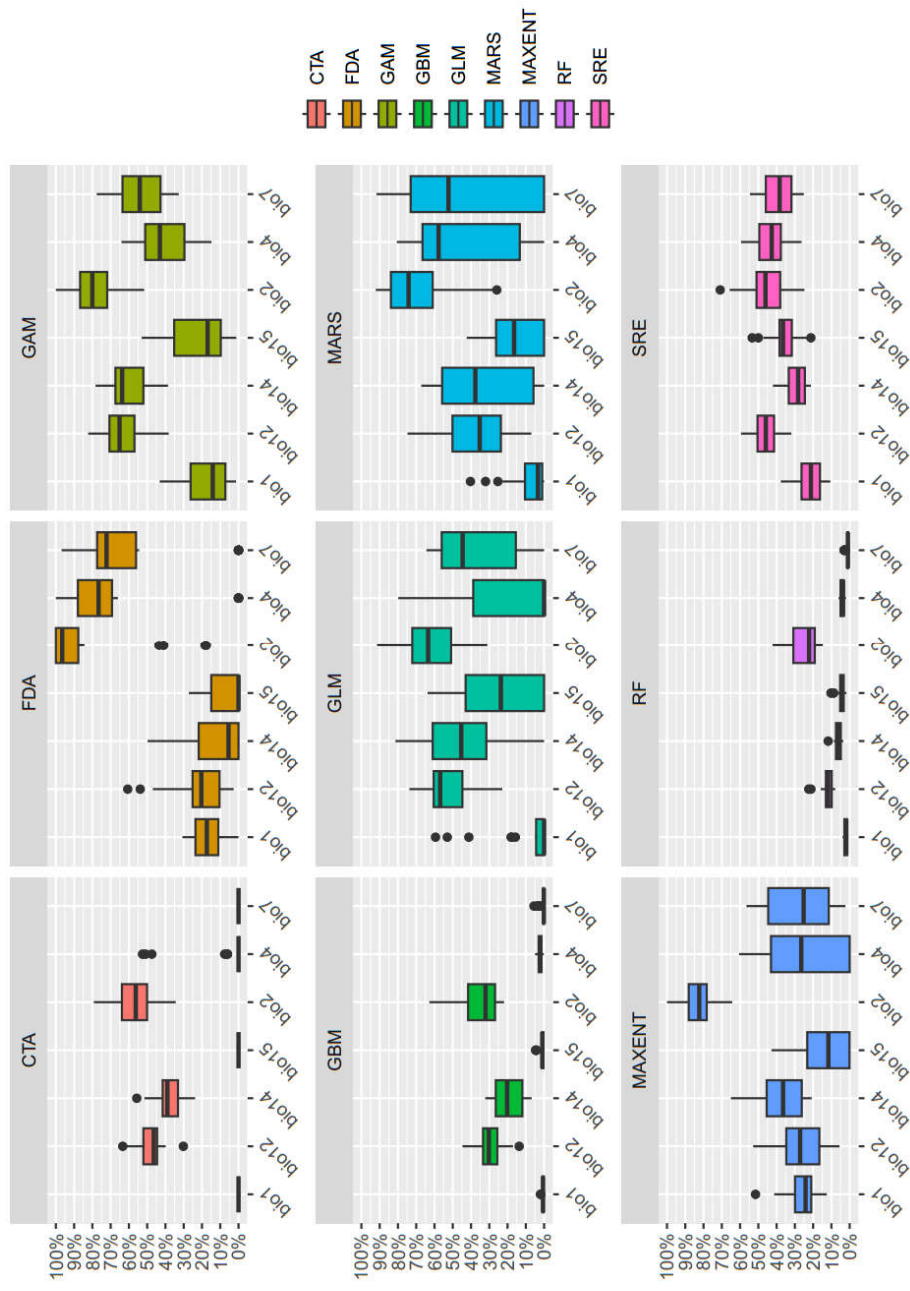


Figure 2-11

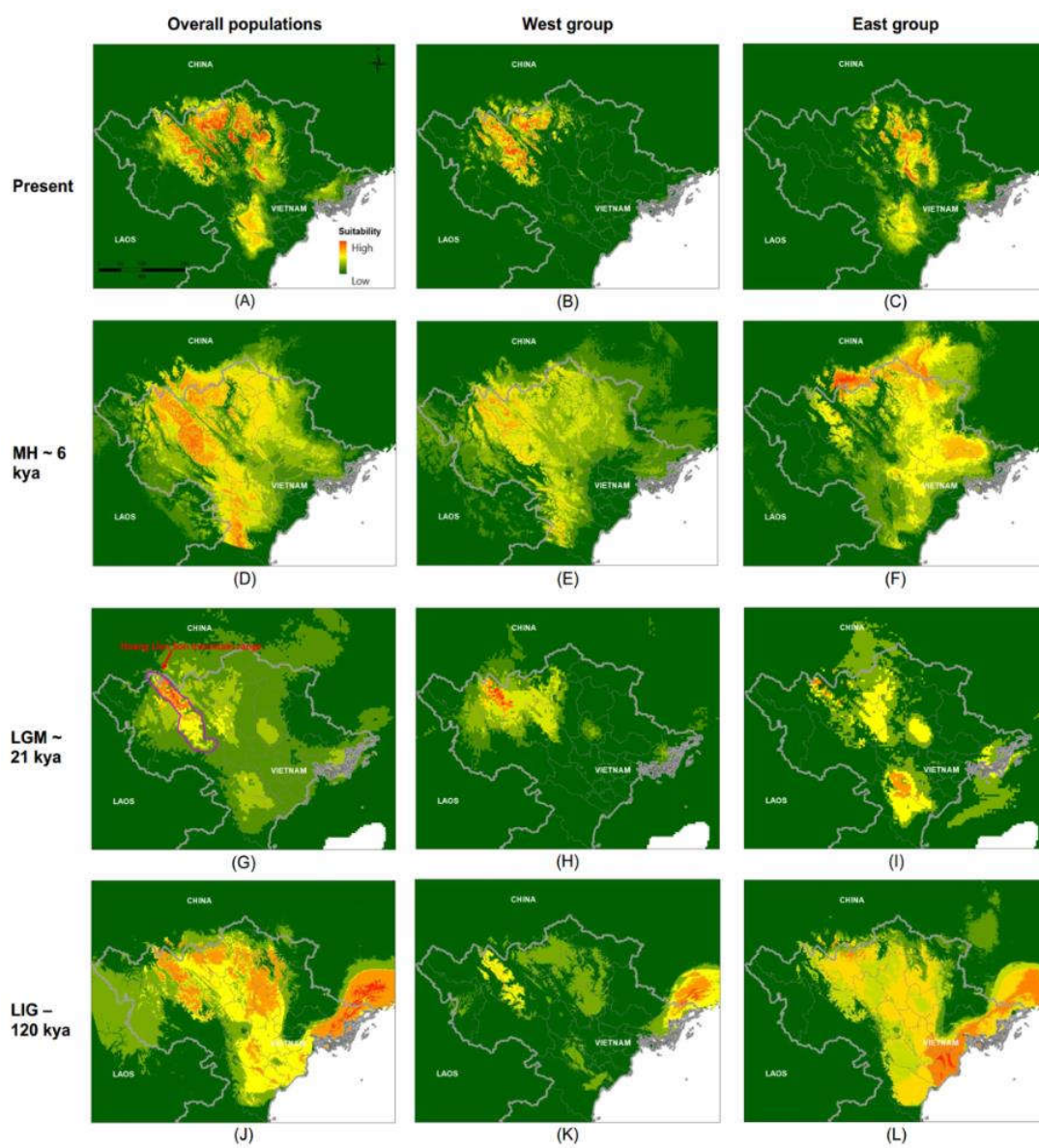


Figure 2-12

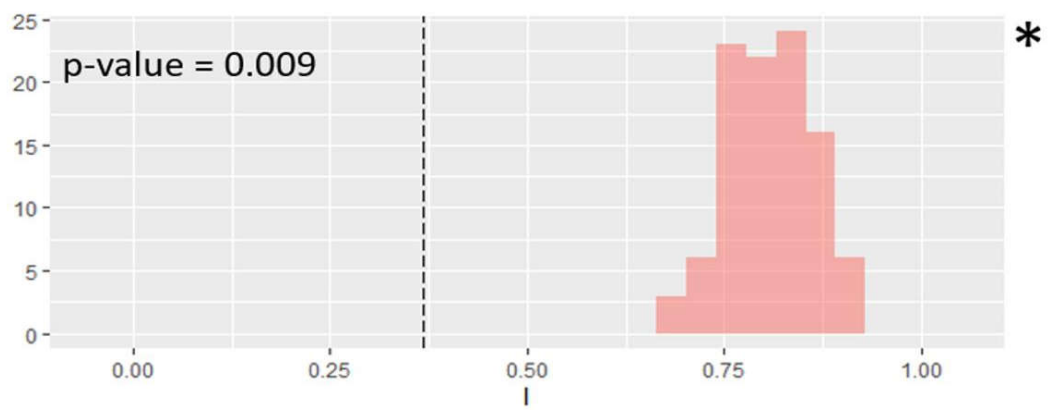
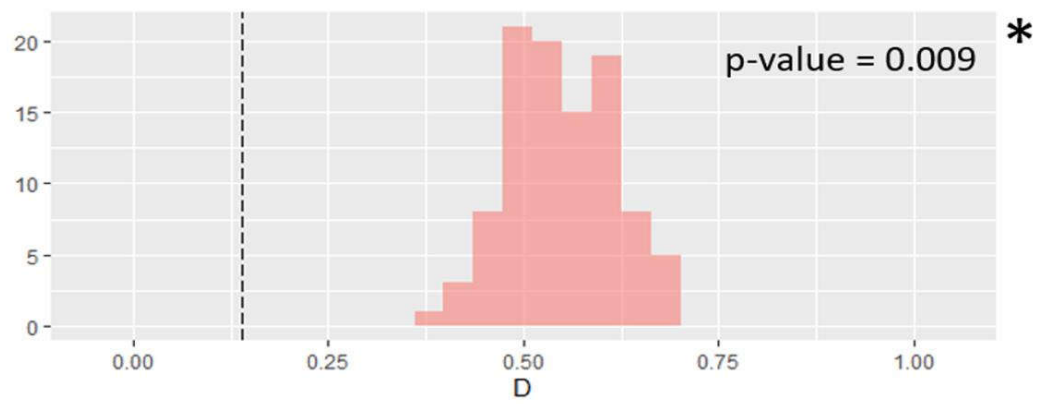


Figure 2-13

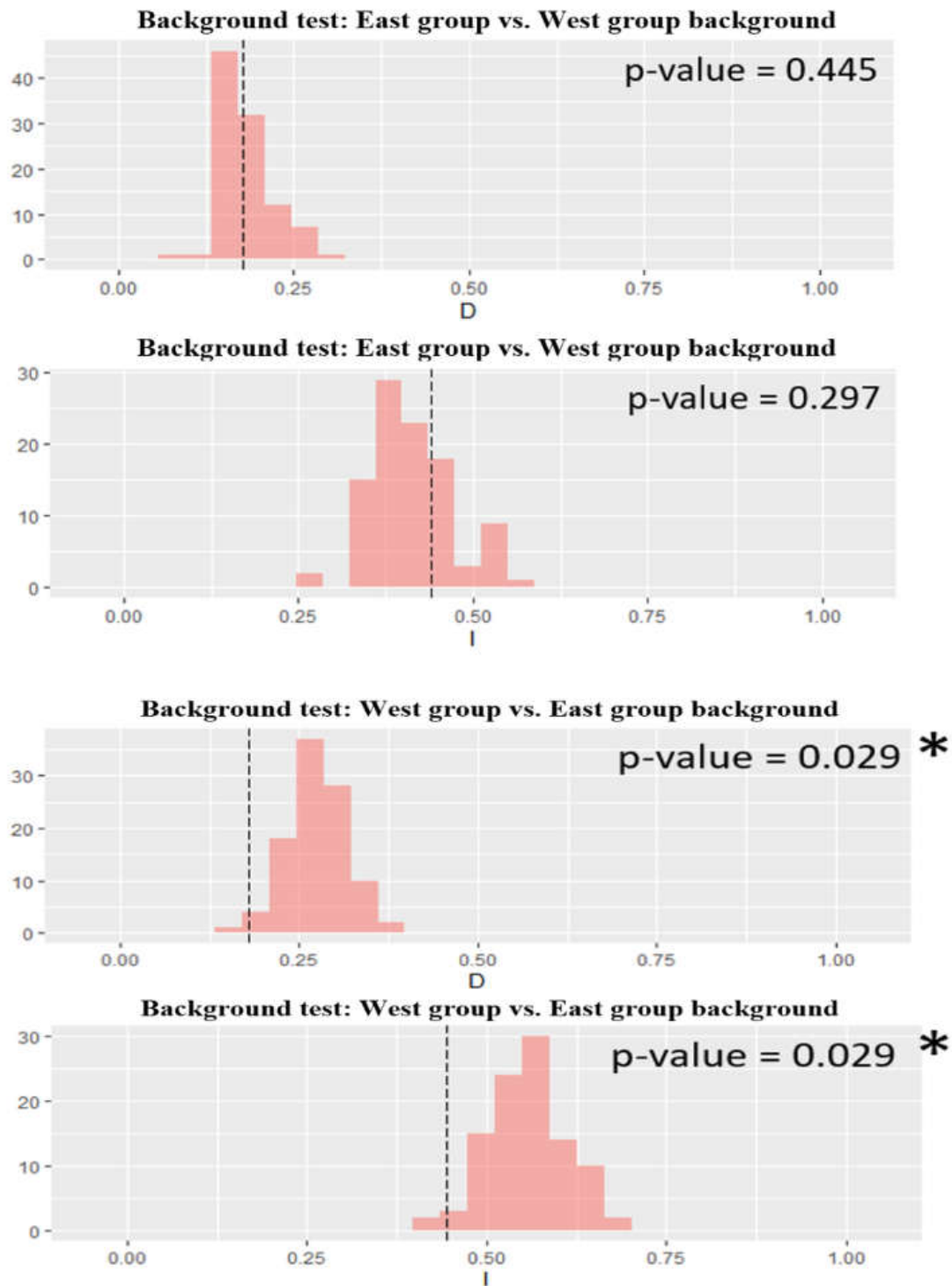


Figure 2-14

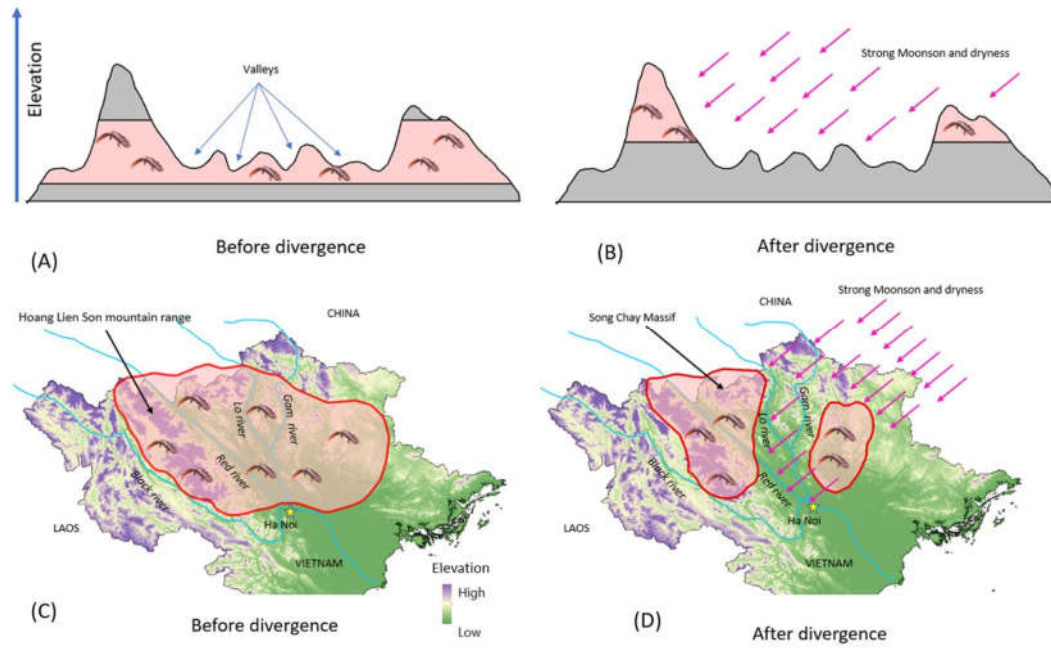
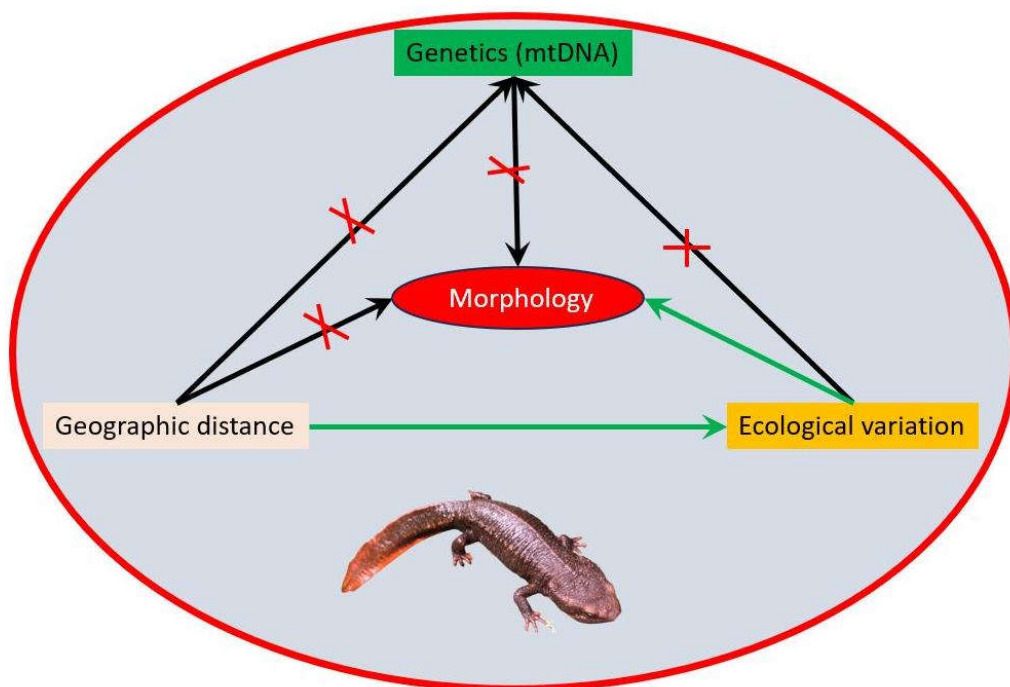


Figure 2-15

CHAPTER 3

Ecological niche variation drives morphological differentiation in tropical stream-dwelling newts from Indochina



In chapter 2, I found genetic variation among populations of Asian warty newts in northern Vietnam through mtDNA analyses. In the chapter, I will examine the morphological and ecological differentiation between the populations.

3-1. Introduction

Morphological variations in organisms can be attributed to several factors other than genetic differentiation (Relethford, 2004; Wright, 1943), including local adaptation (Williams, 2018) and the combination of genetic differentiation and local adaptation (Colombelli-Négrel, 2016). Spatial variation in selection pressure can lead to local adaptation and geographic differences, particularly when gene flow between populations is restricted (Mayr, 1970). However, the divergence of morphological and other phenotypic traits is not always a direct consequence of intra- and interspecific genetic differences (Ujvari et al., 2015). Conversely, local adaptation represents a process whereby each local population evolves specific traits that confer a fitness advantage in response to the prevailing environmental conditions in their respective habitats (Kawecki & Ebert, 2004). Morphological adaptation may also manifest through ecological specialization (Mammola et al., 2020). An exploration of the causes that underlie morphological variation is important for gaining insights into the evolutionary histories of species and their effects on geographic and ecological patterns.

Considering their extensive evolutionary timelines, limited dispersal capabilities, and pronounced environmental constraints, urodeles serve as valuable models for exploring evolutionary history and the relationships of species with their environment (Vieites et al., 2009). In urodeles, morphological traits are frequently influenced by various environmental and ecological factors (Edgington & Taylor, 2019; Nishikawa et al., 2007; Tominaga et al., 2005). For example, terrestrial plethodontid salamanders have longer limbs in comparison to their aquatic counterparts suggesting that their environments have an important role in shaping morphology (Edgington & Taylor, 2019). Furthermore, multiple studies have demonstrated that salamanders and newts exhibit geographical variations in morphological traits (Nishikawa et al., 2007; Sotiropoulos et al., 2008; Šunje et al., 2019; Tominaga et al., 2005). *Paramesotriton*, a genus of Asian warty newts within the family Salamandridae, consists of 15 species distributed in the mountain streams and rivers of southern China and northern Vietnam (Frost, 2023). However, to date, no study has evaluated geographic morphological variation within this genus.

The Species Distribution Modeling (SDM) approach utilizes environmental predictors to determine a potential species distribution based on known occurrence locations (Guisan & Thuiller, 2005). This approach is a powerful tool for predicting the distribution of several organisms, including salamanders and newts (e.g., Luo et al., 2022; Niwa et al.,

2022; Tran et al., 2021; Vaissi, 2021). However, the optimal selection for modeling at a particular taxonomic level remains unclear because the prevalent practice of using species level as the primary unit often overlooks potential intraspecific variations (Smith et al., 2019). It has become increasingly evident that populations of the same species inhabiting geographically and ecologically distinct environments can undergo local adaptation, leading to niche differentiation (Peñalver-Alcázar et al., 2021; Smith et al., 2019; Zhang et al., 2021). Thus, the modeling of ecological niches below the species level can provide more accurate and reliable results concerning species distribution models (Bayliss et al., 2022; Peñalver-Alcázar et al., 2021; Song & Li, 2023).

Here, I examined morphological and ecological variations within two related *Paramesotriton* species (*Paramesotriton deloustali* and *P. guangxiensis*) to assess both intra- and interspecific variation and identify the key factors shaping their morphological characteristics. First, I compared morphological characteristics and the level of differentiation among five populations of these species. Second, I predicted the ecological niches of each group and examined overlaps between the studied groups. Finally, I explored the relationship between morphological differentiation and environmental niche overlap to determine whether local adaptation could explain the observed morphological differences.

3-2. Materials and methods

Study species

Paramesotriton deloustali and *P. guangxiensis* are allopatrically distributed in southern China and northern Vietnam, displaying a preference for cool montane streams within closed-canopy evergreen forest habitats (Sparreboom, 2014). The Vietnam warty newt (*P. deloustali*) was initially identified in Tam Dao National Park and has since been observed in more than 10 regions across northern Vietnam (Sparreboom, 2014; Tran et al., 2023). Conversely, the Guangxi warty newt (*P. guangxiensis*) was first described by Huang et al. (1983) based on a holotype collected from Guangxi Zhuang Autonomous Region, China (hereafter, GX). In Vietnam, the species distribution was suggested to include Cao Bang and Quang Ninh provinces (Tran et al., 2023) (hereafter, CB and QN, respectively). Based on mitochondrial DNA (mtDNA) analyses, Tran et al. (2023) suggested that *P. deloustali* consists of two major groups: West and East. The populations of *P. guangxiensis* in CB and QN are genetically isolated from the populations in GX (Tran et al., 2023). *Paramesotriton deloustali* and *P. guangxiensis* exhibit relatively similar morphological and ecological traits despite their allopatric distribution. Therefore, these species offer a valuable opportunity to explore the mechanisms that underlie morphological adaptation to the local environment.

Morphological analysis

I examined 251 adults from nine populations (Pops. 1–9) of *P. deloustali* and *P. guangxiensis*. Pops. 1–8 were collected during field surveys conducted in 2019–2022, whereas data for Pop. 9 from China were obtained from the work of Huang et al. (1983). Based on the molecular analysis by Tran et al. (2023), I subdivided the populations into five groups (Figure 3-1, Table 3-1). Morphological measurements were directly carried out in the field, and the newts were subsequently released at the respective sites of capture. I measured the snout-vent length (SVL) from the snout tip to the posterior tip of the vent, head length (HL), maximum head width (MXHW), internarial distance (IND; minimum distance between external nares), interorbital distance (IOD; minimum distance between upper eyelids), axilla-groin distance (AGD; minimum distance between the axilla and groin), tail length (TAL), vent length (VL), maximum tail height (MXTAH) at the highest point, forelimb length (FLL), and hindlimb length (HLL). All measurements were recorded to the nearest 0.1 mm using digital calipers (Mitutoyo, Japan). I selected characteristics that covered the overall sizes of newts and could be conveniently measured during field surveys. Newt sex and maturity were identified by observing the swollen cloaca and body size, respectively.

For univariate analyses, I used SVL and its ratios with other morphometric characteristics to compare morphological differentiation (Table 3-2). Sexual differences in SVL were examined using Student's t-test, while character ratios were analyzed using the Mann-Whitney U test. I examined sexual variation through an analysis of covariance (ANCOVA), employing SVL as a co-variable to control for other characteristics. I also computed the sexual dimorphism index (SDI; Gibbon & Lovich, 1990) for each species by dividing the mean SVL of the larger sex by the mean SVL of the smaller sex and subsequently subtracting 1 ($\text{mean_SVL}_{\text{large}}/\text{mean_SVL}_{\text{small}} - 1$). For geographic variation, differences in SVL were analyzed using one-way analysis of variance with the Tukey-Kramer test. The Kruskal-Wallis test with Dunn's multiple comparisons test was used to analyze character ratios according to SVL. For multivariate analyses, I used log-transformed SVL and character ratios as input data. I utilized linear discriminant analysis (LDA) to examine morphological similarities and differences based on pairwise comparisons between groups, then to project multivariate data as a single dimension for maximal separation between groups (Hammer & Harper, 2006). In each group, separate analyses were conducted for males and females because of sexual size dimorphism in both examined species (see Results). P-values < 0.05 were considered statistically significant.

Species distribution modelling

Occurrence data

I gathered data regarding the distribution of the two Asian warty newts in northern Vietnam during field surveys in 2019–2022 (Tran et al., 2023). The distribution of warty newts was determined from previous studies (Nguyen, 2015; Pham et al., 2022) and the Global Biodiversity Information Facility (GBIF, 2023). I divided the locations into five groups (West, East, CB, QN, and GX; Figure 3-2) based on mtDNA analyses conducted by Tran et al. (2023). Because of the limited number of *P. guangxiensis* records in the GX group, I used the distribution map from the IUCN Red List (<https://www.iucnredlist.org/species/59459/63873441>) and adopted the methodology recommended by Hu and Jiang (2012) to systematically select occurrence localities within the distribution map. From these localities, I used the spThin package (Aiello-Lammens et al., 2015) to randomly exclude closely placed points within a 1-km diameter, thus preventing autocorrelation of the occurrence data. Considering the relatively small home ranges exhibited by Asian warty newts, I established a criterion of one km to reduce the number of presence locations, i.e., the estimated average home range of the Hong Kong newt (*P. hongkongensis*) is 0.04 hectares (Lau et al., 2017). My selected localities are presented in Figure 3-2.

Environmental predictors

Climate variables were obtained from the WorldClim database with a resolution of 30 arcsecs (version 2.1; Fick & Hijmans, 2017), including 11 and eight layers of temperature and precipitation, respectively. For the topographic variables, I obtained elevation data from the website of EarthEnv (<https://www.earthenv.org/topography>; Amatulli et al., 2018). Asian warty newts typically prefer small streams in broadleaf forests (Sparreboom, 2014). Therefore, I incorporated three land use variables into my model, including the fractions of forest, wetland, and water in each grid cell (Li et al., 2016). To adjust for the human impact, I gathered human footprint projection data (Venter et al., 2016, 2018). To mitigate autocorrelations among climatic variables, I used ENMTools version 1.4.4 (Warren et al., 2010) for calculation of Pearson's correlation coefficient (r). I applied a threshold of $|r| > 0.80$ and retained only one variable from each highly correlated pair. Subsequently, I selected 12 predictors for inclusion in the final models, including annual mean temperature (Bio1); mean diurnal range (Bio2); temperature seasonality (Bio4); temperature annual range (Bio7); annual precipitation (Bio12); precipitation in the driest month (Bio14); precipitation seasonality (Bio15); elevation; human footprint; and the fractions of forest (Forest), wetland (Wetland), and water (Water) in each grid cell.

Species distribution modelling processes

I used the BIOMOD2 package (Thuiller et al., 2016, 2023) in R software (version 4.2-2; R Core Team, 2023) to generate ensemble models of five separate groups of the two Asian warty newts. I utilized the default settings in accordance with recommendations by Guisan et al. (2017). Initially, I conducted nine preliminary analyses based on suggestions by Vaissi (2021). Because presence-absence data are required for most models, except SRE and MAXENT, I randomly created pseudo-absence localities using the BIOMOD_Formatting Data function in the BIOMOD2 package (Thuiller et al., 2016). The number of pseudo-absence points created was threefold higher than the number of presence points. Furthermore, 10 repetitions were performed, as recommended by the *BIOMOD2* team (Thuiller et al., 2023). I divided the data into model calibration (80%) and performance assessment (20%) datasets. This process was repeated four times to prevent bias caused by data splitting (Guisan et al., 2017; Vaissi, 2021). The Cohen's kappa, area under the receiver operating characteristic curve (AUC), and true skill statistic (TSS) were used to evaluate each model's performance. Kappa and TSS values range from -1 to +1, where a value of +1 indicates perfect performance; values of 0.6–0.9 indicate excellent performance (Allouche et al., 2006). AUC values range from 0 to +1, where values of < 0.6, 0.6–0.9, and > 0.9 indicate poor, moderate, and excellent models, respectively (Peterson et al., 2011). Based on the kappa, TSS, and AUC values, I selected five algorithms (FDA, GBM, MARS, MAXENT, and RF) to generate the final ensemble models.

Niche similarity

I used PCA-env (Broennimann et al., 2012) to determine niche similarity between groups of Asian warty newts found in northern Vietnam and south China. This approach was performed in an environmental space (E-Space), and Schoener's D metric (Schoener, 1968) was used to measure environmental niche similarity. The D value (0–1) represents the degree of overlap in environmental space between two populations: 0 indicates no overlap, suggesting that the two populations occupy distinct environmental conditions, and 1 indicates complete overlap, suggesting that the two populations share the same environmental space. PCA-env employs a kernel smoothing method to compute the occurrence density and environmental factor density along environmental (principal component) axes for each cell. By measuring the densities of occurrences and environmental variables, PCA-env quantifies the level of niche overlap along these axes (Broennimann et al., 2012). The niche similarity test framework (Warren et al., 2008) was used to determine whether the ecological niches of pairwise populations could be explained solely by chance. To perform background tests, I used a background delimited by a minimum convex polygon with a 50-km buffer zone, similar to the method of Hu et al. (2016). When the calculated D value was within the 95% density range of simulated values, the observed niche overlap was not significantly different from the niche overlap explained by chance. In this instance, the

null hypothesis of niche equivalency was not rejected. I performed 100 repetitions of the similarity test to ensure that the null hypothesis was rejected with a high level of confidence. Statistical tests were performed using the *ecospat* package (Di-Cola et al., 2017) in R software (version 4.2-2; R Core Team, 2023).

Relationship between morphological differentiation and ecological niche variation

I used Mahalanobis distance, calculated from the SVL and character ratios in R software (version 4.2-2; R Core Team, 2023), to represent morphometric differentiation among the groups (Nishikawa et al., 2007; Tominaga et al., 2005). The observed niche overlap (D) determined from the similarity test (Warren et al., 2008) was used to represent ecological niche similarity. Geographic distances were measured with the shortest distance between the minimum convex polygon of the presence localities of each group using ArcMap version 10.7 (ESRI). Genetic distances were calculated from the mtDNA sequences reported by Tran et al. (2023), using MEGA software version 7.0 (Kumar et al., 2016). I used the generalized linear model (GLM) to examine the relationships of groupwise morphometric differentiation with ecological niche similarity, geographic distance, and genetic distance. In particular, morphological differentiation was regarded as a response term, whereas ecological niche similarity, geographic distance, and genetic distance were regarded as explanatory terms. Statistical analyses were performed using R software (version 4.2-2; R Core Team, 2023). P-values < 0.05 were considered indicative of statistical significance.

3-3. Results

Morphometric variation

Sexual size dimorphism

My analysis of *P. deloustali* and *P. guangxiensis* showed that, compared with males, females had a significantly larger SVL (Student's t-test, $p < 0.001$ and $p < 0.01$, respectively). The SDI was 0.072 in *P. deloustali* and 0.055 in *P. guangxiensis*, respectively, indicating a tendency towards a female-biased dimorphism. Additionally, the relative TAL and SVL (R_TAL) was higher in females than in males (Mann-Whitney U test, $p < 0.001$ and $p < 0.01$, respectively, Figure 3-4). Conversely, the R_IOD, R_IND, R_VL, and R_MXTAH were significantly higher in males than in females (Mann-Whitney U test, $p < 0.05$). I detected no significant differences in the R_HL, R_MXHW, R_AGD, R_FLL, or R_HLL between females and males (Mann-Whitney U test, $p > 0.05$; Table 3-3A, B and Figure 3-5). The findings were further corroborated by the results obtained from ANCOVA (Table 3-3C).

Morphological differentiation between *P. deloustali* and *P. guangxiensis*

Both sexes of *P. deloustali* were larger than its congener *P. guangxiensis* (SVL: $p < 0.001$; Table 3-4 and Figure 3-6). I also found significant differences between *P. deloustali* and *P. guangxiensis* in terms of R_HL, R_AGD, R_VL, and R_TAL in females, and R_HL, R_IND, R_AGD, R_VL, R_MXTAH, and R_HLL in males (Table 3-4 and Figure 3-6).

Geographic variation

SVL: Among the five examined groups, the mean SVL significantly varied, ranging from 70.1 to 83.7 mm in females and 64.6 to 78.7 mm in males (one-way analysis of variance, $p < 0.001$). Among females, the East group had the largest mean SVL (Table 3-5). Conversely, the QN group had a significantly smaller mean SVL than the remaining newt groups. Among males, the East group had a significantly larger body size than the other groups, except for the West and CB groups. Furthermore, the QN group had a significantly smaller body size than the other groups, except for the GX group (Figure 3-3 and Table 3-5).

Character ratios: Among females, the R_HL showed the largest number of significant differences among comparison pairs (4/6), followed by R_TAL (3/6). No significant differences in R_IND were observed among comparison pairs (Kruskal-Wallis test, $p = 0.077$). Among males, R_AGD had the largest number of significant differences among comparison pairs (7/10), followed by R_HL (6/10). The remaining ratios had at least one comparison pair with significant differences (Table 3-5 and Figure 3-7).

LDA: Based on morphological variation, the examined groups were further divided into four groups of females and five groups of males (Figure 3-8 and Table 3-6). The GX group mainly overlapped with the QN group in both males and females. Furthermore, the CB group significantly overlapped with the East group. In females, eigenvalues of the first axis were 1.85 (61.3%), whereas the second axis were 0.91 (30.2%). In the first axis, the highest absolute magnitudes of standardized canonical discriminant coefficients were 37.6 for R_HL, 9.1 for SVL, and 7.5 for R_AGD. Conversely, SVL (32.7), R_FLL (13.1), and R_HLL (8.3) significantly contributed to the second axis. For males, eigenvalues of the first axis represented 2.44 (50.7%), whereas the second axis represented 1.32 (28.7%). In the first axis, the highest absolute magnitudes of the standardized canonical discriminant coefficients were -81.1 for R_MXTAH, 30.2 for SVL, and 27.1 for R_HL. Conversely, R_TAL (65.2), SVL (31.8), and R_MXTAH (-27.2) significantly contributed to the second axis. The LDA confusion matrix confirmed that the groups had predominantly homogeneous morphological variations (Table 3-6).

Ecological niche variation

Suitable distribution

The Ensemble Ecological Niche Models revealed robust performance among the five selected algorithms in projecting the potential distribution of the five groups of *Paramesotriton*; Kappa, TSS, and AUC values were higher than 0.7 (Figure 3-9). The predicted distribution of the West group comprised the Yen Bai and Lao Cai provinces, along with part of Ha Giang province. The predicted distribution of East group consisted of the Vinh Phuc, Thai Nguyen, Tuyen Quang, Bac Kan, and Cao Bang provinces. The predicted distribution of the CB group was concentrated in Cao Bang Province and the northern Bac Kan Province. The southern Quang Ninh Province was predicted to be a suitable distribution for the QN group. The GX group was predicted to be distributed in the northern Quang Ninh Province in Vietnam and southern Guangxi Province in China (Figure 3-10).

The contributions of environmental predictors to the model differed among groups. The top three contributing variables in the West group were Bio12 ($52.7 \pm 21.0\%$), Bio4 ($26.2 \pm 24.1\%$), and Bio2 ($22.5 \pm 18.9\%$). In the East group, the top three contributing variables were Bio2 ($61.22 \pm 24.06\%$), Bio14 ($22.8 \pm 19.9\%$), and Bio12 ($21.1 \pm 15.5\%$). In the CB group, the top three contributing variables were Bio15 ($46.3 \pm 29.7\%$), Bio1 ($23.0 \pm 25.6\%$), and Bio4 ($22.93 \pm 27.1\%$). In the QN group, Bio2 ($56.6 \pm 26.2\%$), Bio15 ($14.5 \pm 15.8\%$), and Bio14 ($13.7 \pm 16.7\%$) were the top three contributing variables. In the GX group, the top three contributing variables were Bio14 ($44.9 \pm 31.1\%$), Bio4 ($39.9 \pm 21.2\%$), and Elevation ($27.4 \pm 34.4\%$) (Figure 3-11).

Ecological niche similarity

Niche similarity was the highest for the comparison of CB and East (0.2848), followed by West and East (0.2281). The niche similarities of the remaining pairs were minimal, ranging from 0.000 to 0.0317 (Figure 3-12).

Among the 10 comparison pairs, the ordination null test for niche similarity in E-space demonstrated that niches were more similar than random in two reciprocal directions for the comparison of CB and East (Figure 3-12). For the remaining pairs, the observed niche overlap values were within 95% of the confidence limits of the null distribution, suggesting that the ecological differences between groups were not explained by environmental differences. The results also demonstrated environmental heterogeneity between environmental factors across the groups.

Relationship between morphological differentiation and ecological niche variation

In the GLM, morphological differentiation among the analyzed populations was adequately explained by ecological niche similarity, geographic distance, and genetic distance ($R^2 = 0.639$). Ecological niche similarity was a significant explanatory term for the morphological differentiation of newt populations (coefficient = -9.251 , $t = -2.919$, $p = 0.027$). Conversely, geographic distance and genetic distance were not significant explanatory terms (coefficient = -0.006 , $t = -1.294$, $p = 0.243$ and coefficient = -6.423 , $t = -0.712$, $p = 0.503$, respectively). I also found a significant negative correlation between morphological differentiation and niche similarity across the studied populations (Pearson's correlation coefficient: $r = -0.671$, $p = 0.033$; Figure 3-13A and Table 3-7). The correlations between morphological differentiation and both geographic distance and genetic distance were not significant ($p > 0.05$; Figure 3-13B, C).

My analysis revealed a significant negative correlation between ecological niche similarity and geographic distance in the comparison pairs (Pearson's correlation coefficient: $r = -0.653$, $p = 0.040$; Figure 3-13D), but not between ecological niche similarity and genetic distance ($p > 0.05$; Figure 3-13E). Furthermore, the genetic distance was not significantly correlated with geographic distance ($p > 0.05$, Figure 3-13F).

3-4. Discussion

Sexual size dimorphism

My analyses revealed sexual dimorphism regarding the sizes of *P. deloustali* and *P. guangxiensis*, with females exhibiting a significantly larger body size than males. This trend is consistent with previous findings concerning female-biased sexual size dimorphism in urodeles (Amat, 2019; Nishikawa et al., 2007; Salvidio & Bruce, 2006; Shine, 1979; Tominaga et al., 2005) and the family Salamandridae (Altunışık, 2023; Malmgren & Thollessen, 1999; Romer, 1951). The larger body size in females can be explained by female fecundity (Shine, 1979) and an increased clutch size (Nishikawa et al., 2007). Similar to other newts, males of both *P. deloustali* and *P. guangxiensis* can be identified from females by the large size of cloaca. The male cloaca serves as a source for producing material for spermatophores and releasing courtship pheromones (Kühnel et al., 2010; Reinhard et al., 2015). In addition, the shape of the cloaca contributes to increasing male reproductive success (Sever, 2003).

Geographic variation in morphology

Several studies have shown that salamanders and newts exhibit geographic variation in terms of morphological characteristics, for example, in the genera *Hynobius* (Matsui et al., 2009; Nishikawa et al., 2007; Tominaga et al., 2005), *Echinotriton* (Ozaki et al., 2023),

Salamandra (Šunje et al., 2019), *Neurergus* (Rastegar-Pouyani et al., 2005), *Triturus* (Arntzen & Wallis, 1999), and *Mesotriton* (Sotiropoulos et al., 2008). Asian warty newts within the genus *Paramesotriton* are widely distributed in China and northern Vietnam; however, the geographic variation in their morphology has not been extensively studied. My analyses revealed that *P. deloustali* has a larger body size than *P. guangxiensis*. This result is consistent with the finding by Sparreboom (2014) that *P. guangxiensis* resembles a smaller *P. deloustali*.

The morphological characteristics of the West and East groups of *P. deloustali* showed significant differences in terms of SVL, R_HL, R_AGD, and R_FLL in females, and R_HL, R_AGD, R_MXTAH, and R_FLL in males. The East group had larger morphological parameters than the West group. The two groups exhibited genetic diversity at around 1.92 Mya, primarily influenced by historical climate change events, along with differences in historical demography and distributional dynamics (Tran et al., 2023). Additionally, the ordination technique in E-space showed significant differences in ecological niches between the West and East groups. Morphological variables may serve as indications of adaptations to environmental variations across their distribution regions (Huang et al., 2017). Furthermore, morphological variation can occur through diverse mechanisms, including environmental influences, phylogenetic diversification, or their combination (Colombelli-Négrel, 2016; Relethford, 2004; Williams, 2018). I did not detect any significant relationship between the genetic distance from mtDNA and the morphological variation of the examined populations, possibly indicating a minor role of genetics in shaping morphology. Therefore, the morphological variation of the West and East groups is likely driven by ecological niche diversification, or a complex interaction of ecology and phylogeny, similar to other urodeles (Bendik et al., 2013; Huang et al., 2017).

Among the three groups of *P. guangxiensis*, both sexes in the CB group exhibited distinct morphology compared with the other groups (GX and QN). Additionally, the morphological characteristics of the GX group were similar to those of the QN group, as revealed by LDA. I found that geographic distance may indirectly influence morphology through ecological variation (see Figure 3-14). Therefore, the morphology of *P. guangxiensis* groups is likely attributable to the geographic distance. The distance from the CB to GX and QN groups was significantly greater than the distance from the QN to GX groups (Figure 3-1). Similarity tests suggested a significant niche differentiation among the groups. However, mtDNA analyses revealed that the CB and QN groups exhibited a closer relationship than the CB and GX groups and the QN and GX groups (Tran et al., 2023). The morphology of the CB group was relatively similar to that of the East group, despite belonging to different species (*P. guangxiensis* and *P. deloustali*, respectively) (Tran et al., 2023). Based on these findings, I recommend examining nuclear DNA to identify hybridization between the two groups. The congruence between morphological

characteristics and genetic relationships can be disrupted by rapid natural selection in local habitats. My analysis of niche similarities demonstrated that the niches of the CB and East groups were significantly more similar than the null distribution. Additionally, the niche overlap between these two groups was highest among all pairwise comparisons. Nishikawa et al. (2007) also found incongruence between morphological and genetic relationships in the hynobiid salamander *Hynobius boulengeri*. Despite genetic similarities to populations in Kyushu, the Amakusa and Osumi populations (now described as new species; see Nishikawa & Matsui, 2014) exhibited morphological similarities to the Honshu population (*H. boulengeri* sensu stricto), indicating different rates of evolution among populations, possibly because of local selection by environmental factors (Nishikawa et al., 2007).

Ecological niche variation

Traditionally, species distribution modeling has most commonly been conducted at the species level without considering intraspecies variation (Peñalver-Alcázar et al., 2021; Smith et al., 2019). However, the responses of species to environmental changes are significantly influenced by intraspecies variation and local adaptation (DeMarche et al., 2019; Song & Li, 2023; Zhang et al., 2021). Several recent studies have demonstrated that intraspecies genetic variation can provide better and more realizable outputs of species distribution modeling (Nielsen et al., 2021; Peñalver-Alcázar et al., 2021; Song & Li, 2023; Zhang et al., 2021). In the present study, five groups of the two Asian warty newt species from northern Vietnam and southern China were modeled on the basis of mtDNA analyses (Tran et al., 2023). The high kappa, TSS, and AUC values obtained from individual models used in the final ensemble model demonstrated excellent performance in terms of generating potential distributions for both *P. deloustali* and *P. guangxiensis*. Additionally, the ecological niches of the groups were distinctive and covered various geographic distributions, indicating local groupwise adaptations. Therefore, local adaptation can be used at the species level to predict newt distribution.

The distribution patterns of ectothermic species are strongly influenced by climate, considering the significant correlation between body and ambient temperatures (Buckley & Jetz, 2007; Hu & Jiang, 2018; Werner et al., 2013). Most salamanders prefer a cooler climate, including my two study species, which are expected to occur in tropical forests with specifically suitable temperatures and humidity. Ecological niche models indicated that the key contributing variables differed among groups, especially the main contributors were climatic parameters. In *P. deloustali*, Bio12 (annual precipitation) was the top contributing variable in the model of West group, whereas Bio2 (mean diurnal range) was primary contributor in the model East group. I also incorporated variables related to land use, land cover (forest), topography (elevation), and human footprint in the models. Nevertheless, the

results for my models were consistent with the findings of Tran et al. (2023) in a study that only used climate variables. In *P. guangxiensis*, the top contributing variables in the CB, QN, and GX groups were also climatic factors, including Bio15 (precipitation seasonality), Bio2 (mean diurnal range), and Bio14 (precipitation of driest month), respectively. My models suggested that climate plays a key role in determining the distribution of Asian warty newts. These results emphasize the potential impact of ongoing climate changes on tropical amphibians, particularly considering Vietnam's vulnerability to the effects of climate change (World Bank, 2022). Several recent studies have demonstrated that the distributions of amphibians and reptiles in northern Vietnam and southern China may undergo significant reductions or shifts due to climate change effects (e.g., Duan et al., 2016; Luo et al., 2021; Ngo et al., 2022).

My predicted distributions of the groups were allopatric and distinctive, except for the East and CB groups. Although similarity tests in E-space did not reject the null distribution and failed to identify significant similarity or differentiation characteristics, the clear distinctness of the niches indicates that the groups were adapted to local environments. Mitochondrial DNA analysis revealed that the CB and East groups were distinctive (Tran et al., 2023). However, the predicted distribution of the CB group significantly overlapped with the predicted distribution of the East group; the similarity test revealed greater niche similarity than would be expected by chance, probably creating a secondary contact between the two species (East: *P. deloustali* vs. CB: *P. guangxiensis*). Secondary contact can occur between different taxa when their ecological niches exhibit similarity (Hu et al., 2016). My results were supported by the close geographic distribution of the species, as indicated by the significant negative correlation between ecological niche overlap and geographic distance. Additionally, hybridization often occurs because of secondary overlap between two taxa after genetic isolation (Bett et al., 2012; Hu et al., 2016). Ecological selection can lead to the genetic introgression of the nuclear genome (Seixas et al., 2018). Thus, ecological similarity probably led to the introgression of nuclear DNA (but not mtDNA) between the East and CB groups through secondary contact, as suggested by my preliminary study (Tran Van Dung, unpublished data).

Relationship between morphological differentiation and ecological niche variation

In vertebrates, environmental adaptation can occur through diverse mechanisms, including alterations in social organization, behavior, and morphology at different levels (Freudiger et al., 2021). Several taxa exhibit substantial morphological distinctions among populations, primarily influenced by local adaptation (Álvarez et al., 2013; DeWoody et al., 2015; Edgington & Taylor, 2019). As ectothermic animals, amphibians experience significantly greater influence from local environmental conditions, compared with

endotherms, leading to variations in morphology (Lynch, 1981; Nishikawa et al., 2007; Tominaga et al., 2005). My analysis demonstrated a significant relationship between two species of Asian warty newts with similar ecological niches: *P. deloustali* and *P. guangxiensis*. A significant negative correlation was observed between morphological differentiation and ecological niche similarity between the groups. As ecological niche distinction increased, there was a tendency toward greater morphological differentiation, suggesting that newt morphology varied according to environmental parameters. Ecological niche variation is likely to influence overall morphological differentiation of newts, rather than independently acting on individual characteristics. The lack of significant effects on individual morphological traits among the studied groups can be attributed to the taxonomic resolution used at sub-species levels, which contrasts with the broader taxonomic scale used by Edgington and Taylor (2019) in the analysis of plethodontid salamanders.

Furthermore, I did not observe any significant relationships among morphological differentiation, genetic, and geographic distance. Correlations between genetic distance, ecological niche similarity, and geographic distance also were not significant. However, I found a significant negative relationship between ecological niche similarity and geographic distance, suggesting that geographic distance may not directly impact the morphological differentiation of newts, although it can exert an indirect influence through its association with ecological habitats. The lack of a significant relationship between genetic and geographic distances of newts can be attributed to the presence of heterogeneous habitats and physical barriers among groups (Antunes et al., 2021; Goudarzi et al., 2019) in the study areas and the low dispersal capacity in urodeles (Matsui et al., 2006; Wells, 2007). Morphological differences were not solely caused by limited gene flow and genetic drift (Freudiger et al., 2021); but they were also influenced by ecological selection and potentially local adaptation (Conover et al., 2006).

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Table 3-1. Population number (Pop), species and sample size of two Asian warty newts (*P. deloustali* and *P. guangxiensis*) used for morphological analyses.

Pop	Species	Location	Group	Male	Female	Total	Latitude	Longitude	Altitude (m)
1	<i>P. deloustali</i>	Bac Ha, Lao Cai	West	7	8	15	22.47	104.38	948
2	<i>P. deloustali</i>	Xin Man, Ha Giang		11	8	19	22.49	104.46	893
3	<i>P. deloustali</i>	Mu Cang Chai, Yen Bai		16	15	31	21.82	104.33	1584
4	<i>P. deloustali</i>	Van Yen, Yen Bai,		14	12	26	21.87	104.50	636
5	<i>P. guangxiensis</i>	Nguyen Binh, Cao Bang	CB	31	32	63	22.59	105.85	857
6	<i>P. deloustali</i>	Bach Thong, Bac Kan	East	5	7	12	22.26	105.78	722
7	<i>P. deloustali</i>	Tam Dao, Vinh Phuc		19	32	51	21.47	105.60	263
8	<i>P. guangxiensis</i>	Ha Long, Quang Ninh	QN	14	10	24	21.25	107.04	100
9	<i>P. guangxiensis</i>	Guangxi, China	GX	9	1	10	-	-	-
Total				126	125	251			

Table 3-2. Means $\pm$ SD of SVL (in mm), medians of ratios (R) of a metric character (% SVL) of five groups of two Asian warty newts (*P.*

***deloustali* and *P. guangxiensis*) (F: Female; M: Male)**

Species Group	<i>P. deloustali</i>						<i>P. guangxiensis</i>					
	West			East			CB			QN		
	F	M		F	M		F	M		F	M	GX
Number of samples	44	47		39	24		32	31		10	14	9
SVL $\pm$ SD	77.85 $\pm$ 5.91	75.26 $\pm$ 4.48		83.69 $\pm$ 5.56	78.67 $\pm$ 6.27		77.85 $\pm$ 5.93	75.22 $\pm$ 4.78		70.05 $\pm$ 5.82	64.63 $\pm$ 5.98	71 71.86 $\pm$ 2.14
R_HL	27.41	28.27		29.45	30.9		34.22	35.13		29.32	31.08	29.85 30.42
R_MXHW	22.17	24.55		24.53	25.4		24.55	25.79		22.44	23.7	22.67 23.42
R_IOD	14.2	15.21		14.51	15.82		14.62	16.46		12.93	13.71	n/a n/a
R_IND	6.35	6.96		6.48	6.87		6.93	8.18		6.34	6.65	5.91 6.55
R_AGD	48.34	46.76		50.3	50.02		51.24	51.31		51.71	50.58	41.54 41.09
R_VL	7.79	9.99		8.57	11.95		10.29	13.92		8.5	11.46	n/a n/a
R_TAL	96.67	89.27		96.27	93.34		103.3	94.82		90.93	85.23	89.01 83.35
R_MXTAH	17.84	19.47		18.81	21.43		18.93	21.9		17.47	19.93	17.6 20.49
R_FLL	28.95	29.91		30.91	32.98		29.94	30.16		30.68	30.5	29.57 29.44
R_HLL	31.41	32.71		33.42	33.89		31.99	31.31		28.6	31.89	28.03 29.71

Table 3-3. The comparison statistics of morphological characteristics between female and male two Asian warty newts (*P. deloustali* and *P. guangxiensis*).

(Note: The population GX was not in the comparison)

A. P. deloustali

Character	Type for test	F (t/U)	P-value	Significant or ns	Female vs. Male
SVL	t-test student	6.031	<0.001	significant	Larger
R HL	Mann-whitney	2425	0.059	ns	
R MXHW	Mann-whitney	2509	0.113	ns	
R IOD	Mann-whitney	1786	<0.001	significant	Smaller
R IND	Mann-whitney	1927	<0.001	significant	Smaller
R AGD	Mann-whitney	2464	0.08	ns	
R VL	Mann-whitney	1323	<0.001	significant	Smaller
R TAL	Mann-whitney	1683	<0.001	significant	Larger
R MXTAH	Mann-whitney	1550	<0.001	significant	Smaller
R FLL	Mann-whitney	2463	0.08	ns	
R HLL	Mann-whitney	2460	0.05	ns	

B. P. guangxiensis

Character	Type for test	F (t/U)	P-value	Significant or ns	Female vs. Male
SVL	t-test student	2.734	<0.008	significant	Larger
R HL	Mann-whitney	760.0	0.116	ns	
R MXHW	Mann-whitney	660.0	0.015	significant	Smaller
R IOD	Mann-whitney	592	0.003	significant	Smaller
R IND	Mann-whitney	556	0.001	significant	Smaller
R AGD	Mann-whitney	927	0.878	ns	
R VL	Mann-whitney	220	<0.001	significant	Smaller
R TAL	Mann-whitney	599	0.003	significant	Larger
R MXTAH	Mann-whitney	420	<0.001	significant	Smaller
R FLL	Mann-whitney	871	0.530	ns	
R HLL	Mann-whitney	804	0.231	ns	

C. The Analysis of sexual size dimorphism in *P. deloustali* and *P. guangxiensis* using ANCOVA with SVL as co-variable to adjust the characters to body size.

Character adjusted for SVL	<i>P. deloustali</i>		<i>P. guangxiensis</i>	
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
HL	0.561	0.455	0.628	0.43
MXHW	3.004	0.085	4.674	0.033
IOD	4.054	0.046	14.396	<0.001
IND	4.194	0.042	11.8	0.001
AGD	0.021	0.885	2.645	0.107
VL	31.287	<0.001	63.67	<0.001
TAL	21.143	<0.001	16.359	<0.001
MXTAH	30.323	<0.001	18.311	<0.001
FLL	0.049	0.825	0.11	0.741
HLL	0.148	0.701	0.254	0.615

Table 3-4. The comparison statistics of morphological characteristics between two Asian warty newts (*P.deloustali* and *P. guangxiensis*)

A. Female

Character	Type for test	F (t/U)	P-value	Significant or ns	Comparison <i>P. deloustali</i> vs. <i>P. guangxiensis</i>
SVL	t-test student	5.071	<0.001	significant	Larger
R HL	Mann-whitney	389	<0.001	significant	Smaller
R MXHW	Mann-whitney	1724	0.921	ns	
R IOD	Mann-whitney	1614	0.500	ns	
R IND	Mann-whitney	1374	0.054	ns	
R AGD	Mann-whitney	1095	0.001	significant	Smaller
R VL	Mann-whitney	777	<0.001	significant	Smaller
R TAL	Mann-whitney	1317	0.026	significant	Smaller
R MXTAH	Mann-whitney	1420	0.091	ns	
R FLL	Mann-whitney	1729	0.942	ns	
R HLL	Mann-whitney	1374	0.054	ns	

B. Male

Character	Type for test	F (t/U)	P-value	Significant or ns	Comparison <i>P. deloustali</i> vs. <i>P. guangxiensis</i>
SVL	t-test student	3.855	<0.001	significant	Larger
R HL	Mann-whitney	408	<0.001	significant	Smaller
R MXHW	Mann-whitney	1343	0.149	ns	
R IOD	Mann-whitney	1524	0.677	ns	
R IND	Mann-whitney	1027	0.001	significant	Smaller
R AGD	Mann-whitney	837	<0.001	significant	Smaller
R VL	Mann-whitney	609	<0.001	significant	Smaller
R TAL	Mann-whitney	1376	0.209	ns	
R MXTAH	Mann-whitney	1101	0.005	significant	Smaller
R FLL	Mann-whitney	1502	0.588	ns	
R HLL	Mann-whitney	1219	0.032	significant	Larger

Table 3-5. The comparison statistics of morphological characteristics among groups of two Asian warty newts (*P. deloustali* and *P. guangxiensis*)

A. Female; (Note: The population GX was not in the comparison because the data on female is currently not available)

Character	Type for test	F/ Chi-square	p-value	Pair difference (p)
SVL	ANOVA- Turkey	13.237	<0.001	1. CB_QN (0.002) 2. CB_EAST (<0.001) 3. QN_EAST (<0.001) 4. QN_west (<0.001) 5. West_East (0.047)
R_HL	Kruskal-Wallis test	69.581	<0.001	1. West_East (0.004) 2. West_CB (<0.001) 3. East_CB (<0.001) 4. QN_CB (0.028)
R_MXHW	Kruskal-Wallis test	9.845	0.02	1. CB_QN (0.014)
R_IOD	Kruskal-Wallis test	11.195	0.011	1. QN_East (0.011) 2. QN_CB (0.009)
R_IND	Kruskal-Wallis test	6.832	0.077	
R_AGD	Kruskal-Wallis test	19.716	<0.001	1. West_East (0.025) 2. West_CB (<0.001)
R_VL	Kruskal-Wallis test	32.358	<0.001	1. West_CB (<0.001) 2. East_CB (0.002)
R_TAL	Kruskal-Wallis test	19.551	<0.001	1. CB_QN (0.001) 2. CB_East (0.007) 3. CB_West (0.010)
R_MXTAH	Kruskal-Wallis test	13.139	0.004	1. CB_West (0.007)
R_FLL	Kruskal-Wallis test	10.833	0.013	1. East_West (0.007)
R_HLL	Kruskal-Wallis test	14.231	0.003	1. CB_QN (0.045) 2. QN_East (0.001)

B. Male; (Note: The population GX was not compared in R_IOD, R_IND and R_VL because the data on are currently not available)

Character	Type for test	F/ Chi-square	p-value	Pair difference (p)
SVL	ANOVA-Turkey	38.068	<0.001	1. QN _ CB (<0.001) 2. QN _ West (<0.001) 3. QN _ East (<0.001) 4. China _ East (0.014)
R_HL	Kruskal-Wallis test	67.022	<0.001	1. West _ East (0.014) 2. West _ QN (0.014) 3. West _ CB (<0.001) 4. China _ CB (0.026) 5. East _ CB (0.001) 6. QN _ CB (0.046)
R_MXHW	Kruskal-Wallis test	22.467	<0.001	1. CB _ QN (0.003) 2. CB _ West (0.005) 3. CB _ China (0.023)
R_IOD	Kruskal-Wallis test	28.895	<0.001	1. QN _ West (0.008) 2. QN _ East (0.001) 3. QN _ CB (<0.001) 4. West _ CB (0.014)
R_IND	Kruskal-Wallis test	26.472	<0.001	1. QN _ CB (<0.001) 2. East _ CB (<0.001) 3. West _ CB (<0.001)
R_AGD	Kruskal-Wallis test	55.420	<0.001	1. China _ West (0.037) 2. China _ East (<0.001) 3. China _ QN (<0.001) 4. China _ CB (<0.001) 5. West _ East (0.001) 6. West _ QN (0.011) 7. West _ CB (<0.001)
R_VL	Kruskal-Wallis test	45.588	<0.001	1. CB _ QN (0.047) 2. CB _ East (0.006) 3. CB _ West (<0.001)
R_TAL	Kruskal-Wallis test	18.814	0.001	1. China _ East (0.004) 2. China _ CB (0.001)
R_MXTAH	Kruskal-Wallis test	30.136	<0.001	1. West _ east (0.002) 2. West _ CB (<0.001)
R_FLL	Kruskal-Wallis test	16.702	0.002	1. West _ East (0.001)
R_HLL	ANOVA-Turkey	3.815	0.006	1. East _ China (0.005)

Table 3-6. Confusion matrix determined in the linear discriminant analysis for groups of two Asian warty newt (*P. deloustali* and *P. guangxiensis*)

Confusion matrix for Male						
<i>Groups</i>	<i>CB</i>	<i>East</i>	<i>West</i>	<i>QN</i>	<i>GX</i>	<i>Total</i>
CB	28	1	1	1	0	31
East	3	19	1	0	1	24
West	0	4	39	2	2	47
QN	1	0	2	11	0	14
GX	0	0	0	0	8	8
Total	32	24	43	14	11	124
Confusion matrix for Female						
<i>Groups</i>	<i>CB</i>	<i>East</i>	<i>West</i>	<i>QN</i>	<i>GX</i>	<i>Total</i>
CB	28	2	1	1	0	32
East	4	27	7	0	1	39
West	0	3	40	1	0	44
QN	0	0	1	8	1	10
GX	0	0	0	0	1	1
Total	32	32	49	10	3	126

Table 3-7. The Mahalanobis distance, Niche Overlap D, Geographic distance and Genetic distance among comparison pair of two Asian warty newts (*P. deloustali* and *P. guangxiensis*)

Pair	Mahalanobis Distance	Niche Overlap D	Geographic Distance (km)	Genetic Distance
QN vs. GX	4.5191	0.0367	62.06	0.0115
West vs. East	2.3803	0.0207	95.70	0.0212
CB vs. East	2.5489	0.2280	21.51	0.0663
West vs. QN	3.0273	0.2847	264.14	0.0760
CB vs. QN	3.6745	0.0000	181.93	0.0100
East vs. GX	4.1641	0.0000	105.79	0.0628
West vs. GX	4.4090	0.0317	227.80	0.0689
CB vs. West	4.0426	0.0012	136.88	0.0718
East vs. QN	3.8592	0.0174	141.61	0.0679
CB vs. GX	5.7475	0.0009	137.72	0.0118

Figure legends

Figure 3-1. Map of northern Vietnam and south China, showing the location of nine populations of five groups of *Paramesotriton deloustali* (West-W, East-E; dash line), and *P. guangxiensis* (Cao Bang-CB, Quang Ninh-QN, and Guangxi-GX; solid line). The map was created by ArcMap version 10.2 (ERSI). The administrative boundary dataset was downloaded from the GADM (https://gadm.org/download_country_v3.html). The elevation map was extracted from Terrain Elevation Data 2010 (GMTED2010), USGS EROS (<http://eros.usgs.gov/#>).

Figure 3-2. Map of northern Vietnam and south China, showing the occurrence locations (green circles) of five groups of two Asian warty newts (*P. deloustali* and *P. guangxiensis*): West (W), East (E), Cao Bang (CB), Quang Ninh (QN), and Guangxi (GX).

Figure 3-3. Boxplots of the SVL of female (left) and male (right) of five examined groups of two Asian warty newts: *Paramesotriton deloustali* (West [43 females; 48 males] and East group [39 females; 24 males]), and *P. guangxiensis* (CB [32 females; 31 males], QN [10 females; 14 males], and GX group [1 female; 9 males]). The red points showed the mean values, solid lines showed the medians, the edges of boxes are the quartiles, the whiskers are the range of data.

Figure 3-4. Sexual dimorphism of *P. deloustali* (n = 71 males and n = 83 females) and *P. guangxiensis* (n = 54 males and n = 43 females). The selected dimorphic characters were presented: Snout-vent length (SVL), Vent length (VL), tail length (TAL).

Figure 3-5. The boxplot for morphological characteristics between female and male of two Asian warty newts (*P. deloustali* and *P. guangxiensis*).

Figure 3-6. The boxplot for morphological characteristics between two Asian warty newt *P. deloustali* and *P. guangxiensis* (A) Female, (B) Male.

Figure 3-7. The boxplot for morphological characteristics between compared geographic groups of two Asian warty newts (unit: %). West and East groups: *P. deloustali*; CB, QN, GX: *P. guangxiensis*.

Figure 3-8. Linear discriminant analysis (LDA) plot showed the morphological variation among groups for female (above) and male (below) of two Asian warty newts (*Paramesotriton deloustali*, and *P. guangxiensis*). The number samples of each population: West (43 females; 48 males), East group (39 females; 24 males), CB (32 females; 31 males), QN (10 females; 14 males), and GX group (1 female; 9 males). The percentages of the explained variation of each axis were annotated.

Figure 3-9. The value of Cohen's kappa (KAPPA), ROC curve (AUC), and True skill statistic (TSS) of the five selected models Generalized Boosted Models (GBM), Random

Forest (RF), Multivariate Adaptive Regression splines (MARS); Flexible Discriminant Analysis (FDA), and Maximum Entropy (MaxEnt) models projecting potential distribution for groups of two Asian warty newts (*P. deloustali* and *P. guangxiensis*).

Figure 3-10. The potential distribution of five groups two Asian warty newts (*Paramesotriton deloustali*, and *P. guangxiensis*) projected by Ensemble Species Distribution Modeling. The map was created in ArcMap version 10.2 (ERSI). The administrative boundary dataset was downloaded from the GADM (https://gadm.org/download_country_v3.html).

Figure 3-11. The contribution of environmental variables in the five selected models Generalized Boosted Models (GBM), Random Forest (RF), Multivariate Adaptive Regression splines (MARS); Flexible Discriminant Analysis (FDA), and Maximum Entropy (MaxEnt) models projecting potential distribution for groups of two Asian warty newts (*P. deloustali* and *P. guangxiensis*).

Figure 3-12. Niche of the groups of two Asian warty newts (*P. deloustali* and *P. guangxiensis*) in ecological niches comparison pairs from a principal component analysis (PCA-env). Panels (A1 – J1) represent the niche of compared pairs, green and purple colors show the density of the occurrences of group A and B in each pair, respectively. Orange color shows the niche overlap between compared groups. The solid and dashed contour lines represent 100%, and 50% of the available (background) environment of compared group, respectively. The histograms (A2 – J2; A3 – J3) show the observed niche overlap (D) between the two groups (red line with a diamond) and simulated niche overlaps (grey bars) in niche similarity tests. The significance of the test, P-value <0.05.

Figure 3-13. Morphological differentiation revealed a correlation to ecological niche similarity (A) but non-significant correlation to geographic distance (B) and genetic distance (C). Ecological niche similarity revealed a significant correlation to geographic distance (D) but non-significant correlation to genetic distance (E). Genetic distance showed a non-significant correlation to geographic distance (F). The red line showed the model predicted regression line.

Figure 3-14. The relationship between morphology, ecology, genetics, and geographics distance in two Asian warty newts (*Paramesotriton deloustali*, and *P. guangxiensis*).

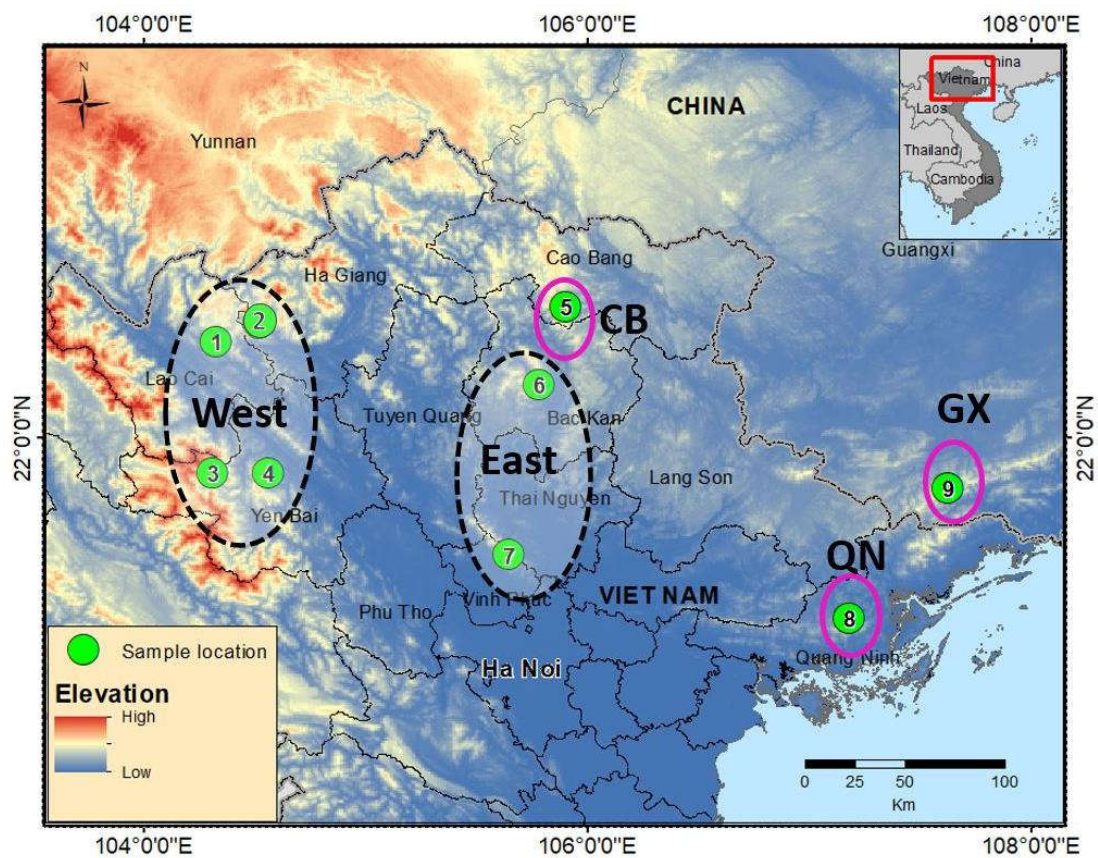


Figure 3-1

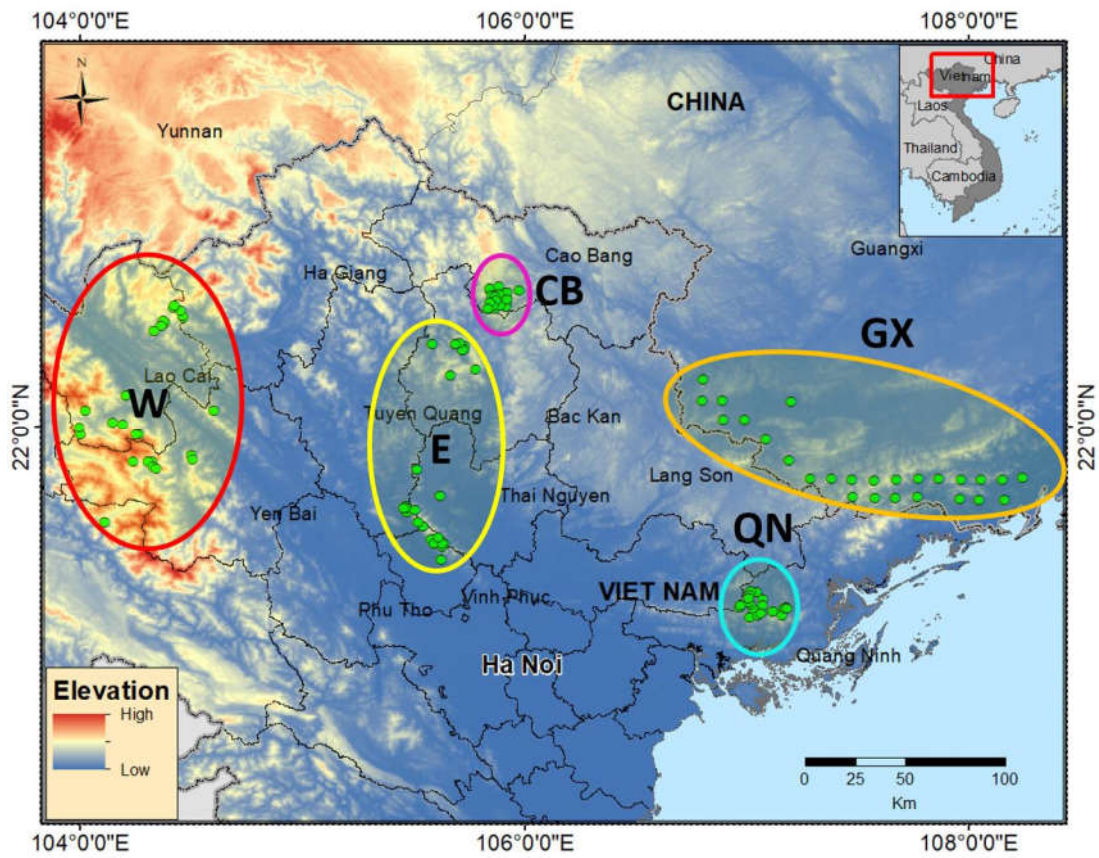


Figure 3-2

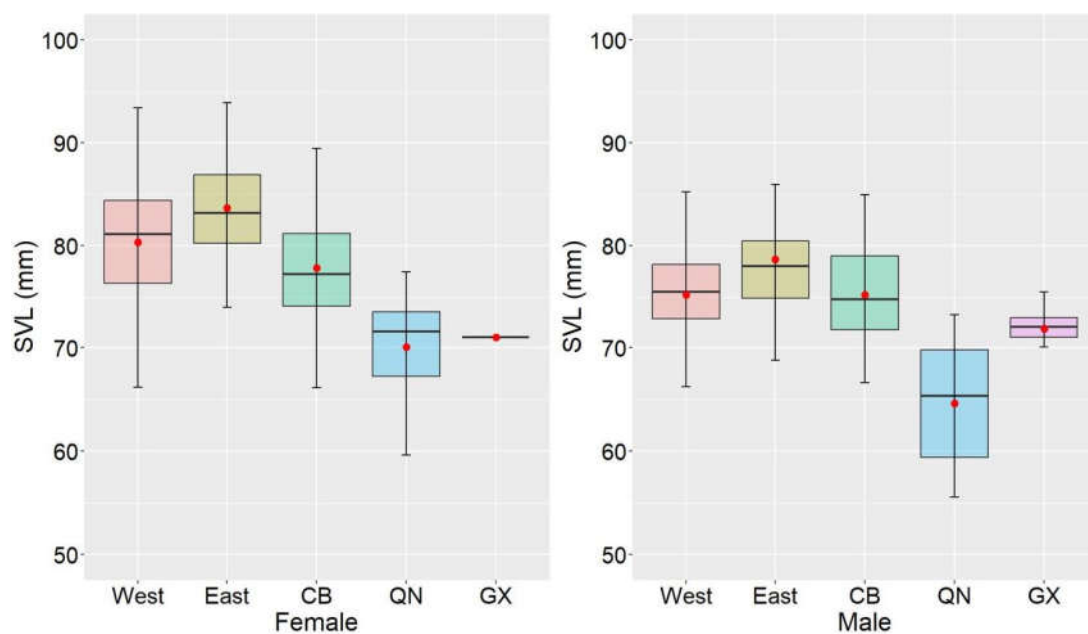


Figure 3-3

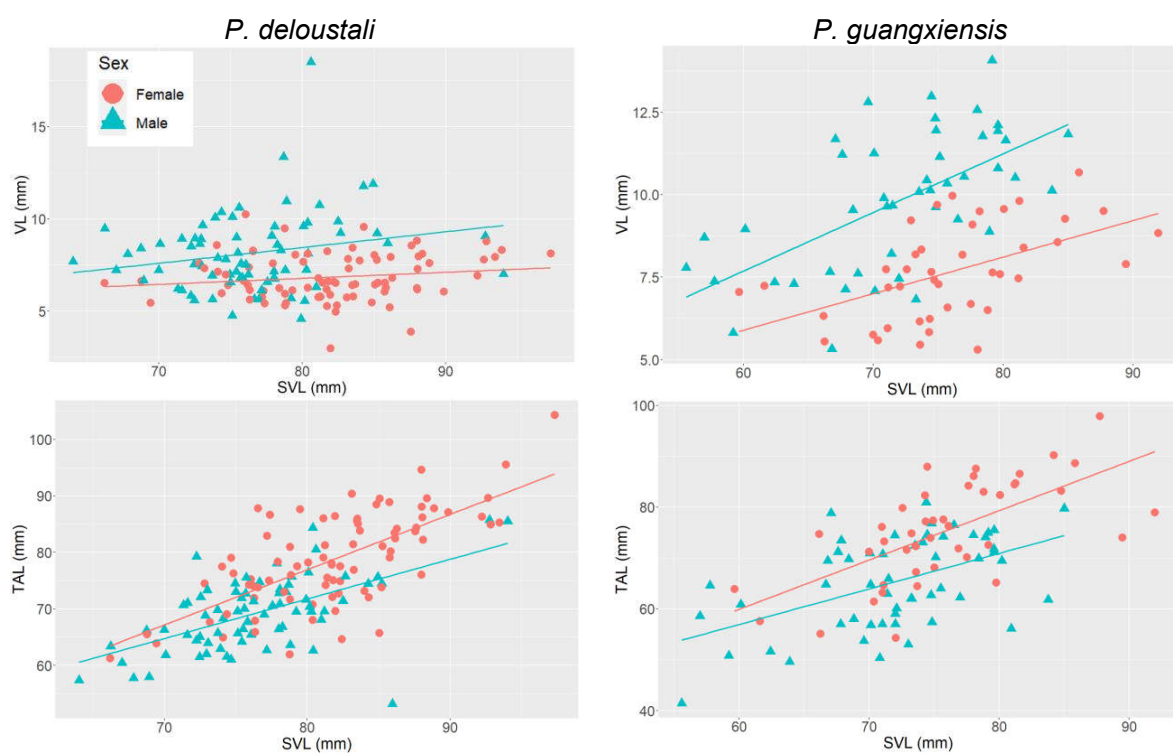
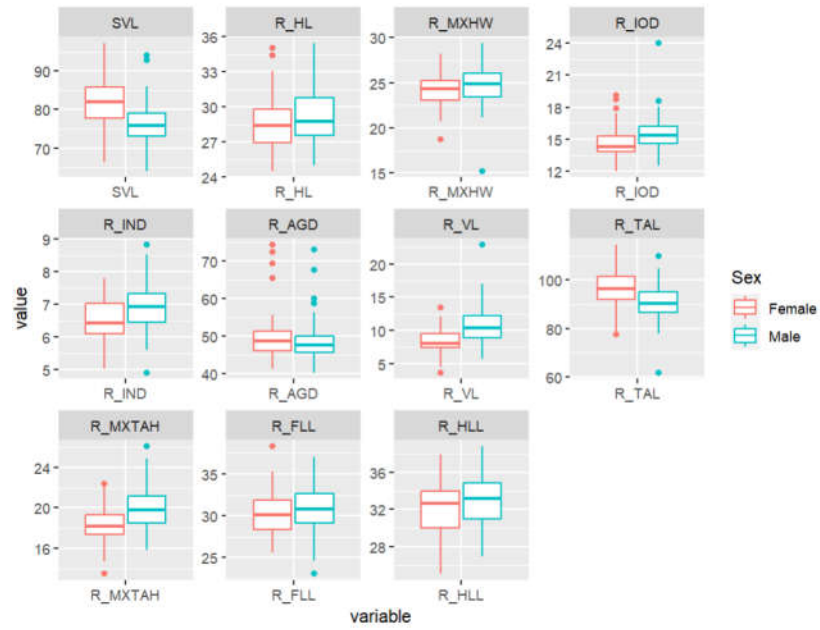
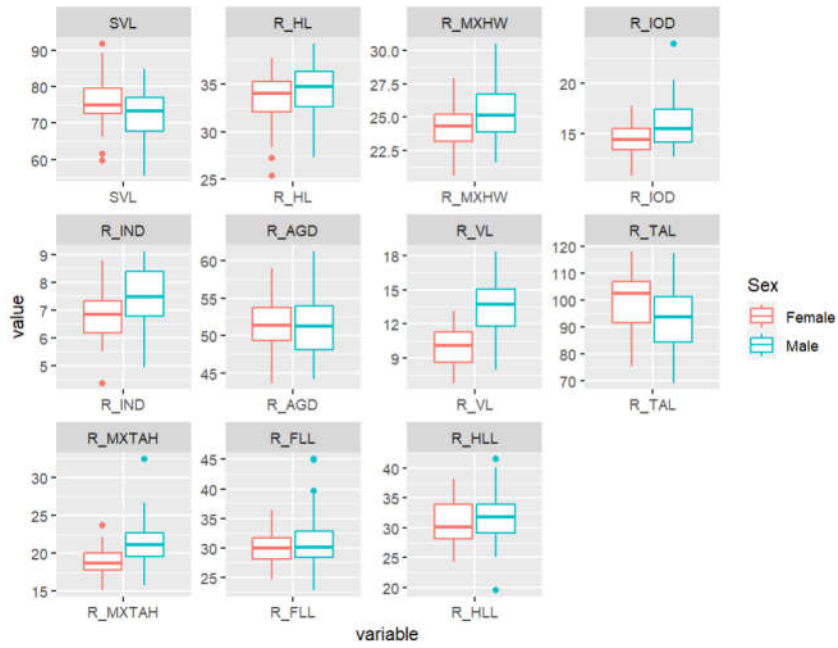


Figure 3-4

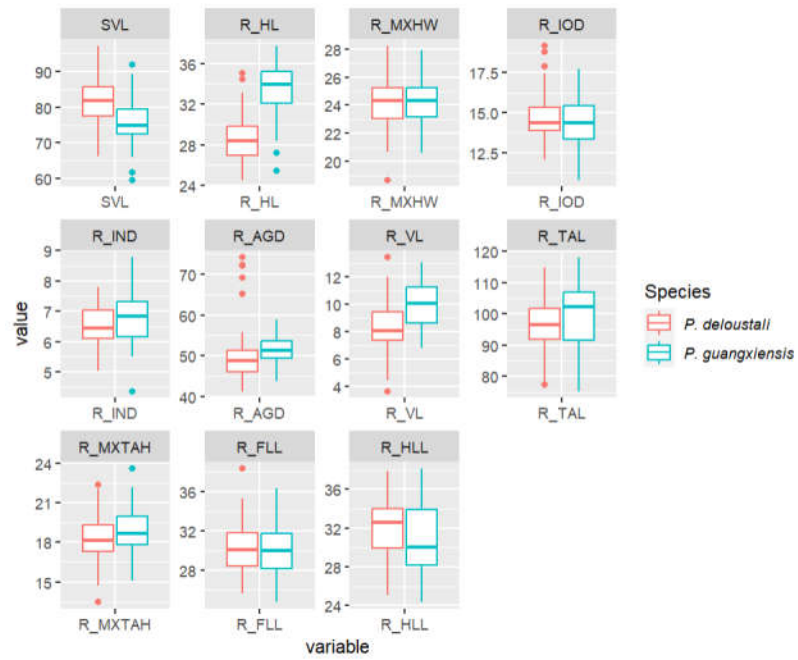


(A) *P. deloustali*

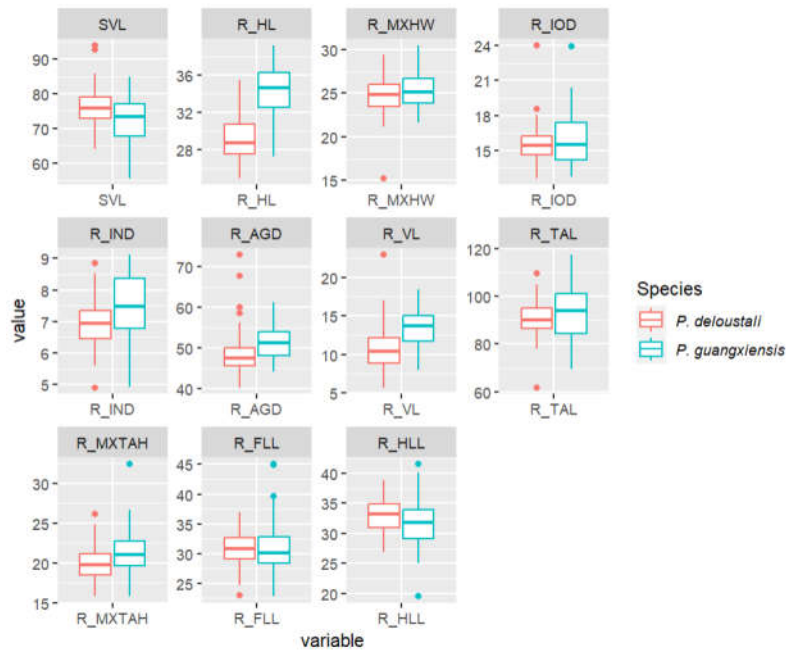


(B) *P. guangxiensis*

Figure 3-5

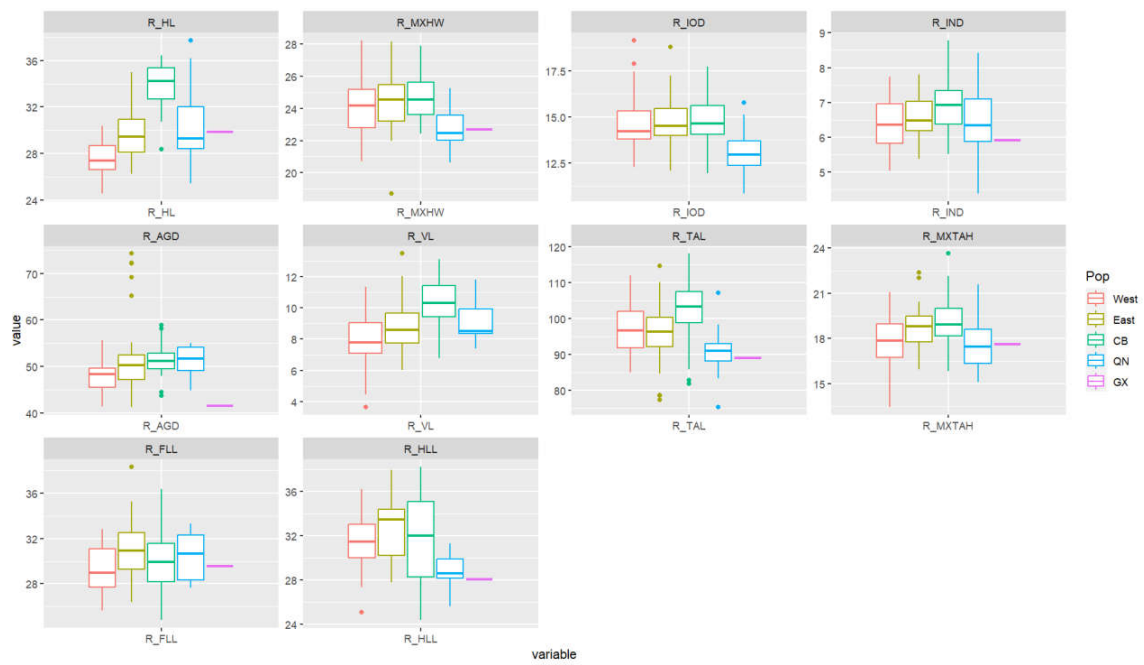


(A) Female

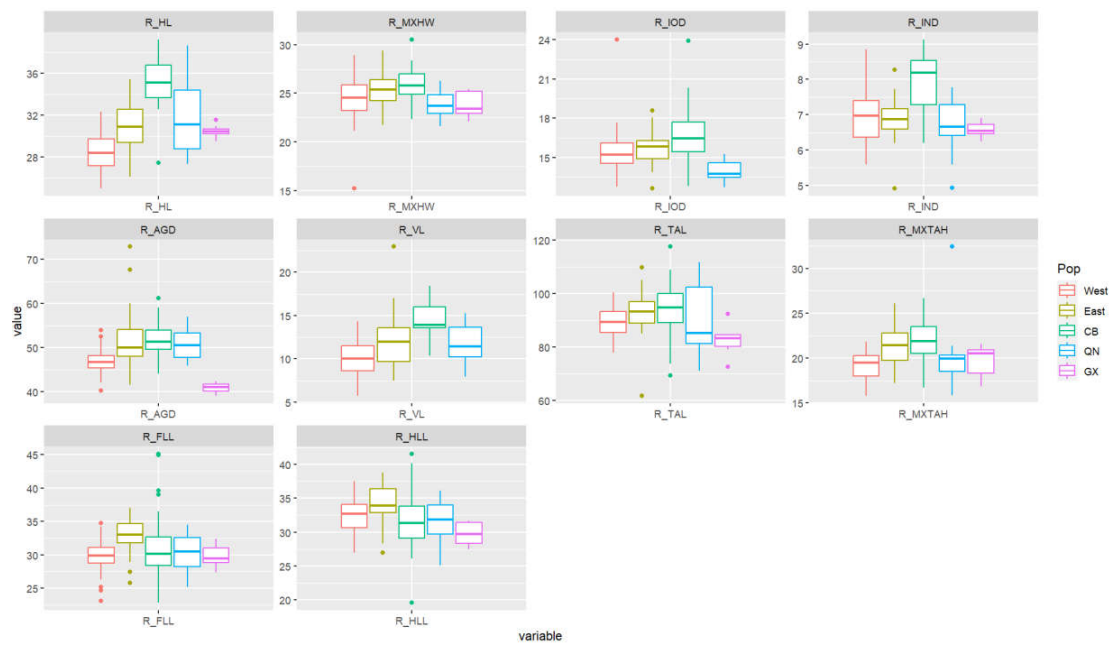


(B) Male

Figure 3-6



A. Female



B. Male

Figure 3-7

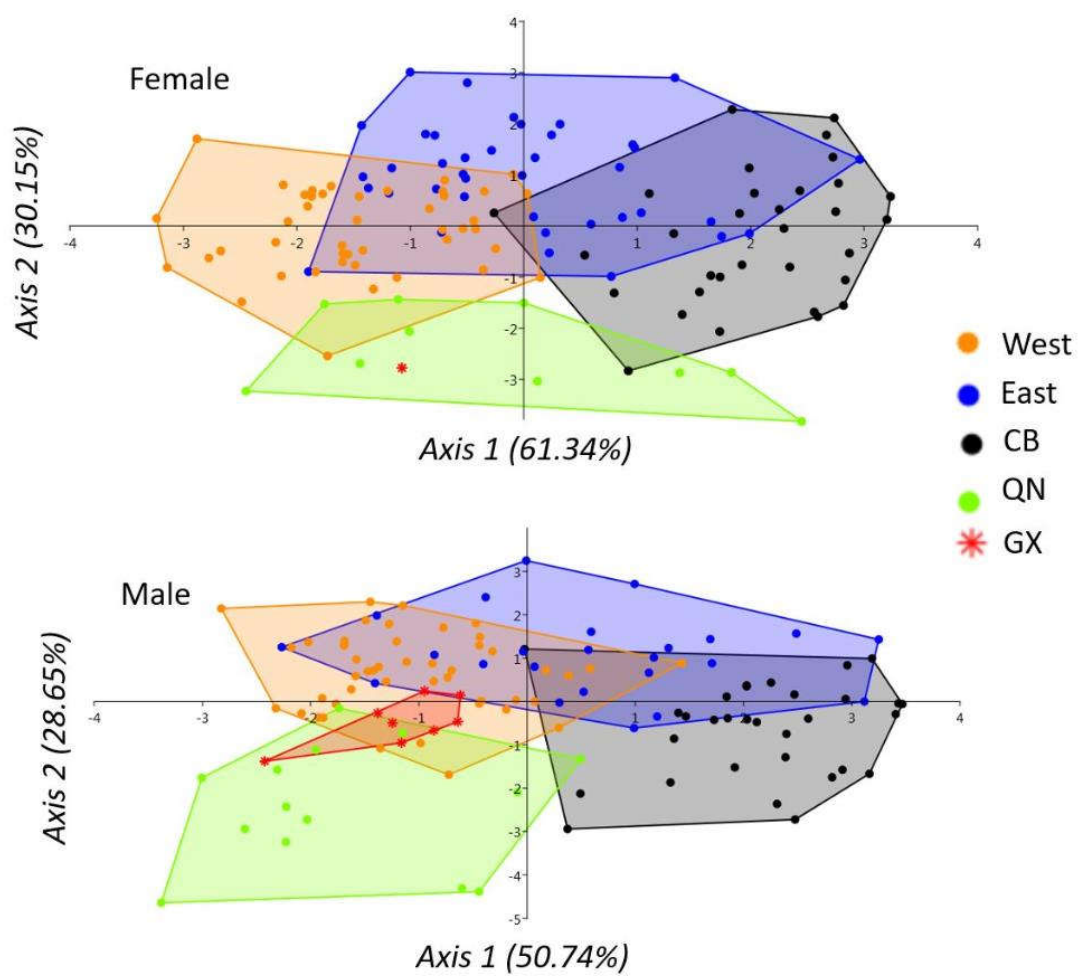
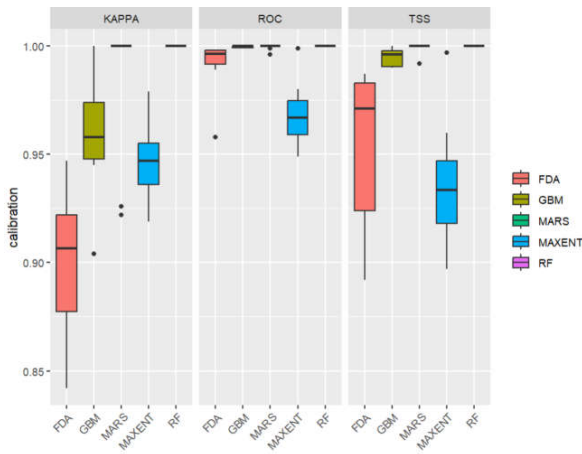
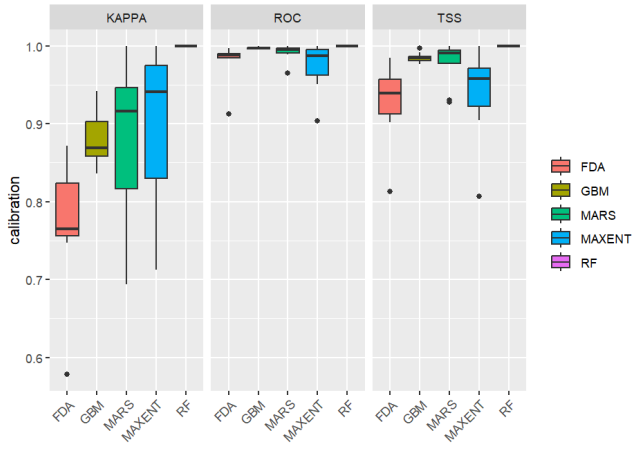


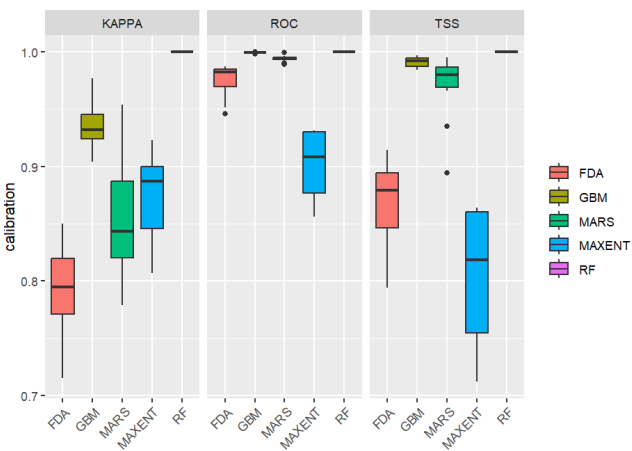
Figure 3-8



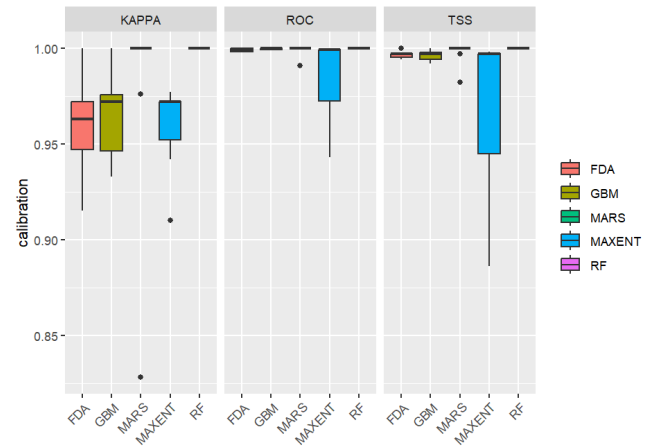
A. CB group



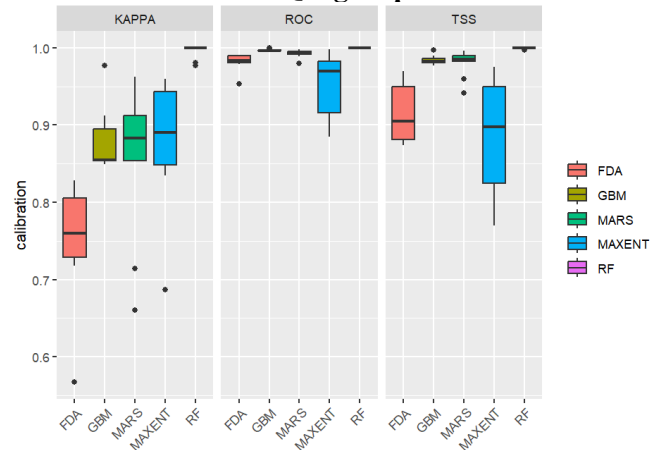
CN group



East group



B. QN group



West group

Figure 3-9

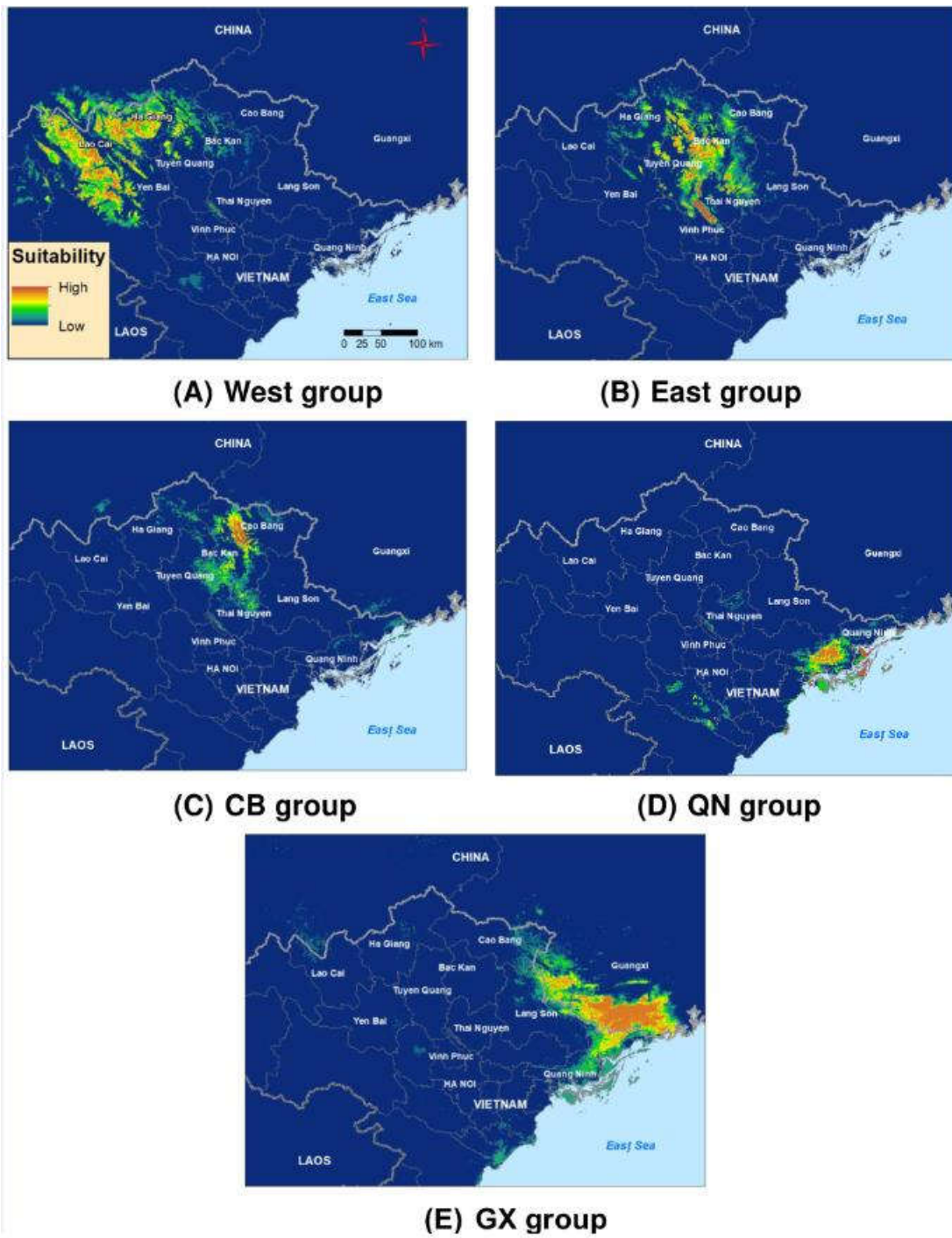
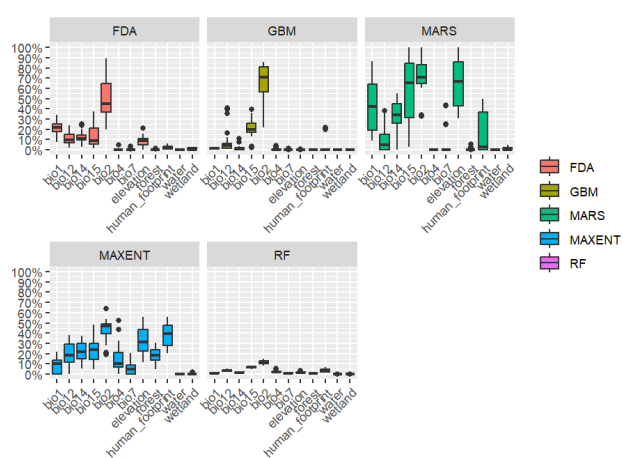
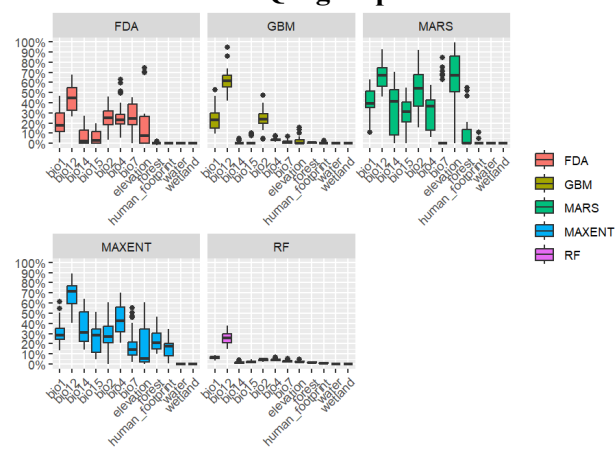


Figure 3-10



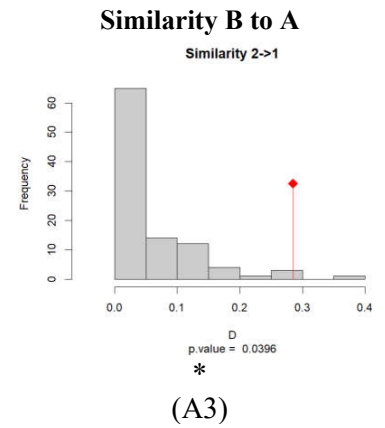
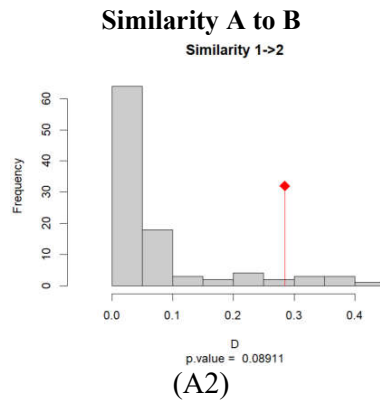
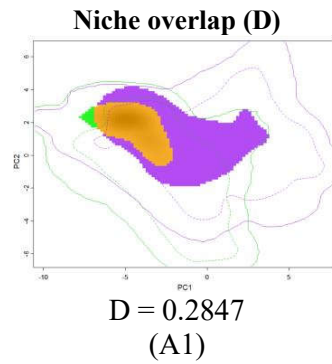
B. QN group



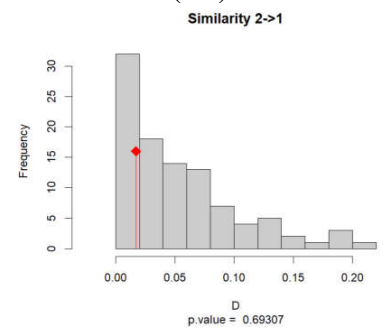
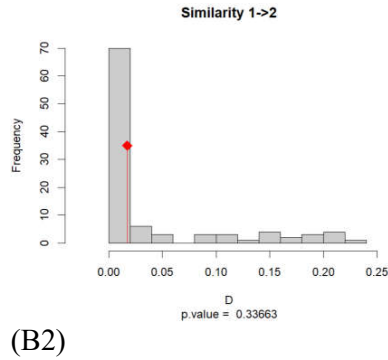
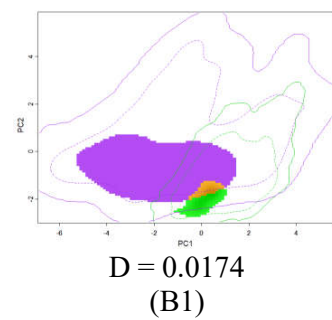
West group



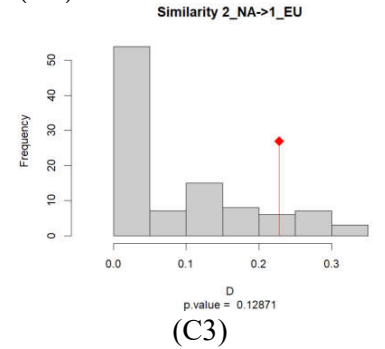
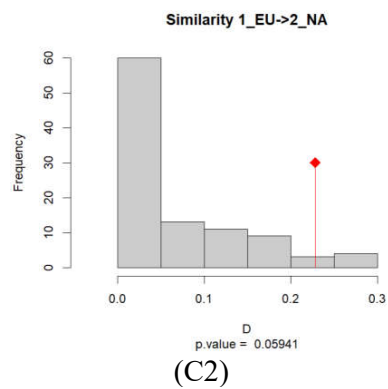
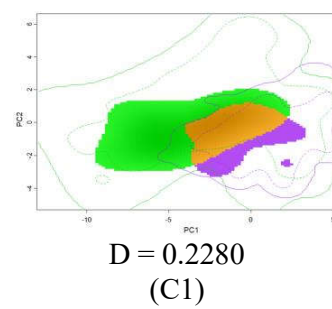
Pairs
CB (A) vs.
East (B)



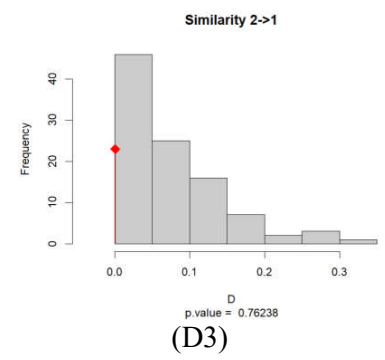
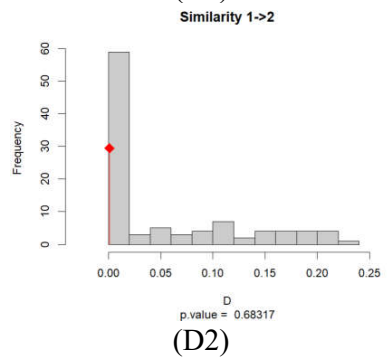
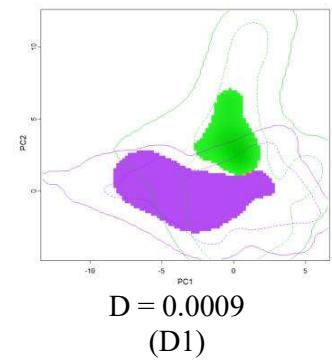
CB (A) vs.
West (B)



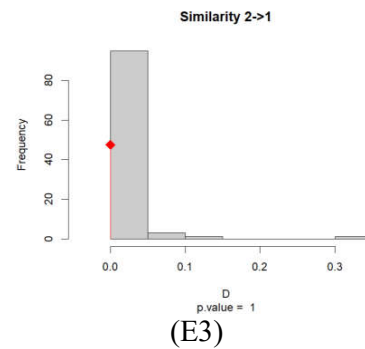
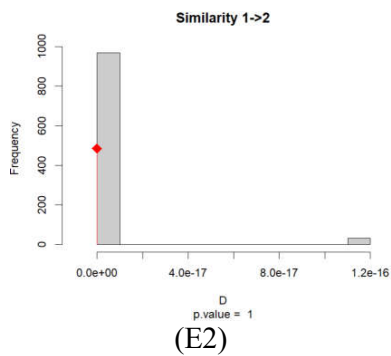
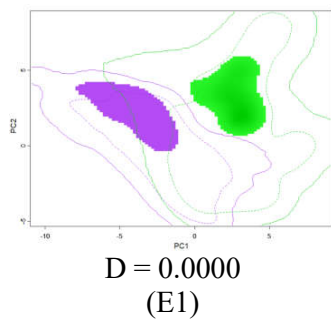
West (A)
vs. East (B)



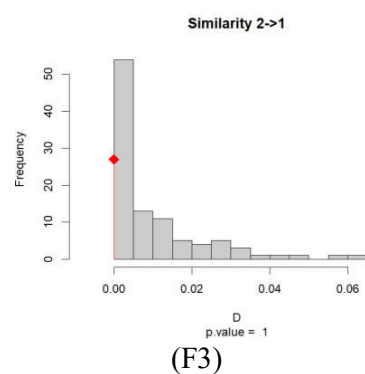
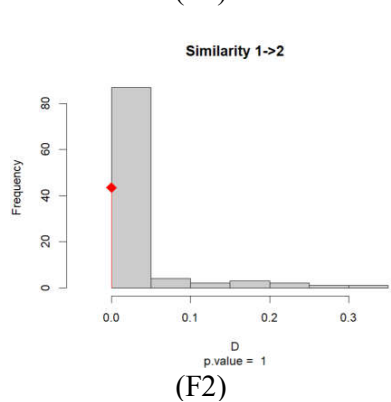
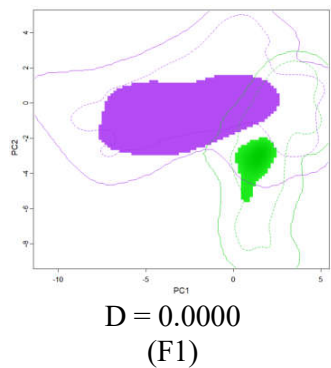
QN (A) vs.
East (B)



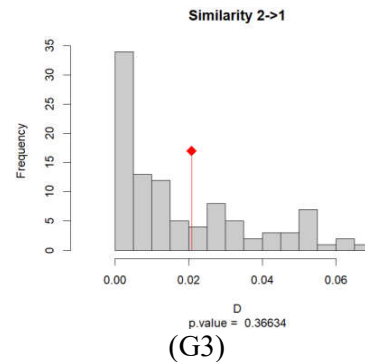
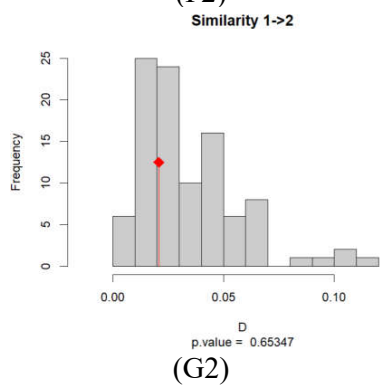
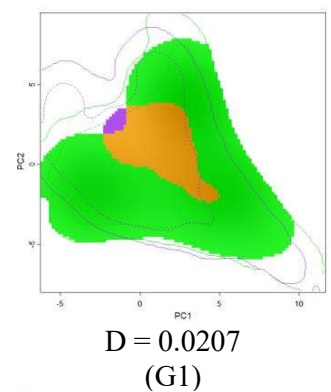
QN (A) vs.
CB (B)



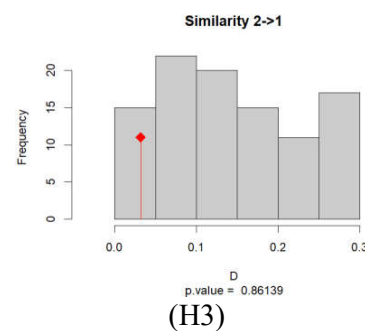
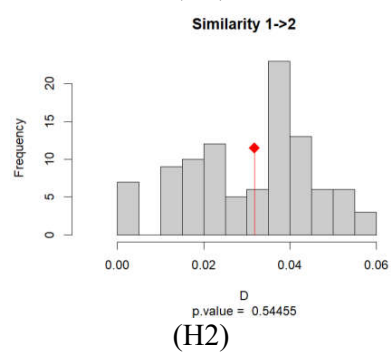
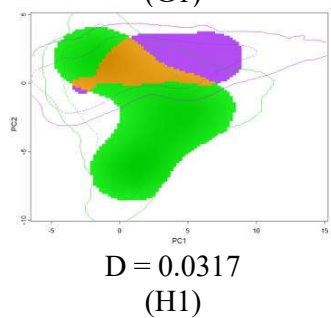
QN (A) vs.
West (B)



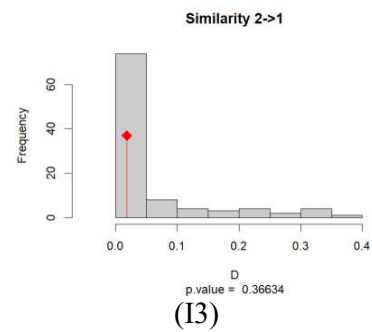
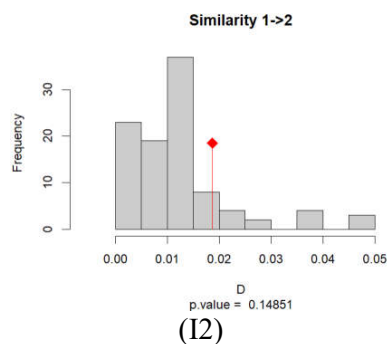
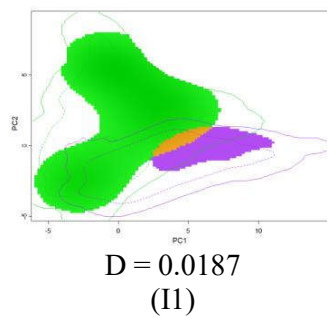
GX (A) vs.
QN (B)



GX (A) vs.
East (B)



GX (A) vs.
CB (B)



GX (A) vs.
West (B)

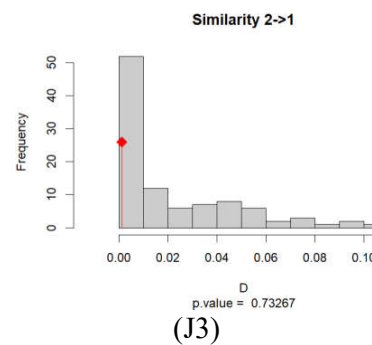
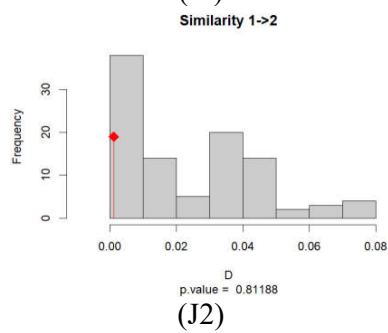
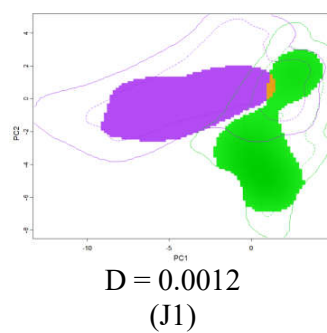


Figure 3-12

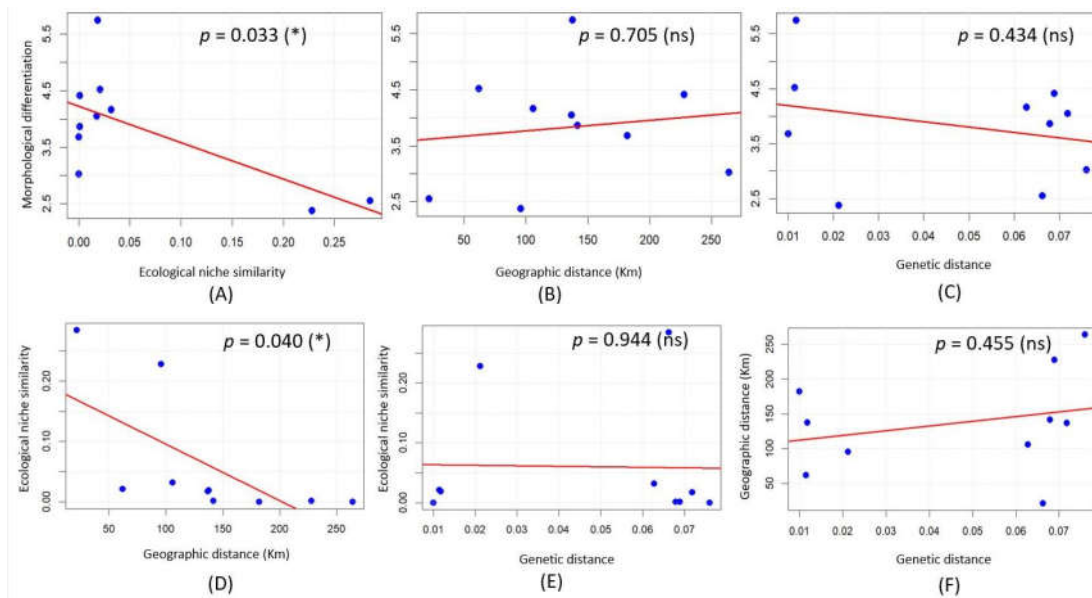


Figure 3-13

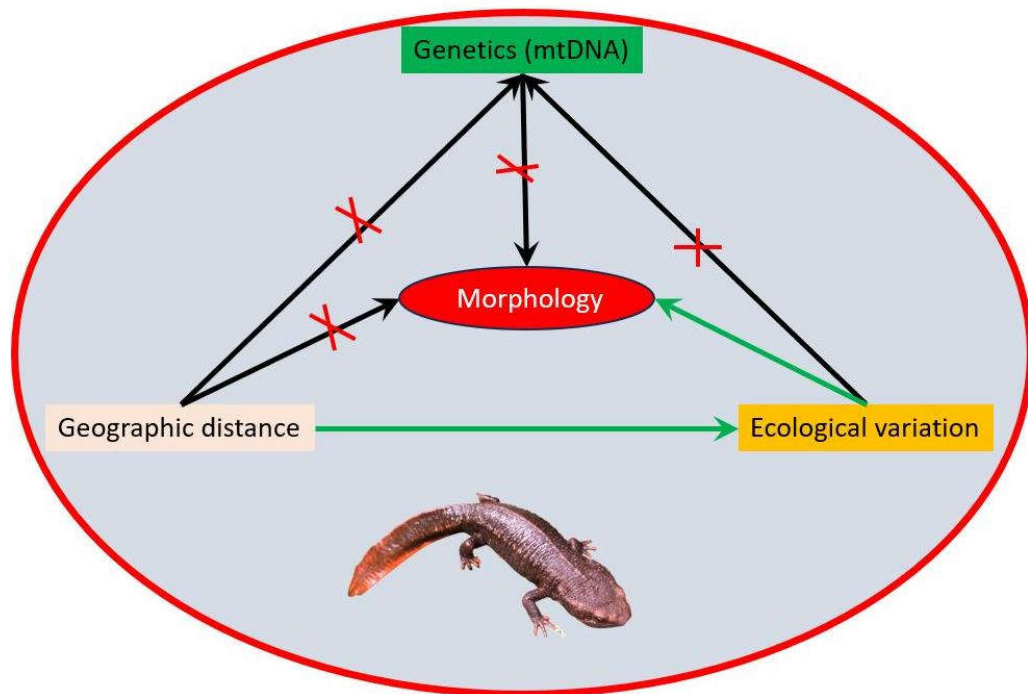
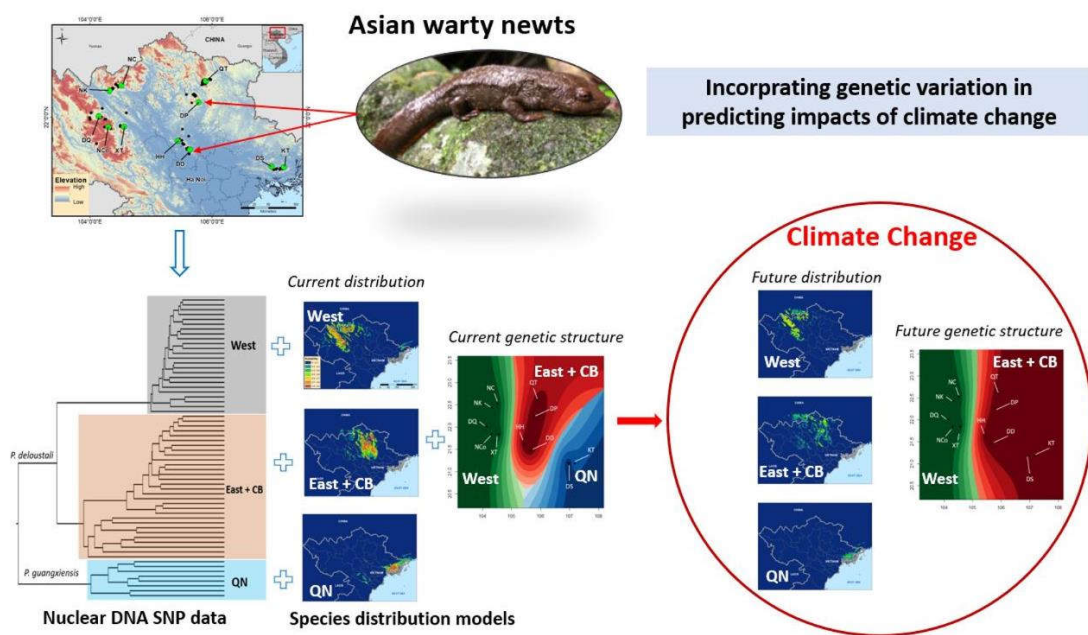


Figure 3-14

CHAPTER 4

Population genetics provides insights into the impact of climate change on the genetic structure and distribution of Asian warty newts



In previous chapters, I observed geographic variations in the mitochondrial DNA, morphology, and ecology of Asian warty newts. Interestingly, I discovered a mismatch between the patterns of these variations. In order to clarify the factor causing this mismatch pattern, I will analyze the nuclear DNA of the populations. Additionally, I will assess the impact of climate change on each population by integrating genetic variation into Species Distribution Models (SDMs).

4-1. Introduction

The global climate is undergoing drastic changes, evidenced by a 0.6°C increase in the world's mean temperature over the last century (Jones et al., 2001). Projections also suggest an expected increase of approximately 1.4–5.8°C by the end of the 21st century (Houghton et al., 2001). Many studies have indicated that climate change is a major threat to global biodiversity (Paterson et al., 2008; Thomas et al., 2004; Trew & Maclean, 2021). More than 20% of plant and animal species are projected to face an elevated risk of extinction in response to climate change (Kaeslin et al., 2012). In addition, many species may be forced to adjust their current distributions and adapt to new locations (Chen et al., 2011). Under such conditions, species typically move toward the north (Root & Schneider, 2002) or relocate to higher altitudes (Li et al., 2013; Wilson et al., 2005). Species with limited capacities for dispersal may be more prone to extinction and population decline compared to itinerant species (Duan et al., 2016).

Amphibians may be particularly susceptible to climate change (Foden et al., 2013; Luedtke et al., 2023; Stuart et al., 2004). Luedtke et al. (2023) demonstrated that 39% of amphibian species were impacted by climate change between 2004 and 2022. Amphibians are characterized by limited mobility and a marked sensitivity to variation in environmental temperature and humidity, due to their permeable skin and biphasic life cycle in water and on land (Duellman & Trueb, 1994; Stuart et al., 2004). The effects of climate change can alter the geographical distribution and abundance of amphibian populations (Carvalho et al., 2010; Chen et al., 2011), and may also result in loss of genetic diversity (Abreu-Jardim et al., 2021). Among amphibians, the urodeles may be particularly vulnerable due to their preference for cooler habitats (Borzée et al., 2019; Sutton et al., 2014; Velo-Antón et al., 2013; Zhang et al., 2020).

The genus *Paramesotriton* (Asian warty newts; family: Salamandridae) currently consists of 15 species and is found exclusively in China and northern Vietnam (Frost, 2024; Sparreboom, 2014). Two species are found in northern Vietnam: *Paramesotriton deloustali* and *P. guangxiensis* (Sparreboom, 2014; Tran et al., 2023a). Based on mitochondrial (mt)DNA analysis, Tran et al. (2023a) identified two genetic groups of *P. deloustali* in the western and eastern parts of northern Vietnam and designated these the West and East

groups, respectively. In addition, two separate populations of *P. guangxiensis* have been identified in Cao Bang (CB) and Quang Ninh (QN; Tran et al., 2023a). However, the genetic structure of Asian warty newts remains unclear. Tran et al. (2023a) also demonstrated that the Asian warty newts in northern Vietnam have been significantly affected by variation in paleoclimate. To date, no study has investigated the impact of climate change on the future distributions of species in northern Vietnam. Although several studies have examined the effects of climate change on the distribution of salamanders and newts (Achour & Kalboussi, 2020; Ashrafzadeh et al., 2019; Borzée et al., 2019; Ebrahimi et al., 2022; Préau et al., 2020; Zhang et al., 2020), most of these studies have focused on temperate rather than tropical species.

Species Distribution Models (SDMs) are widely used to predict the dynamics of species distributions in response to environmental changes by examining the relationship between current distribution data and environmental variables (Guisan & Thuiller, 2005). Such modeling is often performed at the species level (Mota-Vargas & Rojas-Soto, 2016; Smith et al., 2019). However, species exhibit nonuniform characteristics across their geographic ranges, with local adaptations occurring along environmental gradients (Hereford, 2009; Leimu & Fischer, 2008). This intraspecific variation is frequently ignored, potentially leading to over- or underestimation in distribution modeling (DeMarche et al., 2019; Mota-Vargas & Rojas-Soto, 2016; Smith et al., 2019). Integrating genetic structure to include intraspecific variation in SDMs can yield more accurate and meaningful predictions, overcoming this limitation (Bayliss et al., 2022; Claerhout et al., 2023; Hu et al., 2021; Zhang et al., 2021). In addition, combining SDMs with genetic parameters can enhance my understanding of how environmental variables influence genetic diversity and population genetic structure, as well as providing insights into how populations may respond to rapid climate change in the future (Abreu-Jardim et al., 2021; Duan et al., 2016).

In this study, I evaluated the effects of future climate change on the suitable habitats and genetic structure of Asian warty newts in northern Vietnam. To investigate genetic structure and gene flow in these populations, I used genome-wide nuclear (nu)DNA single-nucleotide polymorphism (SNP) data. I integrated data on intraspecific genetic variation from different groups into SDM to predict changes in the suitable habitats for each group under future climate change scenarios. In addition, I modeled the dynamics of genetic clusters conditioned by climatic variables over time.

4-2. Materials and methods

DNA sampling

I collected a single piece of tissue (1 mm × 1 mm) from the tails of each of 62 live newts at 11 locations during field surveys (Figure 4-1). The newts were released after tissue

collection without being euthanized or subjected to anesthesia. All animal procedures and specimen collection protocols were carried out in accordance with the animal experimentation guidelines of Kyoto University, Japan (Nos. 20-A-7 and 22-A-2). Total DNA was extracted from muscle samples using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. To evaluate the genetic structure of the populations, I assessed genome-wide SNPs using multiplexed inter-simple sequence repeat genotyping and the MIG-seq method (Suyama & Matsuki, 2015). I followed the protocols described by Suyama and Matsuki (2015) and Suyama et al. (2022) for DNA library preparation. DNA libraries from each index sample were pooled and sequenced using the HiSeq platform (Illumina, San Diego, CA, USA).

Population genetic analyses

Quality control for the MIG-seq data was performed using FASTP version 0.23.2 (Chen et al., 2018) with the default settings. Sequence reads were trimmed to 100 bp, and the short reads were analyzed using a de novo assembly pipeline and Stacks software version 2.55 (Catchen et al., 2011). When evaluating SNPs in populations using Stacks, data analysis was restricted to only the first SNP per locus (write-single-snp), and loci shared by at least 80% of the individuals across populations were processed (-R 0.8); sites with less than two minor alleles (min-mac 2) and greater than 75% of observed heterozygosity (H_o ; max-obs-het 0.75) were excluded. I also set the maximum distance allowed between stacks (-M) to 2, and the minimum depth of coverage to create a stack (-m) was set to 5 in ustacks. The other data processing parameters were set to default values. I examined the genetic structure of the populations using ADMIXTURE version 1.3 software (Alexander et al., 2009), with the number of clusters (K) = 2 – 11. Cross-validation error plots were drawn using log data. The results of the admixture analysis were visualized using CLUMPAK (Kopelman et al., 2015; <http://clumpak.tau.ac.il>). I also performed principal component analysis (PCA) using TASSEL software version 5 (Bradbury et al., 2007) and visualized the first two eigenvectors in a two-dimensional plot. Phylogenetic trees for the SNP data were constructed using VCF2PopTree software (Subramanian et al., 2019) and the UPGMA tree algorithm. To estimate the bidirectional migration patterns among populations, I calculated relative migration levels between different sampling sites using the DIV-MIGRATE online tool (Sundqvist et al., 2016; <https://popgen.shinyapps.io/divMigrate-online/>) and G_{st} migration indexes (Hedrick, 2005).

The pairwise fixation index (F_{st}) was calculated from nuDNA SNP data using the function pairwise (Weir & Cockerham, 1984) and WCfst from the hierfstat package (Goudet et al., 2015) in R software version 4.2.2 (R Core Team, 2023). As described by Hartl and Clark (1997), F_{st} values were interpreted as follows: values < 0.05 were classified as “little,”

0.05–0.15 as “moderate,” 0.15–0.25 as “great,” and > 0.25 as “very great” genetic differentiation. Correlations between F_{st} and geographical distance between populations were analyzed using the Mantel test function and the APE package in R software version 4.3.0, with 9,999 permutations (Paradis & Schliep, 2019). I also calculated the genetic diversity of each newt population, including H_o , expected heterozygosity (H_e), nucleotide diversity (Π), and inbreeding coefficients (F_{IS}) using the populations program in Stacks (Catchen et al., 2011).

Predicting the dynamics of genetic clusters

To predict the dynamics of genetic clusters conditioned by climate change scenarios, I implemented spatially explicit simulations using POPS software (Jay et al., 2015). This software categorizes samples into genetic clusters according to genetic ancestry and incorporates climatic and landscape variables in these cluster assignments. Then, it forecasts changes in genetic structure in response to environmental changes (Jay et al., 2012). I simulated genetic clusters under present conditions with the same climatic variables for SDMs. To define the number of clusters (K), I ran simulations four times with $K = 2 - 11$. I selected the K values corresponding to the lowest deviance information criterion (Spiegelhalter et al., 2002). The Markov Chain Monte Carlo runtimes in POPS were set to 1,200 sweeps and the burn-in period was 200 sweeps, in accordance with the software recommendations. Finally, I predicted the spatial changes for each cluster under the following future climate change scenarios in 2050 and 2090: shared socioeconomic pathway (SSP) 245 and SSP485.

Predicting the changes in suitable distribution

I collected data for the occurrence of Asian warty newts in northern Vietnam from my field surveys and published documents. After randomly thinning the presence localities to areas with radii of 1 km to prevent autocorrelation of the occurrence data, I gathered population data from 105 localities (Tran et al., 2023a). Climate variables were acquired from the WorldClim database version 2.1, with a spatial resolution of 30 arcs (Fick & Hijmans, 2017). The dataset included 11 layers for temperature and eight layers for precipitation. Elevation data were obtained from the EarthEnv website (<https://www.earthenv.org/topography>; Amatulli et al., 2018). I also downloaded variables for fractions of forest in each grid cell (Li et al., 2016) and human footprint data (Venter et al., 2016, 2018) to represent land use and human impact, respectively. To address autocorrelation among variables, I computed Pearson’s correlation coefficient (r) and systematically removed one variable from each highly correlated pair ($|r| > 0.80$). Finally, I used the following 10 variables to run my models: annual mean temperature (Bio1), mean

diurnal range (Bio2), temperature seasonality (Bio4), temperature annual range (Bio7), annual precipitation (Bio12), precipitation of driest month (Bio14), precipitation seasonality (Bio15), human footprint, fraction of forest, and elevation.

To project future population distributions, I evaluated two SSP scenarios (SSP245 and SSP485) in two periods: 2050 (the mean of 2041–2060) and 2090 (the mean of 2081–2100). I selected two global climate models: ACCESS-CM2 (Bi et al., 2020) and MIROC6 (Tatebe et al., 2019) because they have previously been used to investigate the impact of climate change on organisms in northern Vietnam (Vu et al., 2020; Trinh et al., 2022; Tran et al., 2023b).

The BIOMOD2 package (Thuiller et al., 2016, 2023) in R software version 4.2.2 (R Core Team, 2023) was used to predict the suitable distributions of three groups (West, East + CB, and QN). Based on the population genetics analysis of SNP data, I treated the East and CB populations as one group. The final models were assembled from the following five algorithms: flexible discriminant analysis (FDA), generalized boosted models (GBM), multivariate adaptive regression splines (MARS), maximum entropy (MaxEnt), and random forest (RF). Since presence-absence data are necessary for most models, with the exception of MAXENT, I generated pseudo-absence localities randomly using the BIOMOD_Formatting Data function within the BIOMOD2 package (Thuiller et al., 2016, 2023). The number of pseudo-absence points created was three times higher than the number of presence points. Additionally, I conducted 10 repetitions, following the recommendation of the BIOMOD2 team (Thuiller et al., 2023). Subsequently, I partitioned the data into model calibration (80%) and model testing (20%) datasets. I used Cohen's kappa (KAPPA), the area under the receiver operating characteristic curve (AUC), and the true skill statistic (TSS) to assess the performance of each model.

Niche comparison

I applied n-dimensional hypervolumes (Blonder et al., 2014) to quantify the realized niche space for the different groups (West, East + CB, and QN). The Hypervolume software package (Blonder & Harris, 2018) was used to calculate the shape and volume of hypervolumes for each group using the Gaussian method. The size of the realized niche in multidimensional space was assessed by measuring each hypervolume. I evaluated niche overlap among the different groups using the BAT software package (Cardoso et al., 2021). I measured differentiation between hypervolumes by evaluating both niche shifts, which involved replacing space between hypervolumes, and also niche contraction/expansion, which was the net difference between hypervolumes (Carvalho & Cardoso, 2020; Mammola & Cardoso, 2020). Niche differentiation ranges from zero (indicating complete overlap between hypervolumes) to one (indicating total separation between hypervolumes;

Mammola & Cardoso, 2020).

4-3. Results

Population structure and migration between populations

MIG-seq analysis generated 381 loci consisting of 52,091 sites after filtering. The mean length of the loci was 127 bp. After filtering, I retained 205 variant sites. In PCA analyses, the newts clustered into three distinct groups. Samples from DD, DP, and HH primarily clustered together as the East group, whereas those from QT (the CB group) partially overlapped the East group. The samples from NC, NCo, NK, DQ, and XT clustered as the West group. DS and KT individuals tended to form a separate group (the QN group; Figure 4-2A). MtDNA analysis by Tran et al. (2023a) identified the samples in DD, DP, HH, NC, NCo, NK, DQ, and XT as *P. deloustali*, whereas the remaining samples (DS, KT, and QT) were *P. guangxiensis*. ADMIXTURE analysis showed the smallest cross-validation error for the model with $K = 3$ (Table 4-1). The bar plot mainly corroborated the findings of the PCA. In particular, the East + CB, West, and QN populations were clearly separated. Furthermore, phylogenetic trees constructed from mtDNA and nuDNA SNP data revealed discordance in the CB population. In the mtDNA tree, the CB population clustered with the QN population, identified as *P. guangxiensis* (Figure 4-2C; Tran et al., 2023a). Conversely, in the nuDNA tree, the CB population clustered with the East population (Figure 4-2C).

In addition, I estimated F_{st} values for populations from different species (*P. deloustali* and *P. guangxiensis*) based on nuDNA SNP data. These values ranged from 0.147 to 0.606, whereas those for populations of the same species ranged from 0.045 to 0.491. Notably, the QT population (CB group) had low F_{st} values compared to the others (Figure 4-3, Table 4-2). The Mantel test showed that the populations of *P. deloustali* were significantly isolated by geographic distance ($p < 0.0001$).

The divMigrate analyses only found significant gene flow from the DD (0.54) and DP (0.66) populations in the East group to the QT population in the CB group (Figure 4-1). In addition, I observed no significant migration of populations between the West, East, and QN groups (Figure 4-1).

Genetic diversity

Genetic diversity measures (H_e , H_o , and Π) were highest for the CB group, followed by the QN, West, and East groups (Figure 4-4). The H_e values ranged from 0.05271 (DQ population) to 0.14845 (QT population). The lowest H_o value was 0.06367 (NCo population), whereas the highest was 0.13926 (QT population). The highest Π value was 0.16077 (QT population), whereas the lowest was 0.06439 (DQ population).

In addition, I found that the H_o values were consistently higher than the H_e values across all populations except DS (QN group) and QT (CB group). The F_{IS} values were lower than 0.15 for all populations. An F_{IS} value above 0.15 suggests considerable inbreeding within a population (Hartl, 1988; Wright, 1965). F_{IS} estimates were negative for the DQ and XT populations, with the highest values in the DS (0.06189) and CB (0.05816) populations, and the lowest value in the DQ population (−0.01165; Figure 4-4).

Predicting dynamics in genetic clusters

My analysis revealed three primary clusters among 11 populations of Asian warty newts, correlated with climatic variables ($K = 3$, deviance information criterion = 7199.00; Figure 4-5). The correlation between estimated and predicted admixture coefficients for environmental variables was 0.99, indicating high accuracy. In the current climate, the POPS analysis identified Cluster 1, including populations in DD, HH, DP, and QT. Cluster 2 included populations in NC, NK, NCo, DQ, and XT, whereas Cluster 3 included populations in DS and KT. This pattern closely aligned with the results of the admixture analysis: Cluster 1 = East + CB group; Cluster 2 = West group; and Cluster 3 = QN group. Overall, the distribution of the three clusters was predicted to be susceptible to climate change. The East + CB group exhibited a trend toward expansion across all scenarios, whereas the QN group faced decline in three of the four scenarios. The West group was predicted to remain stable in scenarios SSP245–2090 and SSP485–2050 but to decline in the two remaining scenarios (Figure 4-5).

Niche differentiation

The predicted volume of the realized niche was largest for the West group, followed by the East + CB group, and the QN group (Figure 4-6). Niche differentiation was entirely distinct among all comparison pairs. This dissimilarity was primarily due to niche breadth, with the contribution from niche shifts being very limited (Table 4-3). These results indicate that the suitable distribution of the groups should be modeled separately.

Effects of climate change on suitable distribution

High values for Cohen's kappa, the area under the receiver operating characteristic curve, and true skill statistic metrics were found in all models of the four groups (all values > 0.8; Figure 4-7), indicating strong predictive capacity for the selected algorithms. Under present climate conditions, the regions encompassing Yen Bai, Lao Cai, and Ha Giang in the western part of northern Vietnam were identified as particularly suitable for the West group. Conversely, the eastern region composed of Vinh Phuc, Thai Nguyen, Tuyen Quang,

Bac Kan, and CB were particularly suitable for the East + CB group (Figure 4-8A, B). The areas predicted to be suitable for the West and East + CB groups were similar in size, covering 16,378 km² and 17,252 km², respectively (Table 4-4). Conversely, the area predicted to be suitable for the QN group was mainly within QN province, and only approximately 5,167 km² in size (Figure 4-8C, Table 4-4).

I observed projected declines in the suitable distributions of all groups in response to climate change, except for the East + CB group under scenario SSP245–2050 (Table 4-4). In all scenarios, the expected decrease in the distribution of the West group was more pronounced than that of the East group (Figure 4-8, Table 4-4). Specifically, the suitable habitat of the West group was projected to decrease by 23.2–32.6% under scenario SSP245 and by 34.2–48.4% under scenario SSP485. The suitable habitat for the East + CB group may increase by approximately 21.2% under scenario SSP245 in 2050 but decrease by 15.1% in 2090. Under scenario SSP485, the suitable habitat for the East + CB group is likely to decrease by 7.9–19.5%. The impact of climate change on the suitable habitat for the QN group is also expected to be severe across all scenarios. In particular, the QN groups could almost disappear under climate change scenario SSP485–2090, with more than 90% of their current distribution at risk (Table 4-4).

I also found shifts in the suitable distribution of all three primary groups of Asian warty newts toward higher altitudes under my climate change scenarios (Figure 4-9). The distributions in mountainous regions (e.g., the Hoang Lien Son and Tam Dao mountain ranges) were predicted to remain unaltered, suggesting that these regions may safeguard species from the effects of climate change (Figure 4-7). Furthermore, the centroids of suitable habitats for the West and East + CB groups tended to shift northwest, whereas the centroid of suitable habitats for the QN group tended to shift southwest (Figure 4-10).

4-4. Discussion

PCA and STRUCTURE analyses of nuDNA revealed three primary genetic clusters (West, East + CB, and QN) of Asian warty newts in northern Vietnam. The discordance between the mtDNA and nuDNA SNP data for the CB population suggests contributions from distinct ancestral groups in the East and QN populations. The nuDNA SNP data suggest a close relationship between the CB and East populations. However, in mtDNA analyses, the CB population clustered with the QN population and the GX population in China, reflecting genetic introgression from *P. guangxiensis* (Tran et al., 2023a). Discordance in species delineations determined by mtDNA and nuDNA is common in salamanders and newts (Bisconti et al., 2018; Denton et al., 2014; Fisher-Reid & Wiens, 2011; Tomimori et al., 2023; Zieliński et al., 2013). In this case, mtDNA from the original population may have been replaced entirely with mtDNA from *P. guangxiensis* by

introgression. Partial or complete replacement of mtDNA has been observed in various urodeles (Petit & Excoffier, 2009; Toews & Brelsford, 2012; Zieliński et al., 2013). Here, I provide a similar example from Asian warty newts in northern Vietnam. A paleodistribution analysis of the last interglacial period and last glacial maximum by Tran et al. (2023a) suggested that the mountainous regions of northern Vietnam likely served as refugia for Asian warty newts, potentially fostering introgression by gathering several lineages together. Some of these lineages may have interbred during the glacial periods. As these populations expanded, they may have become separated by geographical barriers such as lowland areas, but could have retained genetic purity within their nuDNA.

The morphology of the CB population was more similar to that of *P. deloustali* (East group) than that of *P. guangxiensis* (QN group). By modeling the suitable distributions of the East and CB groups separately, Tran et al. (2024) observed substantial ecological overlap. There were also noteworthy similarities in the ecological niches of these groups compared to the null distribution. Here, I suggest treating the CB population as a population of *P. deloustali*.

The F_{ST} values between the East and CB populations (including the DP–QT, DD–QT, and HH–QT pairs) were moderate, whereas the values indicating genetic differentiation between the remaining populations were either great or very great. The lower F_{ST} values between the East and CB populations may be due to the geographical proximity of the populations, as the Mantel test shows that the genetic differences significantly correlated with geographic distance. The genetic differentiation between the QN population (including the DS and KT populations) and other populations was considered very great, supporting the hypothesis that the QN population forms a distinct taxon. This is also consistent with the mtDNA data (Tran et al., 2023a). Furthermore, the considerable genetic differentiation observed among the populations likely indicates isolated populations with restricted gene flow (Lovrenčić et al., 2022). This hypothesis is supported by the divMigrate analyses, which revealed significant gene flow only from the East to CB populations.

My analyses of Asian warty newts revealed low genetic diversity within a population in northern Vietnam. For *P. deloustali*, the introgressed CB group displayed higher genetic diversity than the East or West groups. This higher genetic diversity may be attributable to genomes being admixed with *P. guangxiensis*. In the North Hungarian Mountains, a similar situation was observed for a population of the smooth newt *Lissotriton vulgaris*, which also exhibited higher genetic variation due to a genomic admixture (Herczeg et al., 2023). I also found greater genetic variation in the QN group (*P. guangxiensis*) compared to the West and East groups (*P. deloustali*). The low levels of genetic diversity in the West group have been attributed to geographic isolation (Tran et al., 2023a). In addition, the isolation of populations was demonstrated by the absence of gene flow within groups, as revealed by the divMigrate analyses. Although the F_{IS} values of all populations in my study were low ($<$

0.15), there is a risk of inbreeding in the near future if the restricted gene flow persists (Keller & Waller, 2002; Kenney et al., 2014). The limited dispersal capacity of these newts (Matsui et al., 2006; Wells, 2007) and the fragmentation of their suitable habitats (Figure 4-8; Tran et al., 2023a) probably contributes to the isolation of the newt populations.

I evaluated the effects of climate change on the suitable habitats and genetic structure of populations of the tropical warty newts *P. deloustali* and *P. guangxiensis* in northern Vietnam. Overall, my findings suggest that climate change could decrease the geographical distribution of these newts and alter the distribution of genetic clusters. There may be reductions in the size of areas available to these newts across all scenarios. There are projected declines in suitable habitat areas for the QN, West, and East groups of 44.4–96.4%, 23.2–48.4%, and 7.9–19.5%, respectively. Although the suitable distribution of the East + CB group was projected to increase under the SSP245 scenario in 2050, highly suitable areas may become poorly suitable areas, and suitable areas may become fragmented (Figure 4-8E). Therefore, forthcoming climate conditions may not be favorable for tropical Asian warty newts. These results align with findings for other salamanders and newts, and imply that reductions in suitable habitats are an inevitable consequence of climate change for these species due to their sensitivity to environmental conditions and limited capacity for dispersal (Achour & Kalboussi, 2020; Ashrafzadeh et al., 2019; Ebrahimi et al., 2022; Préau et al., 2020; Zhang et al., 2020). For example, Zhang et al. (2020) predicted that the suitable habitat of the Chinese giant salamander (*Andrias davidianus*) could decrease by more than two-thirds. Ashrafzadeh et al. (2019) also projected a 56–98% decrease in the suitable habitat of the Lorestan mountain newt (*Neurergus kaiseri*) by 2050 and 2070. Furthermore, Luo et al. (2022) suggested that historical climate change has likely affected the diversification of the genus *Paramesotriton*. Therefore, I predict that other Asian warty newts in China will also be affected by future climate change.

I observed that the suitable habitat of the West group of *P. deloustali* is likely to be more severely affected by climate change than that of the East + CB group. Analyses of the spatial distribution of genetic clusters conditioned by environmental variation in these two groups suggested opposing trends. The spatial distribution of genetic clusters was projected to expand in the East + CB group but to decrease or remain stable in the West group. Interestingly, all populations in the East + CB group displayed higher genetic diversity (Ho, He, and Π) compared to populations in the West group. Moreover, the East + CB group was introgressed by genomic material originating from *P. guangxiensis*. Populations characterized by low genetic diversity may exhibit increased sensitivity to climate change (Abreu-Jardim et al., 2021; Frankham, 2005; Rizvanovic et al., 2019). Furthermore, introgression can enhance tolerance to environmental change, potentially benefiting populations by mitigating the effects of global warming (Brauer et al., 2023; De-Kayne et al., 2022; Meier et al., 2019). For example, the genetic admixture exhibited by the East +

CB group may make it less vulnerable to climate change. However, climate change-adapted loci need to be identified to improve my understanding of climate adaptations and genomic vulnerability among populations.

Various species are expected to migrate to higher altitudes due to global warming (Li et al., 2013; Wilson et al., 2005). Amphibians may relocate to cooler habitats within their existing distribution range (Bickford, 2005) or seek lower temperatures at higher altitudes (Bickford et al., 2010). Bickford et al. (2010) described at least nine lowland amphibian species that have migrated upward by 500 m or more in Southeast Asia over the past 70 years. Like other urodeles that favor cooler climates in montane habitats (Sparreboom, 2014), Asian warty newts may also move to higher altitudes. In this study, I found that all three primary groups of Asian warty newts in northern Vietnam were predicted to relocate to higher elevations under all climate change scenarios. These locations were similar to the refugia populated by newts during the glacial period (Tran et al., 2023a). Anticipated relocation sites for the West group included the high mountains of the Hoang Lien Son and Tay Con Linh ranges. The East + CB group may move to higher elevations in the Song Gam and Ngan Son ranges, the Tam Dao mountains, and the Phia Oac–Phia Den National Park. The QN group was projected to relocate to the Tay Yen Tu mountainous region.

Centroid migration analysis provides insight into changes in the suitable distributions of species (Wang et al., 2023). The centroid of the suitable range of Asian warty newts shifted due to the predicted impact of climate change. The suitable habitat of the West and East + CB groups shifted northwestward, whereas the distribution of the QN group shifted southwestward. The tendencies observed suggested that the original habitats may no longer be suitable for the newts. Importantly, biodiversity corridors may enable species to migrate toward suitable habitats and enhance gene flow among populations responding to climate change (Sahlean et al., 2020; Vu et al., 2020). These corridors may be particularly crucial for species with limited dispersal capacity, such as amphibians (Sahlean et al., 2020). In northern Vietnam, there are three biodiversity corridors that could link protected areas and mitigate the impact of climate change on biodiversity. These are the Northeast corridor (from Ba Be National Park to Du Gia–Cao Nguyen Da Dong Van National Park), the Northwest corridor (from Cuc Phuong National Park to Ngoc Son–Ngo Luong Nature Reserve), and the Northern coastal corridor (Dong et al., 2013). After considering the suitability of habitats projected by SDM and the gene flow within the East + CB group, I propose that a new corridor through the Ngan Son range to the Phia Oac mountains (Figure 4-8E) could also connect Ba Be National Park and the Phia Oac–Phia Den National Park. Once these corridors are established, they may play important roles in safeguarding species, including Asian warty newts, from the threats posed by climate change.

In conclusion, my population genetics analysis identified three primary groups of Asian warty newts in northern Vietnam: West, East + CB, and QN. Contrary to previous

assumptions that the CB group belonged to the *P. guangxiensis* population, my SNP data suggest that this group should be classified as a population of *P. deloustali* with introgressive mtDNA from *P. guangxiensis*. My results also show that gene flow among populations of Asian warty newts is restricted. I found that both spatial genetic clusters and the suitable habitats of Asian warty newts are vulnerable to the impacts of climate change. Establishing biodiversity corridors connecting the high mountains of northern Vietnam should be considered as a useful measure to safeguard these Asian warty newts from the adverse effects of future climate change.

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Table 4-1. The CV (coefficient of variation) error of ADMIXTURE analysis for

$$K = 2-11$$

CV error (K=2): 0.38748
CV error (K=3): 0.34938
CV error (K=4): 0.37471
CV error (K=5): 0.36091
CV error (K=6): 0.35725
CV error (K=7): 0.41977
CV error (K=8): 0.41219
CV error (K=9): 0.45434
CV error (K=10): 0.46888
CV error (K=11): 0.49933

Table 4-2. The pairwise Fst (below), and geographic distance (above) among the populations of the Asian warty newts in northern Vietnam (The distance unit: km)

Group		East			West					QN		CB
	<i>Pops</i>	<i>DD</i>	<i>HH</i>	<i>DP</i>	<i>NC</i>	<i>Nco</i>	<i>NK</i>	<i>XT</i>	<i>DQ</i>	<i>DS</i>	<i>KT</i>	<i>QT</i>
East	<i>DD</i>	0	23.4	88.6	164.4	140.5	168.7	122	163.2	143.4	163	127.6
	<i>HH</i>	0.052	0	77.3	141	120.2	145.3	101.6	141.6	166.7	187.9	119.8
	<i>DP</i>	0.110	0.141	0	139.2	159	147.9	140.1	170.2	169.6	187.3	40.3
West	<i>NC</i>	0.489	0.444	0.416	0	75.2	11.4	72.7	61.4	293.3	313.9	142.6
	<i>Nco</i>	0.491	0.443	0.423	0.076	0	67.5	20.2	26.2	286.2	308.2	183.8
	<i>NK</i>	0.438	0.369	0.367	0.054	0.051	0	69.0	52.7	307.8	327.1	164.6
	<i>XT</i>	0.466	0.410	0.400	0.116	0.045	0.057	0	41.5	261.4	281.4	162.2
	<i>DQ</i>	0.460	0.421	0.395	0.145	0.073	0.076	0.071	0	308.2	328.2	192.2
QN	<i>DS</i>	0.517	0.440	0.462	0.544	0.547	0.492	0.507	0.506	0	21.3	192.6
	<i>KT</i>	0.555	0.513	0.481	0.606	0.606	0.563	0.589	0.590	0.206	0	204.7
CB	<i>QT</i>	0.221	0.147	0.167	0.383	0.379	0.305	0.333	0.333	0.353	0.335	0

Table 4-3. The niche differentiation among realized niche of the West, East + CB, and QN group from n-dimensional hypervolumes

Niche differentiation		
	West	East + CB
East + CB	1	
QN	1	1
Niche shifts		
	West	East + CB
East + CB	0.034	
QN	0.001	0.055
Niche expansion/contraction (net differences)		
	West	East + CB
East + CB	0.966	
QN	0.999	0.945

Table 4-4. The changes in suitable area for main groups of Asian warty newts in northern Vietnam climate change scenarios SSP245 and SSP485 in 2050 and 2090 (unit: km²)

Group	Present	SSP245-2050		SSP245-2090		SSP485-2050		SSP485-2090	
		Area	Change (%)	Area	Change (%)	Area	Change (%)	Area	Change (%)
West	16378.2	11043.5	-32.6	12581.7	-23.2	10776.2	-34.2	8454.0	-48.4
East + CB	17252.2	20912.6	21.2	14639.9	-15.1	15884.1	-7.9	13893.1	-19.5
QN	5167.0	2873.9	-44.4	2905.5	-43.8	1708.3	-66.9	183.9	-96.4

Figure legends

Figure 4-1. The sampling location for MIG-seq (green circle) and occurrence location (black circle). The West and East populations were identified as *Paramesotriton deloustali*, while CB and QN population were as *P. guangxiensis* by the mtDNA analysis. The direction of gene flow among populations (value > 0.1 are shown) estimated by Div-migrate online was shown by green allows.

Figure 4-2. Results of the (A) PCA analysis of PC1 vs. PC2 with 95% confidence ellipses, (B) ADMIXTURE analysis for $K = 3$, and (C) Two phylogenetic trees constructed from mtDNA and nuDNA (MIG-seq) showed the discordance CB population in relationship with other populations of Asian warty newts in northern Vietnam. The mtDNA tree was modified from Tran et al. (2023a). The nuDNA tree was estimated by VCF2PopTree software (Subramanian et al., 2019). Asterisks indicate adequate statistical support. The GX group was not included in nuDNA tree.

Figure 4-3. Relationship of the F_{st} and geographic distance between populations of the Asian warty newts in northern Vietnam.

Figure 4-4. The diversity genetics across loci in each population of Asian warty newts (H_e : Heterozygosity expected under Hardy-Weinberg equilibrium; H_o : Observed heterozygosity; H : nucleotide diversity; F_{IS} : inbreeding coefficient).

Figure 4-5. The pattern of genetic clusters conditioned to climatic variables projected for West, East + CB, and QN of Asian warty newts under scenarios of climate change SSP245 and SSP485 in 2050 and 2090.

Figure 4-6. The ten-dimensional hypervolume for West, East + CB, and QN groups of Asian warty newts in northern Vietnam. A set of 10,000 randomly sampled points was employed for each hypervolume to delineate their boundaries and shapes.

Figure 4-7. Cohen's Kappa (KAPPA), ROC curve (AUC), True skill statistic (TSS) of five used algorithms predicting the distribution of Asian warty newts in northern Vietnam

Figure 4-8. The suitable distribution of West, East + CB, and QN groups of Asian warty newts in northern Vietnam under climate change scenarios SSP245 and SSP485 in 2050 and 2090.

Figure 4-9. The elevational shift of four main group of Asian warty newts (West, East + CB, and QN) under climate change scenarios.

Figure 4-10. The longitudinal and latitudinal shift of centroid of suitable habitat in Asian warty newts (West, East + CB, and QN) under climate change scenarios.

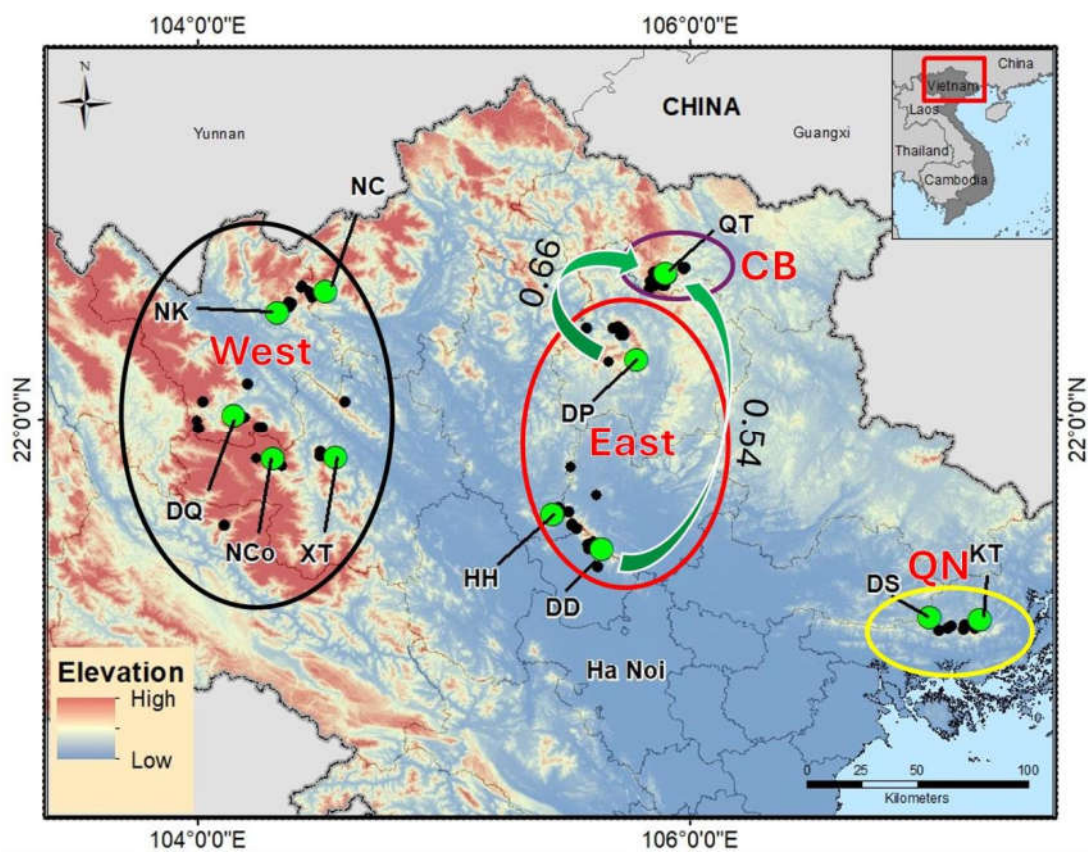


Figure 4-1

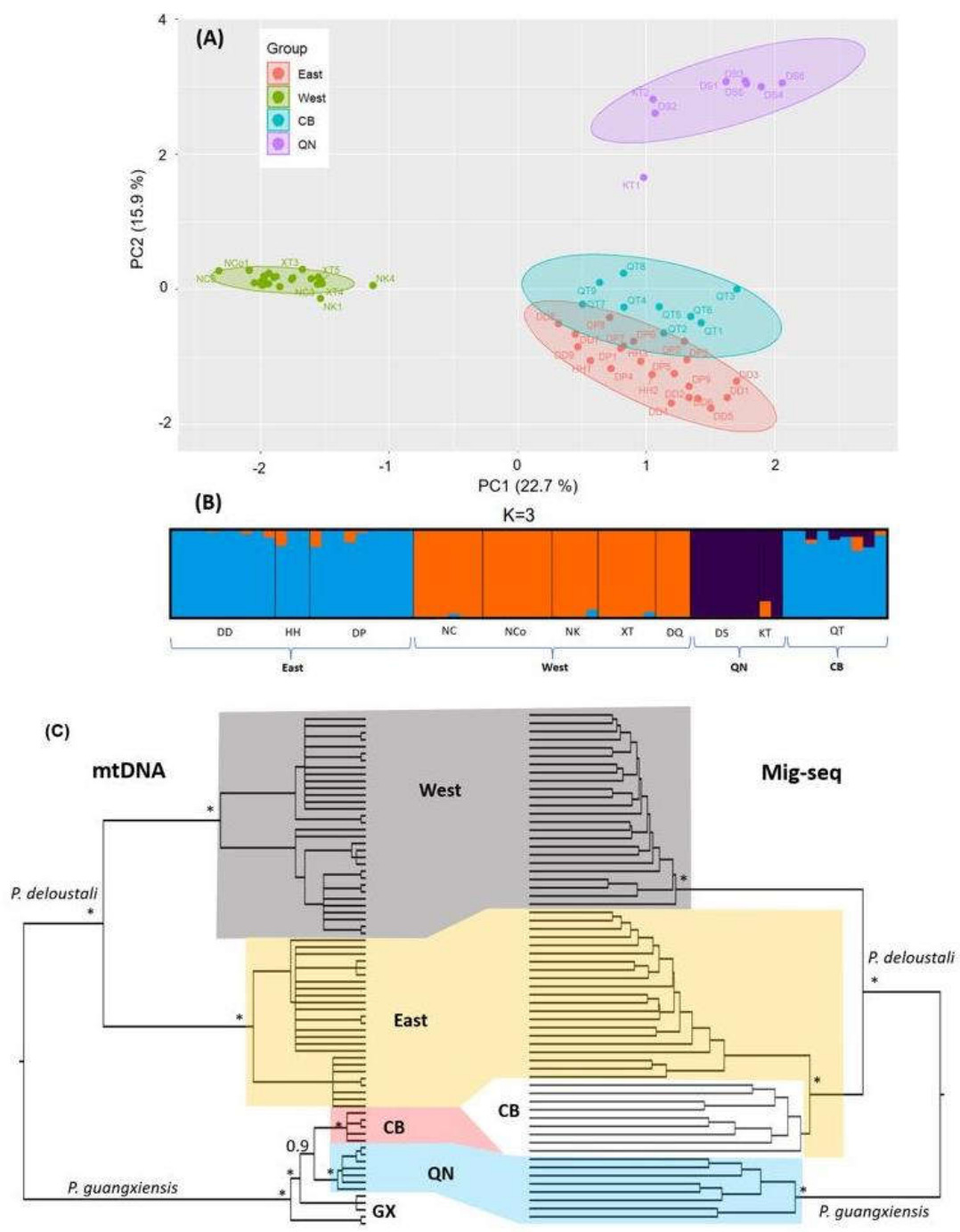


Figure 4-2



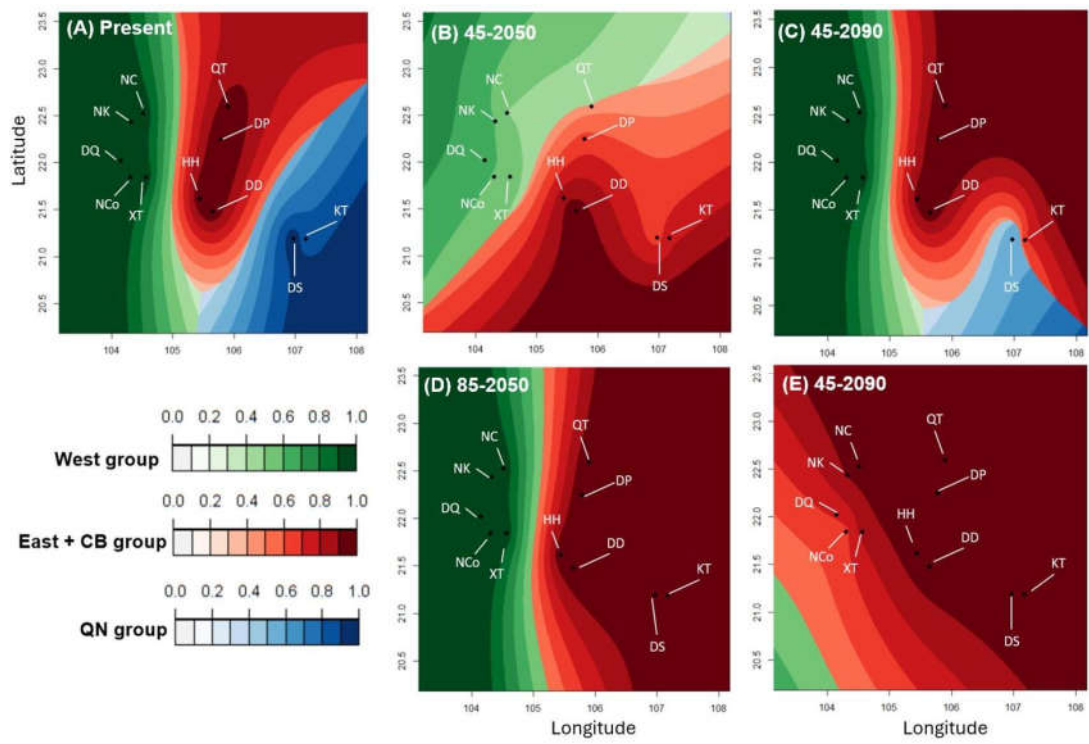


Figure 4-5

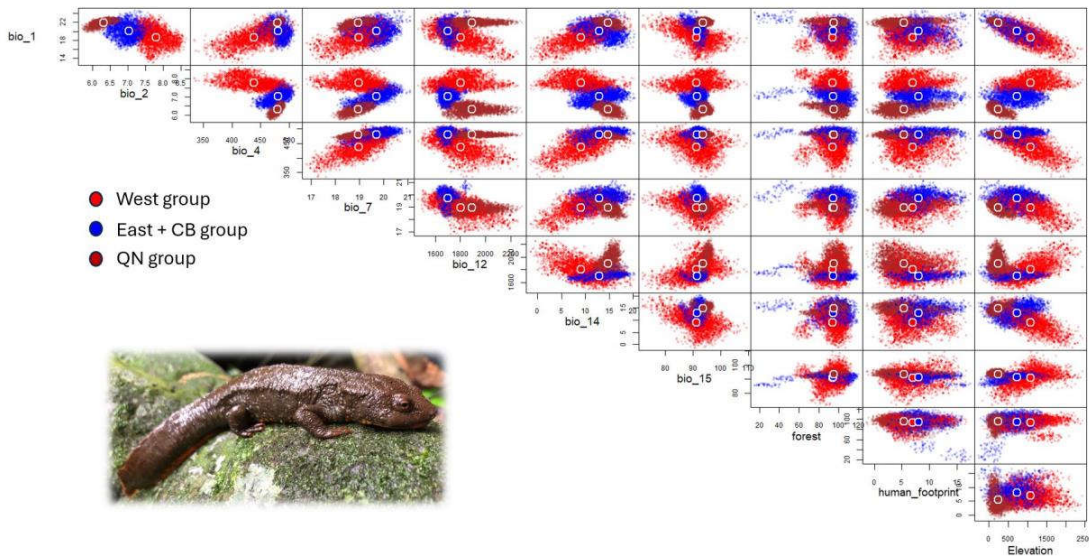
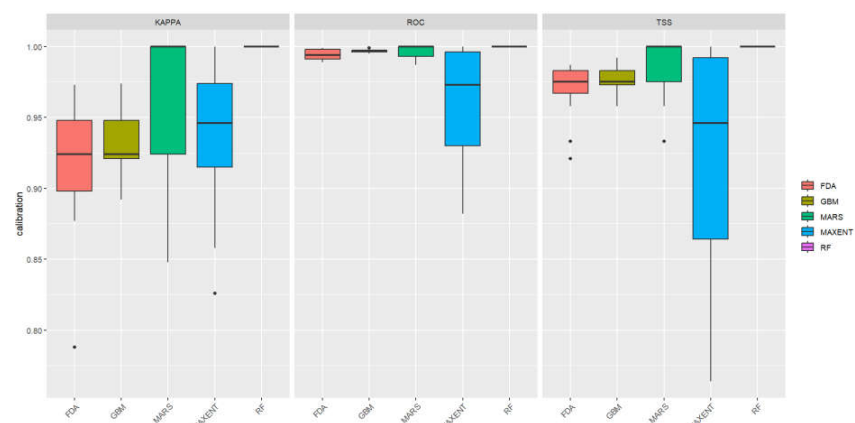
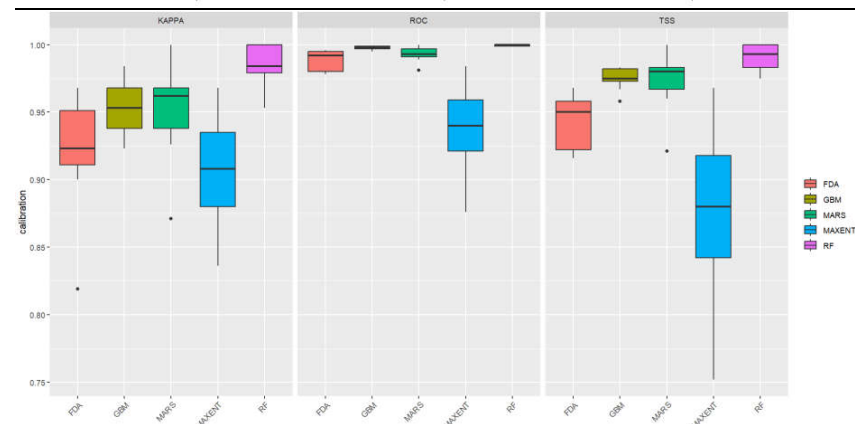


Figure 4-6

West group



East + CB group



QN

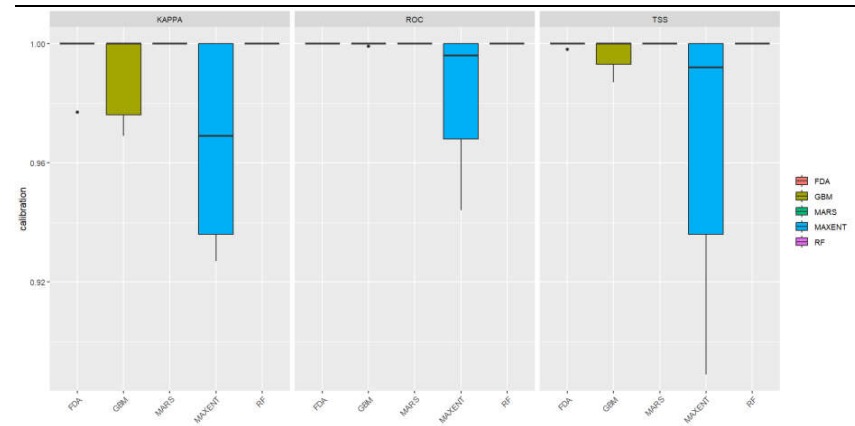


Figure 4-7

West

East + CB

QN

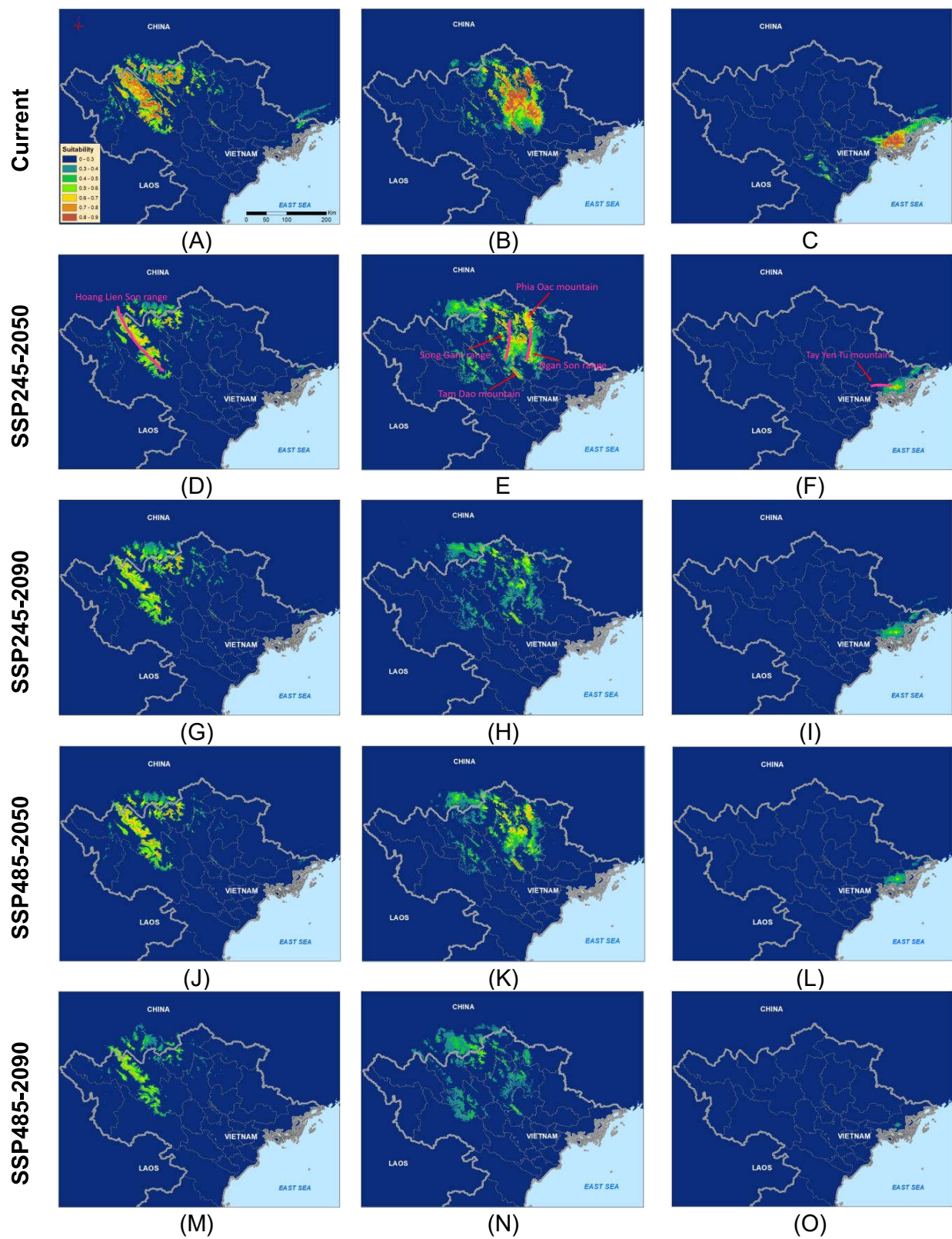


Figure 4-8

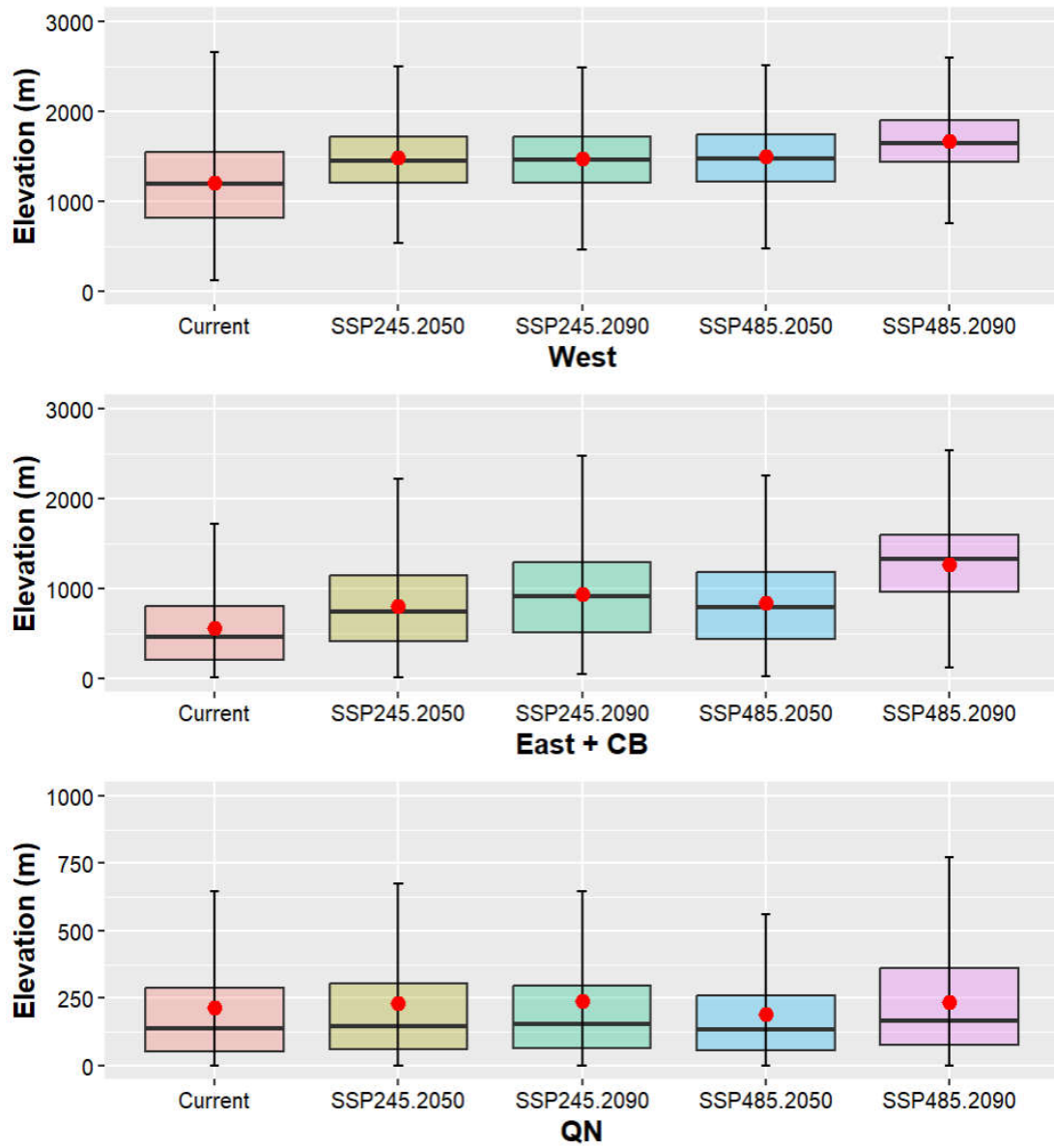


Figure 4-9

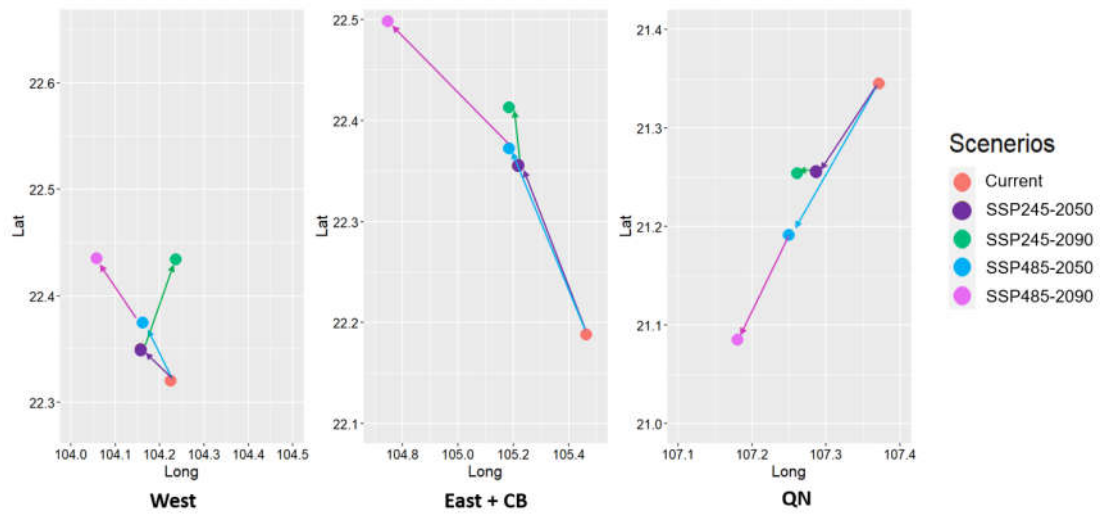


Figure 4-10

CHAPTER 5

Clay model reveals the difference in day and night predation rates on Vietnam warty newt (Caudata: Salamandridae)



In previous chapters, I found that ecological traits significantly influence the morphological variation in Asian warty newts. However, it should be noted that predators can also impact the morphological characteristics of species. In the upcoming chapter, I will identify the potential predators and their attack rates on warty newts towards understanding the predator's effect on the morphological variation.

5-1. Introduction

The predator-prey relationship is one of the major topics in ecology because it occurs globally and has considerable effects on the survival and well-being of numerous species (Berryman, 1992; Caro, 2005; Cooper & Blumstein, 2015). Understanding this relationship could provide valuable insights into the process of evolution (Kuhlmann & Heckmann, 1985) and is essential for development of effective conservation and management strategies (Parsons et al., 2018). However, studying the interactions between predators and their prey is often extremely complex and challenging. Direct observations on the events of predation on amphibians in nature are usually difficult and very rare (Bateman et al., 2017; Niskanen & Mappes, 2005), especially for species with small body sizes, cryptic behaviors and nocturnal habits (Salvidio et al., 2017).

Clay model technique has been widely used to assess the interaction between predators and their prey (Bateman et al., 2017; Röbller et al., 2018; Salvidio et al., 2017). The replicas of prey organisms made from soft, non-toxic modelling clay are exposed in the field for predators to detect and attack. Marks left (teeth, beaks or claws) on the clay models can provide valuable information on the predators (Bateman et al., 2017; Brodie, 1993). Indeed, the method still contains limitations (Bateman et al., 2017; Röbller et al., 2018). For example, lack of odor, detail of shape, reflectance, and movement could negatively affect the detection or attacks of predators, or difficulty in identifying attacked species. However, based on its advantages, such as effectiveness, few legal or ethical requirements, low-cost, and easily shaped and colored clay models, it will continue to be a promising tool for studying predator-prey interactions in nature (Bateman et al., 2017; Costa et al., 2020; Röbller et al., 2018).

For amphibian studies, researchers have successfully used the clay model approach to test the hypothesis on aposematism (Barbieri et al., 2021; McElroy, 2016; Paluh et al., 2014), mimicry (Kraemer & Adams, 2014; Kuchta, 2005), and predator pressure (Mochida, 2011; Velo-Antón & Cordero-Rivera, 2017). For example, Velo-Antón and Cordero-Rivera (2017) implemented a study using clay models and transect surveys in the southwestern Galician coast (Iberian Peninsula). They concluded that the strange diurnal activity of Fire salamander (*Salamandra salamandra*) in the San Martiño was probably affected by strong pressure of nocturnal predators. Otherwise, several similar studies have been conducted to

evaluate the attack rates between daytime and nighttime on reptiles (Costa et al., 2020; Martínez-Freiría et al., 2017; Niskanen & Mappes, 2005). However, the day and night predated proportions of amphibians have not been assessed well.

Vietnam warty newt (*Paramesotriton deloustali*), also known as Tam Dao warty newt, is a native amphibian in Tam Dao National Park (NP), northern Vietnam. The total length is approximately 160–200 mm. The dorsal view has deep olive-brown, while the ventral color is orange-red with a network of black lines (Sparreboom, 2014). The warty newt is predominantly nocturnal and active year-round, and typically prefers to inhabit small streams, pools, and man-made basins within montane evergreen forests (Sparreboom, 2014). In the wild, amphibians are known as preferred foods of several vertebrate and invertebrate animals (Velo-Antón & Cordero-Rivera, 2017; Wells, 2007), and play an important role in many food webs (Hocking & Babbitt, 2014; West, 2018). Furthermore, Vietnam warty newt has been considered as a carnivorous amphibian with several preferred food (e.g. tadpoles, dragonflies, slugs, and worms) (Nguyen et al., 2019; Sparreboom, 2014). However, knowledge of its predators still remains unclear, especially predator composition.

Herein, I applied the clay model method to assess predator pressure on the nocturnal warty newt (*Paramesotriton deloustali*) in Tam Dao NP, northern Vietnam. In particular, I compared the proportions of attacks on newt clay models between the day and night, and determined the rates of attacks by different groups of predators (e.g. mammals and birds). I also evaluated the difference in predated proportions in three habitat types (broadleaf evergreen forest, mixed wood-bamboo forest, and bamboo forest) at different altitudes. Additionally, I employed camera traps to collect data on specific predators of Vietnam warty newt in nature.

5-2. Materials and methods

Study site

My study was conducted in Tam Dao NP, northern Vietnam (21°21'–21°42' N, 105°23'–105°44' E). The national park is a part of Tam Dao mountain range, located in three provinces: Thai Nguyen, Vinh Phuc, and Tuyen Quang, with the highest peak reaching 1,590 m. There are two distinct seasons in Tam Dao NP affected by monsoon winds. The cold and dry season ranges from December to April next year, while the remaining months are in the hot and rainy season. The average temperature is around 23°C, and the annual precipitation is approximately 2600 mm. Here, I designed experiment transects at three study sites, including site A (elevation: 150–300 m a.s.l.), site B (695–720 m asl), and site C (1,179–1,220 m asl; Figure 5-1). The study sites map was created using ArcMap version 10.7 (ESRI, 2019). The elevation data was gathered from GMTED dataset (Danielson & Gesch, 2011) available at <https://earthexplorer.usgs.gov>. The Vietnam warty newt was

found in streams of all three sites. In study site A, the primary habitat is mature forests comprising broadleaf evergreen trees with a top canopy height of 20–30 m, and several shrubs close to streams. The habitat in site B is mainly secondary-growth forest, in which the main component is mixed wood-bamboo forest. The canopy height is around 10–15 m. In site C, the bamboo forest is the majority, with a forest height of approximately 10 m.

Clay model construction

Newt clay models were made from non-toxic plasticine clay with white color (Polymer-Polymax 5) using silicon molds created from a formalin-fixed *P. deloustali* specimen (Nishio Biological Model Co., LTD.). The total length of the model was approximately 180 mm, and the snout-vent length was around 90 mm. Then, the dorsal surface of the clay models was painted with acrylic color (mixing brown and black color 70:30; Mont Marte) to resemble the natural color of the Vietnam warty newt.

Field experiment

From August to September 2022, I conducted field experiments in Tam Dao NP, Vinh Phuc province, Vietnam. I placed 105, 100, and 100 newt clay models in study site A, B, and C, respectively. The clay models were exposed around 5–10 m apart, along streams in the evening of the first day. Then, I checked the displayed model twice per day, at 0700 h and 1700 h for three consecutive days. However, there was an exception for study site A, I had to implement the experiment in four days due to heavy rain and flood on the second day of the experimental period. The data on the second day was not included in my analysis. In site A, I displayed models along streams with a distance of around 0.5–10 m. But in sites B and C, I decided to increase the length (5–30 m) to avoid floods. As part of the control experiment, I displayed 50 plasticine models in site B with different shapes (rectangle, square, and oval) and colors (black, white, and red). These plasticine models were randomly placed with newt clay models. The remaining steps of the control experiment followed the same procedure as that of the newt clay experiment. I scored the model with signs of predator as 1 and without signs as 0. With attacked models, I also categorized the attacked parts (head, torso, tail, and limbs), and potential predators (mammal, bird, undetermined, or arthropod). I detected large tooth marks as signs of mammals, and V-shaped marks of birds bill (Barbieri et al., 2021; Costa et al., 2020; Kuchta, 2005; Niskanen & Mappes, 2005). The arthropod marks were determined when models were attacked with several small bites with various separated pieces of clay around models. I also used photos of attacked marks by mammals and birds in Barbieri et al., (2021); Caideron-Chaico & Putman, (2019); Kuchta (2005); Paluh et al., (2014); Salvidio et al. (2017); Velo-Antón & Cordero-Rivera (2017) as

supporting documents to identify marks of predator groups. I took photos of all attacked models for re-evaluating the marks, then fixed or replaced the damaged models.

I also used four camera traps (three Bushnell, model 19930CWM 2000; and one Covert Illuminator-UV597) to collect data on potential predators by setting the camera traps in front of alive newts and clay models. Camera traps were deployed in each study site for the duration of the newt clay experiment (four days in site A, and three days in sites B and C). I mounted camera traps on the trees at about 20-40 cm above the ground, with following settings: 24 hours/day activation, three photos per trigger, and zero delay between triggers.

Statistical analysis

I took photos of ten randomly selected clay models with a digital camera, then measured the surface area of the head, torso, tail, and limbs by using the ImageJ 1.53k (Schneider et al., 2012). I determined the proportion of expected by chance using average relative proportion on surface area (Costa et al., 2020). Therefore, I applied the proportions 0.164, 0.311, 0.275, and 0.250 for the head, torso, tail, and limbs, respectively, to compare with the observed attacked proportion. Because I checked models at 0700 h and 1700 h each day, I assumed the exposed times were 10 and 14 hours during daytime and nighttime, respectively. Thus, I used the probability of being attacked during the daytime was 0.42 to compare the predated rate of daytime and nighttime.

In the study, I employed Chi-square tests (the significance level at $\alpha = 0.05$) to compare the proportion of attacks between study sites, and the observed predated rate on the body parts (head, torso, tail, and limbs) of clay model with the expected rates based on its surface body areas. The Fisher's exact test was applied for analyzing 2 x 2 tables. The Binomial test ($\alpha = 0.05$) was used to test the difference between attacked proportion during daytime and nighttime. Generally, arthropods do not perceive the clay model as actual prey items (Rößler et al., 2018), then I removed all the arthropod marks from my analysis. All statistical analysis were implemented in SPSS version 20 (IBM Corp, 2011)

5-3. Results

My camera traps at study site A recorded direct attacks by a Greater coucal (*Centropus sinensis*) at 14h58, and a Rat (*Rattus* sp.) at 01h57 on alive warty newt (Figure 5-2A, B). At site B, a Northern treeshrew (*Tupaia belangeri*) attacking warty newt was detected at 17h24 (Figure 5-2C). No recordings of direct attacks were detected by my camera traps at site C. Furthermore, the camera traps installed at sites A and B have detected a high frequency of *Rattus* sp. (Table 5-1).

From the clay model experiments, I detected a total of 45 attacked models: 12/74 (16.22%), 23/97 (23.71%), and 10/98 (10.20%) at study site A, B, and C, respectively (Table 5-2; Table 5-3). The proportions of attacked signs between study sites were significantly different ($\chi^2 = 6.404$, $df = 2$, $p = 0.041$). I did not detect the attacked signs on controlled clay models, except arthropod marks.

My result also showed that the number of attacks in the nighttime was significantly higher than in the daytime: eight versus four attacked models at site A; 18 versus five models at site B, and eight versus two models at site C (Table 5-2). The attacked rates during day and night did not differ between the three study sites ($\chi^2 = 0.798$, $df = 2$, $p = 0.735$). By pooling all attacked models from three sites, I compared the proportion of the day and night attacks. The predation rate of the models differed significantly between daytime (11/45 ~ 24%) and nighttime (34/45 ~ 76%) (binomial test, $p = 0.011$, one-tailed).

Based on the attacked marks on the clay model, I can visually identify the predator groups, for example, mammals (Figure 5-3A), birds (Figure 5-3B), or arthropods (Figure 5-3D). In some cases, precise identification was difficult (Figure 5-3C).

With pooled attacks, my result revealed that the number of attacks on clay models by mammals (25/45) was much higher than the number of attacked models by birds (8/45), or undetermined species group (12/45) (Table 5-2). However, the rates of attacks by predator groups (i.e. mammals, birds, and undetermined) did not show a significant difference between the three study sites, A, B, and C (Fisher's Exact Test, $p = 0.483$; Table 5-4). A similar output was also presented when the attacks of undetermined species were removed from the analysis (Fisher's Exact Test, $p = 0.355$).

For the day versus night proportion of attacks by species group, there was a significant difference between the rate of attacks by mammals between daytime (4/25 ~ 16%) and nighttime (21/25 ~ 84%) with test proportion 0.42 (binomial test, $p = 0.006$, one-tailed). Nevertheless, the attacked proportion by birds did not differ between the daytime (4/8 ~ 50%) and nighttime (4/8 ~ 50%) (binomial test, $p = 0.453$, one-tailed).

Additionally, my experiment showed that there was a highly significant difference between the proportions of observed and expected attacks on model parts (head, torso, tail, and limbs) ($\chi^2 = 56.836$, $df = 3$, $p < 0.001$; Figure 5-4). Significantly, the rate of observed attacks on the head of the newt clay models was three times higher than that of expectation (51.8% observed attacks versus 16.4% of expected predation).

5-4. Discussion

The defensive behaviors to escape from predators have been detected in warty newt (Nishikawa et al., 2014; Tran & Nishikawa, 2021); however, the predation information for

the species has not been officially documented yet. Here, my camera traps provided the evidence for three kinds of predators on Vietnam warty newt (*Paramesotriton deloustali*), including, Northern treeshrew (*Tupaia belangeri*), Rat (*Rattus* sp.), and Greater coucal (*Centropus sinensis*). The species are known as native to Tam Dao NP – the study sites. My finding is consistent with documents on food habits of the predators. Northern treeshrew is primarily crepuscular and omnivorous, eating fruits, seeds, insects, small vertebrates, and bird eggs (Smith & Xie, 2013). *Rattus* species are also mainly nocturnal and omnivorous with a wide variety of food from plants, and animals (Francis, 2019). Another species in the family Salamandridae (Fire salamander, *Salamandra salamandra*) is also recognized as a main prey of *Rattus* species (Velo-Antón & Cordero-Rivera, 2017). Greater coucal is a large bird in the family Cuculidae and is widely distributed in South and Southeast Asia. The bird is an opportunistic predator and feeds on a wide range of foods, including invertebrates and small vertebrates such as amphibians (Choudhary, 2016).

Interestingly, my experiment results showed that the head of the models was the primary target of attacks. The observed attacks on the head were triple those expected by chance based on their relative proportions of surface area (Figure 5-4). It provided evidence that my clay models were perceived as real prey items by predators. Naturally, predators intend to attack the most vulnerable part to kill their preys (Costa et al., 2020), especially dangerous and noxious preys (Kuchta, 2005). A similar tendency was also observed in previous studies applying the clay model approach to assess the predator pressures on salamanders (Barbieri et al., 2021; Costa et al., 2020; Kuchta, 2005) or lizard (Costa et al., 2020). The head covering behavior by raised and coiled tail (Nishikawa et al., 2014; Tran & Nishikawa, 2021) and well-developed frontosquamosal arch (Naylor, 1978) on the skull in *Paramesotriton* must be an advantageous tactics to protect head and eyes from the predators' attack.

The most important finding of the study was that the attacked proportion on the newt clay models in the nighttime was three times higher than that of daytime. The result was consistent with the hypothesis that Vietnam warty newt was under high predator pressures during the nighttime based on its nocturnal behavior (Sparreboom, 2014). My study considered the twilight period of both morning and evening as part of the nighttime. In addition, the composition of predator groups also differed between the daytime and nighttime, with the attacked signs by mammalian species mainly occurred during the nighttime. My camera traps showed that Northern treeshrew and *Rattus* sp. could be major predators on the newts in Tam Dao NP. Both species are primarily active during crepuscular or nocturnal period (Francis, 2019; Smith & Xie, 2013). These reasons could explain the higher rates of predator attacks on warty newt clay models during the nighttime compared to daytime.

My results illustrate that predation proportions on the warty newt clay models were depended on the study sites. It is likely that the attacked pressure on warty newts at the low and medium altitudes (study site A and B) was stronger than that of high altitude (site C). The high attacked proportion probably correlates with the high population density of predators (Velo-Antón & Cordero-Rivera, 2017). In the study, the attacked signs on the newt clay model were mainly by mammals (Table 5-4). Unfortunately, no specific abundance assessment of small mammals has been implemented in Tam Dao NP up to date. Therefore, I did not have enough information about the population of predators of the Vietnam warty newt in natural habitat. However, a high frequency of *Rattus* sp. has been observed through my camera traps (Table 5-1) in study sites A and B. The abundance of small mammals at low altitudes is possibly depended on the food resources availability (Adler et al., 1999; Heaney, 2001; Wiles, 1981). The main habitat in sites A and B are primarily broadleaf evergreen forests, and mixed broadleaf evergreen and bamboo forest. In the habitat type, I believed that small mammals could easily find their preferred foods compared to the bamboo forest. I suggest that a systematic grid of camera traps in the elevation levels and habitat types could be possible to assess the population status and distribution of the newt predators.

Studying predation on organisms in nature is often difficult and may face many challenges (Bateman et al., 2017), particularly in remote and complex geographic areas. Like other studies using the clay model approach, my study also had limitations. I were able to mimic the shape of warty newt by using a mold made from a real specimen. However, I have still used motionless replicas, which could negatively affect the number of attacks (Barbieri et al., 2021; Bateman et al., 2017; Paluh et al., 2014; Rößler et al., 2018). Several predators use the movements to find their preys, especially birds (Paluh et al., 2014; Rößler et al., 2018). Although the study has observed predators (birds and small mammals) attacking the newt clay models, their color reflectance remains unassessed. As avian and mammalian species have different color vision from humans (Bowmaker, 1998), I recommend that the reflectance of the clay models should be measured for future research. Another limitation probably affecting the attack rates of predators was the odor of the clay models (Bateman et al., 2017) when mammals primarily use odor to search their preys (Apfelbach et al., 2005; Costa et al., 2020). Similar to other clay model studies in the tropical rain forest, many of my clay models were damaged because of arthropod attacks, probably leading to the loss of the signs of other predator attacks. I had to replace damaged models with new items causing field cost increases. Furthermore, the frequency disturbances of field researchers could also influence the occurrence of shy and rare predators (Rößler et al., 2018).

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Table 5-1. Number of detections of small mammals in three study sites (A, B, and C) in Tam Dao National Park (Note: 1 hour threshold was used to denote independent detections)

Site	Detected species	Number of detections
A	<i>Ratus</i> sp.	27
	Small-toothed Ferret Badger (<i>Melogale moschata</i>)	1
	Pallas's Squirrel (<i>Callosciurus erythraeus</i>)	1
B	<i>Ratus</i> sp.	22
	Northern Treeshrew (<i>Tupaia belangeri</i>)	3
	Pallas's Squirrel (<i>Callosciurus erythraeus</i>)	2
C	<i>Ratus</i> sp.	6
	Northern treeshrew (<i>Tupaia belangeri</i>)	2
	Striped Squirrel (<i>Tamias</i> sp.)	1
	Pallas's Squirrel (<i>Callosciurus erythraeus</i>)	1

Table 5-2. Newt clay models were displayed and attacked in the three study sites

Site	Displayed model	Attacked by day	Attacked by night	Total attacked model	Not attacked	Missing	Note
A	105	4	8	12	62	31	Heavy rain, flood
B	100	5	18	23	74	3	
C	100	2	8	10	88	2	
Total	305	11	34	45	224	36	

Table 5-3. Information of attacks on newt clay models in Tam Dao National Park

(1: attack)

No	Site	Time of day		Predator group			Attacked region			
		Day	Night	Mammal	Bird	Undeter mined	Head	Torso	Tail	Limbs
1	A	1		1				1		
2	A		1			1			1	
3	A		1	1				1		
4	A		1			1	1			
5	A		1	1				1		
6	A	1			1		1			
7	A	1		1				1		
8	A	1			1			1		
9	A		1	1			1			1
10	A		1			1	1			1
11	A		1	1						1
12	A		1	1			1			
13	B		1	1			1	1		
14	B		1	1			1			
15	B		1	1						1
16	B		1		1			1	1	
17	B		1	1				1		
18	B		1	1			1			
19	B		1	1			1	1		
20	B		1		1		1			
21	B		1	1			1			
22	B		1		1		1			
23	B		1	1			1			
24	B		1	1						1
25	B		1			1	1		1	
26	B	1			1			1		
27	B	1				1	1	1		
28	B	1		1			1	1		
29	B	1			1				1	
30	B		1			1	1			
31	B		1		1		1	1		
32	B		1	1			1	1		
33	B		1			1	1			
34	B		1	1			1			
35	B	1				1	1			
36	C		1	1			1			
37	C		1	1				1		
38	C		1	1			1			
39	C		1			1			1	
40	C		1	1			1	1		
41	C		1			1			1	

No	Site	Time of day		Predator group			Attacked region			
		Day	Night	Mammal	Bird	Undeter mined	Head	Torso	Tail	Limbs
42	C		1			1	1			
43	C		1	1			1			
44	C	1				1	1			
45	C	1		1			1			

(continue Table 5-2)

Table 5-4. The number of attacks on newt clay model by species groups

Site	Mammal attack	Bird attack	Undetermined attack	Total
A	7	2	3	12
B	12	6	5	23
C	6	0	4	10
Total	25	8	12	45

Figure legends

Figure 5-1. The study sites of clay model experiment in Tam Dao National Park, northern Vietnam (elevation data was gathered from <https://earthexplorer.usgs.gov/>, map was created by ArcMap 10.7 (ESRI)).

Figure 5-2. *Paramesotriton deloustali* being preyed by *Centropus sinensis* (A), *Rattus* sp. (B), and *Tupaia belangeri* (C), a clay model was attacked by a *Rattus* sp. (D).

Figure 5-3. Representative predated clay models. (A) Mammalian incisor mark, (B) Bird peck mark, (C) Damaged model due to unknown predator, (D) Damaged model due to arthropod attacks.

Figure 5-4. Comparison of the percentages of expected and observed attacks on newt clay model parts (head, torso, tail, and limbs).

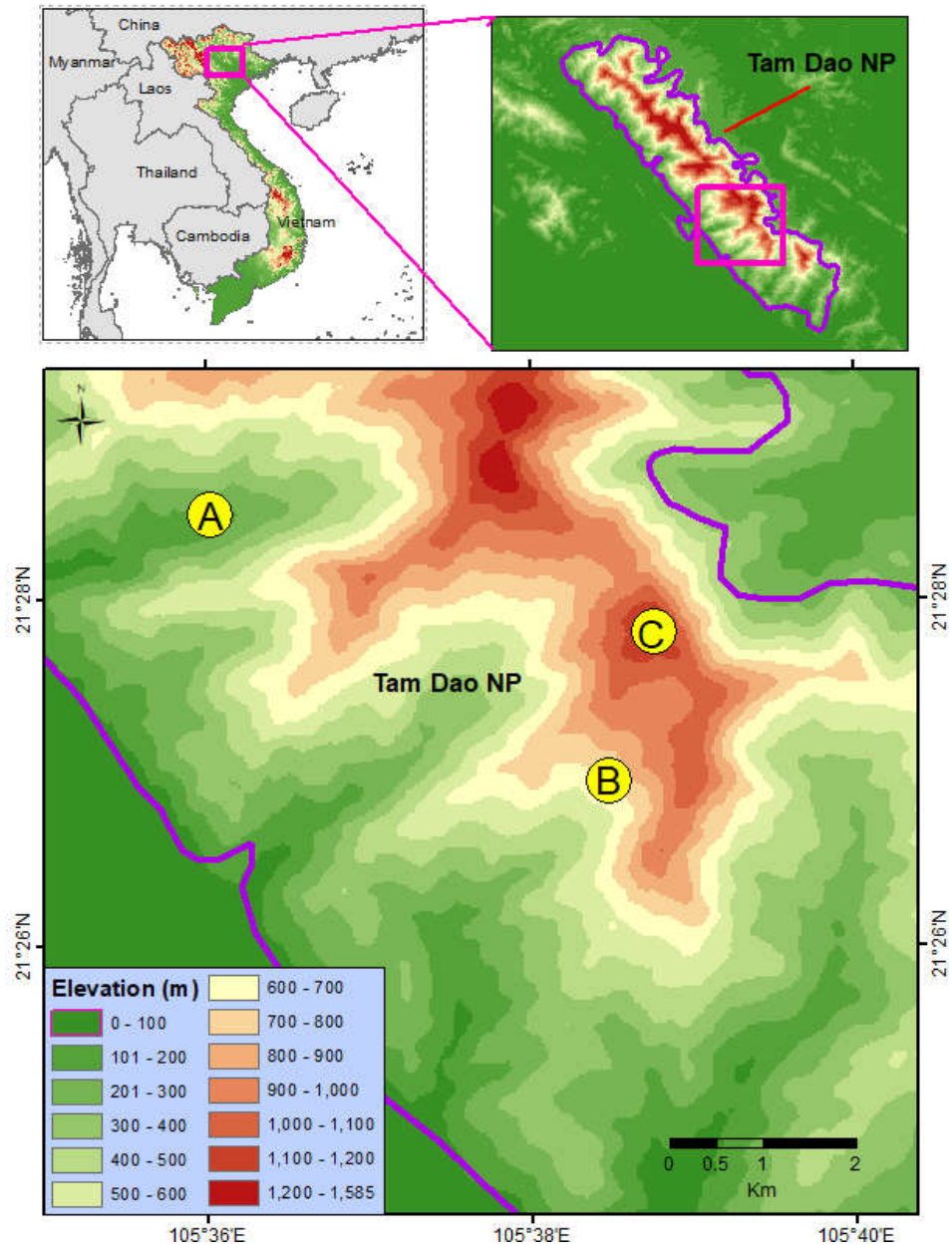


Figure 5-1



(A)



(B)



(C)



(D)

Figure 5-2



(A)



(B)



(C)



(D)

Figure 5-3

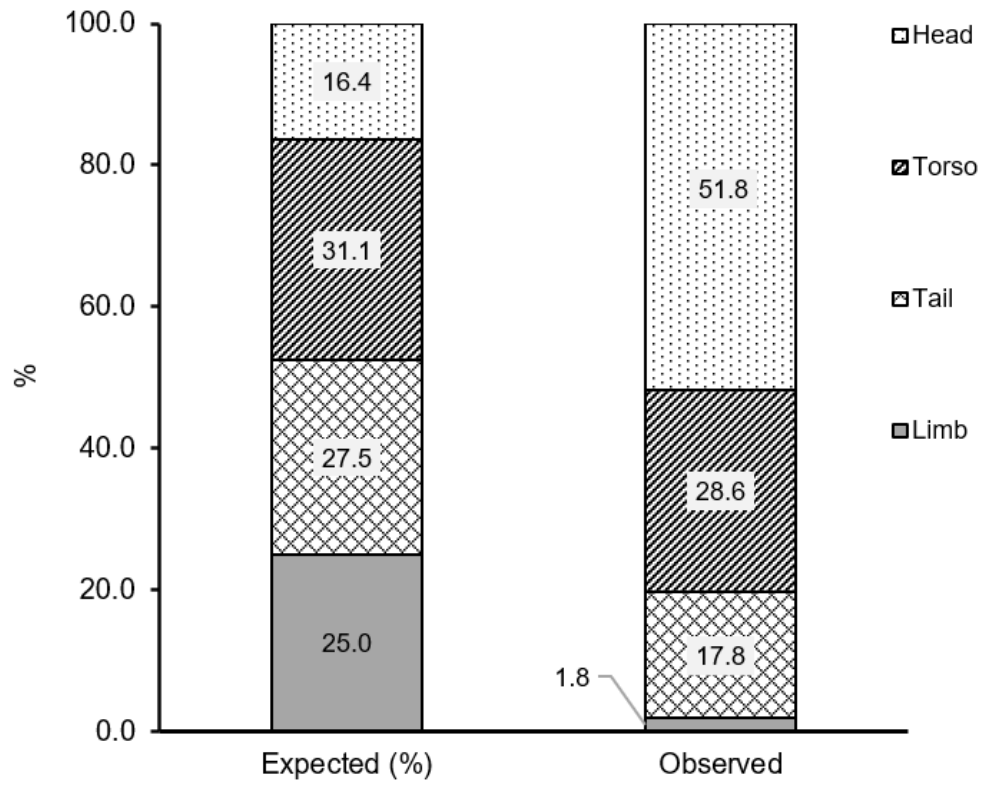


Figure 5-4

CHAPTER 6

Variation in ventral coloration pattern and aposematism in tropical warty newts



In Chapter 5, I identified several predators of Asian warty newts. Here, I will examine geographic variation in aposematism among populations and assess the interaction between predators and the aposematic signals of the warty newts.

6-1. Introduction

Aposematic coloration refers to the conspicuous and distinctive color patterns or markings exhibited by certain animals, commonly including red, yellow, or white patterns on a black background (Cott, 1940). Aposematic organisms possess defensive traits that render them unpalatable and employ warning signals to convey these defenses to their natural predators (Edmunds, 1974; Mappes et al., 2005; Poulton, 1890). Aposematic coloration may be influenced by a complex interplay of multiple factors, including physiological limitations, sexual selection, and the pressures of natural selection (Briolat et al., 2019). For instance, Barzaghi et al. (2022) highlighted that ambient environmental factors and resource availability have a greater impact than local adaptations in shaping differences in aposematic color patterns of the European fire salamander (*Salamandra salamandra*). The evolutionary origins of aposematic coloration are considered among the most complex and challenging to elucidate (Briolat et al., 2019; Endler & Mappes, 2004; Mappes et al., 2005).

Aposematic species can display variation in their aposematic characteristics throughout their natural distribution (Mochida, 2009; Noonan & Comeault, 2009). My knowledge of the factors driving the variation is still significantly limited for many taxa. Amphibians are excellent model species for gaining insights into the relative roles of adaptation and plasticity in shaping the variation of aposematic patterns (Wells, 2007). In this study, I examined the ventral coloration of the Vietnam warty newt (*Paramesotriton deloustali*) and Guangxi warty newt (*P. guangxiensis*) to reveal the aposematic variation among these tropical warty newts. These species exhibit allopatric distribution in southern China and northern Vietnam, and particularly favor cool montane streams located within closed-canopy evergreen forest habitats (Sparreboom, 2014; Tran et al., 2023). The two species share relatively similar morphological and ecological characteristics (Tran Van Dung, unpublished data). Especially, both species possess a red or orange ventral coloration with a black line network, and display aposematic coloration as a defensive behavior (Sparreboom, 2014; Tran & Nishikawa, 2021; Wells, 2007). To display ventral aposematic coloration, newts adopt a distinctive posture by turning the ventral side, curving the body, coiling the tail, and folding limbs to enclose the body (see Tran & Nishikawa, 2021; Wells, 2007).

In the present study, I explored the aposematic ventral coloration of adult warty newts from various populations across different regions in northern Vietnam, aiming to

uncover interpopulation variation. Additionally, I analyzed the connection between the aposematic ventral area and environmental factors to discern the spatial determinants of this variation. I also utilized clay model experiments to test two hypotheses regarding the interaction between predators and the aposematic signals of the warty newts. First, I tested the null hypothesis (H_{10}) that the presence of a red area on the ventral side of warty newts does not significantly deter predator attacks. Rejection of the H_{10} will support the idea that the warty newts are truly aposematic. Second, I hypothesized (H_{20}) that the size of the red ventral area does not have a substantial impact on the rate of predator attacks. If the hypothesis was rejected, I expected that newts with large area of red ventral coloration would be attacked less frequently than those with small red area. Recently, the clay model technique has been extensively employed to evaluate the interaction between predators and their prey (Bateman et al., 2017). It has proven particularly successful in studying urodeles (e.g., Barbieri et al., 2021; Kuchta, 2005; Salvidio et al., 2017).

6-2. Materials and methods

Ventral aposematic traits

Paramesotriton deloustali and *P. guangxiensis* are distributed in northern Vietnam and southern China (Sparreboom, 2014; Tran et al., 2023). To analyse the geographic variation in aposematic coloration of the warty newts, I examined 200 adult individuals from eight populations (Figure 6-1, and Table 6-1) during my field surveys in 2022. I took photographs of the ventral surface of captured newts using a Canon digital camera (model EOS Rebel T6), and measured snout-vent length using digital calipers (nearest 0.1 mm). All the captured newts were then released back to nature at their detected area. The procedures for conducting animal operations followed the guidelines for animal experimentation of Kyoto University, Japan (Nos. 20-A-7 and 22-A-2). The sex of the newts was determined by observing around the opening of the cloaca (swollen in male and not-swollen in female), while maturity was assessed based on body size (> 120 mm and 90 mm in total length as matured for *P. deloustali* and *P. guangxiensis*, respectively; Tran Van Dung, unpublished data). In the study, I selected a region of interest (ROI) at the abdominal part from the pectoral girdle to the pelvic girdle of collected newt for analyzing photographs. I selected the ROI of each photograph using selection tools and counted the number of pixels in Adobe Photoshop CC2017 version. Then, the red areas were selected using the range color tool and the number of pixels was counted. I calculated the relative red ventral area of each newt by the ratio of number of pixels of red area and number of pixels of the ROI.

Clay model experiment for antipredator defense

I examined the antipredator defense of the newts using clay model experiments to mimic the newts. I made newt replicas using the same method that was described in Tran and Nishikawa (2023) for three types of model: one mimicking the dorsal dull newt coloration, and two showing the red and the dark ventral coloration (Figure 6-2). The dorsal models were painted brown color (mixing brown and black color with ratio 70:30; acrylic color Mont Marte) to represent the dorsal surface of the warty newt. The red and black ventral models were painted with orange and black color (acrylic color Mont Marte) to resemble the ventral side of newts with around 70% and 30% red ventral surface area, respectively (Figure 6-2).

Clay model experiments to examine the antipredator defense of *P. deloustali* were conducted at three sites: A, B, and C (see details in Tran and Nishikawa [2023]) in Tam Dao NP, while another experiment was conducted at site D in Dong Son – Ky Thuong NR for *P. guangxiensis* (from July to September 2022). In each site, I displayed 100–110 replicas for each clay model type. Specifically, I used 410, 415, and 417 clay models for dorsal, red, and black ventral type, respectively (for details, see Table 6-2). The clay model types were alternately exposed around 5–10 m apart along streams where I detected the presence of warty newts. In study site A, several models were lost due to a severe flood (see Tran & Nishikawa [2023]), and in site D, some models were removed and collected by local people. For a control experiment, I placed 50 plasticine models at site B, featuring a variety of shapes such as cubes, parallelepipeds, and spheres, and different colors including black, white, and red. I checked models twice per day in the morning (7:00) and afternoon (17:00) for 3 consecutive days. I marked models with predator attacks as 1, while the models without attacks were scored as 0. I identified the sign of attacks by mammals, birds, unidentified, and arthropods following the protocol in Tran and Nishikawa (2023: and see Figure 6-3).

Statistics

I examined the difference of the red ventral area between *P. deloustali* and *P. guangxiensis*, and between male and female individuals using the Mann-Whitney test. I also examined the correlation between the red ventral area of the newt populations and their SVL. Because there was a significant difference between SVL of male and female of warty newts (Tran Van Dung, unpublished data), I applied the standardized SVL (measured SVL/mean SVL of all males or females) for the analysis with the mean SVL of males and females being 74.73 and 79.19 mm, respectively. I also used the one-way Welch test to reveal the interpopulational variation of the newts. Then, to examine the relationship of the red ventral area of newts among a population with environmental variables and standardized SVL, I used a generalized linear model (GLM) by fitting red ventral area as a response term, and

temperature annual range, annual precipitation, and standardized SVL as explanatory terms. The temperature annual range and annual precipitation are available from WorldClim version 2.1 (Fick & Hijmans, 2017). I employed the population mean values for response and explanatory variables (Mochida, 2009).

I compared the number of attacks among three types of newt clay models: dorsal, red, and black ventral types using chi-square tests. I classified all arthropod attacks as “no attack” in my analysis since arthropods typically do not perceive clay models as real prey items (Rößler et al., 2018). The missing models were primarily missing due to flooding and human impacts; consequently, I excluded them from my statistical analysis. Here, I conducted all statistical analyses in R version 4.3 (R Core Team, 2023) with the significance level at $\alpha = 0.05$.

6-3. Results

Ventral aposematic variation

I observed a significant difference in the red ventral area of *P. deloustali* ($58.68 \pm 13.09\%$) when compared to *P. guangxiensis* ($39.86 \pm 15.36\%$; Mann-Whitney U test, $U = 8117$, $p < 0.001$). Among groups under species level, there was no significant difference between the West and East groups of *P. deloustali* (Mann-Whitney U test, $U = 1771$, $p = 0.815$), while the red ventral area was significantly different between the CB and QN groups of *P. guangxiensis* (Mann-Whitney U test, $U = 760$, $p = 0.012$).

Regarding sexual discrimination, my analysis revealed no significant difference in the red ventral area between males and females of *P. deloustali* (Mann-Whitney U test, $U = 2090$, $p = 0.413$). However, I found a significant difference in the relative extent of the red ventral area, with males exhibiting a larger extent compared to females in *P. guangxiensis* (Mann-Whitney U test, $U = 927.5$, $p = 0.017$). Analyzing for each population, I only detected a significant difference between males and females of the CB population (Mann-Whitney U test, $U = 570.5$, $p < 0.0001$). By pooling all data, I found no significant difference in the red ventral area between males and females (Mann-Whitney U test, $U = 5983.5$, $p = 0.308$).

I also found a significant interpopulational variation in the red ventral surface of the warty newts (one-way Welch: $F_{22,367} = 59.032$, $p < 0.0001$). The CB group of warty newts exhibited the lowest red ventral area ($37.60 \pm 9.74\%$), while the NC population had the highest red ventral area ($63.39 \pm 10.06\%$; see Table 6-3). Notably, the red ventral surface of the CB population was significantly different from those of all other populations (pairwise t tests: all $p < 0.001$).

In GLM, the variation in red ventral area among populations was adequately explained by three explanatory variables: temperature annual range, annual precipitation, and standardized SVL ($R^2 = 0.73$). However, only the temperature annual range significantly contributed as an explanatory variable, while annual precipitation and standardized SVL did not reach statistical significance (temperature annual range: $Coef = -18.01$, $t = -3.06$, $p = 0.0280$; annual precipitation: $Coef = -0.01$, $t = -0.65$, $p = 0.541$; standardized SVL: $Coef = 105.72$, $t = 2.20$, $p = 0.078$). I found a negative significant relationship between the red ventral area and temperature annual range among populations (Pearson's correlation coefficient: $r = -0.69$, $p = 0.038$). However, I did not find a significant correlation between red ventral area and standardized SVL (Pearson's partial correlation coefficient: $r = 0.104$, $p = 0.137$).

Potential antipredator defense

To examine the antipredator defense of *P. deloustali*, 931 clay models were used for the experiments in three study sites (A, B, C) in Tam Dao NP; 79 models were attacked, among which 47 (59%), 14 (18%), and 18 (23%) models were attacked by mammals, birds, and unidentified predators, respectively. There were no attacks by predators except arthropods on clay models in the control experiment. Additionally, 57 models were attacked by arthropods, and 107 models were missing. The absence of models was primarily caused by a severe flood (site A) and the involvement of local people (site D). By comparing among types of newt replicas, I found that dorsal type was attacked at higher frequency than both red and black ventral types ($\chi^2 = 12.794$, $df = 1$, $p = 0.0003$; $\chi^2 = 15.755$, $df = 1$, $p < 0.0001$, respectively; Figure 6-3). However, the attacked rates between red and black ventral types were not significantly different ($\chi^2 = 0.053$, $df = 1$, $p = 0.817$; Figure 6-4). For the clay model experiment in Dong Son – Ky Thuong NR (site D), I used 311 clay models, and 28 models were missing, and 24 clay models were attacked by predators. Specifically, 15, five, and five clay models were probably attacked by mammals, birds, and unidentified predators, respectively. The ratio of attacked dorsal models was also significantly different compared to those of red and black ventral models ($\chi^2 = 8.137$, $df = 1$, $p = 0.004$; $\chi^2 = 4.671$, $df = 1$, $p < 0.03$, Figure 6-4). Similar to the experiment in Tam Dao NR, my analysis did not reveal a significant difference between the frequencies of attacks on red and black ventral types ($\chi^2 = 0.179$, $df = 1$, $p = 0.671$).

6-4. Discussion

Aposematic coloration variation can be influenced by various factors, including evolutionary, genetic, ecological, and physiological processes (Ojala et al., 2007; Mochida, 2011; Briolat et al., 2019). My analyses revealed a significant difference in aposematic

coloration between *P. deloustali* and *P. guangxiensis*, with *P. deloustali* exhibiting a larger red area compared to *P. guangxiensis*. The two warty newts represent the southernmost distribution within the genus *Paramesotriton* and exhibit allopatric distribution. I propose that a comprehensive study comparing ventral coloration among species within the genus *Paramesotriton* is necessary to elucidate the relationship between genetics and aposematic coloration in this genus.

Previous research on newts has indicated that males often exhibit a larger bright coloration area compared to females (Balogová & Uhrin, 2015; Kopecký & Šichtař, 2014; Mochida, 2009). This difference can be attributed to the fact that females frequently need to allocate carotenoids to their offspring, which may reduce the availability of carotenoids for aposematic coloration (Hill, 1999; Mochida, 2009). In contrast, the larger aposematic coloration in males can enhance their attractiveness during mating (Balogová & Uhrin, 2015). My study found no significant differences in the red ventral area between males and females, except for the CB population. This variation could potentially be explained by differences in the sex ratio of the sampled newt individuals among populations. In most of my examined populations, I observed a higher number of captured females compared to males (see Table 6-3).

Ventral coloration in newts can be influenced by body size. For instance, the European fire salamander (*Salamandra salamandra*; Barzaghi et al., 2022) has shown a significant correlation between the percentage of yellow coloration and the total length of newts. However, when studying another newt species, *Cynops pyrrhogaster*, Mochida (2009) found no significant link between the red ventral coloration area and body size. In my analyses, I similarly failed to identify any significant correlation between the red ventral area and the standardized SVL of warty newts. This suggests that the body size of the examined warty newt populations does not play a significant role in affecting their aposematic coloration.

Organisms' warning signals can be influenced by the ambient environment (Briolat et al., 2019; Hegna et al., 2013; Lindstedt et al., 2009). In particular, variation in temperature is known to play a significant role in shaping the levels of melanism in aposematic coloration (Briolat et al., 2019; Michie et al., 2011). My study revealed a significant negative correlation between the extent of red ventral coloration and the range of annual temperatures. Populations with high red ventral coloration tend to be more prevalent in regions with a narrower annual temperature range. This finding contrasts with the results of Mochida (2009), who found that thermal selective pressure did not influence the ventral coloration of *C. pyrrhogaster*. Furthermore, the diet of organisms can also impact the size of their aposematic coloration (Barzaghi et al., 2022; Mochida, 2011; Ojala et al., 2007). Invertebrate prey containing carotenoids are responsible for defining the red and yellow patterns in aposematic amphibians (Umbers et al., 2016). Carotenoid productivity can vary

locally in different environments, influenced by temperature differences (Mochida, 2011). However, there is currently no available data indicating a specific relationship between the diet of warty newts and their red ventral coloration. Nguyen et al. (2019) demonstrated that the primary prey items of *P. deloustali* were predominantly invertebrates (e.g., Trichoptera, Ephemeroptera, Coleoptera). It is important to note that fluctuations in temperature can significantly affect the abundance and richness of invertebrate prey (Figueroa et al., 2021), which could indirectly influence warty newt coloration.

Defensive behaviors, involving the display of aposematic coloration, have been documented in *P. deloustali* (Wells, 2007) and *P. guangxiensis* (Tran & Nishikawa, 2021). However, there is currently a lack of empirical data demonstrating the effectiveness of this antipredator strategy in warty newts. My clay model experiments yielded a significantly reduced rate of attacks on ventral models in comparison to dorsal models, providing support for the efficacy of displaying bright ventral coloration as a defense against predator attacks. In other words, my results support the aposematic hypothesis in the warty newts. Predation pressure can significantly influence the extent of aposematic coloration in urodeles (Barzaghi et al., 2022; Mochida, 2009, 2011). For example, Mochida (2009) demonstrated that newts residing on islands displayed more prominent aposematic coloration, likely influenced by variation in the abundance of mammalian predators between island and mainland habitats. However, I did not observe a significant difference in predator attack rates on red and black ventral models across all study sites, leading to acceptance of the null hypothesis (H_{20}). Although comprehensive data on the predators of newts in the entire population region are still lacking, camera traps used by Tran and Nishikawa (2023) revealed a high frequency of small mammals in the areas where my clay model experiments were conducted. Furthermore, my experiments indicated that the majority of attacks on the clay models were carried out by mammals rather than birds. Most mammal predators of amphibians are color-blind, which suggests that visual signals might be less effective in defending against mammalian attacks (Mochida, 2009, 2011). My findings imply that predation pressures from mammals may not be the primary factor influencing the variation in the red ventral coloration area of warty newts.

Implementing clay model in nature can encounter numerous problems due to intricate geography and unpredictable weather conditions. Moreover, the predation rates are often low, therefore, the experiment requires a larger quantity of models to have sufficient samples for demonstrating predation patterns (Noonan & Comeault, 2009), leading to time-consuming and costly for creating clay models. Here are some notes I would like to recommend during conducting clay model experiments in the field. Firstly, I recommend utilizing silicone master molds for producing clay models, as proposed by Yeager et al., (2011), as it proves to be an effective technique for generating multiple models. Following this technique, I and two other field assistants successfully made more than 1200 clay

models in five working days, including both the making and painting of the models. Secondly, it is very important to handle the transportation of clay models with care because the legs and tail of the models are very easy to break. My field assistant (Mr. Ky) and I had an unforgettable experience when I delivered my models to the field by motorbike, covering more than 200 km to Dong Son – Ky Thuong NR. Unfortunately, approximately one-third of the total models suffered damage during this journey. So, I had to make new clay models to replace the broken ones. Consequently, if field locations are situated at a far distance, making clay models in the field using master molds becomes a preferable option. Thirdly, I emphasized the importance of avoiding exposure of clay models to direct sunlight, because it can cause them to be hard and dry. The summer in northern Vietnam is characterized by strong sunlight. On the first day of my experiment, some clay models were left exposed to direct sunlight without the protection of tree canopy. Within a day, these models had become hard and dry, I had to replace them with new ones. Additionally, the safety of both surveyors and clay models during the rainy seasons is also crucial. I made the mistake of placing clay models too close to streams, resulting in the loss of 31 clay models due to flooding at site A of my experiment in Tam Dao NP (see Chapter 5). Last but not least, checking hundreds of clay models in the field twice a day proved to be time-consuming, resulting in signs of attacks not being thoroughly examined. Therefore, after preliminary checks in the fields, I should take photos of all signs of attack to recheck them in the laboratory.

In conclusion, the present study suggested that the variation in the red ventral area of warty newts could potentially be influenced by multiple selection factors, including genetic constraints, sexes, ambient environment (temperature), and diet. My results indicated that body size variation and the pressure of mammal predation may not significantly contribute to the determination of the aposematic variation. However, my study further supported the effectiveness of displaying aposematic coloration in antipredator defense of warty newts.

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Table 6-1. Population number (Pop), species and sample size of warty newts used for red ventral area analyses

Pop	Species	Location	Group (mtDNA)	Number of samples			Latitude	Longitude	Altitude (m)
				Male	Female	Total			
NK	<i>P. deloustali</i>	Bac Ha, Lao Cai, Vietnam	West	4	7	11	22.47	104.38	948
NC	<i>P. deloustali</i>	Xin Man, Ha Giang, Vietnam		11	8	19	22.49	104.46	893
NCo	<i>P. deloustali</i>	Mu Cang Chai, Yen Bai, Vietnam		16	15	31	21.82	104.33	1584
XT	<i>P. deloustali</i>	Van Yen, Yen Bai, Vietnam		9	9	18	21.87	104.50	636
DP	<i>P. deloustali</i>	Bach Thong, Bac Kan, Vietnam	East	5	7	12	22.26	105.78	722
DD	<i>P. deloustali</i>	Tam Dao, Vinh Phuc, Vietnam		5	14	19	21.47	105.60	263
TB	<i>P. deloustali</i>	Tam Dao, Vinh Phuc, Vietnam		5	10	15	21.49	105.62	954
QN	<i>P. quangxiensis</i>	Ha Long, Quang Ninh, Vietnam	QN	8	12	20	21.25	107.04	100
CB	<i>P. quangxiensis</i>	Nguyen Binh, Cao Bang, Vietnam	CB	31	24	55	22.59	105.85	857
Total				94	106	200			

Table 6-2. The number of displayed clay models and attacked model in four study sites to examine the antipredator defense of warty newts in northern Vietnam

Type	Site	Display models	Attacked by mammals	Attacked by birds	Undetermined	By arthropod	Total attacked	Total for calculating	Missing	Note
Dorsal model	A	105	7	2	3	5	12	74	31	(rain)
	B	100	12	6	5	33	23	97	3	
	C	100	6	0	4	7	10	98	2	
	D	105	11	3	2	10	16	97	8	
Sub-total		410	36	11	14	55	61	366	44	
Red ventral model	A	105	3	2	0	20	5	80	25	(rain)
	B	103	6	2	1	40	9	92	11	
	C	103	3	1	0	8	4	98	5	
	D	104	2	1	0	7	3	95	9	
Sub-total		415	14	6	1	75	21	365	50	
Black ventral model	A	100	3	0	0	14	3	70	30	(rain)
	B	110	3	1	2	40	6	107	3	
	C	105	4	0	3	13	7	103	2	
	D	102	2	1	2	19	5	91	11	
Sub-total		417	12	2	7	86	21	371	46	

Table 6-3. Red ventral area, SVL and environmental variables of warty newt populations

Pops	Red ventral area (%)		Mean SVL	Standardized SVL	Sex ratio	Temperature annual range	Annual Precipitation
	<i>Mean</i>	<i>SE</i>					
DP	49.04	9.88	81.46	1.053	0.71	19.7	1789
NCo	61.77	10.65	74.08	0.964	1.07	18.9	1863
NC	63.39	10.06	81.4	1.062	1.38	19.3	1804
DD	62.39	14.97	80.83	1.036	0.36	19.4	1684
TB	61.17	11.56	80.96	1.041	0.5	18.7	1681
XT	51.18	11.34	79.44	1.032	1	19.5	1642
NK	54.96	11.62	78.04	1.006	0.57	19.4	1682
QN	46.06	12.37	70.93	0.918	0.67	19.3	1830
CB	37.6	9.74	75.29	0.982	1.29	19.8	1728

Figure legends

Figure 6-1. Sampling locations of warty newts in northern Vietnam. The map was created using ArcMap version 10.2 (ERSI). The administrative boundary dataset was downloaded from the GADM (https://gadm.org/download_country_v3.html). The based map was extracted from the climatic variable Bio7-Temperature annual range (°C; <http://www.worldclim.com/>, version 2.1; Fick & Hijmans, 2017).

Figure 6-2. Dorsal, red, and black ventral sides of warty newts (A, B, C, respectively), and dorsal, red, and black clay model types (D, E, F, respectively).

Figure 6-3. Attacks of mammals (A, C, D), birds (B), and arthropods (E, F) on displayed clay models of newt

Figure 6-4. Number of predation attacks on each type of clay model (dorsal, red, black ventral model) in four study sites.

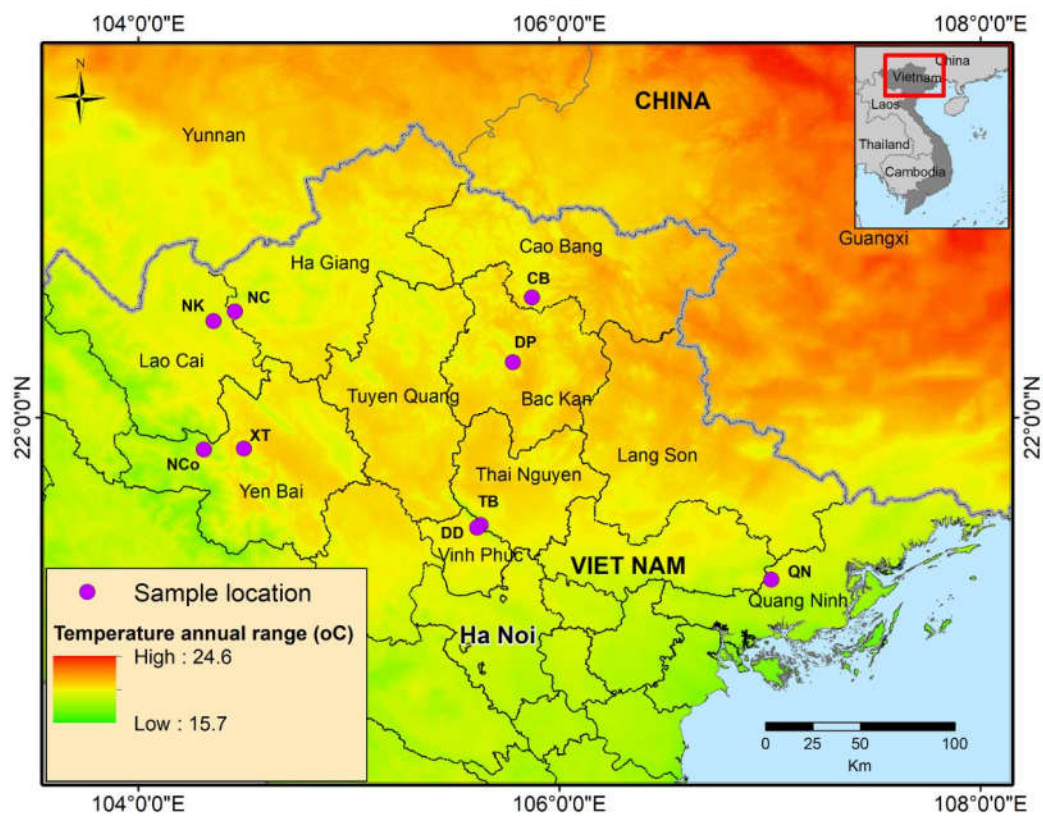


Figure 6-1

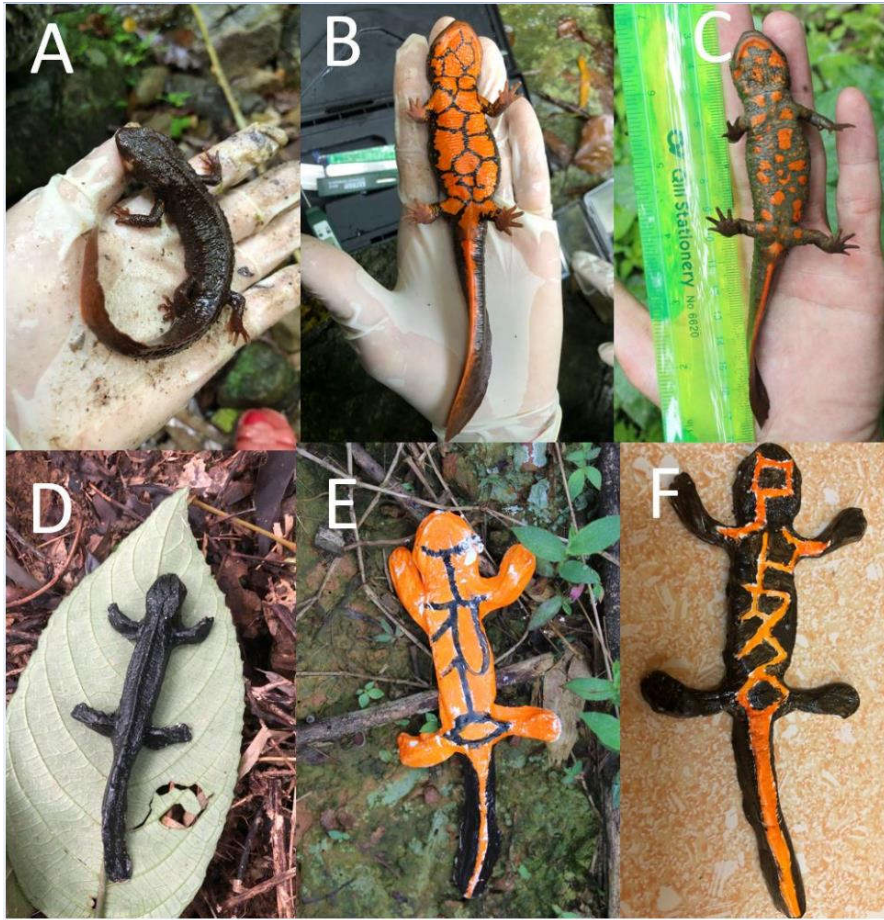


Figure 6-2



A



B



C



D



E



F

Figure 6-3

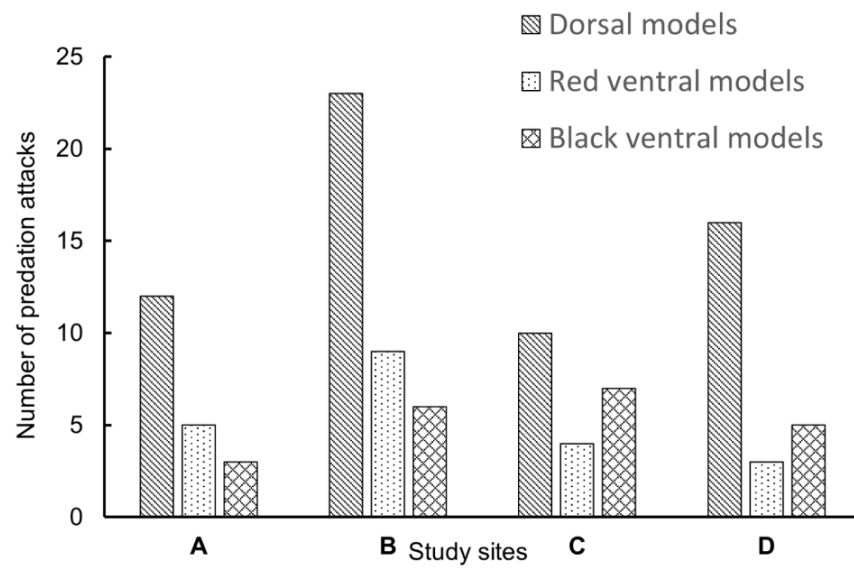


Figure 6-4

CHAPTER 7

General Discussion



In this thesis, I combined experimental works, molecular analyses, ecological niche modeling to reveal the geographic variation in genetics, morphology, and ecology of Asian warty newts in northern Vietnam. Furthermore, I also assessed the impact of climate change both in the past and future on distribution of the species. Here, I discussed the major results from my research.

7-1. Revising species delineation based on geographic variation studies

The mtDNA analyses showed evidence for taxonomy of Cao Bang (CB) population as *P. guangxiensis* (Chapter 2). However, I found a significant overlap in the predicted distribution of the East group (*P. deloustali*) and the CB group (Chapter 3). Furthermore, individuals in both the East and CB groups exhibited similar body sizes (Chapter 3). This finding prompted us to examine the genetic structure of the groups using nuDNA SNP data. Subsequently, I observed genetic similarity between both groups, but there was evidence of introgression in the genetics of the CB group (Chapter 4). Therefore, I suggested that the CB group should be treated as *P. deloustali*. Using only mtDNA for species identification can lead to problematic because it represents only a single genetic locus and may not accurately reflect the whole evolutionary history of the species (see a case in Japanese salamander: Tomimori et al., 2023). My analysis therefore supports the idea that multiple genetic locus, including both mitochondrial and nuclear DNA, as well as morphological and ecological characteristics, should be considered to accurately identify species before implementing further analyses. Comprehensive data analyses are indispensable for accurately delineating distribution ranges. This multi-approach provides a deep understanding of evolutionary relationships and reduces the probability of misidentification. Additionally, DNA collection may not always be feasible for certain species, morphological and ecological data can provide valuable support in such cases. Therefore, I also would like to emphasize the crucial role of morphological and ecological data alongside genetic information when determining species distribution.

This is the first time, mito-nuclear DNA discordance has been found in tropical newts, whereas it is common in urodeles in temperate zone (Bisconti et al., 2018; Denton et al., 2014; Fisher-Reid & Wiens, 2011; Tomimori et al., 2023; Ziełiński et al., 2013). The phenomena can be occurred due to mitochondrial introgression, where mtDNA from one species is incorporated into another through either hybridization (Gompert et al., 2008; Hill, 2019), or incomplete lineage sorting, where ancestral genetic variation is retained within species (DeBiasse et al., 2014; DeRaad et al., 2023). In the present case, it was likely due to past hybridization, because incomplete lineage sorting scenario often occur between close sister lineages (Maddison & Knowles, 2006).

7-2. Geographic variation

In the study, I found geographic variation in genetics, morphology, and ecology at both intra- and interspecific levels of Asian warty newts in northern Vietnam (Chapter 2, 3, 4, and 6; Figure 7-1). Previous studies have revealed geographic variation in urodeles, however, they mainly focused on only single dimension. For example, Cox et al. (2021) highlighted genetic variation in the Great crested newt (*Triturus cristatus*). Tran et al. (2024) found ecological niche differentiation among four main clades of Japanese fire-bellied newt (*Cynops pyrrhogaster*). Morphological variation was also demonstrated in *Lissotriton* spp. (Urošević et al., 2024), Japanese crocodile newt, *Echinotriton andersoni* (Ozaki et al., 2023), Paddletail newt, *Pachytriton labiatus* (Nishikawa et al., 2009). To date, not many studies have concentrated on multiple dimensions of geographic variation. In my study, I emphasized that genetic, morphological and ecological niche variation of Asian warty newts, and their relationships have been investigated for tropical newts.

I also highlighted that environment could play a great impact on morphological characteristics through ecological niche of species (Chapter 3; Figure 7-1). Furthermore, ecological factors can change rapidly over time, leading to morphological adaptations to new environments (Edgington & Taylor, 2019; Freudiger et al., 2021). Therefore, when considering geographic variation, it is important not only to rely on genetic and morphological data but also to incorporate ecological data.

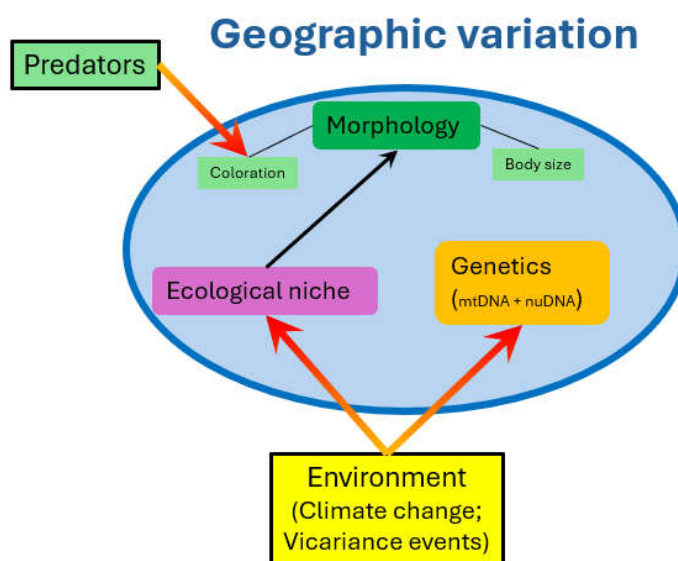


Figure 7-1. The relationship among genetic, morphological, and ecological variation of warty newts, and the factors associating the variation.

Regarding the interspecific level, I demonstrated the geographic variation between *P. deloustali* and *P. guangxiensis*. The body size of *P. deloustali* is larger than that of *P.*

guangxiensis. It supported to the observation of Sparreboom (2014) that *P. guangxiensis* resembles a juvenile of *P. deloustali*. Nevertheless, it should be noted that Sparreboom's statement was derived from singular observation lacking supporting data. In the current study, I provided comprehensive data supporting this conclusion. Additionally, I presented a significant interpopulational variation in the red ventral surface of the warty newts (Chapter 6). I also successfully used clay models to test hypotheses on interaction between predators and red ventral coloration (aposematism) in Asian warty newts (*P. deloustali* and *P. guangxiensis*; Chapter 6). The study is the first to provide scientific evidence of the aposematic hypothesis in warty newts.

Aposematism serves as a defense mechanism for organisms against predator attacks (Caro & Ruxton, 2019; Mappes et al., 2005; Ruxton et al., 2019; Chapter 6), thus aposematic groups are less likely to face extinction and more likely to undergo speciation (Przeczek et al., 2008). This suggests that conspicuous coloration could enhance the speciation rate, leading to the possibility that aposematic groups may be more diverse than non-aposematic lineages. The validity of this hypothesis was initially tested through sister-group analysis by Przeczek et al. (2008), and reconfirmed by Arbuckle & Speed (2015). In the study, I observed that the local fauna also could influence the attacked rate (Chapter 5), and aposematism of warty newts (Chapter 6, Figure 7-1). Based on these findings, I would like to propose a new hypothesis that components of local predators could drive macroevolutionary patterns, in particularly influencing the rate of speciation through aposematic traits.

At the intraspecific level, I focused on the West and East populations of *P. deloustali*. Historical climate change, and geography between two sides of West and East part of northern Vietnam possibly played a main role in driving their divergence (Chapter 2). The finding was consistent to the diversification of Pigmy narrow-mouth frogs (*Microhyla fissipes* complex), an amphibian in northern Vietnam due to both geographic barrier and Pleistocene climatic fluctuations (Yuan et al., 2016). By examining morphological characteristics, I found that the East group exhibited larger body size than the West group (Chapter 4). Morphological variables may serve as indications of adaptations to environmental variations across their distribution regions (Huang et al., 2017). In fact, the West group distributed in the higher elevation those of East group, possibly leading to lower temperature (Chapter 2). The finding was consistent with the trend found by Olalla-Tárraga & Rodríguez (2007) that thermoconformers (urodeles) would tend to be larger in hotter environments (the converse Bergmann's rule).

The differentiation in ecological niches between two lineages of a species was also found in Bosca's newt (*Lissotriton boscai*; Peñalver-Alcázar et al., 2021). Another example is in Japanese fire-bellied newt, *Cynops pyrrhogaster*. Tran et al. (2024) used climate-based

ecological niche models to identify differentiation between four lineages of *C. pyrrhogaster* across the Japanese archipelago. Intraspecific variation in ecological niches could improve the accuracy of predicting the separate distributions of each lineage (Peñalver-Alcázar et al., 2021; Tran et al., 2024). The study is also the first applying under species level into ecological niche models for tropical newts. The result had supported that intraspecific approaches could be effective to examining the ecological niches in populational level and could contribute to evolutionary studies in small geographic scales.

I found geographic intraspecific variation in ventral coloration of Asian warty newts (Chapter 6). However, numerous prey species utilize colorful "aposematic" signals to advertise their toxicity (Blount et al., 2009; Skelhorn et al., 2016). Therefore, I also would like to suggest studying the defensive chemical and toxin pattern in Asian warty newts. Tetrodotoxin (TTX) and its analogues have been confirmed in modern Asian newts, including *P. deloustali* and *P. guangxiensis* (Brodie et al., 1974; Yotsu-Yamashita et al., 2017). From interviews conducted during field surveys, local people confirmed that some persons had died after using the newt as medicine. I doubt that this phenomenon could be related to the presence of TTX. However, our knowledge of their toxins is very limited. The geographic variation of toxins could be influenced by prey community composition or the pressure of predator (Daly et al., 2008; De Meester et al., 2021; Longson & Joss, 2006; Saporito et al., 2012). In other words, the variation possibly depend on the balance between the costs and benefits of toxins (Blennerhassett et al., 2019). The results on predators of warty newts from clay model experiments and camera traps (Chapter 5, 6) could provide valuable information for the future studies on toxins of these newts.

7-3. Incorporating genetic variation in assessing impact of climate change

For salamanders and newts, several studies have assessed the effects of climate change on their distribution (e.g., Achour & Kalboussi, 2020; Ashrafzadeh et al., 2019; Borzée et al., 2019; Ebrahimi et al., 2022; Préau et al., 2020; Zhang et al., 2020). Most of the studies concentrated on temperate species, while the assessments of climate change on tropical salamanders and newts were very limited. In this study, I integrated spatial genetic structure into Species Distribution Models to evaluate the influence of climate change on the distribution of tropical Asian warty newts in northern Vietnam. I found that the newts are likely affected significantly by both historical and future climate change (Chapter 2, and 4; Figure 7-1). This combined approach has demonstrated its advance and reliability in determining the effects of climate change on organism distribution (Aguirre-Liguori et al., 2021; Benito-Garzón et al., 2019; Collart et al., 2021; Chapter 4). Furthermore, local morphological adaptations have been occurred in the Asian warty newts due to the differentiation of ecological niches (Chapter 3). Here, I highly recommend incorporating not

only genetic variation but also morphological variation into studies of assessing the impact of climate change. The morphological assessment has a greater advantage in terms of cost and speed than the genetic and/or ecological ones, which is very important to assess large areas especially in developing countries.

In this study, climate change-adapted loci related to climate change adaptation has not been identified, because the urodelan genome is one of the largest in the terrestrial tetrapods and most of them are unresolved yet. However, the information on adaptive loci could allow us to improve our understanding of climate adaptations and genomic vulnerability among populations (Brauer et al., 2023; Chen et al., 2022; Tigano et al., 2024). Genome level approach related to climate change was left in future task in the Asian warty newts.

7-4. Conservations for Asian warty newts

As amphibian species face numerous serious threats, mainly due to anthropogenic disturbance and climate change (i.e., agriculture, timber and plant harvesting, and infrastructure development, and pollution [Luedtke et al., 2023]). Among these species, Asian warty newts are particularly vulnerable. Through the present studies, I suggest implementing both international and local actions at macro- and microscale levels, respectively, to conserve these species and their habitats.

At the international scale, I revealed that future climate change can significantly affect the genetic structure and suitable distribution of the newts in northern Vietnam and southern China (Chapter 4). Cross-border cooperation for conservation management will grow increasingly crucial in the face of climate change (Blair et al., 2022). In the context, transboundary cooperation between the governments of Vietnam and China is essential to develop a shared conservation plan for these species, encompassing both technical and policy actions. In the conservation plan, it is important to highlight that high mountains in northern Vietnam predicted as potential shelters under the impact's future climate change. High priority of surveys and conservation efforts should be given to the areas. Establishing ecological corridors should be considered as an effective solution to facilitate the movements of Asian warty newts to move towards suitable habitats and enhance gene flow among populations in response to climate change.

Furthermore, *P. deloustali* and *P. guangxiensis* are listed as Least Concern (LC) and Vulnerable (VU), respectively (IUCN SSC Amphibian Specialist Group, 2017, 2022), however, their distribution was shown to be wrong by my taxonomic study. Taxonomic and distributional information of these newts should be reconsidered immediately.

For local scale, protecting populations of the newts and their habitat is very important to conserving genetic diversity inside the species. In 2022, through the cooperation between the Vietnam National University of Forestry and Phia Oac – Phia Den NP, I organized three meetings to raise awareness among local communities in the buffer zone of Phia Oac – Phia Den NP (Figure 7-2). During these meetings, my team discussed with local authorities and communities the conservation of the newts (CB population of *P. guanxiensis*: see Chapter 2; East population of *P. deloustali*: see Chapter 4), including status of newts, the role of local communities in biodiversity conservation, and current regulation of Vietnamese government to the protection of newts and other wildlife. However, similar activities were still very limited in Vietnam. In the West and East population, the two major genetic groups of *P. deloustali* (Chapter 2, 4) and QN population of *P. guangxiensis*, we have no such activities. At least, the genetic groups recognized in my study must be protected in order to conserve genetic diversity in the species.



Figure 7-2. Raising awareness on Asian warty newts conservation for location communities

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SUMMARY

Understanding the patterns and processes of geographic variation in organisms is crucial for advancing our knowledge of biodiversity and natural systems. Urodeles are ideal models to explore geographic variation due to their long evolutionary history, low dispersal abilities, and high environmental constraints. However, most studies on geographic variation have been conducted in temperate zones, leaving our knowledge of geographic variation in tropical newts greatly limited. The primary aim of my dissertation was to determine the geographic variation pattern in tropical warty newts and reveal the relationships between these variations and their environment. To achieve this, I conducted a study on the geographic variation in tropical warty newts in northern Vietnam, integrating field experiments, molecular analyses, and ecological niche modeling.

In chapter 2, I explored genetic variation among populations of two species of Asian warty newts distributed in northern Vietnam (*Paramesotriton deloustali* and *P. guangxiensis*). I also discovered that historical climate change played a significant role in driving the genetic divergence of the warty newts.

In chapter 3, I uncovered variations in morphology and ecology among newt populations. A significant relationship between morphological differentiation and ecological niche variations across populations was revealed, indicating that newt morphology responds to environmental factors. This finding demonstrates that morphological variation is not only caused by limited gene flow and genetic drift, but also influenced by ecological variation.

In chapter 4, for the first time mito-nuclear DNA discordance has been observed in tropical newts. Morphological and ecological niche data further supported the nuclear DNA findings. Therefore, I emphasized the crucial role of morphological and ecological data alongside genetic information in identifying species delineation. By integrating genetic variation into ecological niche modeling, my analysis indicated that these newts are vulnerable to future climate change in both suitable habitat and genetic structure.

In chapter 5, my work was the first to determine potential nocturnal predators and reveal their great attack rate on warty newts. I also found interspecific variation in aposematism of warty newts.

Finally, in chapter 6 clay model experiments supported the effectiveness of displaying aposematic coloration as an antipredator defense mechanism. The present dissertation comprehensively revealed the geographic variation in genetics, morphology, and ecology of *P. deloustali* and *P. guangxiensis*. This research advanced our understanding of the natural history of tropical stream-dwelling newts and provided updated information to safeguard these species in the face of future drastic climate change.

論文要旨

生物の地理的変異のパターンとプロセスを理解することは、生物多様性と自然界の体系的構造の知識を深める上で重要である。サンショウウオ類やイモリ類などを含む有尾類は、その進化の歴史の長さ、分散能力の低さ、好適な環境が限られているために、地理的変異を研究する上で理想的なモデルとなる生物である。しかし、地理的変異に関するほとんどの研究は温帯地域で行われており、熱帯イモリの地理的変異に関する知識が限られている。本論文の主な目的は、熱帯のコブイモリの地理的変異パターンを調査し、この変異と環境の関係を明らかにすることである。この目的を達成するために、北ベトナム産のコブイモリの地理的変異に関して、野外実験、分子解析、生態的ニッチモデリングを統合して研究した。

第 2 章では、北ベトナムに分布する 2 種類のコブイモリ（ベトナムコブイモリとグアンシーコブイモリ）の集団間の遺伝的変異を調査した。そして、歴史的気候変動がイモリの遺伝的分化に重要な役割を果たした可能性があることを発見した。

第 3 章では、イモリの集団間で形態的・生態的な変異の実態を明らかにした。集団間で形態と生態的ニッチの違いの間には有意な関係があり、イモリの形態が環境要因に応答することを示した。この発見は、限られた遺伝子流動と遺伝的変動だけにより形態的変異が生じるのではなく、生態的条件の変動によっても形態が影響を受けることを示している。

第 4 章では、熱帯のコブイモリで初めてミトコンドリアと核 DNA との間での種の境界の不一致が観察された。形態学および生態的ニッチのデータは、核 DNA の結果の方を支持したため、形態学および生態学的データは遺伝情報と並んで種の区別を行う上で重要な役割を果たすことを示したと言える。遺伝的変異のデータを生態的ニッチモデリングの分析に統合することにより、これらのイモリが将来の気候変動により適した生息地と遺伝的多様性の両方を失う可能性の高いことが示された。

第 5 章では、本論文は熱帯産コブイモリの潜在的な捕食者を初めて特定し、夜間における被攻撃率を明らかにした。また、コブイモリの種間においても警告色に差が見られることを明らかにした。

最後に、第 6 章では、粘土モデル実験の結果は、警戒色が捕食者から身を守る防御メカニズムとして機能していることを支持した。本論文は、インドシナ産の 2 種類のコブイモリについて遺伝的、形態的、生態的な地理的変異を総合的に調査してその実態を明らかにしたと言える。この研究は、熱帯の河川に生息するコブイモリ類の自然史の理解を大きく進め、これらの種を将来の急激な気候変動から保護するために必要な新たな情報を提供した。

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