Differentiated historical demography and ecological niche forming present distribution and genetic structure in coexisting two salamanders (Amphibia, Urodela, Hynobiidae) in a small island, Japan

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Background. The climatic oscillations in the Quaternary period considerably shaped the distribution and population genetic structure of organisms. Studies on the historical dynamics of distribution and demography not only reflect the current geographic distribution but also allow us to understand the adaption and genetic differentiation of species. However, the process and factors affecting the present distribution and genetic structure of many taxa are still poorly understood, especially for endemic organisms to small islands.

Methods. Here, we integrated population genetic and ecological niche modelling approaches to investigate the historical distribution and demographic dynamics of two co-existing salamanders on Tsushima Island, Japan: the true *H. tsuensis* (Group A), and *Hynobius* sp. (Group B). We also examined the hypothesis on the equivalency and similarity of niches of these groups by identity and background tests for ecological niche space.

Results. Our result showed that Group A is considered to have undergone a recent population expansion after the Last Glacial Maximum while it is unlikely to have occurred in Group B. The highest suitability was predicted for Group A in southern Tsushima Island, whereas the northern part of Tsushima Island was the potential distribution of Group B. The results also suggested a restricted range of both salamanders during the Last Interglacial and Last Glacial Maximum, and recent expansion in Mid-Holocene. The genetic landscape-shape interpolation analysis and historical suitable area of ecological niche modelling were consistent, and suggested refugia used during glacial ages in southern part for Group A, and in northern part of Tsushima Island for Group B. Additionally, we found evidence of nonequivalence for the ecological niche of the two groups of the salamanders, although our test could not show either niche divergence or conservatism based on the background tests. The environmental predictors affecting the potential distribution of each group also showed distinctiveness, leading to difference in selecting suitable areas. Finally, the combination of population genetics and ecological modeling has revealed the differential demographic/historical response between coexisting two salamanders on a small island.

1 **Differentiated historical demography and ecological niche forming present**

2 **distribution and genetic structure in coexisting two salamanders (Amphibia,**

3 **Urodela, Hynobiidae) in a small island, Japan**

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ABSTRACT

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39   distribution of each group also showed distinctiveness, leading to difference in selecting suitable

40   areas. Finally, the combination of population genetics and ecological modeling has revealed the

41   differential demographic/historical response between coexisting two salamanders on a small

42   island.

44   **INTRODUCTION**

45   Qualifying the interspecific variation in geographic distribution is central to the

46   understanding of the drivers constraining organism pattern in a region (*Brown, Stevens &*

47   *Kaufman, 1996*). Currently, several studies have concentrated on exploring variation in

48   geographic ranges by comparing the ecological niches of given species. The differentiation in

49   ecological niches may provide evidence for evolution and species delineation. Particularly, these

50   species might show niche conservatism, that means the species retain ecological characteristic

51   from common ancestors, leading to allopatric range (*Wiens & Graham, 2005*) or overlap

52   distribution in secondary contacting (*Bett, Blair & Sterling, 2012*). Otherwise, niche divergence

53   of two species might be promoted by the reproductive isolation to adapt to contrasting

54   environmental space (*Schluter, 2009*), leading to occur as sympatric distribution (*Via, 2001*) or

55   allopatric distribution (*Kozak & Wiens, 2006*). In some cases, the counter-intuitive result might

56   occur when compared the ecological niches among species due to the heterogeneity of

57   environmental conditions that explain the factors limiting the expansion/contraction of species

58   (*Zhang et al., 2014*; *Tran et al., 2021*).

59   The climatic oscillations in the Quaternary period affected considerably on shaping

60   distributional pattern and population genetic structure of organisms from boreal, temperate to

61   tropical regions (*Hewitt, 2000, 2011*). Studies on the historically dynamics of distribution and

62   demography not only reflect the current geographic distribution but also allow us to understand

63   the adaption, genetic change of species. The knowledge is also useful for predicting the future

64   potential evolution, then proposing the appropriate species management strategies. The empirical

65   studies showed that several species in temperate zone generally showed a trend on contracting

66   population size and/or distribution into refugia and then expanding again (*Tominaga et al., 2013*;

67   *Aoki et al., 2019*; *Matsui et al., 2020*). However, species might response the great climatic

68   changes (e.g., the Last Glacial Maximum) by different scenarios (*Hewitt, 2000*). The process and

69   factors affecting on the present pattern and genetic structure of many taxa are still poorly

70   understood, especially for endemic organisms to small islands.

71   Recently, molecular analysis and ecological niche models (ENMs) on population level

72   have been integrated as a powerful approach to reveal the historical distribution formation and

73   population dynamics that might shape the current distribution (*Chang et al., 2012*; *Pahad,*

74   *Montgelard & Jansen, 2019*; *Ren et al., 2020*). ENMs have been widely used for predicting the

75   ecological niche of species at different scale of space and time. By conjunction the current

76   climatic condition and paleoclimate, the projection of ENMs can present the historically

77   potential pattern and refugia of species during Quaternary period, for example, the Last Glacial

78   Maximum or Mid-Holocene (*Aoki et al., 2019*; *Ren et al., 2020*). On the other hand, populational

79   genetic data can reveal genetic diversity and structure of species and estimate the demographic

80   dynamics (*Tominaga et al., 2013; Jeon et al., 2021*). Consequently, the integrating of these

81   approaches has supported for each other and shed enlightenment on the evolutionary process of

82   species (*Pahad, Montgelard & Jansen, 2019*). However, most of these studies subjected a large

83   geographic range and a few studies on a small island were conducted (e.g., *Igawa et al., 2013*).

84   The Tsushima Island is a small island (696 km^2), located between the Japanese

85 Archipelago and Korean Peninsula, from 34º05' to 34º42' N latitude, and 129º10' to 129º29' E

86 longitude (Fig. 1A). The island includes two major parts, including Kamijima (northern part) and

87 Shimojima (southern part), and mainly dominated by mountainous geography and a few small

88 plains near the sea (*Nagaoka, 2001*). Historically, the formation of the straits between Kyushu

89 Island (Japan) and Korean Peninsula was estimated to connect at least five times (*Kitamura &*

90 *Kimoto, 2006*). According to *Emerson (2002)*, the biography of species in islands can be clearly

91 explained by natural events in the past. It can be seen that Tsushima Island is a good example to

92 show the biographic dynamics of organisms because of its unique geography, location, and

93 historical connections. In fact, herpetofauna on the island is unique because it is categorized as

94 four types by biogeographic affinities: (1) species distributed on Korean Peninsula, Tsushima

95 Island, and the Japanese Archipelago (the frog *Dryophytes japonicus* [*Matsui & Maeda, 2018*]);

96 (2) on Korean Peninsula and Tsushima Island (the frog *Rana uenoi* [*Matsui & Maeda, 2018*], the

97 skink *Scincella vandenburghi* [*Chen et al., 2001*], the lizard *Takydromus amurensis*, the snake

98 *Lycodon rufozonatus rufozonatus* [*Herpetological Society of Japan (ed.). 2021*]); (3) on

99 Tsushima Island and Japanese Archipelago (the snake *Elaphe climacophora* [*Herpetological*

100 *Society of Japan (ed.). 2021*]); (4) endemic to Tsushima Island (the salamander *Hynobius*

101 *tsuensis* [*Sato, 1943*], the frog *Rana tsushimensis* [*Matsui & Maeda, 2018*], the viper *Gloydius*

102 *tsushimaensis* [*Herpetological Society of Japan (ed.). 2021*]).

103  *Figure 1 inserted here*

104   The Tsushima salamander (*Hynobius tsuensis*), an endemic salamander to Tsushima

105 Island, thus categorized as (4) noted above, was described by *Abe (1922)*. Recently, *Niwa, Kuro-*

106 *o & Nishikawa (2021)* showed that there are two distinct groups within *H. tsuensis* based on

107 mitochondrial cytochrome b (cyt *b*) gene and seven nuclear microsatellite loci, including Group

108 A (probably, true *H. tsuensis*), and Group B (*Hynobius* sp.). Group B was phylogenetically

109 closer to *Hynobius nebulosus* sensu stricto which distributed on Kyushu and its adjacent islands

110 (excluding Tsushima Island) than to Group A (*Niwa, Kuro-o & Nishikawa, 2021*). Furthermore,

111 the body coloration of the two groups is also distinctive. Group A showed a dark brownish

112 dorsum with yellowish spots or a yellowish dorsum with dark spots, and the upper and lower

113 edges of the tail were bright yellow, while Group B presented a brownish dorsum with dark

114 stipples, and tail without bright yellow color (*Niwa, Kuro-o & Nishikawa, 2021*). Although the

115 two groups were discovered syntopically from same site (within 5 m × 5 m) in the several

116 streams in Tsushima Island, hybrids (including introgressive hybridization) between them were

117 not found. Thus, these two groups (Group A and Group B) were considered to be actually

118 different species (*Niwa, Kuro-o & Nishikawa, 2021*). *Niwa, Kuro-o & Nishikawa (2021)* was the

119 first report on the co-existing of two lotic salamanders of *Hynobius* in a small island. However,

120 the geographic distribution, population genetic, and underlying factors affecting the current

121 distribution of these groups are still unclear.

122 In this study, we investigated the ecological niche and population genetic of two groups

123 of *Hynobius* on Tsushima Island that could be useful to infer the fluctuation of historical

124 distribution and demographic dynamics of these two co-existing salamanders on the island. The

125 main objectives of this study include (1) examining the population dynamics between two

126 salamanders from Tsushima Island; (2) elucidating the distribution pattern between two

127 salamanders on Tsushima Island; (3) revealing the ecological niche differentiation of two

128 salamanders on Tsushima.

129

MATERIALS AND METHODS

Population genetics

Sample collection

Forty-eight specimens of Group A from 27 localities and 20 specimens of Group B from 12 localities were sampled for population genetic analyses (Table S1). Since to be endangered salamanders, we could not collect multiple specimens in several localities. Thus, we sampled one to three specimens per locality in each group, because it was necessary to decrease sampling bias among localities. Tissues were preserved in 96–99% ethanol and stored in freezer at -18 ºC. Specimen collection protocols and animal operations followed the guideline of animal experiments in Kyoto University (approval nos. 29-A-7 and 30-A-7). All specimens were stored in graduate school of Human and Environmental studies, Kyoto University. Total DNA was extracted from muscle or liver tissues using DNeasy Blood & Tissue Kit (Qiagen). Then, all specimens (48 of Group A and 20 of Group B) were sequenced for the partial cyt *b* gene of 413 base pairs (bp), and methods of PCR, purification of PCR product, and sequencing were the same as those described in *Aoki, Matsui & Nishikawa (2013)*. Cytochrome b gene was widely used for genetic studies on salamanders of *Hynobius* (e.g., *Okamiya et al., 2018*; *Matsui et al., 2019, 2020*; *Suk et al., 2020*).

Prior to analyses, we added the published sequences of 39 specimens (25 of Group A and 14 of Group B) from DDBJ (DNA data bank of Japan) to our data set. In total, 73 individuals of Group A from 37 localities and 34 individuals of Group B from 17 localities were used in the following analyses (Fig. 1B; Table S1). Of these localities, Group A and Group B were discovered syntopically at nine localities (Fig. 1B; Table S1).

152 *Demographic history*

153   Genetic diversity in each group (Group A and Group B) were calculated with respect to

154 haplotype diversity (h; *Wenink, Baker & Tilanus, 1993*) and nucleotide diversity (π; *Nei &*

155 *Tajima, 1981*). To infer demographic history in each group, we conducted mismatch distribution

156 analysis showing the frequency distribution of pairwise differences (*Rogers & Harpending,*

157 *1992*). We tested if the observed data fit to models of either sudden population expansion or

158 spatial population expansion by calculating goodness of fit on the basis of the sum of square

159 deviations (SSD) with 10,000 replicates. To assess potential expansion, neutrality tests in each

160 group were conducted with 10,000 simulations based on two index, Tajima's *D* (*Tajima, 1989*)

161 and Fu's *Fs* (*Fu, 1997*). Significant negative values of the tests tell us to have been occurred the

162 past demographic expansion. These analyses were performed using Arlequin ver. 3.5 (*Excoffier*

163 *& Lischer, 2010*). Furthermore, genetic landscape-shape interpolation analysis in each group

164 were conducted using sequence (partial cyt *b* gene, 413 bp) and locality data with Alleles In

165 Space (*Miller, 2005*). The locality data, latitude and longitude were converted into XY

166 coordinates for the analysis. Although this analysis was generally used to visualize spatial

167 patterns of genetic diversity (*Miller, 2005*; *Miller et al., 2006*), in the several phylogeographic

168 studies, the analysis has been applied to research past refugia (e.g., *Nunome et al., 2010*;

169 *Tominaga et al., 2013*). Finally, we inferred the dynamics of effective population size through

170 time in each group using the Bayesian skyline plot method (*Drummond et al., 2005*) as

171 implemented in BEAST v2.6.2 (*Bouckaert et al., 2019*). Using Kakusan4 (*Tanabe, 2011*),

172 Hasegawa-Kishino-Yano (HKY; *Hasegawa, Kishino & Yano, 1985*) model was selected as site

173 model for both groups. The substitution rate of cyt *b* gene sequence for strict clock model was set

174 as 0.64% substitutions per million years (MY) per lineage. Although this value is rate of

175 mitochondrial ND2 gene of Salamandrid (*Weisrock et al., 2001*), it has widely been applied to

176 phylogenetic studies on Japanese salamanders of *Hynobius* (*Tominaga et al., 2006*; *Aoki, Matsui*

177 *& Nishikawa, 2013*; *Matsui et al., 2017, 2019, 2020*; *Niwa, Kuro-o & Nishikawa, 2021*). An

178 MCMC run for 30 million generations was conducted with sampling every 5000 generations.

179 Convergence of the parameters was ensured using Tracer ver. 1.6 (*Rambaut et al., 2014*) with

180 effective sample size (ESS) > 200. After determining burn-in size with Tracer ver. 1.6., the initial

181 10% generations were discarded as burn-in. The Bayesian skyline plot was reconstructed with

182 Tracer ver. 1.6 to visualize historical demographic pattern.

183 **Ecological niche models**

184 *Occurrence data*

185   Present records of two groups were collected from our field survey in 2015–2018. To

186 prevent spatial autocorrelation of occurrence data, we randomly selected points within 100

187 meters, with ten replicates from others by using "spThin" package (*Aiello-Lammens et al., 2015*).

188 Consequently, 37 localities of Group A (Fig. 2A) and 17 of Group B (Fig. 2B) were employed in

189 the final model.

190   *Figure 2 inserted here*

191 *Environmental variables*

192   Here, we constructed ENM for the two salamanders using environmental variables from

193 various sources. For climate data, we used 19 bioclimatic data at 30-arc-second (approximately 1

194 km) resolution from the WorldClim database (*Fick & Hjimans, 2017*). We also collected LAI

195 (Leaf Area Index), EVI (Enhanced Vegetation Index), and NDVI (Normalized Difference

196 Vegetation Index) in January and June at NASA LPDAAC collection from the MODIS database

197   (https://lpdaac.usgs.gov). To present land cover on Tsushima Island, we gathered the high

198   resolution of Land use and Land cover map products with 30 m resolution from ALOS Science

199   Project (https://www.eorc.jaxa.jp/ALOS/en/lulc/lulc_index.htm). The forest height data was

200   downloaded from the website of the Global Land Analysis & Discovery:

201   https://glad.umd.edu/dataset/gedi/ (*Potapov et al., 2021*). In addition, the Shuttle Radar

202   Topography Mission (SRTM) at 30 x 30 m resolution (downloaded from

203   https://earthexplorer.usgs.gov/) was used as elevation variables. We, then, calculated the slope

204   and aspect of Tsushima Island from the elevation layer by applying ArcMap 10.6 (ESRI). The

205   variable of pH of water (pH_H_2O) of Tsushima Island was gathered from the SoilGrids database

206   that available from ISRIC-World Soil Information: https://soilgrids.org// (*Hengl et al., 2017*).

207   Totally, we collected 31 environmental layers for initial analysis. To avoid

208   autocorrelation among variables, we calculated correlation and subsequently reduced the variable

209   pairs with high correlation (|r| > 0.85) by ENMTools version 1.4.4 (*Warren, Glor & Turelli,*

210   *2010*). Finally, 16 variables were selected for running ecological niche models (Table 1).

211   *Table 1 inserted here*

212   To project the historical distribution of the salamanders on Tsushima Island, we used

213   climate reconstruction with a Global Climate Models of MIROC-ESM (*Sueyoshi et al., 2013*) for

214   the Mid-Holocene (~6000 years ago), the Last Glacial Maximum (LGM) (~22,000 years ago),

215   and the Last Interglacial (LIG) (~120,000 - 140,000 years BP; *Otto-Bliesner et al., 2006*),

216   available from the WorldClim database. We kept the data of topography and landcover as

217   constant variables for historical projections because the historical data is only available for

218   climate data at this time.

219   *Ecological niche model processing*

220   We predicted ecological niches for salamanders on Tsushima Island by using Maxent

221   (version 3.4.1; *Phillips, Anderson & Schapire, 2006*). The method uses present data and

222   environmental conditions to estimate the unknown probability distribution defining a species'

223   range (*Phillips, Dudik & Schapire, 2004*; *Phillips, Anderson & Schapire, 2006*). Comparing to

224   other algorithms, Maxent is shown to perform well, especially for small occurrence sample size

225   (*Elith et al., 2006*; *Wisz et al., 2008*; *van Proosdij et al., 2016*). For Group A, we ran models by

226   using 10-folds cross-validation to evaluate model, while the number of occurrence localities of

227   Group B was limited (n = 17), thus we applied the jackknife method for a small sample size

228   (*Pearson et al., 2007*). To select the optimal model for the species, we applied the ENMval

229   package in R (*Muscarella et al., 2014*). The package built a series of model by turning six

230   featured class (L, LQ, H, LQH, LQHP and LQHPT (L = linear, Q = quadratic, H = hinge, P =

231   product and T = threshold) combined with eight regularizations from 0.5 to 4 (interval = 0.5). In

232   addition, we also adjusted the regularization parameter for each species by ranging from 1 to 10,

233   interval = 0.5 (*Bett, Blair & Sterling, 2012*), and kept feature class as auto selection. A total of

234   58 candidate models were analyzed, then, we chose the best model with the minimum value of

235   AICc (*Muscarella et al., 2014*), and based on the highest AUC (area under the curve; *Phillips,*

236   *Anderson & Schapire, 2006*). From candidate models, we selected the best model with

237   regularization value 1 for Group A, and 2 for Group B, and auto feature class. For other

238   parameters, we used default set up as a maximum of 500 iterations, convergence threshold 10^{-5}

239   (*Phillips, Anderson & Schapire, 2006*). To determine suitable or unsuitable area, we applied the

240   minimum training presence threshold (the lowest presence threshold) for both species.

241   **Niche equivalency and similarity comparison**

242   Observed niche overlap values for salamanders on Tsushima were calculated by using ENMtools

243   (*Warren, Glor & Turelli, 2010*) with Schoeners's D and Hellinger's I niche similarity metrics.

244   These indices range from 0 (no overlap) to 1 (identical niche models), which predicted the

245   similarity of ecological niche between species (*Warren, Glor & Turelli, 2008*).

246   The "Identity test" (also called equivalency test) was used to test whether the ENM of

247   Group A is equivalent to Group B (*Warren, Glor & Turelli*, *2008*). The test creates a null

248   distribution by pooling the occurrence points for both species, randomizing the species identities

249   of the localities, and creating two new samples of the same sizes as the original samples without

250   consideration of suitable habitat to either species. Then, we compared observed niche overlap

251   values to the null distribution of 100 pseudo-replicate niche overlap values by one-side test with

252   an alpha level of 0.05. We considered that these ecological niches were not equivalent if the

253   observed value fell within the bottom 5% of the null distribution. The test was implemented by

254   using the package ENMTools 1.0 (*Warren et al., 2021*) in R version 4.1.0 (*R Core Team, 2021*).

255   We also applied the "background test" in ENMtools version R (*Warren et al., 2021*) to

256   test for niche conservatism or divergence of two species. The test compared an ENM of Group A

257   against a null distribution generated from random points selected within the geographic range of

258   Group B. We used 100 replicates for the test. The opposite direction, comparing an ENM of

259   Group B to and ENMs generated randomly within the ranges of Group A, also was implemented.

260   Then, the test compared the observed niche overlap value between Group A and B to the null

261   distribution by a two-sided test and alpha level of 0.05. We determined that when the observed

262   niche overlap value between two species was above the 95% confidence interval of the null

263   distribution, it might support niche conservatism. By contrast, the observed value was below the

264   95% confidence interval, supporting niche divergence (*Warren, Glor & Turelli*, *2008*). In the

265   case of the null hypothesis was supported, the niche overlap might be explained by regional

266   similarities in the habitat available to each group. If the background test is only significant in one

267   direction but not for the remaining direction, we could reject the null hypothesis that similarity

268   (or divergence) between group is less than expected based on the availability of habitat (*Warren,*

269   *Glor & Turelli, 2008*).

270   In addition, we also applied a "PCA-env" framework of *Broennimann et al. (2012)* that

271   uses kernel density to estimate the environmental distribution of species and the distribution of

272   available environment to quantify niche overlap in a two-dimensional environmental space.

273   Then, the data were used to implement hypothesis tests similar to the approach in *Warren, Glor*

274   *& Turelli (2008)* (called ecospat-identity test, and ecospat-background test). Here, we used

275   functions *enmtools.ecospat.id* and *enmtools.ecospot.bg* in ENMTools version R (*Warren et al.,*

276   *2021*) to conduct the tests. The functions automatically ran principal components analysis to

277   reduce the predictors to a two-dimensional space (*Warren et al., 2021*) because we employed

278   more than two predictors in the analysis.

279

280   **RESULTS**

281   **Demographic history**

282   Partial cyt *b* gene sequences (413 bp) were determined for 68 specimens (Group A [$n = 48$],

283   Group B [$n = 20$]) and deposited in DDBJ (accession numbers: LC638502–LC638569). Twenty-

284   nine haplotypes and nine haplotypes were observed in 73 individuals of Group A and 34

285   individuals of Group B, respectively (Table 2). Thirty-one and nine polymorphic sites were

286   detected in Group A ($n = 73$) and Group B ($n = 34$), respectively. A3, A24, and [A2, A17, A19]

287   haplotypes were the most (34.2%: 25 of 73 individuals), second (8.2%: six individuals), and third

288   (5.5%: four individuals) dominant haplotypes in Group A, respectively. Also, of 34 Group B

289   individuals, B7, [B3, B6], and B1 haplotypes were the most (23.5%: eight individuals), second

290   (20.6%: seven individuals), and third (14.7%: five individuals) abundant haplotypes, respectively.

291   One (A3) and four (B1, B3, B6, and B7) major haplotypes (frequency within each group > 10%)

292   were detected in Group A and Group B, respectively. Twenty and four haplotypes were found

293   from one individual in Group A and Group B, respectively. Genetic diversity was higher in

294   Group A (h ± SD: 0.8702 ± 0.0347; π ± SD: 0.00585 ± 0.00356) than in Group B (0.8520 ±

295   0.0278; 0.00547 ± 0.00343). Mean number of pairwise differences were larger in Group A

296   (2.4140 ± 1.3256) than in Group B (2.2603 ± 1.2739). One and three differences were the most

297   in Group A and Group B, respectively (Fig. 3). Mismatch distribution test of Group A could not

298   rejected significantly the null hypothesis of population expansion ($P > 0.05$ in both sudden

299   expansion and spatial expansion models), showed a unimodal shape and fitted the curves of both

300   the sudden expansion (Tau = 1.447, SSD = 0.00035184) and spatial expansion models (Tau =

301   1.328, SSD = 0.00038387) (Fig. 3). These suggests the occurrence of past demographic

302   expansion in Group A. On the other hands, Group B showed a different result; not suggests the

303   occurrence of population expansion in the sudden expansion model (Tau = 2.910, SSD =

304   0.02556232, $P < 0.05$), but suggests that in spatial expansion model (Tau = 2.814, SSD =

305   0.02246269, $P > 0.05$) (Fig. 3). In the neutrality tests, Group A showed a significant negative

306   value for both Tajima's D and Fu's Fs tests ($P < 0.01$), which suggested to have been occurred a

307   recent population expansion in this group. On the other hands, results of Group B were not

308   significant for both neutrality tests ($P > 0.05$) (Table 2).

309   *Table 2 inserted here*

310   *Figure 3 inserted here*

311   Genetic landscape-shape interpolation analysis illustrated geographic patterns of genetic

312   diversity in each group (Fig. 2). The relatively high peaks (i.e., high genetic diversity) were

313   detected at the high mountainous areas in southern part of Tsushima Island for Group A (Fig.

314   2A), while the results of Group B showed the high diversity in northern part of the island (Fig.

315   2B).

316   Bayesian skyline plot presented different curves between Group A and Group B in

317   change of effective population size. It had been increased constantly at least ca. 300,000 to

318   50,000 years ago in Group A (Fig. 4A). There was no drastic change for the effective population

319   size of Group B during ca. 200,000 to 100,000 years ago, but the occurrence of a recent

320   expansion was estimated from ca. 75,000 years ago to the present (Fig. 4B). Group A had a

321   larger effective population size than in Group B.

322   *Figure 4 inserted here*

323   **Ecological niches of two salamanders**

324   The Maxent models presented strong ability to generate potential distribution of salamanders on

325   Tsushima Island particularly, the AUC for training Group A = 0.895 ± 0.006, and testing AUC =

326   0.775 ± 0.068, and for Group B was = 0.908 ± 0.008, and testing AUC = 0.908 ± 0.231. The

327   potential distribution of Group A resulting from our models covered most of Tsushima Island,

328   but the high suitable area showed a concentration on the southern part of Tsushima Island. On

329   the contrary, the predicted distribution of Group B was fragmented and mostly restricted to the

330   northern region. The overlap area between the two groups was fragmented and mainly occurred

331   in the northern part of the island (Fig. 5).

332   *Figure 5 inserted here*

333   The top three variables contributing the model of Group A account for a total of 63.5%,

334   including Bio12 (24.5%), NDVI_Jun (22.4%), and Landcover (16.6%). For Group B, the

335   variables related to topography were the most important, and the contribution of Elevation and

336   Slope were equal with 28.8%. The Bio12 had the third largest contributor to the Group B model

337   at 15.3% (Table 3).

338   *Table 3 inserted here*

339   The shared high contributing variables of both groups were Bio12 (annual precipitation

340   with 24.5% for Group A and 15.3% for Group B) and Elevation (with 10.6% and 28.8% for

341   Group A and Group B, respectively). Interestingly, the response curve of the shared predictors

342   presented distinctive trends. In particular, Group A preferred a higher annual precipitation area,

343   roughly 2050 mm, whereas the most suitable annual precipitation of Group B was around 1900

344   mm (Fig. 6). The elevation showed peak suitability around 100 meters and 80 meters for Group

345   A, and Group B, respectively. However, the habitat suitability of Group A decreased gently

346   while that of Group B presented a significant decrease after peaking (Fig. 6).

347   *Figure 6 inserted here*

348   **Historical potential distribution**

349   The projected distribution of the two salamanders on paleo-climate reconstructions showed the

350   fluctuation following the time period, and was contrasting compared to the current distribution.

351   Particularly, the potential distributions of both species were increased presently compared to the

352   Last Interglacial, and Last Glacial Maximum (Fig. 7). The distribution of Group A was mostly

353   restricted in the southern part, while the range of Group B concentrated around the isthmus in the

354   center of the island on the Last Interglacial. For the Last Glacial Maximum, the distribution of

355 salamanders tended to move toward the north, Group A focused roughly on the isthmus, whereas

356 Group B moved greatly to the northern tip of the island. On the other hand, the projection on

357 Mid-Holocene increased compared to the present (Fig. 7). Furthermore, it can be seen that the

358 predicted ranges on Mid-Holocene overlapped significantly at the current time for both groups.

359 *Figure 7 inserted here*

360 **Niche equivalency and similarity tests**

361 Our identity test showed that the two salamanders had nonequivalent ENMs (Fig. 8), with

362 Hellinger's-based I ($P < 0.05$), and Schoener's D ($P < 0.05$), leading to the rejection of the null

363 hypothesis of equivalency test. The background tests indicated that our null hypothesis could not

364 be rejected due to non-significant for both directions of the comparison ($P > 0.05$ for Hellinger's-

365 based I; $P > 0.05$ for Schoener's D; Fig. 8). The results of ecospat-identity test and ecospat-

366 background test also showed similar trends. The ecospat-identity test rejected the null hypothesis

367 of equivalent niches ($P < 0.05$) while both direction of ecospat-background test could not reject

368 the null hypothesis of niche similarity ($P > 0.05$ for Hellinger's-based I; $P > 0.05$ for Schoener's

369 D; Fig. 9).

370 *Figure 8 inserted here*

371 *Figure 9 inserted here*

372

373 **DISCUSSION**

374 **Demographic history of two salamanders on Tsushima Island**

375 Both haplotype and nucleotide diversities of Group A was higher than those of Group B in our

376 genetic analysis (Table 2), although number of samples was different between two groups (73

377   specimens in Group A and 34 in Group B), which was reflected by differences on number of

378   localities (37 localities in Group A and 17 in Group B). ENM analyses also inferred that the

379   suitable habitats of Group A were larger than those of Group B at the Last Interglacial (LIG),

380   Mid-Holocene, and current scenario (Fig. 7), and the results of mismatch distribution analysis

381   (Fig. 3) and neutrality test suggested that Group A have undergone a recent population expansion.

382   Group A seemed to invade (or isolate) into Tsushima Island earlier than Group B (*Niwa, Kuro-o*

383   *& Nishikawa, 2021*) and the range is separated into two areas (i.e., the northern and southern

384   areas of Tsushima Island) due to split by the intermediate lowland (Fig. 5A). Further, the genetic

385   landscape-shape interpolation analysis showed that high genetic diversity was detected in the

386   southern part of Tsushima for Group A (Fig. 2A). The two areas are not severely isolated but

387   could provide gene flow between the northern and southern populations of Group A. Thus,

388   Group A retains higher haplotype and nucleotide diversity than Group B. Such high intraspecific

389   genetic diversity may enable Group A much more adapted to the variable climate conditions and

390   elevations than Group B. On the basis of ENM analyses, Group A had a restricted distribution in

391   the Last Glacial Maximum (LGM), but a large highly suitable distribution in the Mid-Holocene

392   (Fig. 7). Further, the highly suitable distribution area under current environmental conditions was

393   continuous within Tsushima Island except for the isthmus between the northern and southern

394   areas (Fig. 5A). These suggest that recent population expansion of Group A rapidly occurred on

395   Tsushima during LGM to the Mid-Holocene, which might be caused by ecological adaptation

396   and its genetic basis, e.g., large genetic diversity in Group A.

397   On the contrary, Group B has a relatively small distribution range in the current climatic

398   condition (Fig. 7) and our ENM analyses suggested the shrinking of their habitat in the LIG and

399   LGM (Fig. 7). In addition, it seems that Group B has not been split into multiple populations (Fig.

400   5B). Because Group B was closely related to *H. nebulosus* from Kyushu Island (*Niwa, Kuro-o &*

401   *Nishikawa, 2021*), the ancestral population of Group B is suggested to invade to Tsushima from

402   Kyushu Island relatively recently. If such invasion occurred by small-sized populations, genetic

403   diversity in Group B is expected to be small by the founder effect. Such small genetic diversity

404   will prohibit Group B to expand its range as to cover all the island area.

405   Results of the Bayesian skyline plot indicated a distinctive population dynamics between

406   two groups; Group A was thought to have increased constantly at least ca. 300,000 to 50,000

407   years ago, while Group B was considered to have undergone no clear changes in the effective

408   population size during ca. 200,000 to 100,000 year ago and then a recent population expansion

409   during last 75,000 years (Fig. 4). Furthermore, the effective population size of Group A was

410   larger than that of Group B (Fig. 4). These would suggest Group A was more suitable to

411   environment and/or geography of Tsushima Island than Group B, which may have been caused

412   by the earlier invasion (or isolation) into Tsushima in Group A than in Group B.

413   In the mismatch distribution, Group B was suggested to experience the occurrence of

414   population expansion not in both expansion models, and the frequency of number of pairwise

415   difference was the highest at the three differences (Fig. 3). Also, results of Group B were not

416   significant in the neutrality tests, which means that demographic expansion would not have

417   occurred. On the other hand, occurrence of recent population expansion in Group B was inferred

418   by the result of the Bayesian skyline plot (Fig. 4). Interestingly, Group B has four major

419   haplotypes (B1, B3, B6, and B7), in contrast Group A has only one major haplotype (A3). Group

420   B is thought to have diverged from *H. nebulosus* relative recently (*Niwa, Kuro-o & Nishikawa,*

421   *2021*), thus it is possible that Group B still has retained ancestral haplotypes to the present. Such

422   ancestral variation may affect the results of mismatch distribution and neutrality tests.

423   Interestingly, the effective population sizes of both two groups did not decrease around

424   the LGM (ca. 22,000 years ago; Fig. 4), although suitable distribution areas of them shrank at

425   that time (Fig. 7). These mean that population density of two groups was high at the LGM.

426   Salamanders of *Hynobius* are widely distributed in East Asia and some species occurs on the

427   subarctic zone (e.g., *Hynobius retardatus* on Hokkaido Island of Japan; *Hynobius leechii* in the

428   north-eastern China) (*Sparreboom, 2014*) and up to high mountains at 2600 m in elevation

429   (*Hynobius nigrescens* in Honshu Island of Japan; *Matsui et al., 2020*). Salamanders of *Hynobius*

430   are cold-resistant animals judged by these facts. Therefore, two groups on Tsushima would have

431   not decreased in the effective population sizes even in the LGM.

432   **Distributional pattern of two salamanders on Tsushima Island**

433   Our results on ENMs of two salamanders from Tsushima Island suggested that both salamanders

434   have their own geographic ranges and unique ecological niches (Figs. 5, 6, 7). The potential

435   distribution of Group A is larger (Fig. 5), and suitable range in environmental variables also is

436   broader than those of Group B (Fig. 6), indicating that the Group A had a wider ecological niche

437   of environmental variables compared to Group B. In other words, it means that the Group A was

438   more tolerant than Group B.

439   The contribution of environmental predictors to models of each group also showed

440   distinctive (Table 3), and the response curves of the shared high contribution variables presented

441   considerably different trends (Fig. 6), indicating that they had different correlation with the set of

442   available conditions. The distribution of Group A was affected by precipitation, vegetation, and

443   land cover, which suggests that Group A has been adapted to climate and biological conditions in

444   Tsushima Island. On the contrary, the distribution of Group B was affected by elevation and

445   slope, which suggests that Group B has selected a given habitat based on topography (i.e., low

446   altitudinal and flat lands). Group B may have been less adapted to the climate and biological

447   conditions in the island because of the relatively recent invasion to the island.

448   Group A and Group B were found syntopically at nine localities (Fig. 1B), where their

449   larvae inhabited the same mountain streams. Furthermore, two groups were phylogenetically

450   close each other and genetic distance of them was not so large (uncorrected *p*-distance = 9.2%),

451   although they were not sister species (*Niwa, Kuro-o & Nishikawa, 2021*). Therefore, ecological

452   habits (e.g., prey item) may be similar with each other. Some biological factors such as

453   competition also may affect the formation of current distributions of two groups. However, the

454   knowledge about competition between two groups is lacked, further studies will be required to

455   clarify factors affecting their distribution patterns.

456   Our models on historical distribution for the two salamanders suggested a significant

457   contraction during Last Interglacial (LIG) and Last Glacial Maximum (LGM) compared to the

458   current distribution (Fig. 7). Our finding revealed that the distribution of these salamanders

459   might have been affected by the climate change during the Quaternary. The climate in the LIG

460   and LGM were colder and drier than the Mid-Holocene and current conditions (*Tsukada, 1983*;

461   *Takahara & Kitagawa, 2000*). Particularity, the global cooling during LGM caused by reduction

462   of sea level and atmospheric CO_2 likely led to the smaller potential distribution for both groups

463   than that of current models (Fig. 7). In which, we also found that the suitable area of Group B

464   tends to move to the northern part of Tsushima Island in the LGM. It can be explained by a

465   latitudinal gradient temperature on Japanese Archipelago at this period (*Tsukada, 1983*). By

466   contrast, the Mid-Holocene model showed a suitable area larger than the predicted by present

467   model for both groups (Fig. 7), supporting by wetter and cooler to warmer climate (*Takahara &*

468   *Kitagawa, 2000*; *Lutaenko et al., 2007*).

469   Historical refugia of species enable us to expand knowledge on ecological resilience,

470   migration rates in response to shifting climates, and enhance our understanding of how

471   population may react to future climate change (*Wielstra et al., 2010*). In our result, the genetic

472   landscape-shape interpolation analysis was relatively consistent with the historical suitable area

473   of ENMs, and which suggested refugia used during glacial ages. In Group A, the high genetic

474   diversity and high suitable distribution at the LIG and LGM were projected in southern part (Figs.

475   2A, 7), suggesting that the past refugia for Group A have existed on the southern part of

476   Tsushima. On the other hand, the relatively high genetic diversity for Group B could be observed

477   in genetic landscape-shape interpolation analysis in northern part of the island (Fig. 2B). The

478   result of the ENM based on LIG and LGM climate projection also showed that suitable areas of

479   Group B concentrated in the northern part (Fig. 7). The location of historical refugia existed

480   within the present distribution of both species suggested that the current ranges of these

481   salamanders were promoted from their refugia during historical ice ages.

482   **Ecological niche differentiation of two salamanders on Tsushima Island**

483   As expected, we found evidence of nonequivalence for the ecological niche of the two groups of

484   the salamanders on Tsushima Island from both identity test and ecospat-identity test (Figs. 8, 9),

485   presenting a lack of exchangeability ecology between them (*Blair et al., 2013*). Otherwise, the

486   result of the background test and the ecospat-background test showed that the null hypothesis

487   was not rejected, meaning the comparison pairs do not show either niche divergence or

488   conservatism based on the background test. The background test could not reveal the

489   divergence/conservatism of the ecological niches between Group A and Group B (Figs. 8, 9),

490   which tells that the niche difference is not so great between them. In fact, the two groups were

491   found syntopically at nine localities from the central to northern parts of Tsushima (Fig. 1B).

492   Probably, another factor may involve determining the distributional difference in the two

493   salamanders. Furthermore, the environmental variables within the selected background sites for

494   these groups were relatively similar, especially for climate data when the area of Tsushima is

495   only a small island (area ~ 696 km^2), probably leading to the accepted null hypothesis in

496   background tests. Moreover, nonbiological factors (e.g., level of resolution, or methods of

497   selection of environmental predictors) might also lead to a weak power in the statistic test (*Blair*

498   *et al., 2013*). In the present study, it is worth comparing ecology, breeding habits, and life

499   history among syntopic and allopatric areas of the two salamanders. *Niwa, Kuro-o & Nishikawa*

500   *(2021)* reported Group B breed earlier than Group A and such difference in breeding habit enable

501   them to occur syntopically. In a preliminary survey by one of the authors (K Niwa, 2015–2018,

502   personal observation), Group A tends to breed in the fast-flowing stream but Group B does in the

503   slow-flowing one, although sometimes they breed in the same stream. Group A might be more

504   adapted to the stream than Group B, which is supported by the wider niche in Group A than

505   Group B and the longer divergence from lentic-breeding ancestors in Group A than Group B in

506   phylogeny (*Niwa, Kuro-o & Nishikawa, 2021*). The similar result also was detected in other newt

507   species, such as an endemic newt (*Lissotriton boscai*) in Iberian Peninsula (*Peñalver-Alcázar,*

508   *Jiménez-Valverde & Aragón, 2021*), or *Lissotriton italicus* and *L. vulgaris meridionalis* in Italian

509   peninsula (*Iannella, Cerasoli & Biondi, 2017*), or European plethodontid salamanders (genus

510   *Hydromantes*) (*Ficetola et al., 2018*). Niche divergence of two groups could be explained by the

511   heterogeneous habitat in environmental space available to each group (*Warren, Glor & Turelli,*

512   *2008*; *Blair et al., 2013*).

513   Generally, the body size of closely related organisms correlates with their distribution,

514   species with larger body size possess the widely distributional range due to their competitive

515   relationship in food and/or optimal habitat (*Costa et al., 2008*; *Penner & Rödel, 2019*). However,

516   our results showed a different trend when Group A has significantly wider distribution on

517   Tsushima Island, but the body size of two species is mostly equivalent (K Niwa, 2021,

518   unpublished data). Thus, it can be seen that the ecological niche of the two salamanders on the

519   small island were not clearly correlated with their body size. One possible reason to explain the

520   result is the adaptation capacity of each species due to the different time invaded to Tsushima

521   Island. Group A was isolated on Tsushima Island earlier than that of Group B, ca. 3.5 to 3.2

522   MYA and ca. 1.5 to 1.4 MYA, respectively (*Niwa, Kuro-o & Nishikawa, 2021*) and occupied

523   mainly the island, including high mountainous area. However, studies on the interspecies

524   interaction and behavior plasticity in both larva and adult period should be conducted to explore

525   the exact mechanisms of ecological relationship between two groups.

526   **Why do two salamanders have the differential demographic/historical patterns?**

527   The present study showed the differential demographic/historical response between two

528   salamanders, although they were mostly sympatric in the small island. One possible reason to

529   explain these differential response between the two groups could be attributed to the different

530   times of invasion to Tsushima Island. Group A is considered to have invaded the island earlier

531   than Group B (*Niwa, Kuro-o & Nishikawa, 2021*). The different times of invasion may have

532   promoted the different levels of adaptations to the mountainous/lotic environment of the island

533   (i.e., niche differentiation) and those of demographic variation between Group A and Group B.

534   The Group A invaded earlier thus to be better adapted in the island than Group B. However,

535   there are no diverse habitats (e.g., breeding site) available to be completely separated by each

536   group in the small (696 km^2) and mountainous (a few plains) (*Nagaoka, 2001*) island. Therefore,

537   it is possible that the ecological niches were not split between two groups and the current

538   sympatric (syntopic) distribution of two salamanders has been retained.

539

540   **CONCLUSION**

541   Our study on integrating population genetics and ecological niche modeling suggested the

542   fluctuation in demography and distribution for two co-existing salamanders on Tsushima Island.

543   Results of genetic analyses indicated the different genetic structure and demographic patterns

544   between two salamanders. The population of both salamanders shrank considerably during LIG

545   and LGM, then expanded in Mid-Holocene. The genetic landscape-shape interpolation analysis

546   and ENM results on past climatic projection were consistent in revealing the different historical

547   refugia of these species that probably promoted the present distribution. Our model also

548   predicted the current distribution of Group A mainly focusing on southern part of Tsushima

549   Island, while northern part of Tsushima Island was suitable habitat of Group B. The different

550   effects of environmental predictors to models indicated that each group selects a different set of

551   available conditions. The background test could not reveal the divergence/conservatism of the

552   ecological niches between the two groups. Thus, we suggested that other factors may involve

553   determining the distributional difference such as micro-habitat selection and interspecific

554   relationship. The result of the study enables us understand distributional and populational

555   dynamics of salamanders in a limited area like Tsushima Island, and may aid the conservation

556   and sustainable management of these unique salamanders.

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562

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800

Figure 1

The position of Tsushima Island, Japan (A) and sampling localities on Tsushima Island (B).

(B) Red triangles and blue inverse triangles show the collection localities of Group A and Group B, respectively. The shaded areas indicate elevations of 200 m or more.

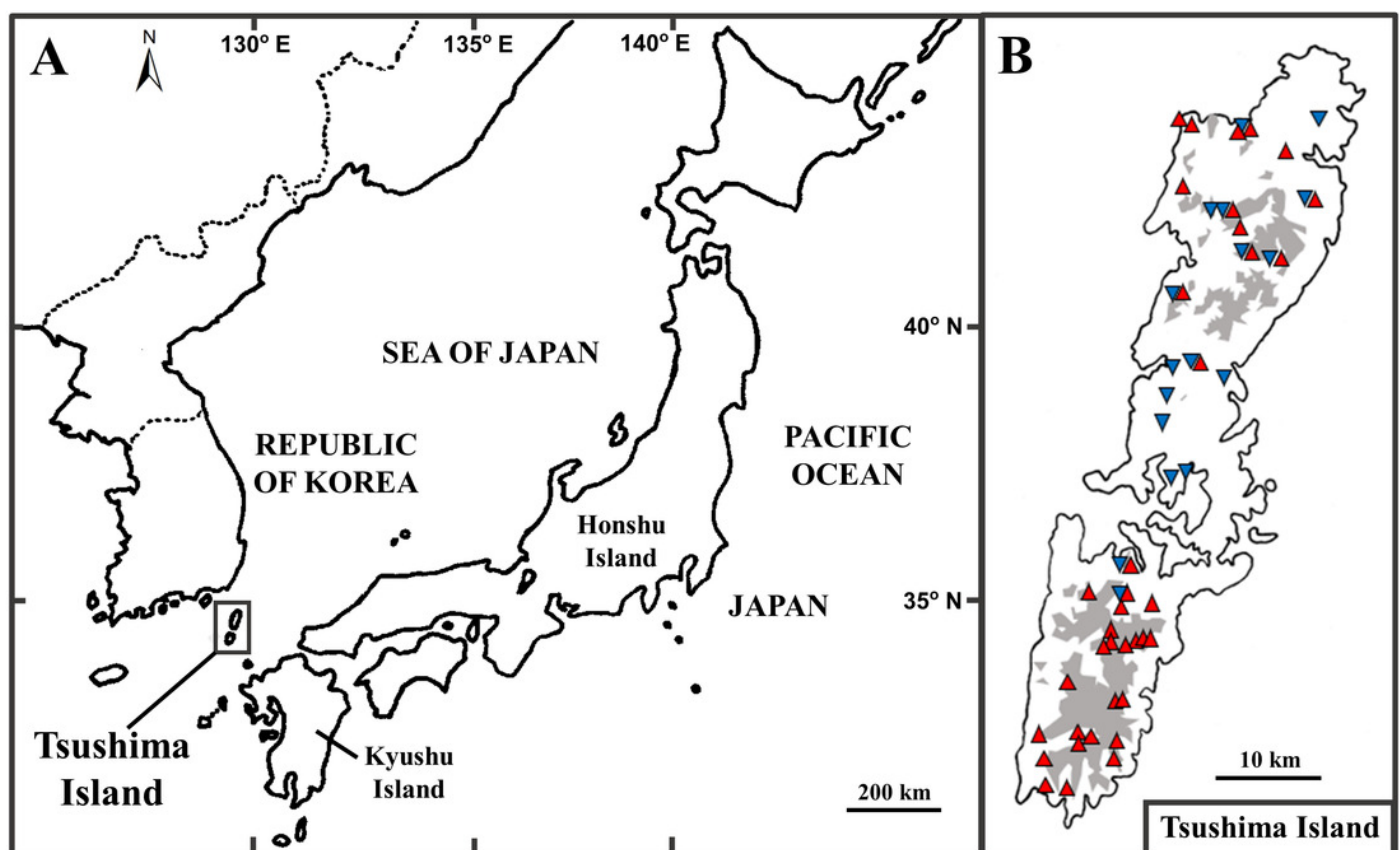



Figure 2

Upper: A lateral view of the result of a genetic landscape-shape interpolation analysis for Group A (A) and Group B (B). Lower map: Sampling localities of Group A (red triangles) and Group B (blue inverse triangles).

Right and left tips of the upper figures correspond to the northernmost and southernmost localities of Group A (red triangles) and Group B (blue inverse triangles) in Tsushima Island (lower maps). High peaks showed high genetic diversity in the upper figures. Red triangles and blue inverse triangles in the lower maps correspond to "Locality" in Table S1. The shaded areas indicate elevations of 200 m or more.

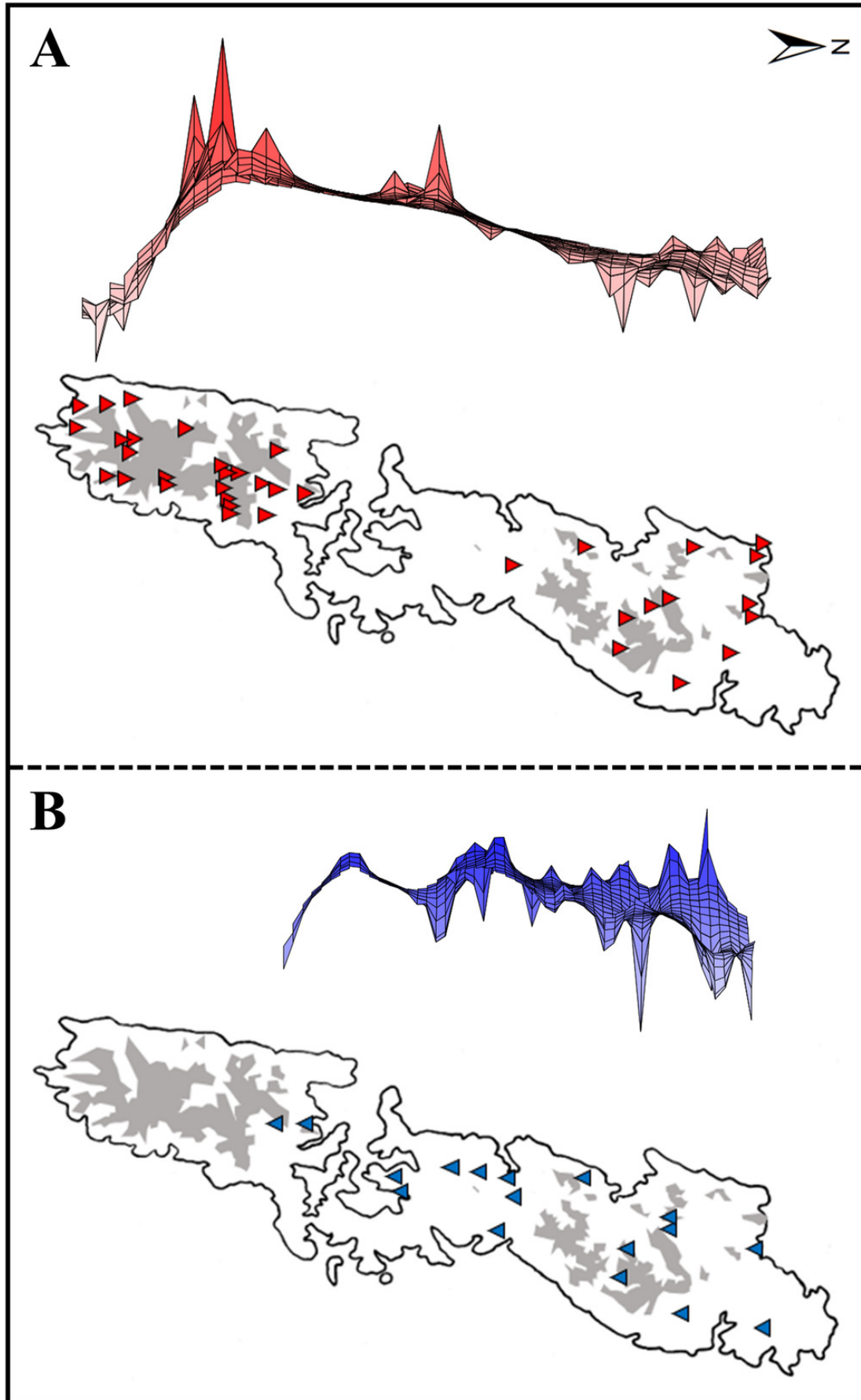



Figure 3

Mismatch distribution analyses based on the partial cyt *b* sequences (413 bp).

Dashed and solid lines indicate the sudden expansion and spatial expansion models, respectively.

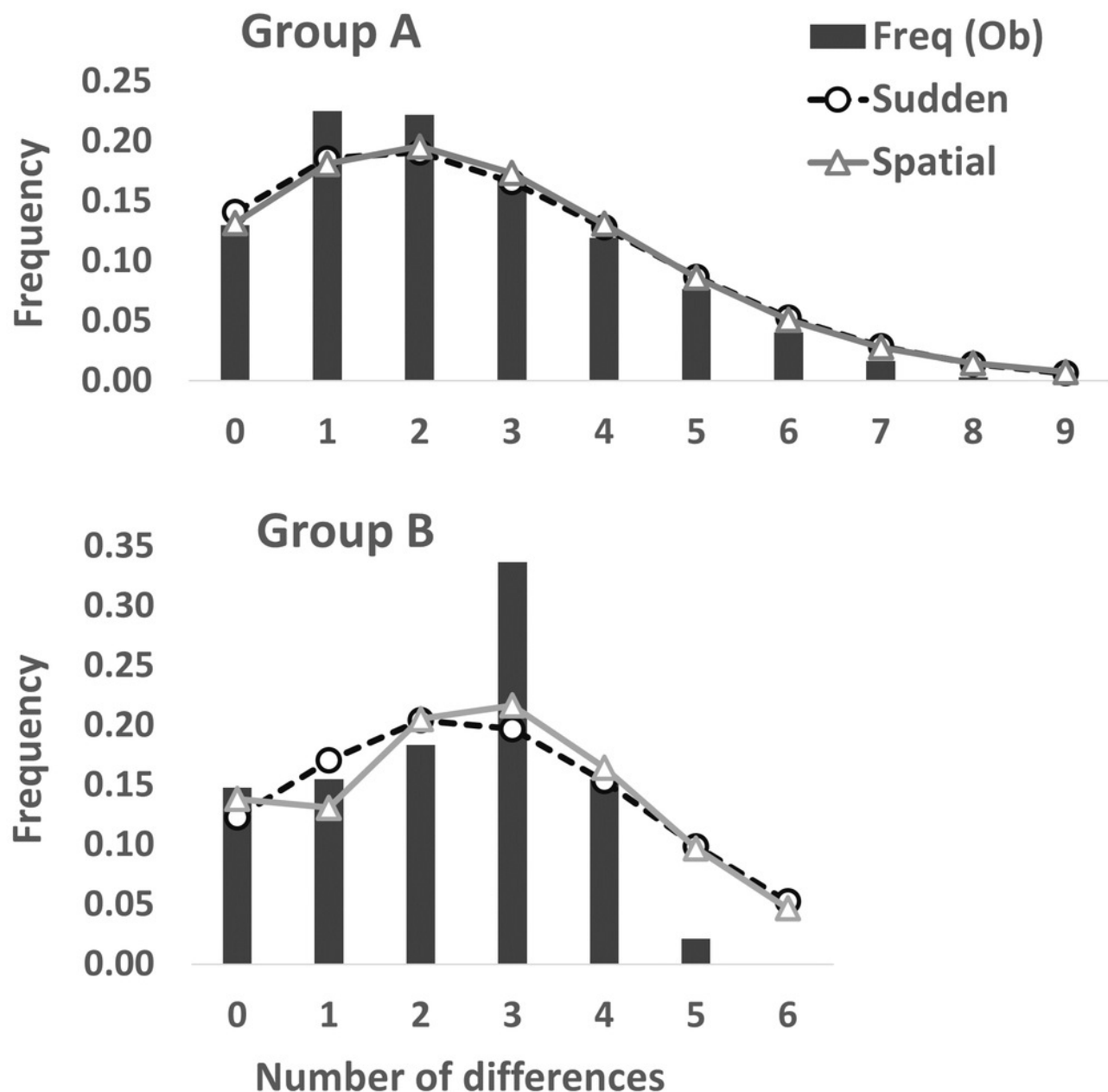



Figure 4

The Bayesian skyline plot based on the partial cyt *b* sequences (413 bp).

Population dynamics of Group A (A) and Group B (B).

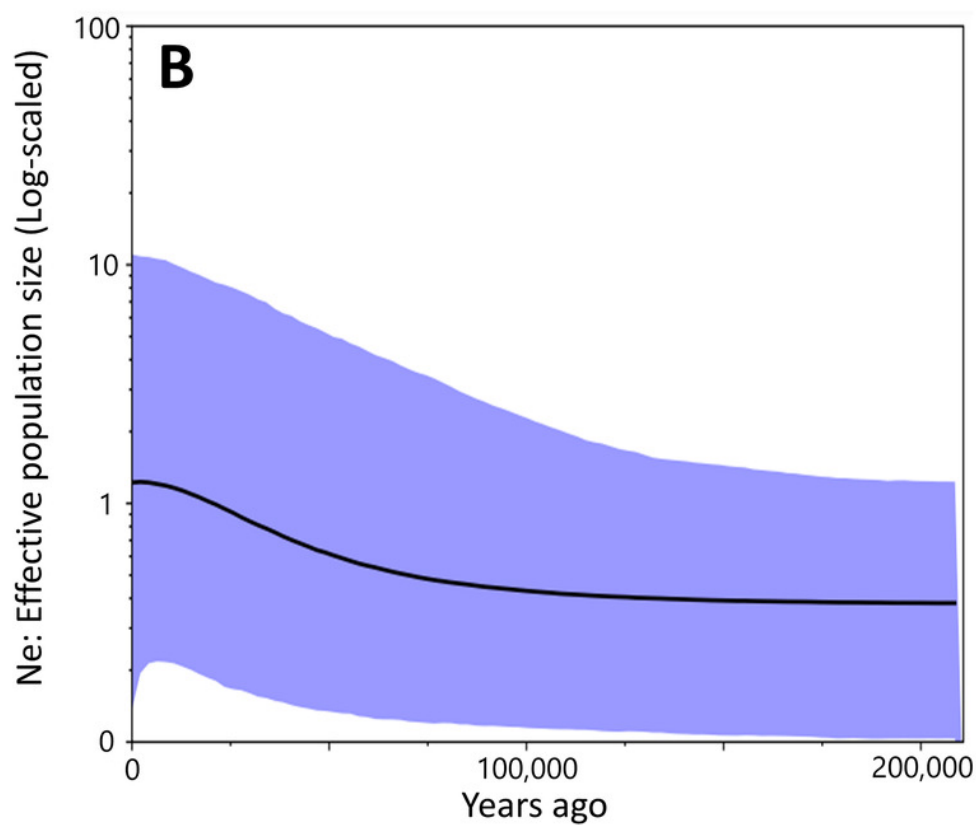
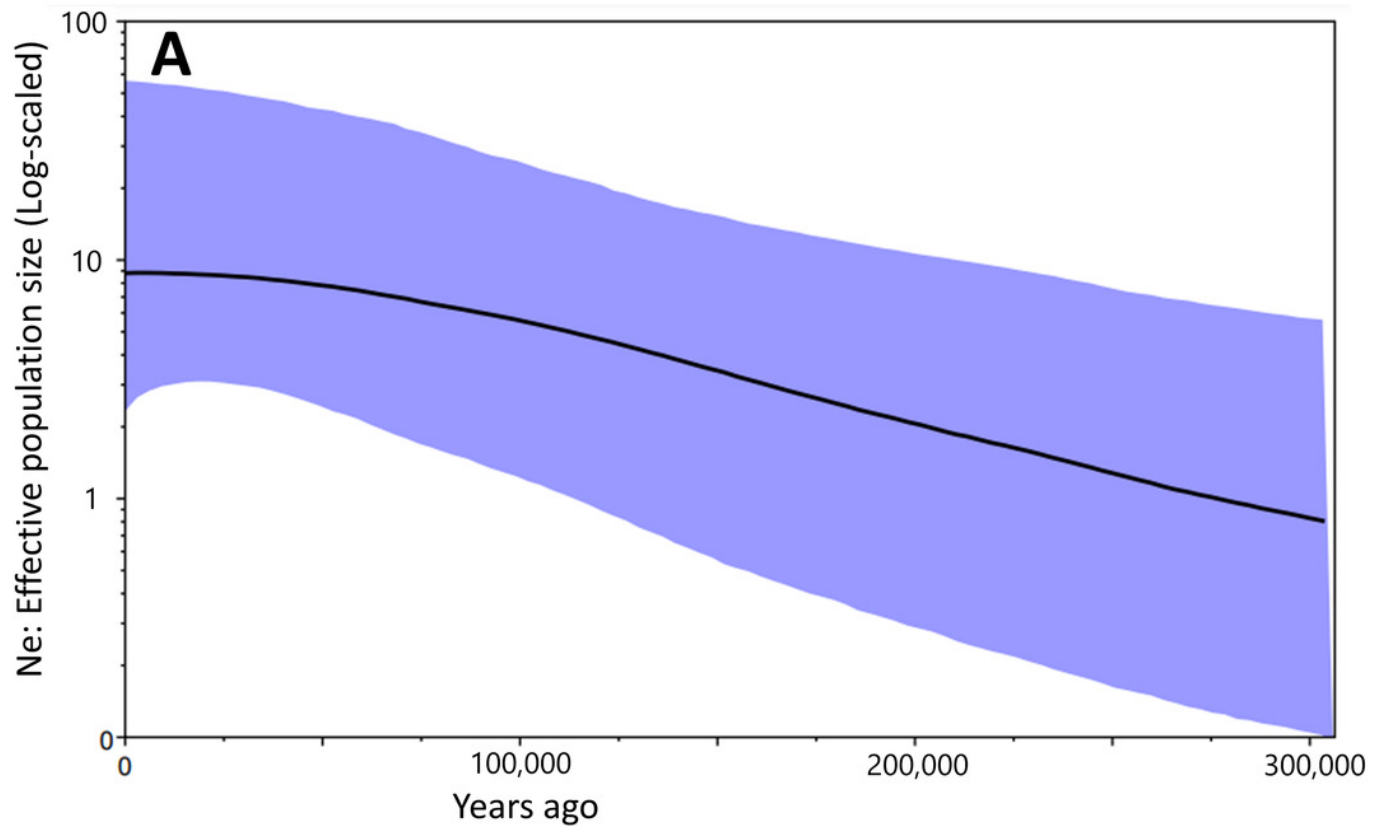



Figure 5

The potential distribution of Group A (A), Group B (B) and overlap area between Group A and Group B (C) under current environmental conditions from Maxent model.

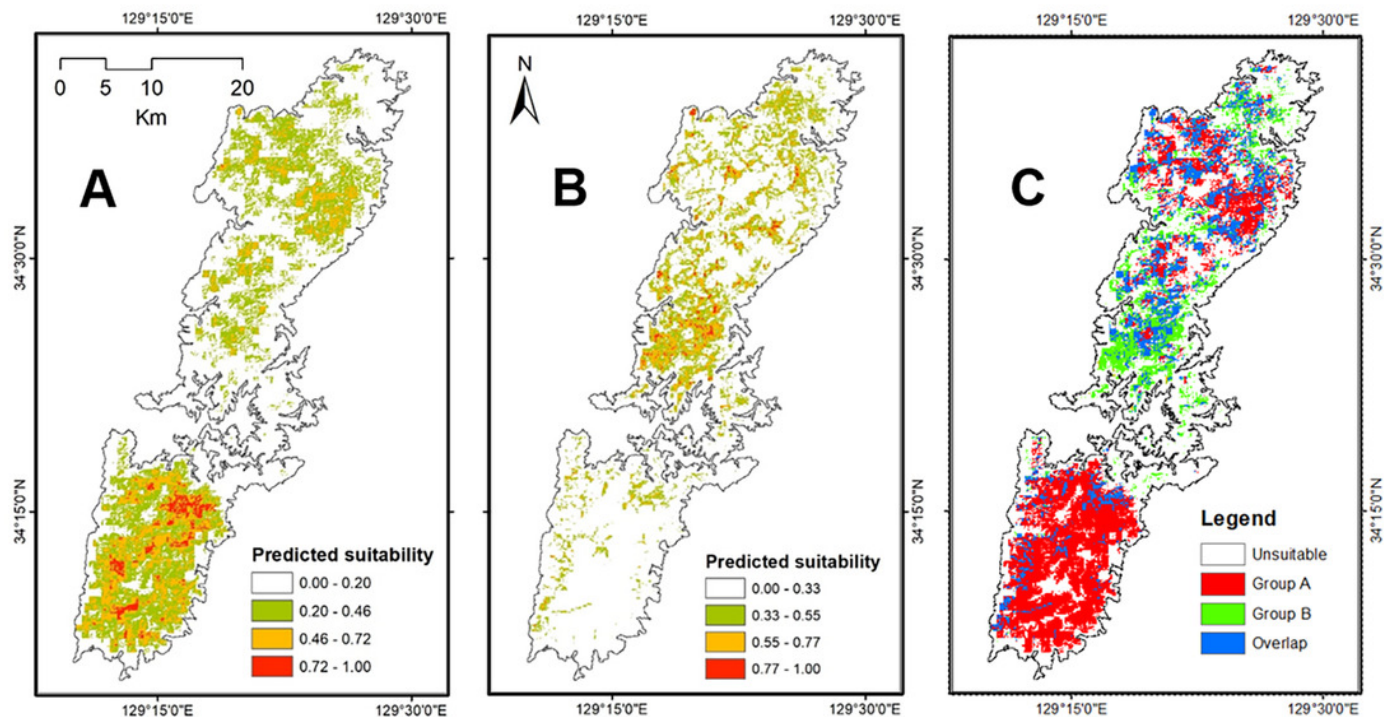



Figure 6

Left: The response curves of the Annual precipitation (upper) and Elevation (lower) of Group A (red solid line) and Group B (blue dashed line). Right: Distribution of the variables on Tsushima Island.

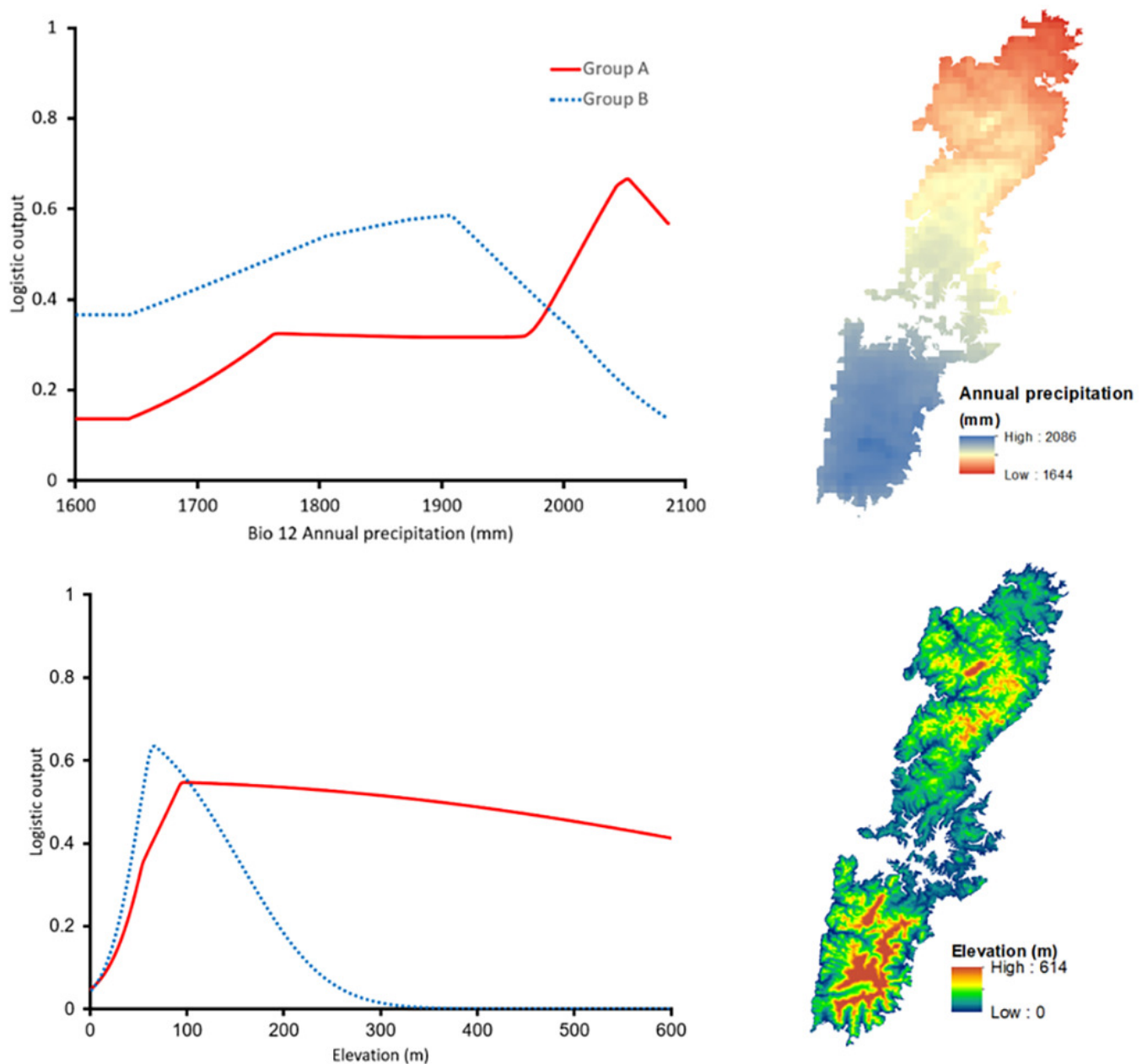



Figure 7

The potential distribution of Group A (upper) and Group B (lower) for Last Interglacial (LIG), Last Glacial Maximum (LGM), Mid-Holocene (Mid-Holo), and present scenarios.

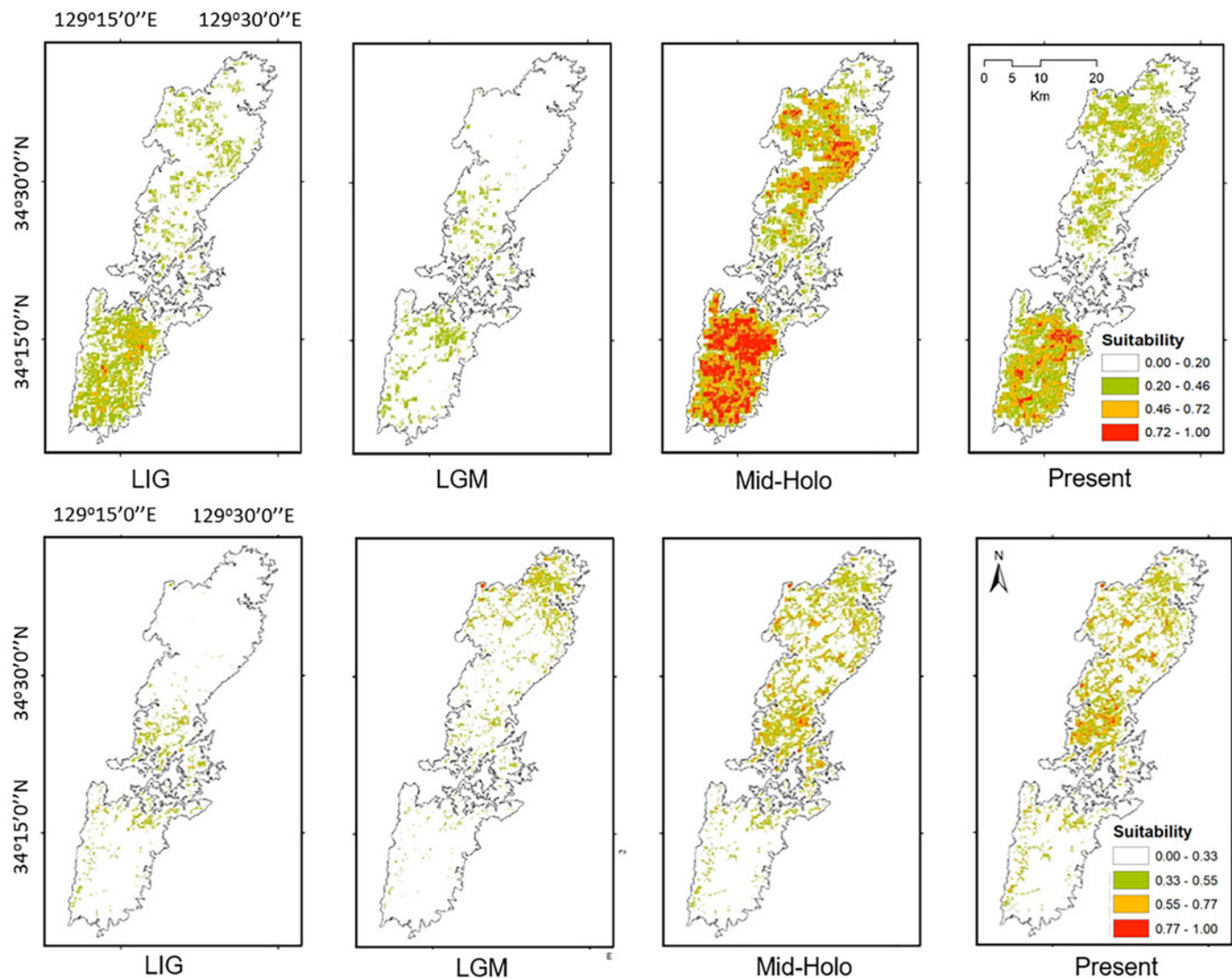
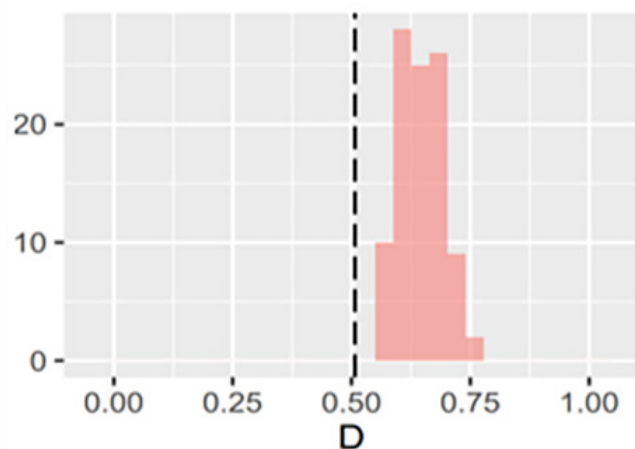
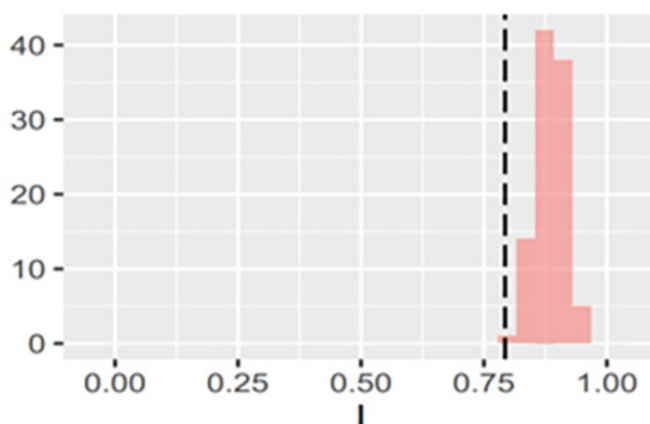
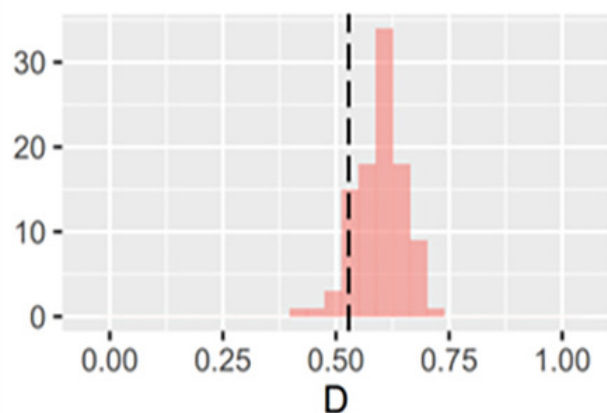
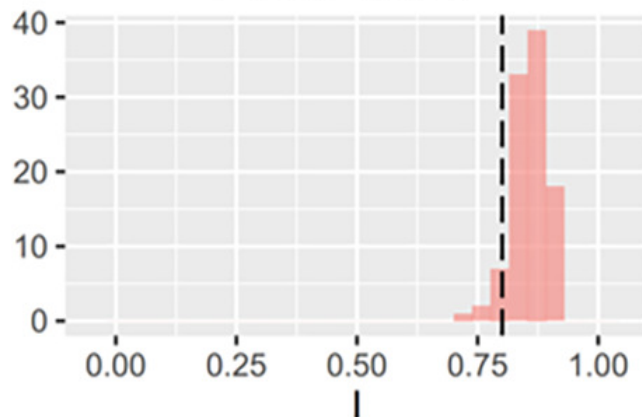
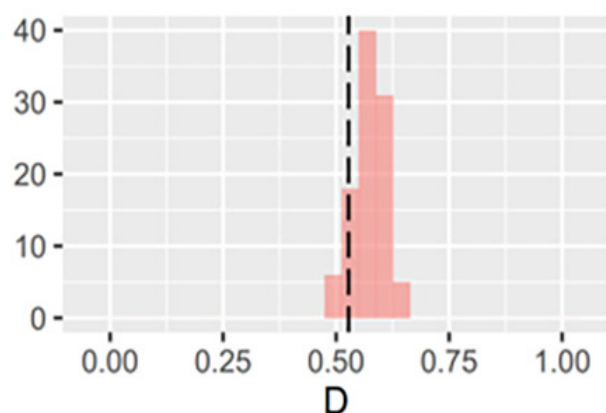



Figure 8

The results of identity test, background tests of Group A and Group B by ENMTools version R .

Black dashed line indicates the results of niche overlap representing the true calculated niche overlap. Red columns show the result of 100 replicates. The left plots showed the Schoener's D index, and the right plots indicated the Hellinger's-based I.

Identity test: Group A vs. Group B
P-value = 0.0198

Identity test: Group A vs. Group B
P-value = 0.0198

Background test:
Group A vs. backgound of Group B
P-value = 0.0693

Background test:
Group A vs. backgound of Group B
P-value = 0.0693

Background test:
Group B vs. backgound of Group A
P-value = 0.1386

Background test:
Group B vs. backgound of Group A
P-value = 0.1584

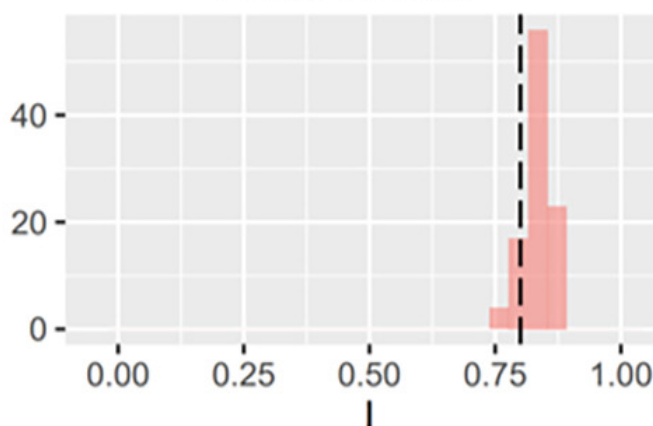
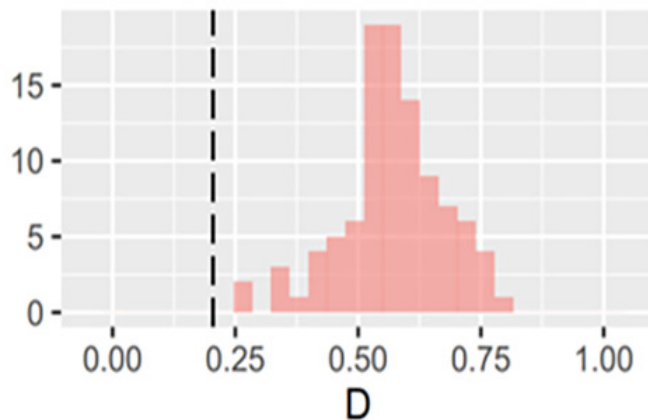
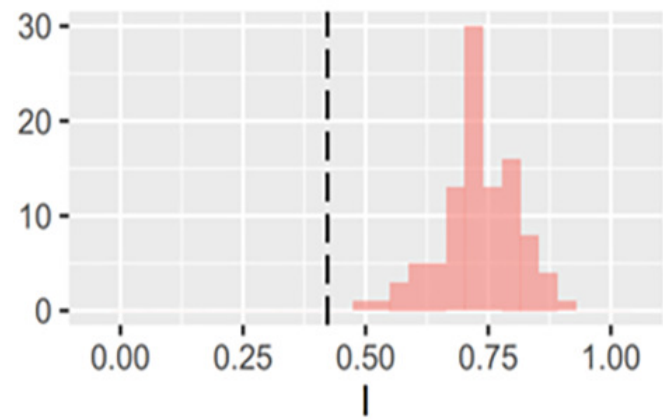



Figure 9

The results of ecospat identity test, ecospat background tests of Group A and Group B by ecopat function in ENMtools version R.

Black dashed line indicates the results of niche overlap representing the true calculated niche overlap. Red columns show the result of 100 replicates. The left plots showed the Schoener's D index, and the right plots indicated the Hellinger's-based I.

List of environmental variables.

No.	Name	Sources	Description
1	Bio02		Mean diurnal range (Mean of monthly =max temp - min temp)
2	Bio03		Isothermality (BIO2/BIO7) (* 100)
3	Bio05	WorldClim	Max temperature of warmest month
4	Bio06		Min temperature of coldest month
5	Bio12		Annual precipitation
6	Bio15		Precipitation seasonality (Coefficient of variation)
7	Elevation		Height above sea level
8	Slope	USGS EROS	Degree of rise/run
9	Aspect		Direction a slope face
10	pH_H_2O	Soilgrids	Mean of Soil pH in H2O (at depth: 0-5cm)
11	Forest height	GLAD	Forest canopy height (m)
12	NDVI_Jan		Normalized Difference Vegetation Index of the area in January 2019.
13	NDVI_Jun	NASA LPDAAC collection	Normalized Difference Vegetation Index of the area in June 2019.
14	EVI_Jan		Enhanced Vegetation Index of the area in January 2019.
15	LAI_Jan		Leaf Area Index (LAI) is the one-sided green leaf area per unit ground area in January 2019.
16	Land Cover	ALOS Science Project	The high resolution of Land use and Land cover map products.

Number of individuals (N), number of haplotypes (Nh), haplotype diversity (h), nucleotide diversity (π), Tajima's D, and Fu's Fs.

Double asterisks (**) indicate a significant support ($P < 0.01$).

Group	N	Nh	$h \pm \mathrm{SD}$	$\pi \pm \mathrm{SD}$	Tajima's D	Fu's Fs
Group A	73	29	0.8702 ± 0.0347	0.00585 ± 0.00356	-1.97036**	-24.75337**
Group B	34	9	0.8520 ± 0.0278	0.00547 ± 0.00343	0.08118	-1.43238

The contribution of environmental variables to suitable distribution of Group A and Group B by MaxEnt model.

No.	Group A		Group B	
	Environmental variables	% contribution	Environmental variables	% contribution
1.	Bio12	24.5	Elevation	28.8
2.	NDVI_Jun	22.4	Slope	28.8
3.	Land cover	16.6	Bio12	15.3
4.	Elevation	10.6	Forest height	5.3
5.	Bio5	9.1	pH_H$_2$O	5.3
6.	LAI_Jan	3.2	Land cover	4.8
7.	Bio3	3.0	NDVI_June	4.6
8.	pH_H$_2$O	2.7	Bio6	3.6
9.	Slope	2.3	Aspect	1.5
10.	Bio6	2.0	LAI_Jan	0.8
11.	Aspect	1.2	Bio2	0.3
12.	Forest height	1.2	EVI_Jan	0.3
13.	Bio15	1.0	NDVI_Jan	0.2
14.	NDVI_Jan	0.2	Bio5	0.2
15.	EVI_Jan	0.0	Bio15	0.2
16.	Bio2	0.0	Bio3	0.0