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 affected considerably on the shaping distributional pattern 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 pattern and genetic structure of many taxa are still poorly understood, especially for endemic organisms to small islands.

Methods. Here, we integrated the population genetics and ecological niche modelling approach 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 niches of these species 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 occur in Group B. The high suitable predicted distribution of Group A mainly focused on the southern part of 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 nonequivalent 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 fine genetic structure and historical dynamics of 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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16 **ABSTRACT**

17 **Background.** The climatic oscillations in the Quaternary period affected considerably on the

18 shaping distributional pattern and population genetic structure of organisms. Studies on the

19 historical dynamics of distribution and demography not only reflect the current geographic

20 distribution but also allow us to understand the adaption and genetic differentiation of species.

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23 **Methods.** Here, we integrated the population genetics and ecological niche modelling approach

24 to investigate the historical distribution and demographic dynamics of two co-existing

25 salamanders on Tsushima Island, Japan: the true *H. tsuensis* (Group A), and *Hynobius* sp. (Group

26 B). We also examined the hypothesis on the equivalency and similarity niches of these species

27 by identity and background tests for ecological niche space.

28 **Results.** Our result showed that Group A is considered to have undergone a recent population

29 expansion after the Last Glacial Maximum while it is unlikely to occur in Group B. The high

30 suitable predicted distribution of Group A mainly focused on the southern part of Tsushima

31 Island, whereas the northern part of Tsushima Island was the potential distribution of Group B.

32 The results also suggested a restricted range of both salamanders during the Last Interglacial and

33 Last Glacial Maximum, and recent expansion in Mid-Holocene. The genetic landscape-shape

34 interpolation analysis and historical suitable area of ecological niche modelling were consistent,

35 and suggested refugia used during glacial ages in southern part for Group A, and in northern part

36 of Tsushima Island for Group B. Additionally, we found evidence of nonequivalent for the

37 ecological niche of the two groups of the salamanders, although our test could not show either

38 niche divergence or conservatism based on the background tests. The environmental predictors

39   affecting the potential distribution of each group also showed distinctiveness, leading to

40   difference in selecting suitable areas. Finally, the combination of population genetics and

41   ecological modeling has revealed the fine genetic structure and historical dynamics of two

42   salamanders on a small island.

43

INTRODUCTION

45   Qualifying the interspecific variation in geographic distribution is central research of

46   ecologists to understand 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   (*Nakazato, Warren & Moyleet, 2010*; *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 also provides foundation for predicting

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

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

66   contracting population size and/or distribution into refugia and then expanding again (*Hewitt,*

67   *2000*; *Tominaga et al., 2013*; *Aoki et al., 2019*). However, species might response the great

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

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

70   poorly 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 (*Carstens & Richards, 2007*; *Aoki et al., 2019*; *Ren et al., 2020*). On

79   the other hand, populational genetic data can reveal genetic diversity and structure of species and

80   estimate the demographic dynamics (*Avise, 2010*; *Tominaga et al., 2013*). Consequently, the

81   integrating of these approaches has supported for each other and shed enlightenment on the

82   evolutionary process of species (*Pahad, Montgelard & Jansen, 2019*).

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

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

85   longitude. The island includes two major parts, including Kamijima (northern part) and

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

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

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

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

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

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

92   historical connections.

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

94   Island, was described by *Abe (1922)*. Recently, *Niwa, Kuro-o & Nishikawa (2021)* showed that

95   there are two distinct lineages of *Hynobius* on Tsushima Island based on mitochondrial

96   cytochrome b (cyt *b*) gene and seven nuclear microsatellite loci, including Group A (probably,

97   true *H. tsuensis*), and unknown species of Group B (*Hynobius* sp.). Furthermore, the body

98   coloration of the two groups is also distinctive. Group A showed a dark brownish dorsum with

99   yellowish spots or a yellowish dorsum with dark spots, and the upper and lower edges of the tail

100   were bright yellow, while Group B presented a brownish dorsum with dark stipples, and tail

101   without bright yellow color (*Niwa, Kuro-o & Nishikawa, 2021*). The study was the first report on

102   the co-existing of two lotic salamanders of *Hynobius* in a small island. However, the geographic

103   distribution, population genetic, and underlying factors affecting the current distribution of these

104   species are still unclear.

105   In the contribution, we investigated the ecological niche and population genetic of two

106   groups of *Hynobius* on Tsushima Island that could be useful to support the hypothesis on the

107   fluctuation of historical distribution and demographic dynamics of these two co-existing

108   salamanders on the island. The main objectives of this study include (1) examining the

109   population dynamics of two salamanders from Tsushima Island; (2) constructing ecological

110   niche models of the two salamanders under present and Quaternary climate; (3) qualifying the

111   ecological niches of these groups by using equivalence and similarity tests in order to explain the

112   present distribution pattern of the two sympatric salamanders.

113

114   **MATERIALS AND METHODS**

115   **Population genetics**

116   *Sample collection*

117   Forty-eight specimens of Group A from 27 localities and 20 specimens of Group B from

118   12 localities were sampled for population genetic analyses (Table 1). Tissues were preserved in

119   96–99% ethanol and stored in freezer at -18 ℃. Specimen collection protocols and animal

120   operations followed the guideline of animal experiments in Kyoto University (approval nos. 29-

121   A-7 and 30-A-7). All specimens were stored in graduate school of Human and Environmental

122   studies, Kyoto University. Total DNA was extracted from muscle or liver tissues using DNeasy

123   Blood & Tissue Kit (Qiagen). Then, all 68 specimens were sequenced for the partial cyt *b* gene

124   of 413 base pairs (bp), and methods of PCR, purification of PCR product, and sequencing were

125   the same as those described in *Aoki, Matsui & Nishikawa (2013)*.

126   *Table 1 inserted here*

127   Prior to analyses, we added the published sequences of 39 specimens from DDBJ (DNA

128   data bank of Japan) to our data set. Totally, 73 individuals of Group A from 37 localities and 34

129   individuals of Group B from 17 localities were used in the following analyses. Of these localities,

130   Group A and Group B were discovered syntopically at nine localities (Table 1).

131   *Demographic history*

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

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

134   *Tajima, 1981*). Neutrality tests in each group were conducted based on two index, Tajima's *D*

135   (*Tajima, 1989*) and Fu's *Fs* (*Fu, 1997*). These analyses were performed using Arlequin ver. 3.5

136   (*Excoffier & Lischer, 2010*). Furthermore, genetic landscape-shape interpolation analysis was

137   conducted using Alleles In Space (*Miller, 2005*). Although this analysis was generally used to

138   visualize spatial patterns of genetic diversity (*Miller, 2005*; *Miller et al., 2006*), in the several

139   phylogeographic studies, the analysis has been applied to research past refugia (e.g., *Nunome et*

140   *al., 2010*; *Tominaga et al., 2013*).

141   **Ecological niche models**

142   *Occurrence data*

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

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

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

146   Consequently, 37 localities of Group A (Fig. 1) and 17 of Group B (Fig. 2) were employed in the

147   final model.

148   *Figure 1 inserted here*

149   *Figure 2 inserted here*

150   *Environmental variables*

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

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

153   1km) resolution from the WorldClim database (*Fick & Hjimans, 2017*). We also collected LAI

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

169   *2010*). Finally, 16 variables were selected for running ecological niche models (Table 2).

170   *Table 2 inserted here*

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

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

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

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

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

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

177   climate data at this time.

178   *Ecological niche model processing*

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

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

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

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

183   other algorithms, Maxent showed to perform well, especially for small occurrence sample size

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

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

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

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

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

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

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

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

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

193   58 candidate models were analyzed, then, we chose the best model with the minimum value of

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

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

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

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

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

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

200   **Niche equivalency and similarity comparison**

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

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

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

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

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

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

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

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

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

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

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

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

213   using the R version of ENMTools (*Warren et al., 2021*).

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

215   test for niche conservatism or divergence of two species. The test created a null distribution by

216   comparing an ENM of Group A to an ENM generated from random points selected within the

217   geographic range of Group B. We used 100 replicates for the test. The opposite direction,

218   comparing an ENM of Group B to and ENMs generated randomly within the ranges of Group A,

219   also was implemented. Then, the test compared the observed niche overlap value between Group

220   A and B to the null distribution by a two-sided test and alpha level of 0.05. We determined that

221   when the observed niche overlap value between two species was above the 95% confidence

222   interval of the null distribution, it might support niche conservatism. By contrast, the observed

223   value was below the 95% confidence interval, supporting niche divergence (*Warren, Glor &*

224   *Turelli, 2008*). In the case of the null hypothesis was supported, the niche overlap might be

225   explained by regional similarities in the habitat available to each species. If the background test

226   is only significant in one direction but not for the remaining direction, we could reject the null

227   hypothesis (*Warren, Glor & Turelli, 2008*).

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

229   using kernel density estimates environmental distribution of species and the distribution of

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

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

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

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

234   *2021*) to conduct the tests. The functions automatically ran principal components analysis to

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

236   more than two predictors in the analysis.

237

238   **RESULTS**

239   **Demographic history**

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

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

242   nine haplotypes of Group A and nine haplotypes of Group B were observed (Table 3). Genetic

243   diversity was higher in Group A (h ± SD: 0.8702 ± 0.0347; π ± SD: 0.00585 ± 0.00356) than in

244   Group B (0.8520 ± 0.0278; 0.00547 ± 0.00343). In the neutrality tests, Group A showed a

245   significant negative value for both Tajima's D and Fu's Fs tests ($P < 0.01$). On the other hands,

246   results of Group B were not significant for both neutrality tests ($P > 0.05$) (Table 3).

247   *Table 3 inserted here*

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

249   diversity in each group (Figs. 1, 2). The relatively high peaks (i.e., high genetic diversity) were

250   detected at the high mountainous areas in southern part of Tsushima Island for Group A (Fig. 1),

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

252   **Ecological niches of two salamanders**

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

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

255   0.775 ± 0.068, and for Group B was = 0.908 ± 0.008, and testing AUC = 0.908 ± 0.231. The

256   potential distribution of Group A resulting from our models covered almost Tsushima Island, but

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

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

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

260   in the northern part of the island (Fig. 3).

261   *Figure 3 inserted here*

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

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

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

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

266   at 15.3% (Table 4).

267   *Table 4 inserted here*

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

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

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

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

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

273   mm (Fig. 4). The elevation showed peak suitability around 100 meters and 80 meters for Group

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

275   while that of Group B presented a significant decrease after peaking (Fig. 4).

276   *Figure 4 inserted here*

277   **Historical potential distribution**

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

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

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

281   Last Interglacial, and Last Glacial Maximum (Figs. 5A, 5B, 5D). The distribution of Group A

282   was mostly restricted in the southern part, while the range of Group B concentrated around the

283   isthmus in the center of the island on the Last Interglacial. For the Last Glacial Maximum, the

284   distribution of salamanders tended to move toward the north, Group A focused roughly on the

285   isthmus, whereas Group B moved greatly to the northern tip of the island. On the other hand, the

286   projection on Mid-Holocene increased compared to the present (Fig. 5C). Furthermore, it can be

287 seen that the predicted ranges on Mid-Holocene overlapped significantly at the current time for

288 both groups.

289   *Figure 5 inserted here*

290 **Niche equivalency and similarity tests**

291 Our identity test showed that the two salamanders had nonequivalent ENMs (Fig. 6), with

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

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

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

295 based I; $P > 0.05$ for Schoener's D; Fig. 6). The results of ecospat-identity test and ecospat-

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

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

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

299 D; Fig. 7).

300   *Figure 6 inserted here*

301   *Figure 7 inserted here*

302

303 **DISCUSSION**

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

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

306 genetic analysis (Table 3). ENM analyses also inferred that the suitable habitats of Group A were

307 larger than those of Group B at the Last Interglacial (LIG), Mid-Holocene, and current scenario

308 (Figs. 5A, 5C, 5D), and the neutrality test suggested that Group A have undergone a recent

309   population expansion. Group A seemed to invade (or isolate) into Tsushima Island earlier than

310   Group B (*Niwa, Kuro-o & Nishikawa, 2021*) and the range is separated into two areas (i.e., the

311   northern and southern areas of Tsushima Island) due to split by the intermediate lowland (Fig.

312   3A). Further, the genetic landscape-shape interpolation analysis showed that high genetic

313   diversity was detected in the southern part of Tsushima for Group A (Fig. 1). The two areas are

314   not severely isolated but could provide gene flow between the northern and southern populations

315   of Group A. Thus, Group A retains higher haplotype and nucleotide diversity than Group B.

316   Such high intraspecific genetic diversity may enable Group A much more adapted to the variable

317   climate conditions and elevations than Group B. On the basis of ENM analyses, Group A had a

318   restricted distribution in the Last Glacial Maximum (LGM) (Fig. 5B), but a large highly suitable

319   distribution in the Mid-Holocene (Fig. 5C). Further, the highly suitable distribution area under

320   current environmental conditions was continuous within Tsushima Island except for the isthmus

321   between the northern and southern areas (Fig. 3A). These suggest that recent population

322   expansion of Group A rapidly occurred on Tsushima during LGM to the Mid-Holocene, which

323   might be caused by ecological adaptation and its genetic basis, e.g., large genetic diversity in

324   Group A.

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

326   condition (Fig. 5D) and our ENM analyses suggested that shrinking of their habitat in the LIG

327   and LGM (Figs. 5A, 5B). In addition, it seems that Group B has not been split into multiple

328   populations (Fig. 3B). Because Group B was closely related to *H. nebulosus* from Kyushu Island

329   (*Niwa, Kuro-o & Nishikawa, 2021*), the ancestral population of Group B is suggested to invade

330   to Tsushima from Kyushu Island relatively recently. If such invasion occurred by small-sized

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

332   small genetic diversity will prohibit Group B to expand its range as to cover all the island area.

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

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

335   have their own geographic ranges and unique ecological niches (Figs. 3, 4, 5). Our results

336   showed that the high suitable predicted distribution of Group A mainly focused on southern part

337   of Tsushima Island, while northern part of the island was the major ecological niche of Group B

338   (Figs. 3A, 3B), supporting the north-to-south gradient in salamander distribution on Japanese

339   archipelago (*Yoshikawa et al., 2008*; *Li, Fu & Lei, 2011*). The potential distribution of Group A

340   is larger (Fig. 3), and suitable range in environmental variables also broader than those of Group

341   B (Fig. 4), indicating that the Group A had a wider ecological niche of environmental variables

342   compared to Group B. In another word, it means that the Group A was more tolerance than

343   Group B.

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

345   distinctive (Table 4), and the response curves of the shared high contribution variables presented

346   considerably different trends (Fig. 4), indicating that they had different correlation with the set of

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

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

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

350   slope, which suggests that Group B has selected a given habitat based on topography where

351   Group A has not or less occurred. Such unsuitable topography for Group A is low altitudinal and

352   flat lands. Group B has been less adapted to the climate and biological conditions in the island

353   because of the relatively recent invasion to the island.

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

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

356   current distribution (Fig. 5). Our finding revealed that the distribution these salamanders might

357   have been affected by the climate change during the Quaternary. The climate in the LIG and

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

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

360   of sea level and atmospheric CO_2 likely led to the smaller potential distribution for both species

361   than that of current models (Fig. 5). In which, we also found that the suitable area of Group B

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

363   latitudinal gradient climate on Japanese Archipelago at this period (*Tsukada, 1983*). By contrast,

364   the Mid-Holocene model showed a suitable area larger than the predicted by present model for

365   both species (Fig. 5C), supporting by wetter and cooler to warmer climate (*Takahara &*

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

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

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

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

370   landscape-shape interpolation analysis and historical suitable area of ENMs relatively resemble

371   and suggested refugia used during glacial ages. In Group A, the high genetic diversity and high

372   suitable distribution at the LIG and LGM were projected in southern part (Figs. 1, 5A, 5B),

373   suggesting that the past refugia for Group A have existed on the southern part of Tsushima. On

374   the other hand, a tendency for Group B could be observed in genetic landscape-shape

375   interpolation analysis in northern part of the island (Fig. 2). The result of the ENM based on LIG

376   and LGM climate projection also showed that suitable areas of Group B concentrated in the

377   northern part (Figs. 5A, 5B). The location of historical refugia existed within the present

378   distribution of both species suggested that the current ranges of these salamanders were

379   promoted from their refugia during historical ice ages.

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

381   As expected, we found evidence of nonequivalent for the ecological niche of the two groups of

382   the salamanders on Tsushima Island from both identity test and ecospat-identity test (Figs. 6, 7),

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

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

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

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

387   divergence/conservatism of the ecological niches between the two species (Figs. 6, 7), which

388   tells that the niche difference is not so great between them. In fact, the two species were found

389   syntopically at nine localities from the central to northern parts of Tsushima (Table 1). Probably,

390   another factor may involve determining the distributional difference in the two species.

391   Furthermore, the environmental variables within the selected background sites for these groups

392   were relatively similar, especially for climate data when the area of Tsushima is only a small

393   island (area ~ 696 km^2), probably leading to the accepted null hypothesis in background tests.

394   Moreover, nonbiological factors (e.g., level of resolution, or methods of selection of

395   environmental predictors) might also lead to a weak power in the statistic test (*Blair et al., 2013*).

396   In the present study, it is worth to compare ecology, breeding habits, and life history among

397   sympatric and allopatric areas of the two salamanders. *Niwa, Kuro-o & Nishikawa (2021)*

398   reported Group B breed earlier than Group A and such difference in breeding habit enable them

399   to occur sympatrically. In a preliminary survey by one of the authors (K Niwa, 2015–2018,

400 unpublished data), Group A tends to breed in the fast-flowing stream but Group B does in the

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

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

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

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

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

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

407 peninsula (*Iannella, Cerasoli & Biondi, 2017*). Niche divergence of two groups could be

408 explained by the heterogeneous habitat in environmental space available to each group (*Warren,*

409 *Glor & Turelli, 2008*; *Blair et al., 2013*).

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

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

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

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

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

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

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

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

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

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

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

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

422 the exact mechanisms of ecological relationship between two species.

423

424   **CONCLUSION**

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

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

427   The population of both salamanders shrank considerably during LIG and LGM, then expanded in

428   Mid-Holocene. The genetic landscape-shape interpolation analysis and ENM results on past

429   climatic projection were consistent in revealing the different historical refugia of these species

430   that probably promoted the present distribution. Our model also predicted the current distribution

431   of Group A mainly focusing on southern part of Tsushima Island, while northern part of

432   Tsushima Island was suitable habitat of Group B. The different effects of environmental

433   predictors to models indicated the distinctively selecting the set of available conditions for each

434   species. The background test could not reveal the divergence/conservatism of the ecological

435   niches between the two species. Thus, we suggested that other factors may involve determining

436   the distributional difference such as micro-habitat selections and interspecific relationship. The

437   result of the study enables us understand distributional and populational dynamics of

438   salamanders in a limited area like Tsushima Island, and may aid the conservation and sustainable

439   management of these unique species.

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445

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Figure 1

Upper: A lateral view of the result of a genetic landscape-shape interpolation analysis using partial cyt *b* gene sequences of 413 bp for Group A. Lower: Collection localities of Group A.

Red triangles correspond to "Locality" in Table 1. The map was created by processing the

Geospatial Information Authority of Japan (GSI) Maps (https://www.gsi.go.jp/) .
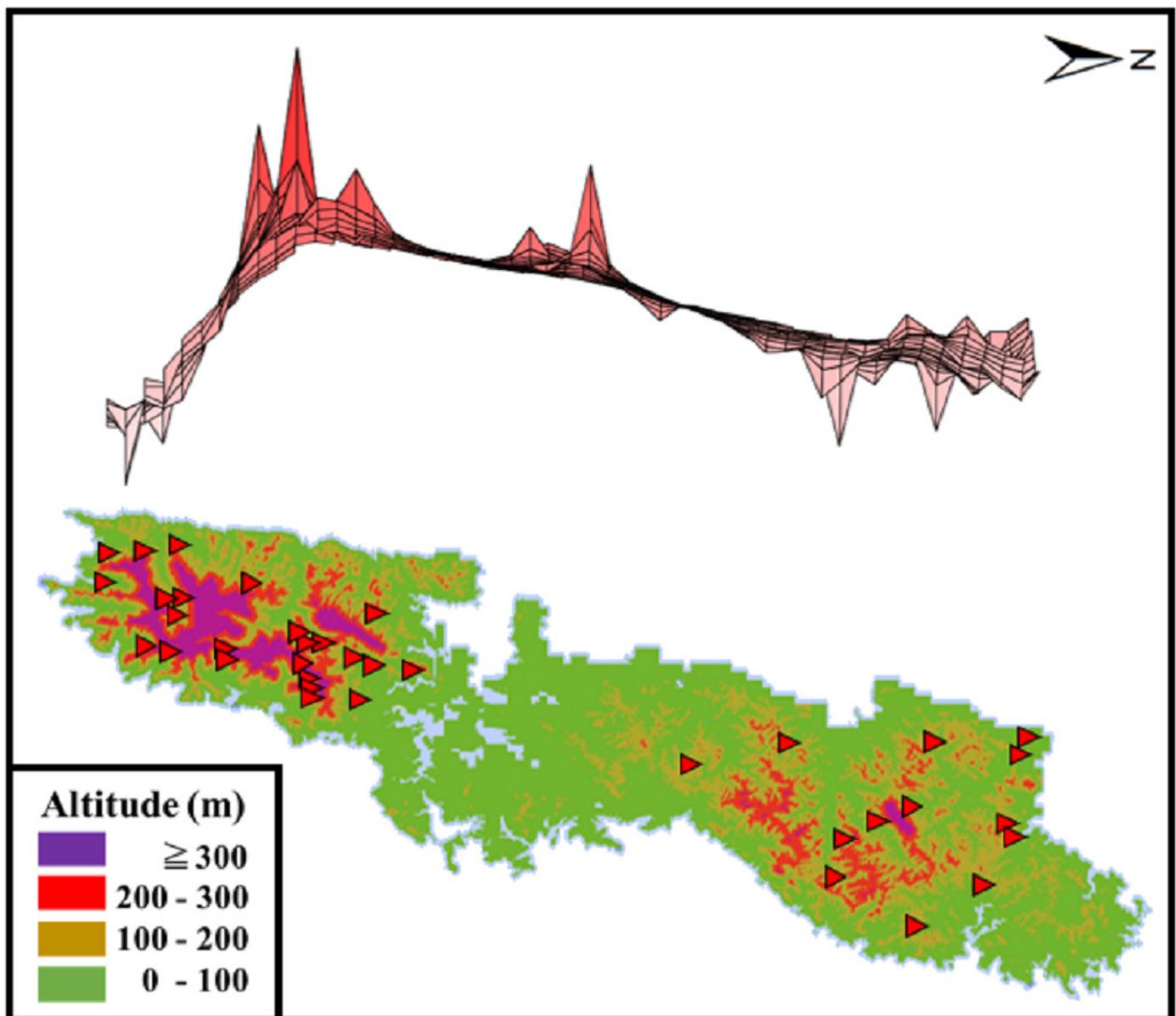
Figure 2

Upper: A lateral view of the result of a genetic landscape-shape interpolation analysis using partial cyt *b* gene sequences of 413 bp for Group B. Lower: Collection localities of Group B.

Blue inverse triangles correspond to "Locality" in Table 1. The map was created by

processing GSI Maps (https://www.gsi.go.jp/).
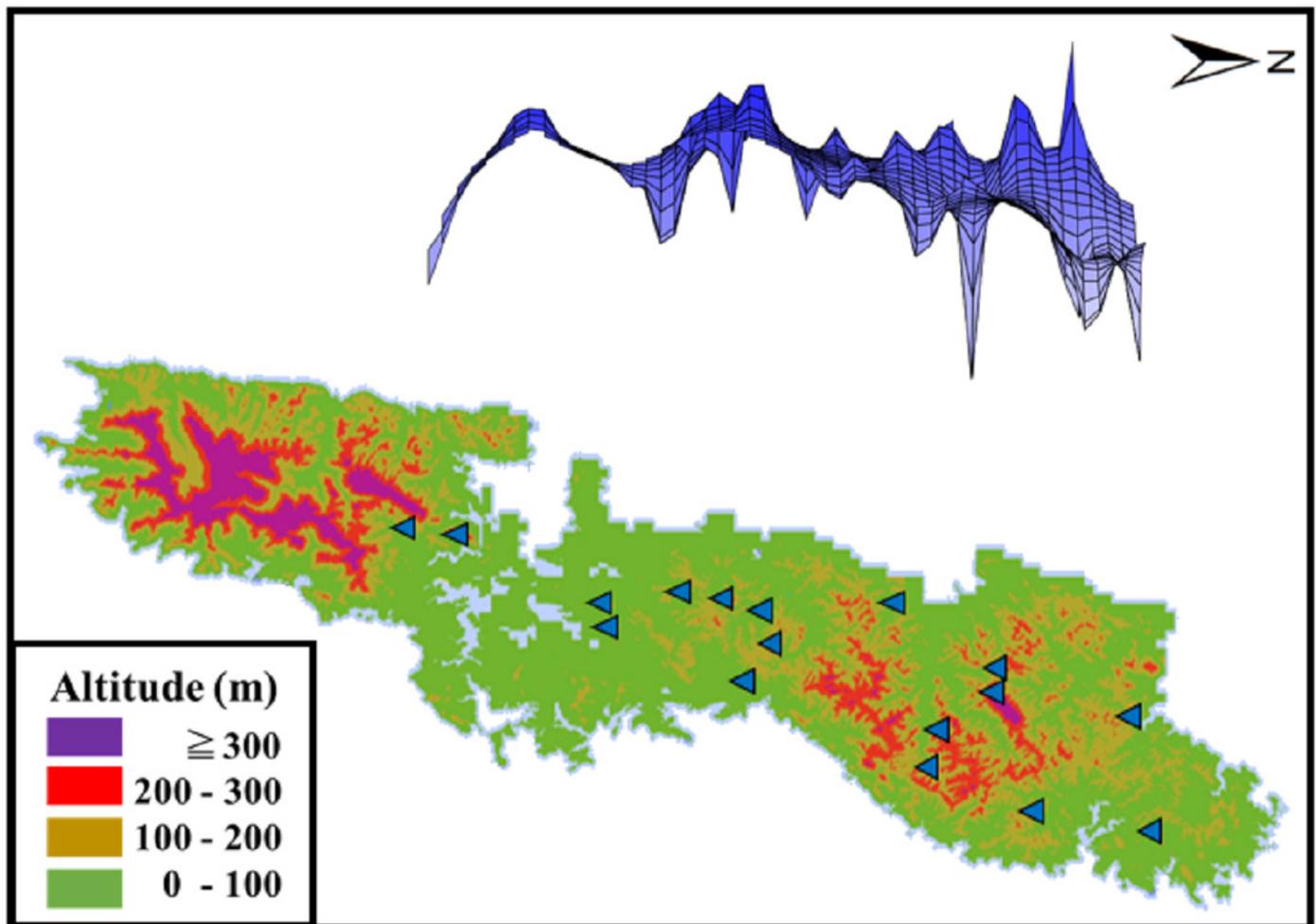
Figure 3

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.
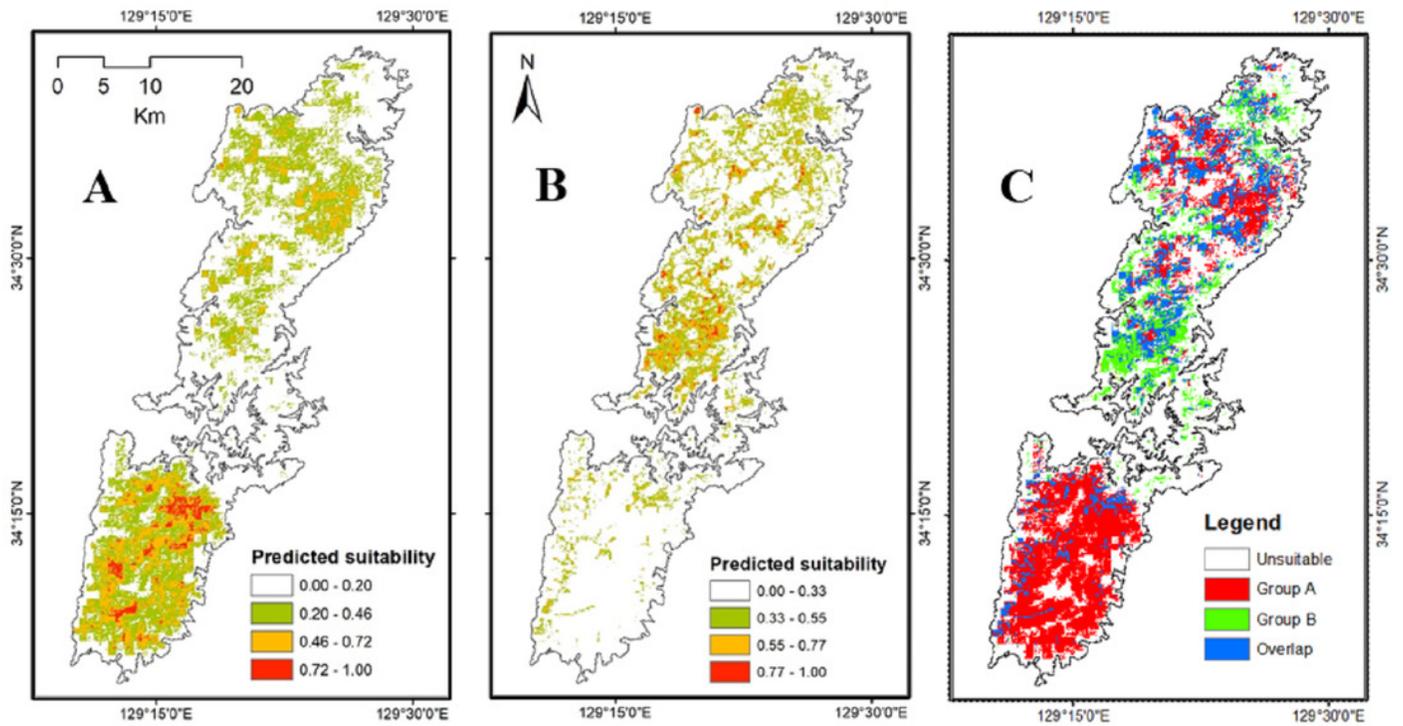
Figure 4

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.
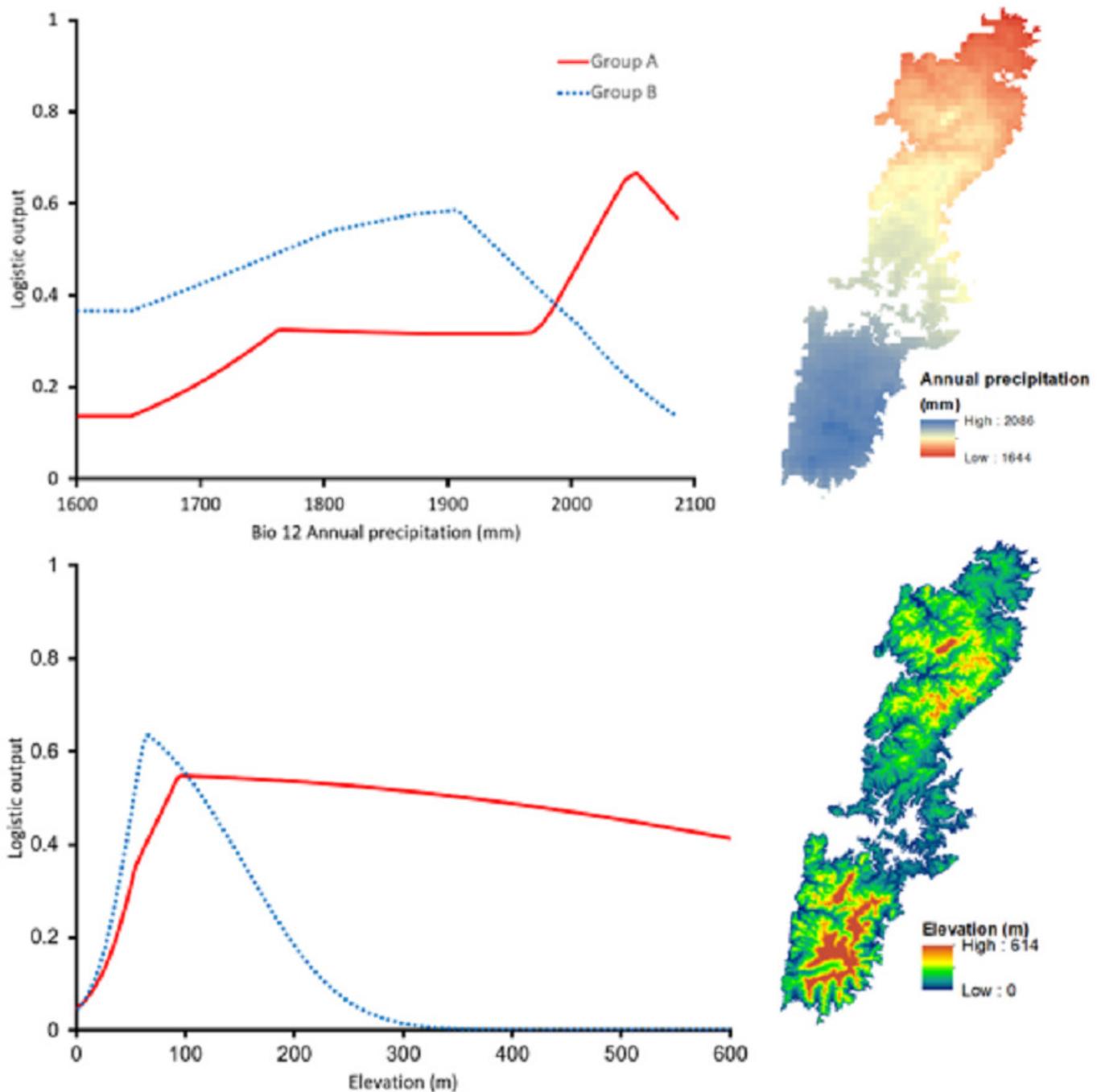
Figure 5

The potential distribution of Group A (left) and Group B (right) for Last Interglacial (LIG) (A), Last Glacial Maximum (LGM) (B), Mid-Holocene (C) and current scenarios (D).
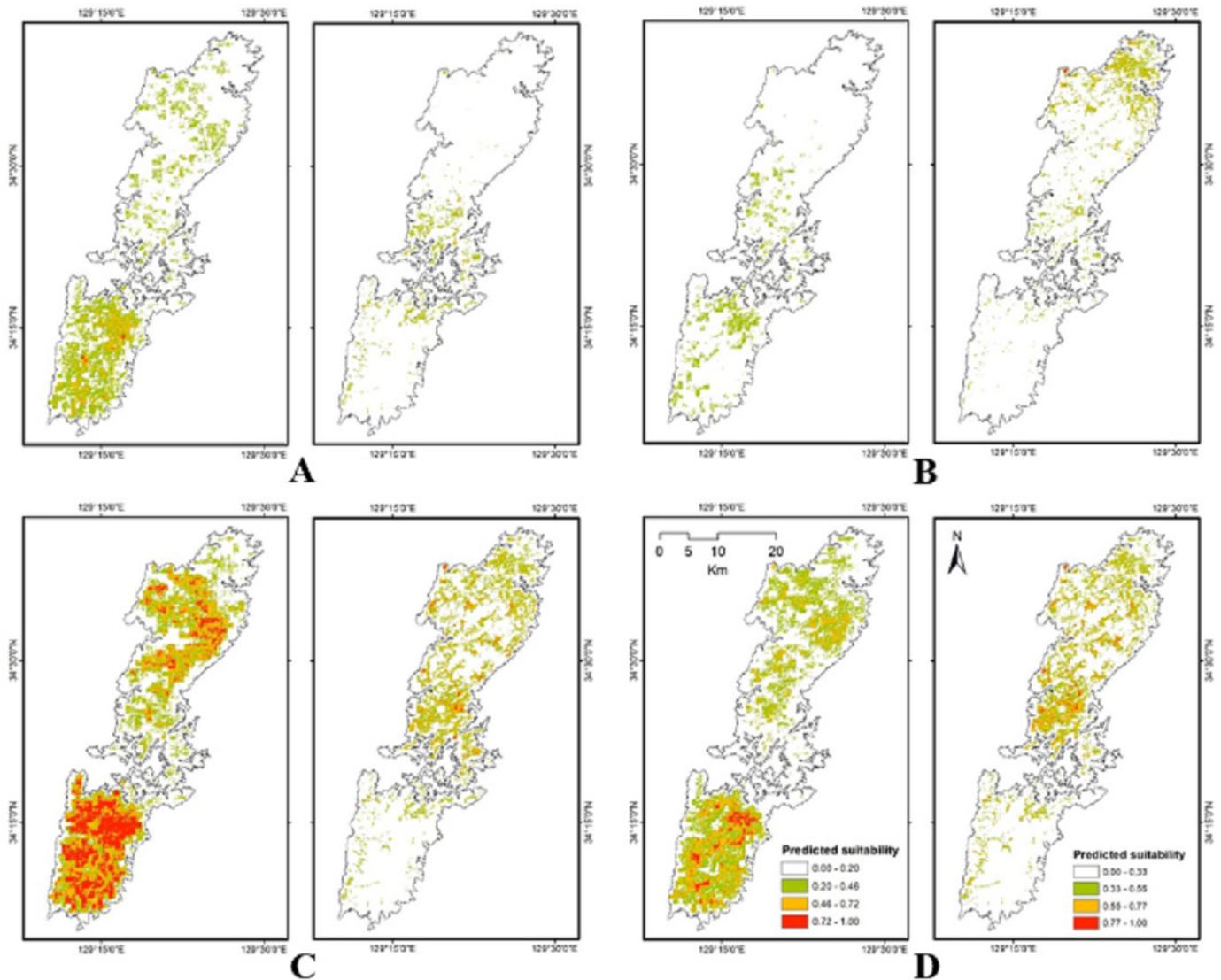
Figure 6

Figure 6: 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.
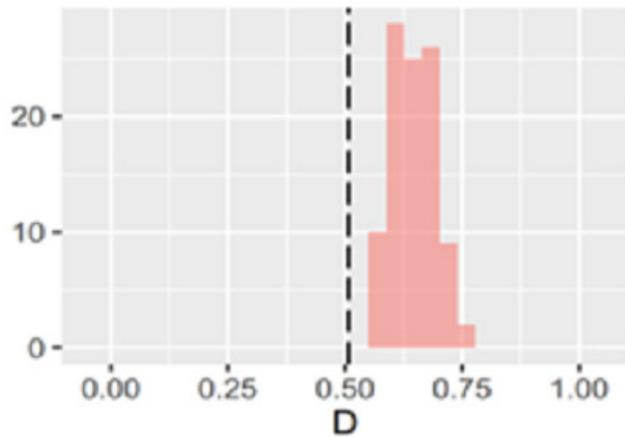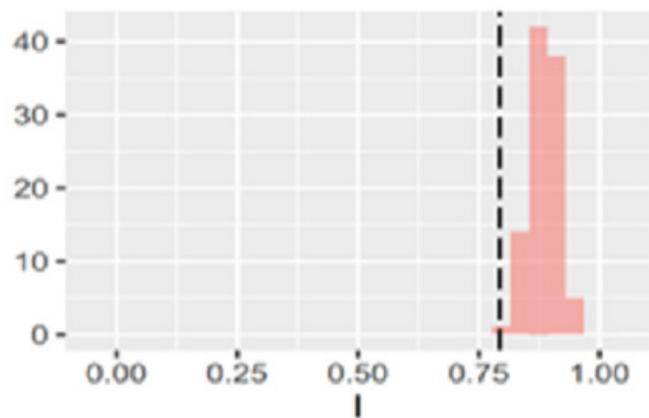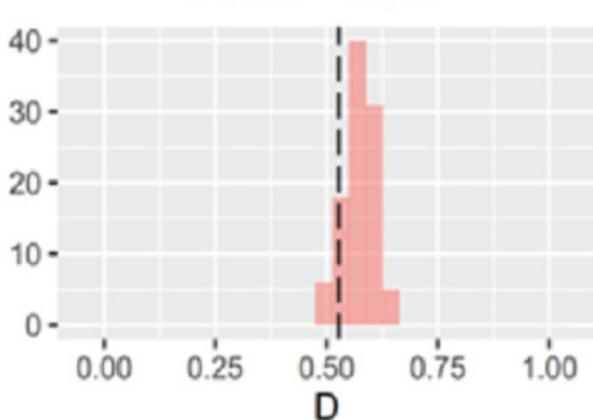
Figure 7

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.

Ecospat Identity test:
Group A vs. Group B
P-value = 0.0099
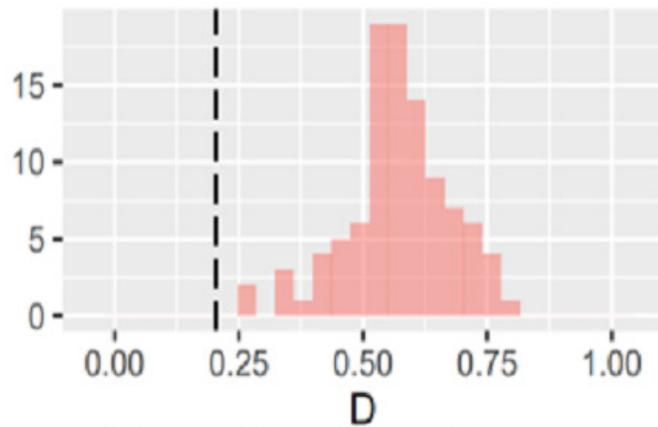
Ecospat Identity test
Group A vs. Group B
P-value = 0.0099
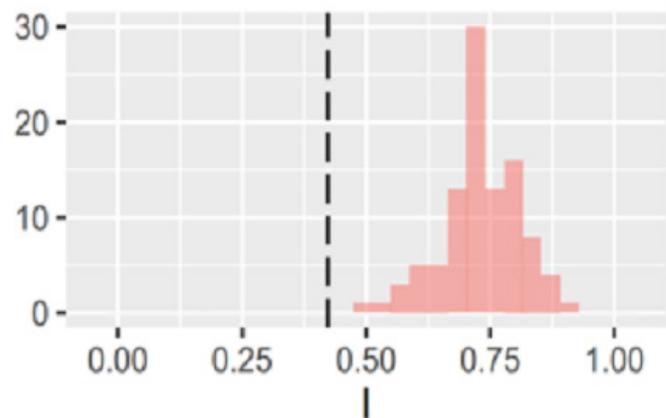
Ecospat background test:
Group A vs. backgound of Group B
P-value = 0.3960
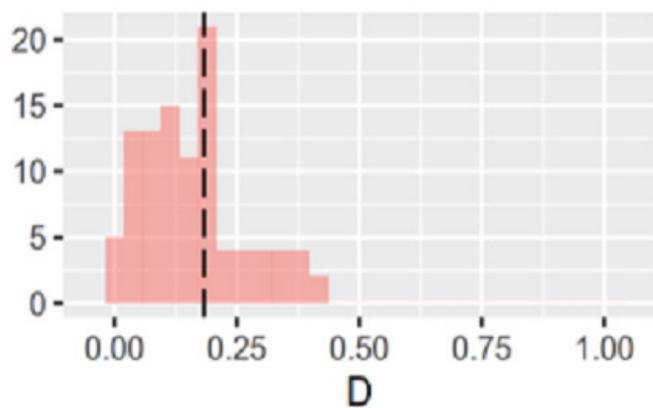
Ecospat background test:
Group A vs. backgound of Group B
P-value = 0.3267
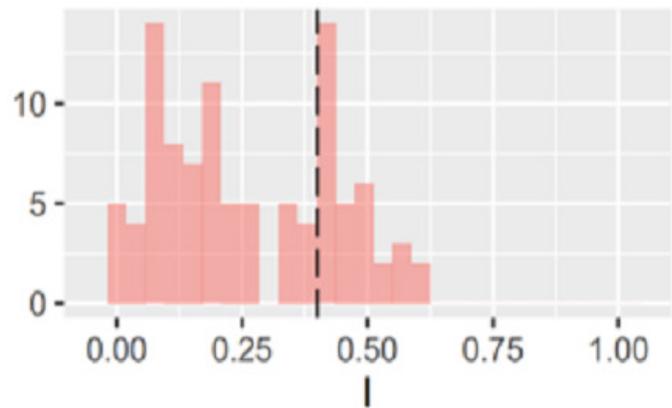
Ecospat background test:
Group B vs. backgound of Group A
P-value = 0.0990
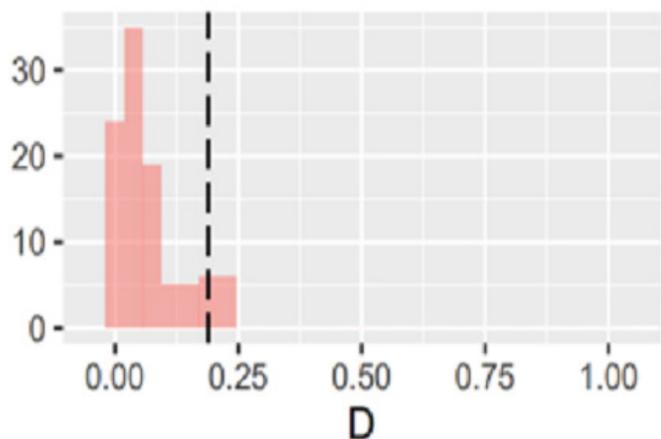
Ecospat background test:
Group B vs. backgound of Group A
P-value = 0.1089
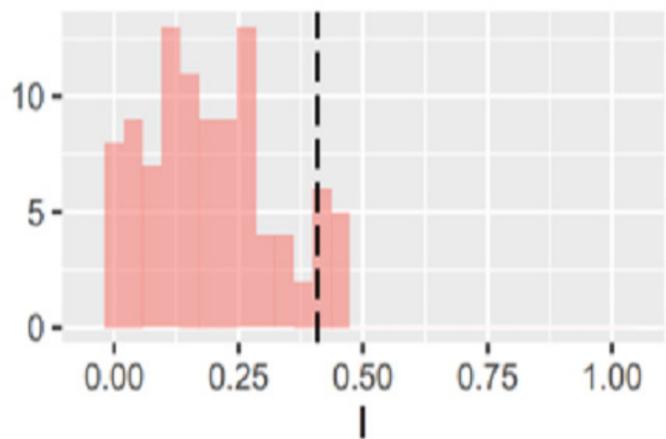
Table 1 (on next page)

Sampling localities, number of individuals (N), number of haplotypes (Nh), haplotype No., accession No., and sources.

Asterisks indicate that Group A and Group B were discovered syntopically.

Group	Locality	N	Nh	Haplotype No.	DDBJ accession No.	Source
Group A	Tsutsu	3	2	A2, A3(2)	LC638502-LC638504	This study
	Tsutsuse	2	1	A2(2)	LC156103, LC156104	Niwa et al. (2021)
	Azamo	3	1	A3(3)	LC156105-LC156107	Niwa et al. (2021)
	Agami	2	2	A3, A4	LC638505, LC638506	This study
	Kuwa	1	1	A3	LC638507	This study
	Taterasan1	1	1	A3	LC638508	This study
	Taterasan2	1	1	A3	LC638509	This study
	Uchiyama	2	2	A5, A6	LC638510, LC638511	This study
	Kunehama	2	1	A3(2)	LC638512, LC638513	This study
	Shiine	1	1	A7	LC638514	This study
	Kitazato1	2	2	A1, A3	LC156108, LC156109	Niwa et al. (2021)
	Kitazato2	2	2	A3, A8	LC638515, LC638516	This study
	Kitazato3	1	1	A9	LC638517	This study
	Kitazato4	1	1	A10	LC638518	This study
	Shimobaru1	2	2	A3, A11	LC156110, LC156111	Niwa et al. (2021)
	Shimobaru2	2	2	A12, A13	LC583751, LC583752	Niwa et al. (2021)
	Shimobaru3	2	2	A14, A15	LC583753, LC583754	Niwa et al. (2021)
	Shimobaru4	2	1	A3(2)	LC638519, LC638520	This study
	Shimobaru5	1	1	A2	LC638521	This study
	Sumo1*	4	3	A3(2), A10, A22	LC156112, LC156113, LC156115, LC156116	Niwa et al. (2021)
	Sumo2	2	2	A3, A17	LC638522, LC638523	This study
	Kashi	3	2	A3(2), A16	LC638524-LC638526	This study
	Mikata*	2	2	A10, A16	LC638527, LC638528	This study
	Kechi	3	3	A3, A16, A20	LC638529-LC638531	This study
	Mine*	3	3	A3, A17, A22	LC638532-LC638534	This study
	Shishimi*	1	1	A24	LC156132	Niwa et al. (2021)
	Kaidokoro*	3	3	A18, A19, A27	LC156123-LC156125	Niwa et al. (2021)
	Seta	1	1	A19	LC638535	This study
	Mitake2*	2	2	A23, A24	LC638536, LC638537	This study
	Saozaki	2	1	A26(2)	LC638538, LC638539	This study
	Sago1	2	2	A21, A24	LC638540, LC638541	This study
	Sago2	1	1	A24	LC638542	This study
	Shitaru	1	1	A3	LC638543	This study
	Sasuna*	1	1	A19	LC638544	This study
	Oshika*	2	2	A17, A24	LC638545, LC638546	This study
	Kin*	4	4	A17, A24, A28, A29	LC156133-LC156135, LC156137	Niwa et al. (2021)
	Shushi	3	3	A3, A19, A25	LC638547-LC638549	This study

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Group	Locality	N	Nh	Haplotype No.	DDBJ accession No.	Source
Group B	Sumo1*	3	1	B1(3)	LC156114, LC638550, LC638551	Niwa et al. (2021), this study
	Mikata*	2	2	B1, B2	LC638552, LC638553	This study
	Itose	3	2	B3(2), B4	LC583773, LC156117	Niwa et al. (2021)
	Nii	1	1	B3	LC638554	This study
	Otsuna	2	1	B3(2)	LC638555, LC638556	This study
	Yoshida1	2	2	B3, B5	LC156118, LC156119	Niwa et al. (2021)
	Yoshida2	1	1	B7	LC638557	This study
	Mine*	2	2	B3, B4	LC496473, LC430988	Niwa et al. (2021)
	Kushi	1	1	B4	LC638558	This study
	Shishimi*	1	1	B1	LC638559	This study
	Kaidokoro*	3	1	B6(3)	LC583775	Niwa et al. (2021)
	Mitake1	1	1	B8	LC638560	This study
	Mitake2*	3	1	B6(3)	LC638561-LC638563	This study
	Sasuna*	2	1	B7(2)	LC638564, LC638565	This study
	Oshika*	2	2	B6, B9	LC638566, LC638567	This study
	Kin*	3	1	B7(3)	LC583776	Niwa et al. (2021)
☐	Hamakusu	2	1	B7(2)	LC638568, LC638569	This study

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Table 2(on next page)

List of environmental variables.

1

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.

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Table 3(on next page)

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 ($P < 0.01$).

1

Group	N	Nh	$h \pm SD$	$\pi \pm SD$	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

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Table 4(on next page)

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

1

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

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