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臺灣 *Petaurista* 屬飛鼠的
親緣地理學、族群數量變遷、與環境適應

Phylogeography, demography, and environmental adaptation of *Petaurista* flying squirrels in Taiwan

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本論文係 Carina Lee Terry 君（R10H44009）在國立臺灣大學生物多樣性國際碩士學位學程、所完成之碩士學位論文，於民國 112 年 07 月 04 日承下列考試委員審查通過及口試及格，特此證明

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Petaurista lena, illustrated by my mother Mary Noer

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Abstract

Double digest restriction site associated DNA sequencing (ddRAD-seq) is an increasingly accessible tool for phylogenetic studies, with a broad range of applications, including the characterization of past population dynamics and prediction of future responses to change. However, this tool has yet to be applied to small mammal species of Taiwan, where previous phylogenetic studies have used exclusively mitochondrial DNA. The aim of my research was to assess the potential of genomic SNP data to provide a deeper understanding of small mammal phylogeography in Taiwan. Using ddRAD-seq, I explored the phylogeography of two endemic species of giant flying squirrel: *Petaurista lena*, the white-faced giant flying squirrel, and *Petaurista grandis*, the red giant flying squirrel. Contrary to previous findings, both species demonstrated significant population structure along the North-South axis. There was a strong correlation between genetic distance and spatial distance for both species; however, I found no strong relationship between genetic variation and climate or landscape factors. Demographic analyses indicated that *P. lena* experienced population expansion during the glacial period MIS 6 but has been declining since the early-mid Holocene. *P. grandis* demonstrated the opposite reaction, experiencing expansion during the climate warming of the early Holocene. These findings tell a different story than previous mitochondrial analyses, providing novel insight into the population structure and demography of *Petaurista* species in Taiwan. Overall, ddRAD-seq proved effective for detecting and explaining genetic variation across Taiwan; future analyses of small mammal species should consider incorporating genomic SNP data, particularly for species whose phylogeography is not well explained.

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Introduction

As climate change and human disturbances continue to alter the environment, the survival of small mammal species increasingly depends on their ability to either 1) adapt to a changing niche, 2) disperse to regions where they can maintain their original niche, or 3) exhibit phenotypic plasticity. For researchers and conservationists, determining the potential of species to adapt or disperse is a key part of understanding which species are likely to face extinction in the future. Population genetics studies are a common way to determine a species' reaction to past climate changes, as well as its current health and likely selection pressures; this knowledge can then be used to predict future survival (i.e., Buckley & Kingsolver, 2012; Kuntner et al., 2014; P.-W. Sun, 2022).

Double digest restriction site associated DNA sequencing (ddRAD-seq) is an increasingly accessible method used to accomplish such analyses. A form of reduced-representation sequencing, this method allows for the detection of tens of thousands of SNPs across the genome while avoiding the often-prohibitive costs of whole-genome sequencing (Peterson et al., 2012; Puritz et al., 2014). ddRAD-seq has been shown to accurately reflect the diversity and phylogenetic relationships within a population, and has demonstrated greater sensitivity and accuracy compared with microsatellite data (Fischer et al., 2017; Zimmerman et al., 2020). It has the advantage over mitochondrial DNA of having a greater number of data points and not being limited to the maternal lineage; previous studies have found that ddRAD-seq can detect phylogenetic relationships missed by mtDNA analyses (Dong et al., 2022; Lavretsky et al., 2019).

Predicting the survival of montane and islandic species has been a focus of the ecological community in recent years, as these species are particularly vulnerable to extinction (Ricketts et al., 2005). Montane species are largely experiencing upwards

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shifts in their elevational range in response to climate warming (I.-C. Chen et al., 2011; Lenoir et al., 2008; Moritz et al., 2008; Sekercioglu et al., 2008); however, the effects of this shift on a species total range area and population connectivity depends on the topography of the specific mountain range and the coincidence of human settlements with elevation (Elsen et al., 2020; Elsen & Tingley, 2015). Island ecosystems are disproportionately affected by threats such as biodiversity loss, invasive species, and sea level rise(Loope & Giambelluca, 1998; Macinnis-Ng et al., 2021; Veron et al., 2019).

As a geologically young, continental island with a large number of endemic species (Ali, 2017; Feng et al., 2016; Lei et al., 2003), Taiwan is an optimal region in which to study the effects of past climate changes on a wide variety of taxa. Many studies have been conducted on the genetics of Taiwan species in order to reconstruct their history and determine the effects of glaciation (He et al., 2018). However, the vast majority of these studies have exclusively used mitochondrial DNA; despite the utility of ddRAD-seq, few studies of Taiwan fauna have made use of this technology. Small mammal species of Taiwan are particularly neglected, with no ddRAD-seq studies to date.

The phylogeography of mammal species is heavily dependent on their specific life history, dispersal ability, and range (Montgelard et al., 2014). As such, small mammal species in Taiwan demonstrate several different patterns of phylogeography. There is a general trend towards species with more restricted highland or lowland elevational ranges demonstrating greater population differentiation (Yu, 1995). *Microtus kikuchii*, an endemic vole that lives above elevations of 2500 meters, can be divided into four major groups living in the heights of different mountain groups (Wang, 2012), while the Pallas squirrel *Callosciurus erythraeus* lives below 2000 meters and can also be divided into four major groups (Oshida et al., 2006). For mid-elevation species, population structure

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likely depends heavily on their capacity and tendency towards dispersal. *Anourosorex yamashinai* , the Taiwanese mole shrew, is restricted between elevations of 1000 – 2500 meters, and has two major phylogroups with a north/south split, as well as one minor phylogroup centered around Mount Houhuan (Yuan et al., 2006). The Formosan wood rat *Apodemus semotus* and Formosan white-bellied rat *Niviventer culturatus* both live in an elevation range of around 1500 – 3600 meters; however, *A. semotus* can be divided into 2 major phylogroups with a north/south split (Hsu et al., 2001), while the larger *N. culturatus* shows high connectivity and little population structure (Hsu et al., 2000).

The endemic giant flying squirrels of Taiwan, *Petaurista lena* and *Petaurista grandis,* have been previously thought to show phylogenetic patterns similar to *N. culturatus*, with high connectivity across the island. A study by Oshida et al. (2011) found no evidence of major phylogroups in either species, and only minor association between geographical association and population structure in *P. lena*, with no such biological interpretations found for *P. grandis*. They also found that both species demonstrated a historical population expansion, and estimated the timing around 0.16 mya for *lena* and 0.06 mya for *grandis*, but could not find an explanation for the differences in this expansion time. However, I theorize that due to their life history and dispersal ability, this assessment may not accurately reflect the genetic structure and historical demography of these species.

Both are mid elevation species; of the two, *P. lena* has a wider range, living between 800 – 3500 meters elevation, while *P. grandis* can be found between 100 – 2500 meters (P. F. Lee et al., 1986). Giant flying squirrels are capable of gliding over 100 meters in a single jump; *Petaurista petaurista* has been observed making glides of up to 150 meters (Thorington & Heaney, 1981), and glides of up to 115 meters have been

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recorded for *Petaurista leucogenys* (Andō & Shiraishi, 1993). However, such long jumps are rare, and *Petaurista* species seem to largely prefer shorter glides between 10 – 50 meters (Andō & Shiraishi, 1993). *Petaurista petaurista* specifically most frequently makes glides between 20 – 40 meters (Krishna et al., 2016), while *Petaurista philippensis* most frequently makes glides between 11 – 20 meters and has not been observed gliding further than 35 meters (Koli et al., 2011). Flying squirrels are well adapted for a gliding lifestyle; they have the lowest body weight relative to size when compared to ground or tree squirrels, and have the largest tail/body ratio, aiding in their stability and maneuverability during glides (Hayssen, 2008; Scheibe & Robins, 1998). However, while these adaptations make gliding largely energetically efficient and can increase flying squirrels' ability to disperse across forested areas, it also increases the energetic cost of terrestrial locomotion (Flaherty et al., 2008). This increased cost can make it difficult for them to cross large forest gaps; even for gaps that fall within the upper range of their gliding ability, long glides can put squirrels at greater risk of predation or severe weather (Asari et al., 2007). In addition to the difficulties of gap-crossing, *Petaurista* species do not generally disperse far from their home ranges, which comprise a few hectares in area (Baba et al., 1982).

Given these limitations on their dispersal ability, I expect that there is more variation within giant flying squirrel populations than was discovered by Oshida et al (2011). I expect that ddRAD-seq data can provide insight into more subtle population differences that may have been missed by mitochondrial data; I also expect that ddRAD-seq data can construct a more detailed demographic history and better explain differences in this history between each species. Finally, I expect that ddRAD-seq data can provide

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deeper insight into how these *Petaurista* species' population structures are related not only to distance but to climate and land-use factors.

With these expectations, I asked the following questions:

1 – Do *P. lena* and *P. grandis* demonstrate distinct population structure? Is this structure related to geographic location and/or elevational range?
2 – What is the demographic history of these species in Taiwan?
3 – How does the genetic variation of these species relate to climate and land-use factors? Are they at risk from future climate change?

P. lena and *P. grandis* are commonly hunted species by indigenous communities in Taiwan (Fan et al., 1992; Simon, 2010), and are a primary food source for certain montane raptors (Y.-H. Sun et al., 2009). Neither species is currently classified as threatened; however, it is unclear whether their populations will be affected by climate changes and human disturbance in the future. Their gliding ability and flexible diet should support their ability to adapt and disperse, but their montane distribution, limited ability to glide across open areas, and status as a hunted species create limitations. Understanding the phylogenetic history and spatio-genetic distribution of these species can help determine their future reactions to disturbance, and indicate whether conservation action will be necessary for these species. In this study, I hope to both develop such an understanding for *P. lena* and *P. grandis*, and demonstrate how ddRAD-seq methods can be used in the future to better understand the phylogenetics of small mammal species across Taiwan.

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Methods

Data sources

Flying squirrel tissue samples were obtained from three sources: the Endemic Species Research Institute, the National Museum of Natural Science, and indigenous hunters in the Hualien region. The majority of samples were muscle tissues, although there were several that consisted of liver, toe, or ear. Samples were sourced from a wide range of locations across Taiwan (Figure 1). A complete record of sample information with source, collection location, collection date can be found in Supplementary Table 1.

DNA Processing

DNA was extracted from tissue samples using the Favorgen Tissue Genomic DNA Extraction kit (Favorgen Biotech Corp., Ping Tung, Taiwan). After extraction, DNA quality and quantity were assessed using gel electrophoresis, NanoDrop 2000 Spectrophorometers (Thermo Fisher Scientific, Wilmington, DE, USA), and Qubit 2.0 Fluorometers (Life Technologies, Carlsbad, CA, United States). Following this quality control, 115 samples were selected to proceed with sequencing. Samples were double-digested using enzymes SbfI and MseI, and were pooled in 6 libraries. Fragments were ligated to Illumina sequencing adaptors including an individual barcode and library-specific index. Fragments were then sequenced with Illumina Novaseq 6000 (Illumina, USA).

Using software Stacks (J. Catchen et al., 2013; J. M. Catchen et al., 2011), reads were demultiplexed and then assembled using the *de novo* mapping pipeline. A secondary assembly was also performed using the reference-based mapping pipeline, with the genome of *Glaucomys volans* used as the reference (Wolf et al., 2021). SNPs were filtered and exported as VCF files using Stacks function populations. Inclusion of SNPs was

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based on the following criteria: a minor allele frequency of at least 0.05; a maximum observed heterozygosity of 0.7; and a missing rate of less than 20%. For analyses requiring filtering for linkage disequilibrium, SNPs were exported by selecting a single random SNP from each locus.

Population analyses

Before proceeding with analyses, I removed all duplicate samples and samples with an anomalous location, and removed one of each pair of samples with a kinship coefficient greater than 0.031 (half-cousins). I also randomly selected 5 samples collected from Hualien in 2021, and removed the remaining samples to avoid over-sampling this region and year. A total of 42 samples of *P. lena* and 36 samples of *P. grandis* were included in the final analyses; the list of excluded samples and their reason for removal can be found in Supplementary Table 1. I then used software Admixture (Alexander et al., 2009) to assess population structure, testing K values 1 through 10 and calculating the cross-validation error. After the first run of Admixture, I found that four samples had been labeled with the wrong species name; I corrected these and re-did the analysis.

Using the software R (R Core Team, 2021) and packages ape, pegas, and vcfR (Knaus & Grünwald, 2017; Paradis, 2010; Paradis & Schliep, 2019), I then calculated a matrix of p-distances and constructed a neighbor-joining tree. A principal coordinate analysis (PCoA) was performed for both species grouped together as well as each species separately. I calculated nucleotide diversity (pi) and Tajima’s D for each species as a whole and for the putative Northern and Southern subpopulations. Using the package dartR (Mijangos et al., 2022*),* I performed an AMOVA and calculated the Phi ST values between northern and southern populations.

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To assess past population demography, I used Stairway Plot v2 (Liu & Fu, 2020; Lu & Fu, 2015), a software that infers past population size based on site frequency spectrum (SFS). Assuming a mutation rate of 5.4e-9 per site per generation based on the findings of Uchimura et al. (2015) and a generation time of 2.25 years based on the age at sexual maturity of *P. leucogenys* and an estimated lifespan in the wild of 6 years (Kawamichi, 1997), I performed this analysis using the folded SFS for each species as a whole, as well as for each putative subpopulation individually. I also used software fastsimcoal2 (Excoffier et al., 2013, 2021) to simulate three past demographic scenarios for each species: past divergence and size change of the putative northern population, past divergence and size change of the putative southern population, and past divergence and size change of both populations (Figure 2). Finally, using software Maxent (Phillips et al., n.d.), I created a model of present species niche, and used this model to predict species niche during the last glacial maximum (~22 kya). To obtain an accurate species distribution, I combined data from GBIF with the occurrence records from this study and thinned the locations with too-high sample density (*GBIF Occurrence Download*, 2023a; *GBIF Occurrence Download*, 2023b). I used bioclimatic variables 1-19 from WorldClim version 1.4 (R. Hijmans et al., 2005), the most recent WorldClim version to provide ancient climate data. (Descriptions of each variable are available in Supplementary Table 2). Data was downloaded at a resolution of 2.5 minutes (approximately 20 km^2). I first ran models with all variables, then removed any variables that had a permutation importance <5 or that had past predicted values substantially outside of the range of training values. I repeated this process with a permutation importance threshold of 10, and continued to repeat until all variables fell within the criteria.

Spatial analyses

For the spatial analyses, I used only individuals that had precise location information (35 samples of *P. lena*, 29 samples of *P. grandis*). I developed two spatial models to explain the relationship between landscape and genetic distance: straight-line geographic distance and isolation-by-resistance using environmental and land-use factors. To obtain a resistance matrix from climate factors, I again created a model of species niche for both species using software Maxent (Phillips et al., n.d.), using the same species data but employing climate data from WorldClim version 2.1 (Fick & Hijmans, 2017). Data was downloaded at a resolution of 30 seconds (approximately 1 km^2). I first ran Maxent models with all variables included, then removed any variables with a permutation importance <5. I ran the models again and filtered for permutation importance <10, and repeated this filtering process until all variables met this criterion. Next, I used R packages raster and usdm to compare the selected variables and check for correlation (R. J. Hijmans, 2023; Naimi et al., 2014); if a comparison showed an $|r| > 0.7$, I removed the variable with lower permutation importance and re-ran the model in Maxent.

To add in land-use factors, I used data from Chen et al. (2019) characterizing each 500 x 500 m portion of land in Taiwan as either forest, grassland, agriculture land, built-up land, inland water, or bare soil. I assigned forested land and grassland a value of NA, while agriculture, built-up land, inland water, and bare soil were given a value of 0.01. This matrix was then resampled to a resolution of 1 km^2 and overlaid with the climate suitability matrix, and the minimum value for each cell was selected. Finally, the resistance matrix was created by subtracting this suitability matrix from 1.

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After establishing friction matrices, I calculated the least-cost paths using R package *dartR* (Gruber et al., 2018; Mijangos et al., 2022). Additionally, I created a matrix simply indicating whether each pair of samples was from the same population (0) or different populations (1). Using R package vegan (Oksanen et al., 2022), I performed a Mantel test to compare the p-distance values with distance values from each spatial model, as well as a partial Mantel test between each pair of models to determine the contribution of each predictor. I also used Mantel tests to check for specific genome-environment relationships. For each species, the values of environmental variables 1-19 were scaled to a mean of 0 and standard deviation of 1. Distance matrices were then constructed for each variable. These distances were compared with p-distances using both a simple Mantel test and a partial Mantel test with geographic distance. Each test was done with 1000 permutations to test for significance, and for all Mantel tests, pairs with a geographic distance less than 5 km were excluded. Finally, I created two additional Maxent models for each species: one for each putative population (North and South). I used the same variables identified by the previous filtering process.

I conducted a genome-wide association study using bioclimatic variables 1-19 from WorldClim as the “trait values” (Fick & Hijmans, 2017). Trait values were again scaled to a mean of 0 and standard deviation of 1. I used sequences aligned to the *G. volans* reference genome, and performed the analysis in software TASSEL (Bradbury et al., 2007; Wolf et al., 2021). From the results of this analysis, I selected SNPs with a -log10(p) value greater than 2 from any variable to proceed with a gradient forest analysis. I then conducted the gradient forest analysis using R package gradientForest (Ellis et al., 2012), using only bioclimatic variables selected for each species by the previous Maxent analysis, and scaling these values to a mean of 0 and standard deviation of 1.

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Results

Population analyses

After filtering each set of sequences for a minor allele frequency of 0.05, a maximum observed heterozygosity of 0.7, and a maximum missing proportion of 0.2, I found 133,275 variant sites for both species sequenced together, 73,564 for *P. lena*, and 46,063 for *P. grandis*. After removing sites in linkage disequilibrium, 26,835 sites were retained for both species together, 25,946 retained for *P. lena,* and 20,010 retained for *P. grandis.* The mean effective per-sample coverage was 32.9x, 32.6x, and 34.8x, respectively (Table 1). Results from the reference-based alignment to the *G. volans* genome can be found in Supplementary Table 3.

From the admixture analysis, the cross-validation error for both species together was lowest for K=2 at 0.184, with K=3,4 also relatively low at 0.192 and 0.195, respectively. The cross-validation error for *P. lena* was lowest for K=1 at 0.560, with K=2 also relatively low at 0.621. The cross-validation error for *P. grandis* was also lowest for K=1 at 0.601, with K=2 also relatively low at 0.649. A complete record of cross validation error can be found in Supplementary Table 4, while plots of cross validation error can be found in Supplementary Figure 1.

The neighbor-joining tree and PCoA performed on both species together indicated a clear separation with no admixture between species (Supplementary Figure 2). The first principal component (PC1), along which the species were separated, accounted for 98.9% of variation, while PC2 accounted for 0.095% of variation and PC3 accounted for 0.082%. The neighbor-joining tree and PCA performed for each species individually indicated a clear North/South split in populations of both species, as well as a more minor East/West split in *P. grandis*, with the third cluster centered around Hualien. The North/South split

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fell along PC1, explaining 9.2% and 14.2% of variation in *P. lena* and *P. grandis*, respectively; The East/West split fell along PC2, explaining 8.5% of variation in *P. grandis* (Figure 3,4).

Both species demonstrated negative Tajima's D values in all populations (Table 2). *P. grandis* showed little difference between populations, while *P. lena* was more strongly negative in the Southern population compared to the Northern. However, neither species demonstrated significance in these values. Nucleotide diversity (pi) was similar between species and populations, approximating 0.29 for *P. lena* and 0.31 for *P. grandis.* The mean FIT value across loci was higher for *P. grandis,* approximating 0.099, while the value for *P. lena* was 0.078 (Table 3).

The AMOVA between North and South populations indicated significant differences between populations for both species; however, the fraction of variation between populations was relatively small compared to the variation within populations (Table 4). Approximately 11.6% of variation was explained by between-population differences in *P. grandis*, while 4.0% of variation was explained by between-population differences in *P. lena.* The AMOVA performed between populations clustered into 3 groups was also significant, but explained a smaller percentage of variation for both species compared to the 2-group AMOVA.

From the stairway plot analysis, I found a substantial historical population expansion for both species. For *P. lena*, this expansion appears to have occurred around 200 kya (CI: 100-300 kya), around the start of Marine isotope stage 6 (MIS6; Lisiecki & Raymo, 2005). For *P. grandis*, the expansion appears to have occurred later, around 12 kya (CI: 10-20 kya), near the start of MIS 1 (Figure 5). The fastsimcoal analysis provided fairly similar results; the best model for *P. lena* was DS01, indicating past population

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divergence and subsequent size change in both Northern and Southern populations. The divergence time of *P. lena* was estimated around 170 kya, and corresponded to a population size increase in both populations; this change was followed by a decrease in population size, occurring around 40 kya for the Northern population and around 6 kya for the Southern population (Table 5). The best model for *P.* grandis was DS0, indicating past population divergence and subsequent size change of the Northern population. The divergence time of *P. grandis* was estimated around 50 kya, corresponding to a large decrease in the Northern population; this change was followed by an increase in the Northern population around 15 kya (Table 5).

I found that bioclimatic variables 10 and 16 were the best predictors of past species niche for *P. lena*, while variables 5, 18, and 19 were the best predictors for *P. grandis*. The species niche of *P. lena* during the last glacial maximum (22 kya) appears substantially larger compared to present day, while the niche of *P. grandis* shows an expansion along the edges of the species range, but a decrease of suitability along the center of the range (Figure 6).

Spatial analyses

For present-day species niche, I found that bioclimatic variables 9, 13, and 19 are the best predictors for *P. lena,* while variables 5, 17, and 18 are the best predictors for *P. grandis*. Both species demonstrate a strong correlation between genetic and geographical distances. Least-cost path distance was a better predictor for *P. lena*, while straight-line distance was a better predictor for *P. grandis*, although the overall performance of each model was similar (Table 6). Population category (same vs. different) was the strongest predictor for both species, and both species demonstrated a slightly bimodal distribution of genetic distance that corresponds closely to population category (Figure 7). All mantel

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R values were higher for *P. grandis* compared to *P. lena* (Table 6). Several climatic variables demonstrated a significant correlation with genetic distance from the basic Mantel tests; however, from the partial Mantel tests in which geographic distance was accounted for, this significance disappeared for all but one comparison (Table 7). Maxent predictions of niche for North and South populations appeared substantially different for both species (Figure 8).

No loci in either species met the significance threshold or the suggestive threshold of the GWAS analysis for any bioclimatic predictor (Supplementary Figure 3). However, all predictors had a very high heritability of 0.99 (Supplementary Table 5). I selected loci with a -log10(p) value greater than 2 for any predictor to proceed with for the gradient forest analysis. From the gradient forest analysis, Bio 13 was the best predictor of change in *P. lena* loci, followed by Bio 19 and Bio 9, with accuracy importance values of 0.0487, 0.0381, and 0.0329 respectively (Figure 9). A total of 112 loci demonstrated significantly positive R2 values. Bio 18 was the best predictor of change in *P. grandis* loci, followed by Bio 17 and Bio 5, with accuracy importance values of 0.0484, 0.0426, and 0.0163 respectively (Figure 9). A total of 136 loci demonstrated significantly positive R2 values.

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Discussion

Both species of giant flying squirrel in Taiwan demonstrate distinct population structure along the North-South axis; the separation can be seen in the bimodal distribution of the genetic variation, the significant variation shown by the AMOVA, and the clustering in the PCA. This type of division between subpopulations is frequently seen in Taiwanese fauna, but generally falls along the lines of a clear geographic barrier. Lower elevation species, such as Pallas's squirrel, are often separated by the mountain ranges spanning Taiwan, particularly the Central mountain range (Oshida et al., 2006); higher elevation species, such as the Taiwan vole, are isolated by the valleys separating major mountain ranges (Wang, 2012). However, for *P. lena* and *P. grandis*, there is no clear barrier between populations; both species are distributed across Taiwan, have a fairly wide elevational range, and have an ability to glide past gaps in the canopy < 100 m.

There are several possible explanations for this genetic split. One possibility is that the northern and southern populations of these species could be under selection for different traits. My analysis of the two populations in Maxent showed that the climate variables in the range of each population are very different, and the mantel tests between genetic distance and environmental distances indicated a significant correlation for several environmental variables; in addition, the gradient forest analysis showed relationships between most predictor variables and alleles. Despite the lack of identified peaks from GWAS, it was also demonstrated that all bioclimatic variables have a high heritability, implying a strong basis for selection.

However, when spatial distance was accounted for through partial mantel tests, the correlations between genetic distance and environmental distances did not remain significant. The variables assessed with gradient forest demonstrated a fairly linear

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increase in cumulative importance, showing no particular threshold at which a division occurred. These results imply that the relationships between genetics and environmental characteristics are likely a result of spatial divisions, rather than being a demonstration of selection. It is still possible that selection is present; a different selection of explanatory variables could perhaps better explain the North/South split. Forest type may be a superior predictor, although I expect this variable to be highly correlated with climate factors. It is also possible that a greater sequencing depth may be necessary to detect signatures of selection in this species. However, from my data I cannot conclusively say that selection has caused the observed population divisions.

A more compelling explanation is that these North/South divisions are an artefact of population changes in response to past glacial cycles, as is demonstrated in species such as the mole-shrew and field mouse (Hsu et al., 2001; Yuan et al., 2006). I expected that the two flying squirrel species would have demonstrated opposite reactions to glacial cycles; that *P. grandis* would have been isolated in intermontane refugia during glacial periods, while *P. lena* would have experienced a range expansion; and conversely, that *P. grandis* populations would have expanded during interglacial periods, while *P. lena* became isolated in separate highland regions.

According to my analysis of past demography, this hypothesis is likely accurate. *P. lena* experienced a population expansion at approximately 200 kya, around the beginning of the glacial MIS 6. The population size does not appear impacted by any interglacial substages during MIS 5, and seems fairly stable throughout the Weichselian glacial period. The population then begins decreasing around the beginning of the Holocene, when the climate began warming again. This reaction is consistent with the

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models of past vs present species niche; the range of *P. lena* was substantially larger during the last glacial maximum than the present day.

The present division of *P. lena* populations appears to follow the lines of the Zhuoshi River and the Hualien River valleys. These rivers are a common divider for high elevation species, and the effect is not limited to genetics; a study of sarcoptic mange occurrence on the Formosan Serow found that infestations were limited to populations south of these rivers (C.-C. Chen et al., 2012). The least-cost-path analysis indicated that to disperse latitudinally, individuals likely travel along a slightly more narrow highland area to avoid the lower elevation areas created by these rivers (Supplementary Figure 4). This hourglass- shaped suitable area likely inhibits gene flow more than simple straight-line distance. Though it does not completely isolate the northern and southern populations in the present day, this pattern was likely more extreme during past interglacial areas, and could have created the North/South split that now lingers in their genes. Given the current trend of population decline and climate warning, it also seems likely that the range and population size of *P. lena* will continue to decrease with the warming climate, and this species may once again see a more severe separation between northern and southern populations.

P. grandis demonstrated the opposite response to past climate changes. My least cost path analysis was not significant compared to straight-line distance, indicating a lack of current geographical separation, despite the apparent barrier of the Central Mountain Range. Instead, I found that *P. grandis* demonstrated a past expansion at approximately 12 kya, around the beginning of the present interglacial period. From the model of past species niche, it appears that during the last glacial maximum, the suitable niche of *P. grandis* expanded along the edges of its range, where the boundaries of the Taiwan

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coastline were extended; however, in the central part of its range, the suitable niche was reduced, creating a greater separation between Northern and Southern populations. This separation is consistent with the findings of my fastsimcoal analysis, which indicated that *P. grandis* experienced a population division and decline around 50 kya, during the glacial MIS3.

The expansion pattern of *P. lena* is not too different from previous estimates of this species; Oshida et al. (2011) calculated an expansion time of 160 kya, which also fits an expansion during the glaciation of MIS 6. However, my findings for *P. grandis* are very different from previous estimates. Oshida et al. estimated this species' expansion time to be around 60 kya, during the Weichselian glaciation, but could not explain why these species had two different expansion times during different glaciations. I determined that *P. grandis* instead likely experienced population isolation during the Weichselian glaciation, and expanded during the subsequent warming of the Holocene. This finding would explain the otherwise strange difference in expansion time between *P. lena* and *P. grandis.* This finding also changes the likely future distribution of *P. grandis*: rather than expecting a decrease in species range and population size similar to *P. lena*, it is possible that *P. grandis* could experience another population expansion as their range expands with the warming climate.

Giant flying squirrels lead a relatively adaptable lifestyle. They demonstrate flexibility in their diet, consuming primarily new leaves but also berries, nuts, insects, and old leaves by availability (Kuo & Lee, 2003); they have a bi-annual breeding season (P.-F. Lee et al., 1993), indicating that they are not strongly limited by seasonal variation of resources, and although they do not tend to stray far from their home range, their arboreal nature gives them the ability to easily disperse throughout forests. Although these factors

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bode well for their ability to adjust to future climate change, researchers should monitor populations to ensure their continued health; *P. lena* in particular may be at risk from the warming climate. These species are valuable to the ecosystem as both prey for montane raptors (Y.-H. Sun et al., 2009), and a source of food and traditional medicine for indigenous communities (Fan et al., 1992; Simon, 2010). As folivores, a role as ecosystem engineers is also a potential but under-explored aspect of their niche (Chapman et al., 2013; Schowalter et al., 1986).

More research is necessary to improve the understanding of giant flying squirrels in Taiwan. The life history of these species is not well understood; in particular, the lifespan and age at sexual maturity have not been studied for either species. These traits are important for the estimation of past demography, and while estimates based on traits of related species are generally acceptable, studying the specific traits of these species would further knowledge of their demographic history. The creation of a reference genome and collection of morphological data would also be highly beneficial for future studies of adaptation in this species.

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Conclusions

My findings provide a novel understanding of *Petaurista* phylogeography in Taiwan. Through ddRAD-seq analysis, I was able to explore genetic variation to a greater level of detail and detect variation that was missed by mitochondrial DNA analyses. I was also able to further explore the demographic history of these populations, and explain the difference in past expansion time between *P. lena* and *P. grandis*. A similar analysis could be done of other species, such as *Niniventer culturatus,* that have been previously found to demonstrate no population structure across Taiwan. Even for species whose population structure has been previously detected, ddRAD-seq data could be used to confirm and explain these structures in greater detail, and model more complex demographic changes. This process will no doubt play a large role in future studies of mammal phylogeography in Taiwan.

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Figures and Tables

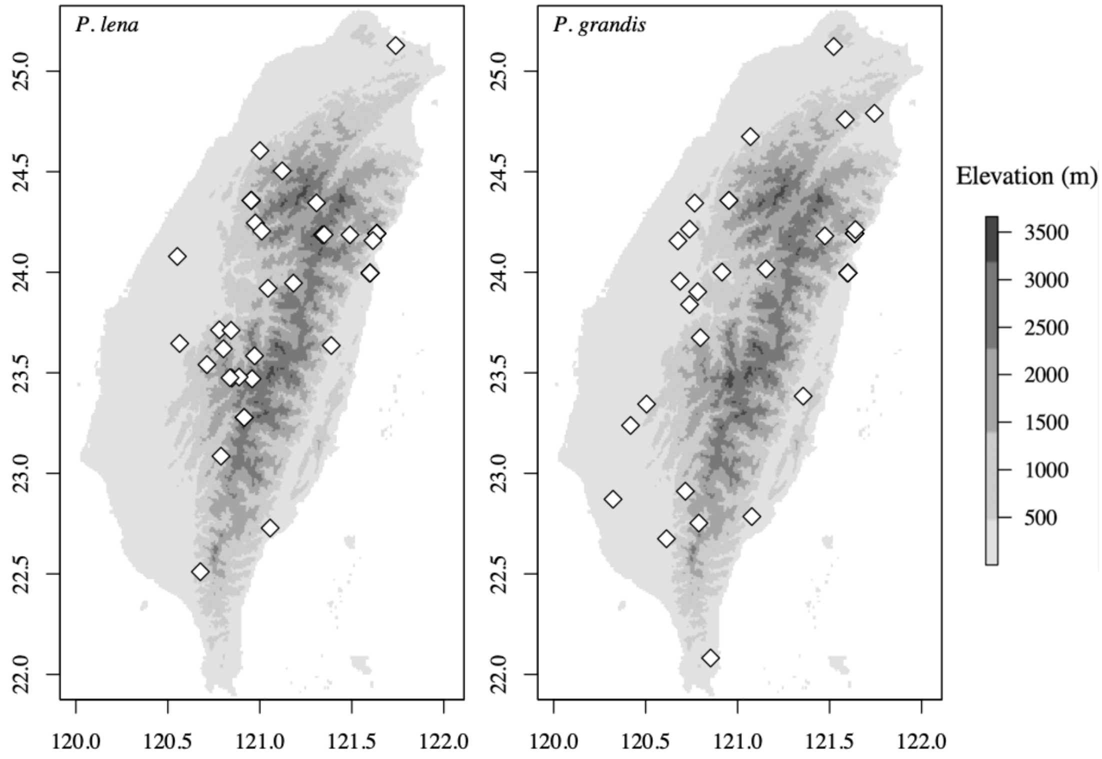

Figure 1: Sampling locations of *Petaurista lena* (left) and *Petaurista grandis* (right).

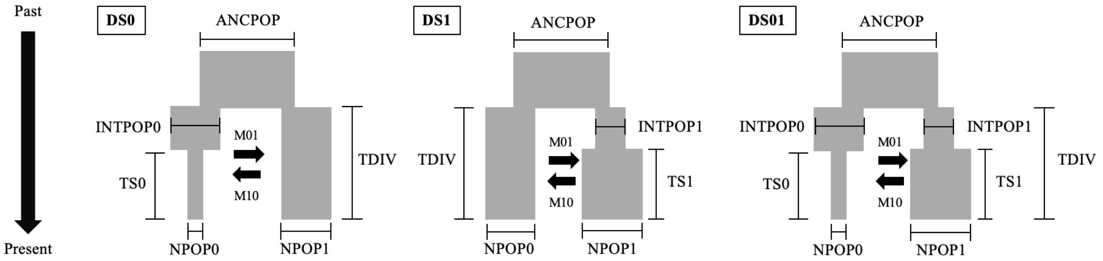

Figure 2. Three scenarios simulated in Fastsimcoal2: historical divergence with subsequent size change of population 0 (DS0), historical divergence with subsequent size change of population 1 (DS1), and historical divergence with subsequent size change of both populations (DS01). All scenarios include migration between the populations.

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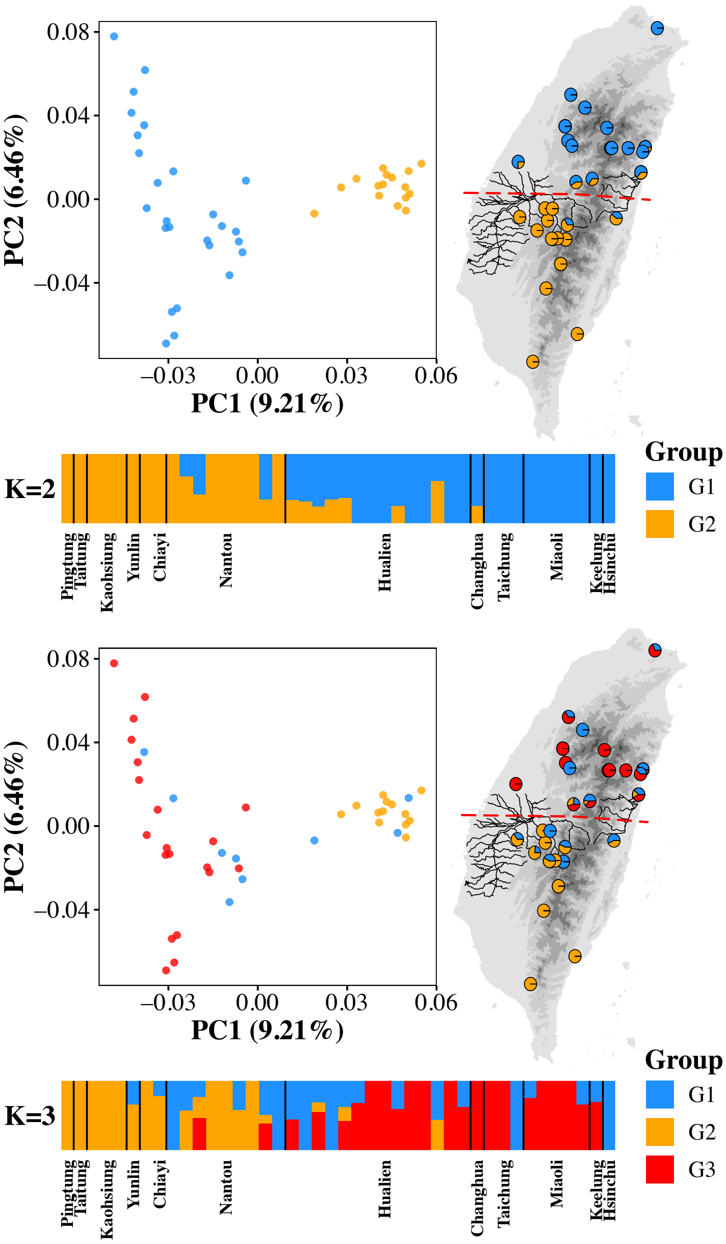

Figure 3. Genetic admixture, principal coordinates analysis, and geographic locations of *P. lena* samples, for two and three assumed population clusters (top and bottom, respectively). Black lines on the map indicate the Zhuoshui and Hualien River systems, and red dashed lines indicate the geographic division of populations.

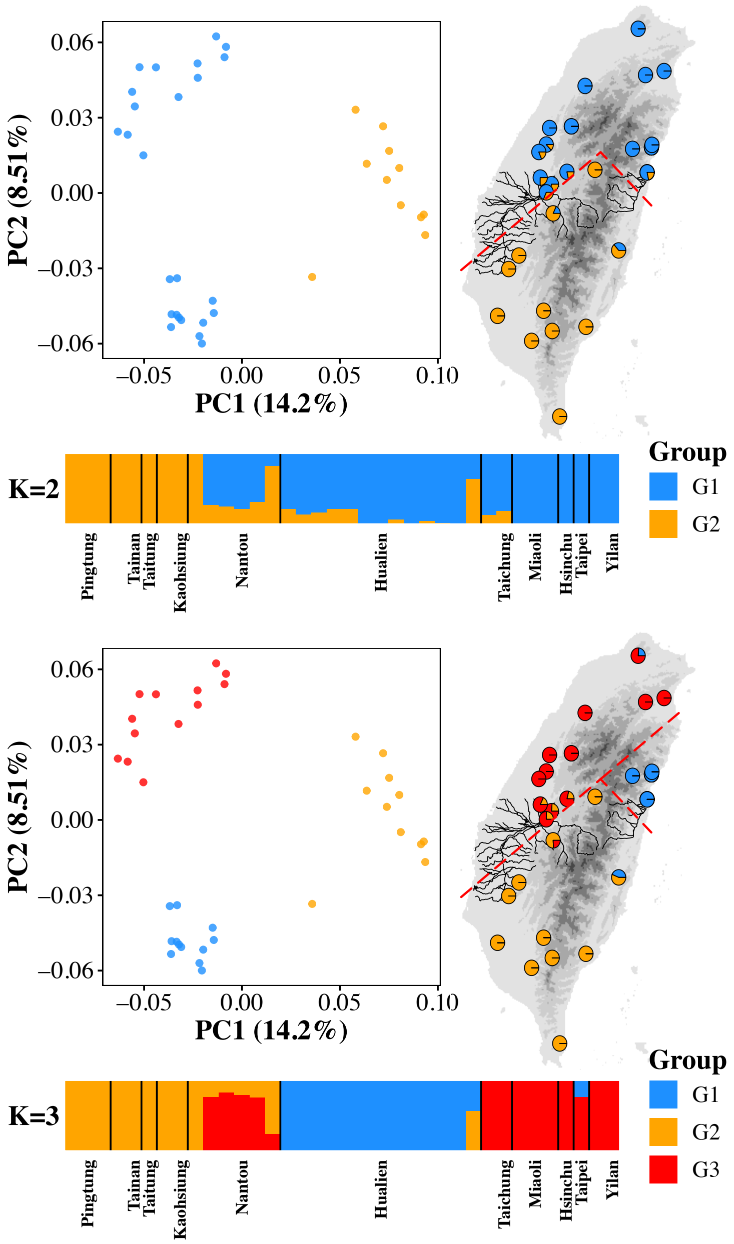

Figure 4. Genetic admixture, principal coordinates analysis, and geographic locations of *P. grandis* samples, for two and three assumed population clusters (top and bottom, respectively). Black lines on the map indicate the Zhuoshui and Hualien River systems, and red dashed lines indicate the geographic division of populations.

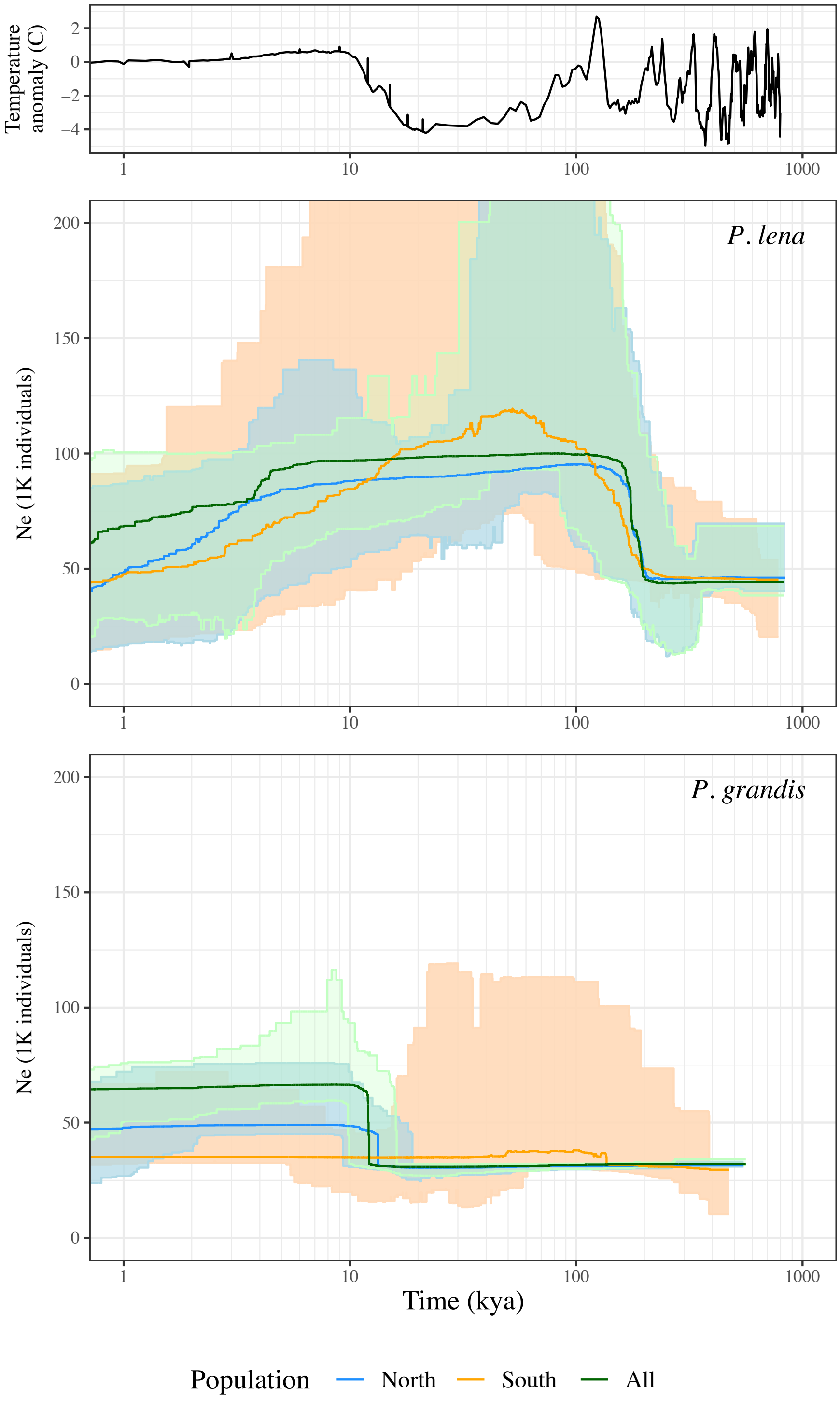

Figure 5. Past demography of *P. lena* and *P. grandis* estimated through Stairway Plot v2, with historical temperature anomaly plotted for comparison. Lines indicate median estimates, while shaded areas indicate the 95% confidence interval. Temperature data is sourced from Snyder (2016), Marcott et al. (2013), and Moberg et al. (2005), and uses the

spatially weighted global average, with the anomaly scaled to a glacial-interglacial range of 4°C. The average value from 1951 – 1980 is used as the base value from which to calculate the temperature anomaly.

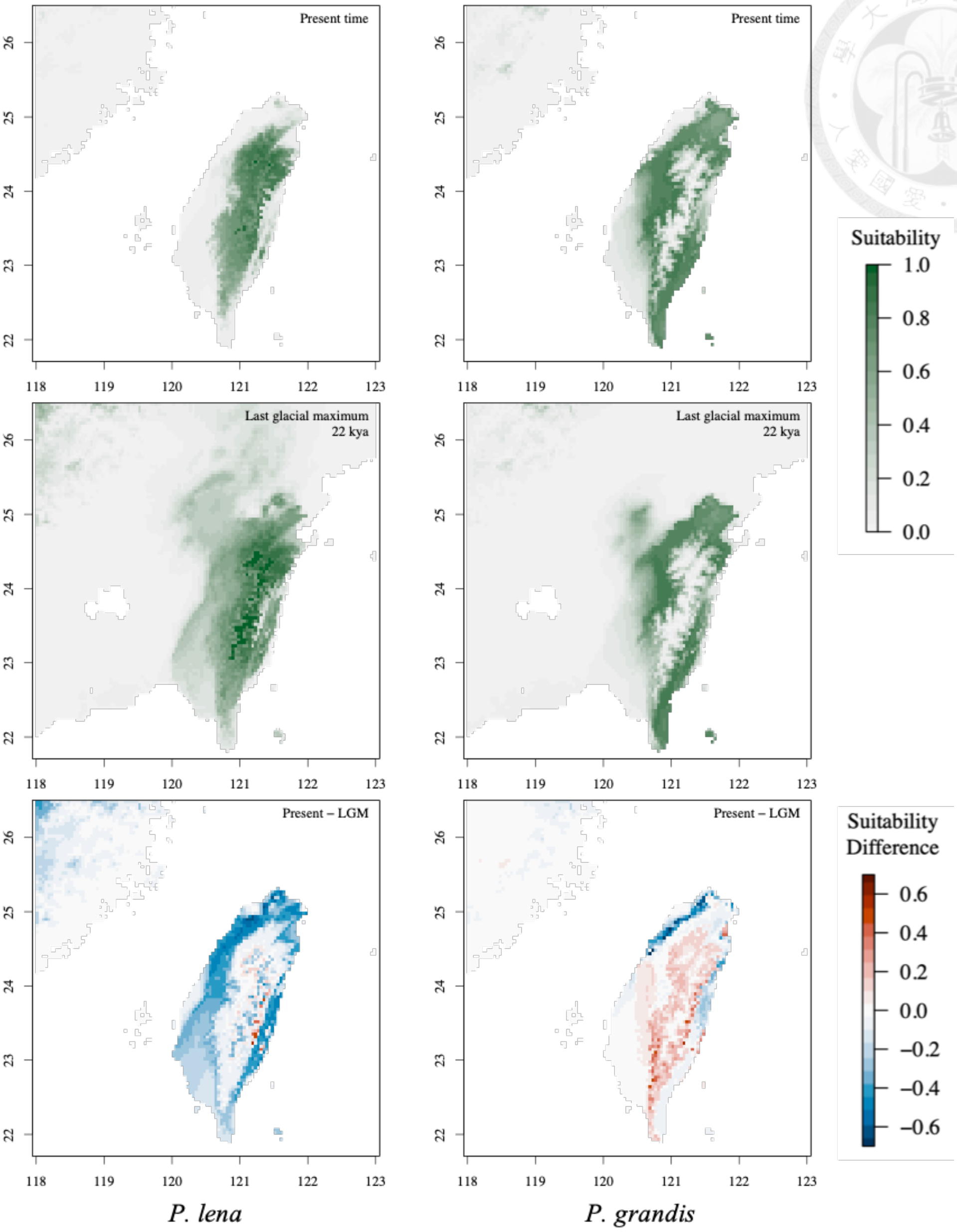

Figure 6. Present, historical (22 kya), and delta species niche estimated in Maxent for *P. lena* (left) and *P. grandis* (right). Delta species niche was determined by subtracting historical niche suitability from present niche suitability. Bioclimatic variables 10 and 16 were selected as predictors for *P. lena*, while variables 5, 18, and 19 were selected as predictors for *P. grandis* (Fick & Hijmans, 2017).

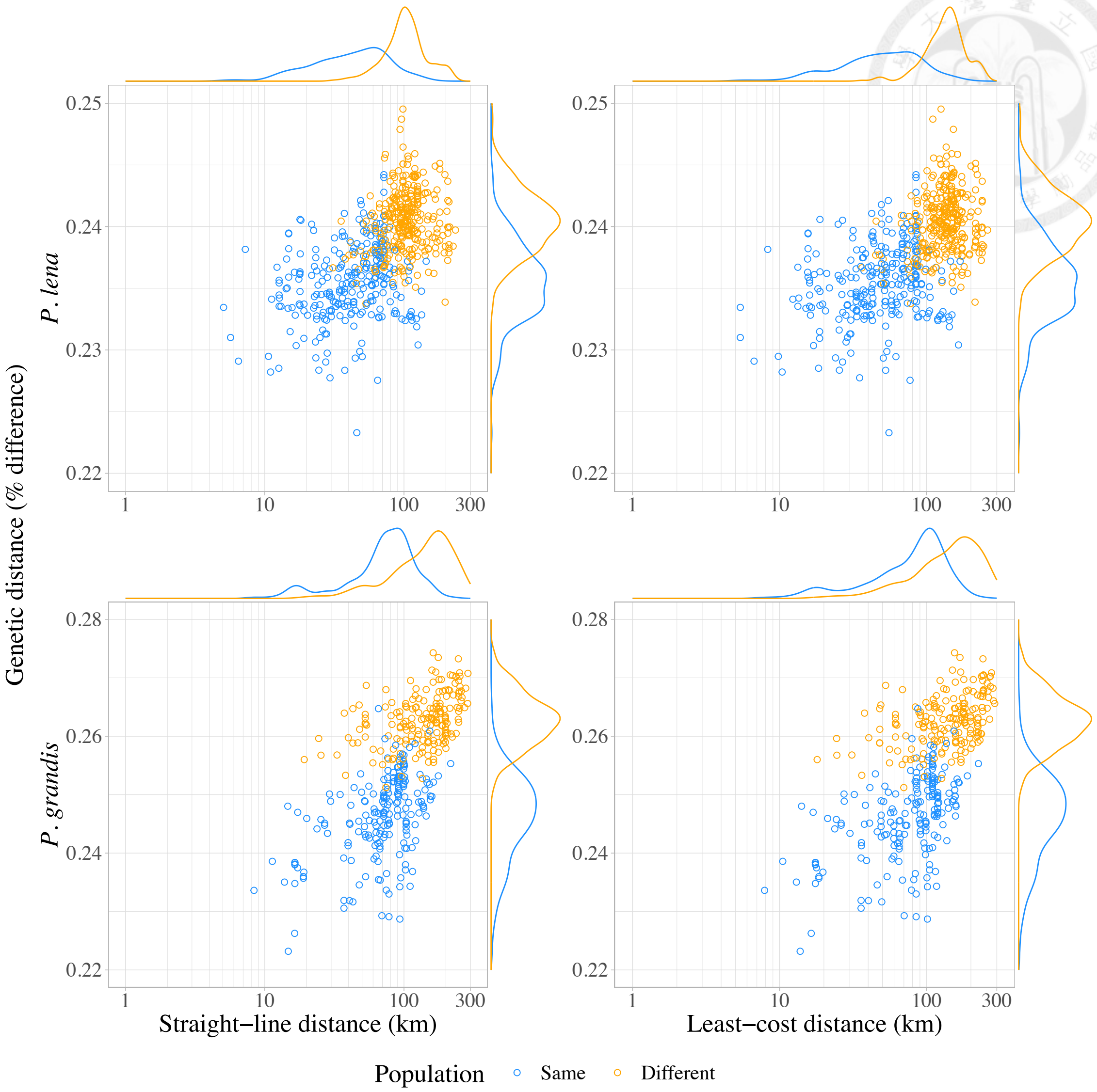

Figure 7. Genetic difference vs. geographic distance, with marginal distributions plotted along each axis. P-distance was used to represent genetic distance, while straight-line (left) and least-cost (right) distances were used to represent geographic distance. Blue points indicate individuals sourced from the same population, while orange points indicate individuals sourced from different populations.

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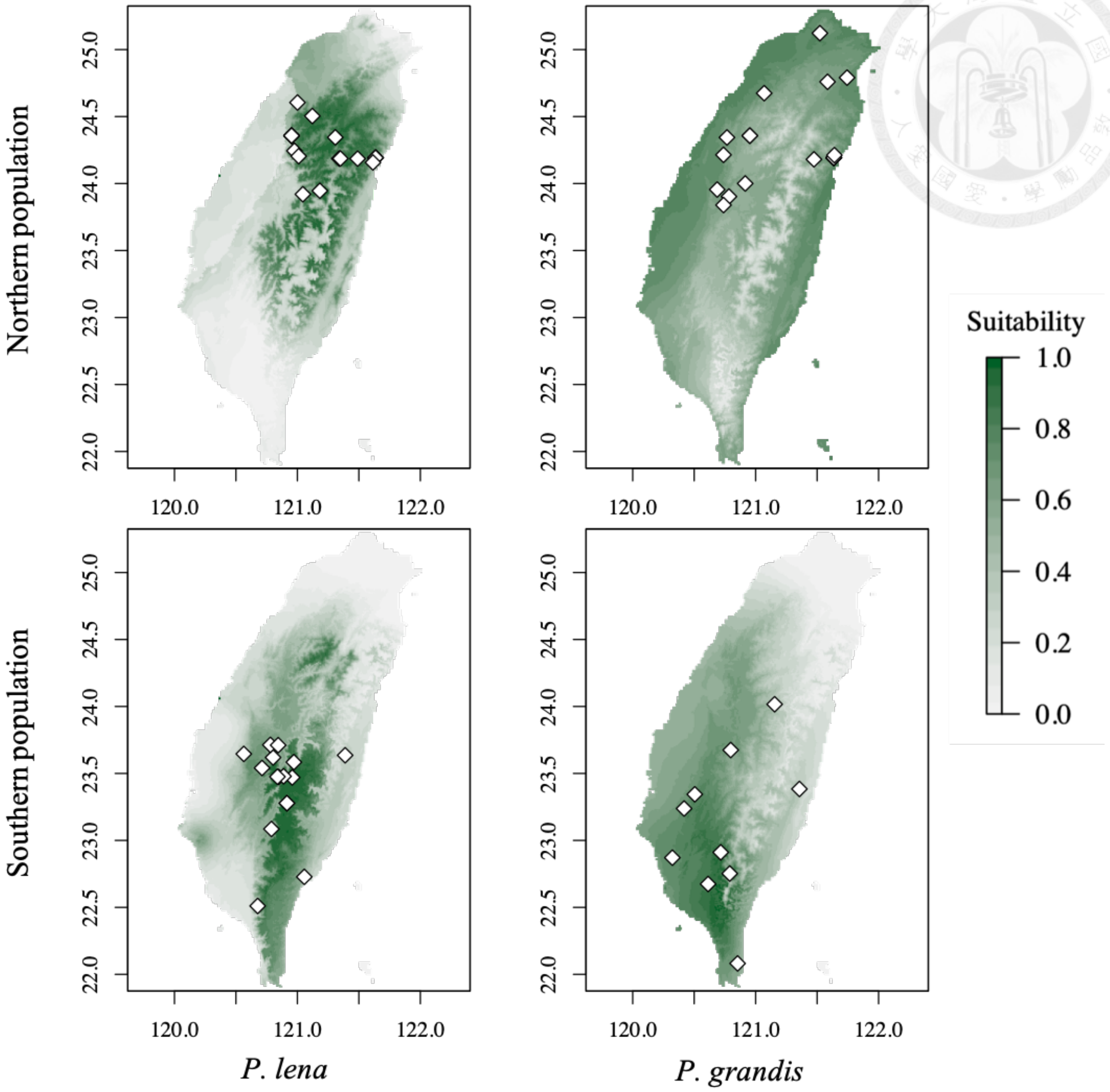

Figure 8. Species niche estimated in Maxent from putative Northern (top) and Southern (bottom) populations of *P. lena* (left) and *P. grandis* (right). Bioclimatic variables 9, 13, and 19 were selected as predictors for *P. lena*, while variables 5, 17, and 18 were selected as predictors for *P. grandis* (Fick & Hijmans, 2017).

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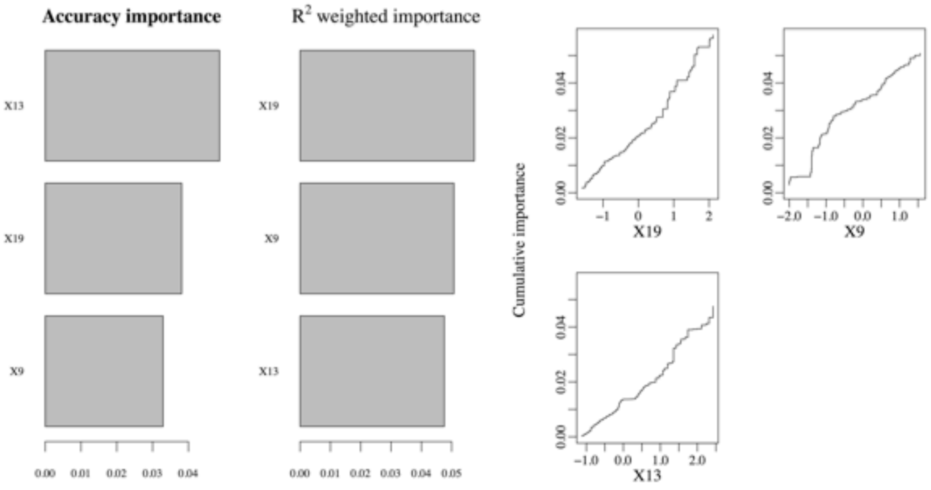
P. lena



P. grandis

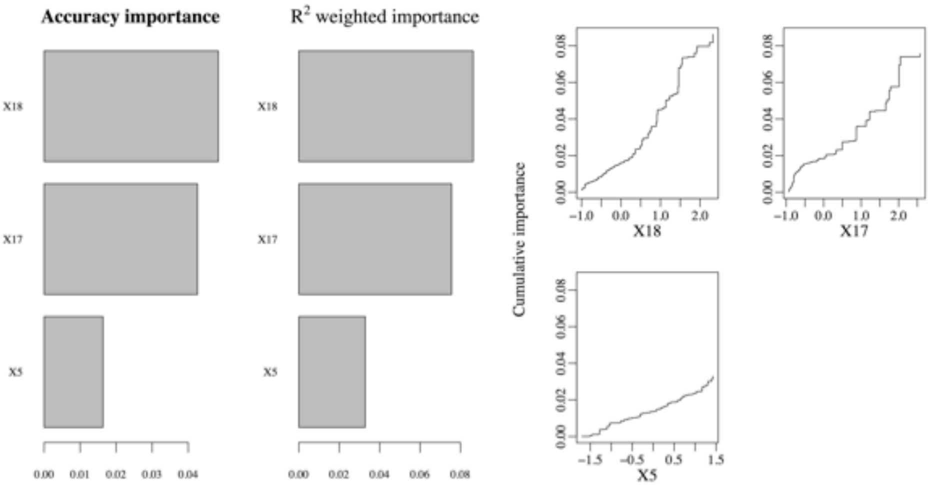


Figure 9. Accuracy importance, R2 weighted importance, and cumulative importance reported from gradient forest analyses for *P. lena* (top) and *P. grandis* (bottom). Bioclimatic variables 9, 13, and 19 were used as predictors for *P. lena*, while variables 5, 17, and 18 were used as predictors for *P. grandis*. SNPs with a GWAS -log10(p) value of 2 or greater were used as the “species” in this analysis.

Table 1. Results of *de novo* sequencing in Stacks 2 for *P. lena*, *P. grandis*, and both species together.

	P. lena	*P. grandis*	Both species
Mean effective per sample coverage	32.6x	34.8x	32.9x
Loci	35537	37173	28597
Total sites	9829174	10364847	7858780
Variant sites	73564	46063	133275
Variant sites after filtering for LD	25946	20010	26835

Table 2: Number of samples, nucleotide diversity, and Tajima's D calculated for populations of *P. lena* and *P. grandis*. All values of Tajima's D are not significant.

		All	North	South
P. lena	Number of samples	42	26	16
	Nucleotide diversity (pi)	0.290	0.291	0.286
	Tajima's D	-0.716	-0.485	-0.876
P. grandis	Number of samples	36	25	11
	Nucleotide diversity (pi)	0.307	0.299	0.303
	Tajima's D	-0.449	-0.476	-0.440

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Table 3: F statistics calculated between North and South populations of *P. lena* and *P. grandi*s.

	F_{ST}	F_{IT}	F_{IS}
P. lena	0.020	0.078	0.059
P. grandis	0.035	0.099	0.069

Table 4. AMOVA Phi ST and P values for subpopulations of *P. lena* and *P. grandis*

	Number of subpopulations	Phi ST	P value
P. lena	K=2	0.040	<0.001
	K=3	0.029	<0.001
P. grandis	K=2	0.116	<0.001
	K=3	0.110	<0.001

Table 5. Model performance and estimated parameters of Fastsimcoal2 simulations. Three models were simulated: historical divergence with subsequent size change of population 0 (DS0), historical divergence with subsequent size change of population 1 (DS1), and historical divergence with subsequent size change of both populations (DS01). DS01 was the best performing model for *P. lena*, while DS0 was the best performing model for *P. grandis*.

	Model	AIC	Delta LH	NPOP0	NPOP1	INTPOP0	INTPOP1	ANCPOP	TS0	TS1	TDIV	M01	M10
P. lena	DS0	1647074	143	69509	77953	132660	NA	89221	13367	NA	162119	3.22E-05	2.18E-05
	DS1	1647073	142	63371	71391	NA	171050	88643	NA	9371	182779	2.62E-05	3.11E-05
	DS01	1647054	138	71404	60132	130235	124723	86004	41882	6089	173601	3.44E-05	2.36E-05
P. grandis	DS0	1006766	220	65032	57361	9838	NA	64824	15595	NA	50078	9.22E-06	2.03E-05
	DS1	1007269	329	51433	54719	NA	45385	62378	NA	6077	27209	1.50E-05	1.32E-05
	DS01	1006785	223	68618	63578	11516	50620	66792	14382	13678	54466	9.70E-06	2.00E-05

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Table 6. Mantel test results comparing genetic p-distance with geographic distance. Models in the format "A|B" indicate that the performance of predictor A is being assessed while variance from predictor B is partitioned out.

Model	*P. lena*		*P. grandis*	
	R statistic	P value	R statistic	P value
Straight line	0.562	0.001	0.653	0.001
Least cost	0.581	0.001	0.634	0.001
Population	0.668	0.001	0.814	0.001
Least-cost | Straight line	0.176	0.005	-0.076	0.903
Straight line | Least cost	-0.015	0.563	0.213	0.005
Population | Straight line	0.471	0.001	0.733	0.001
Straight line | Population	0.199	0.003	0.464	0.001
Population | Least cost	0.441	0.001	0.753	0.001
Least cost | Population	0.190	0.001	0.483	0.001

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Table 7. Mantel test results comparing genetic p-distance with distance calculated from environmental variables. Partial Mantel tests were conducted with least-cost path distance as the secondary variable for *P. lena* and straight-line geographic distance as the secondary variable for *P. grandis*, controlling for the effect of geographic distance. Highlighted cells indicate significance.

Variable	*P. lena*				*P. grandis*			
	basic		partial		basic		partial	
	R statistic	P value	R statistic	P value	R statistic	P value	R statistic	P value
1	-0.068	0.881	-0.096	0.964	0.184	0.013	0.038	0.247
2	-0.015	0.561	-0.112	0.948	0.159	0.018	-0.008	0.551
3	0.344	0.001	0.125	0.027	0.368	0.001	-0.033	0.656
4	0.236	0.002	0.032	0.311	0.295	0.001	0.009	0.421
5	-0.053	0.820	-0.070	0.907	0.050	0.189	0.043	0.207
6	-0.066	0.858	-0.100	0.957	0.306	0.003	0.059	0.210
7	0.154	0.015	-0.029	0.644	0.235	0.003	0.020	0.346
8	-0.058	0.824	-0.078	0.906	0.083	0.101	0.035	0.252
9	-0.071	0.906	-0.107	0.961	0.121	0.032	-0.057	0.830
10	-0.054	0.784	-0.076	0.894	0.070	0.136	0.045	0.200
11	-0.073	0.911	-0.113	0.974	0.303	0.002	0.028	0.299
12	-0.067	0.812	-0.043	0.712	0.135	0.068	-0.038	0.681
13	0.129	0.047	-0.082	0.872	0.439	0.001	0.119	0.097
14	0.022	0.329	-0.064	0.883	-0.005	0.464	-0.387	1.000
15	0.092	0.073	-0.201	1.000	0.355	0.002	-0.228	0.998
16	0.079	0.121	-0.080	0.866	0.472	0.001	0.145	0.070
17	0.027	0.301	-0.077	0.934	0.013	0.411	-0.393	1.000
18	0.099	0.092	-0.075	0.839	0.491	0.001	0.141	0.054
19	-0.018	0.586	-0.095	0.924	0.096	0.132	-0.406	1.000