Genetic diversity and demography of *Bufo japonicus* and *B. torrenticola* (Amphibia: Anura: Bufonidae) influenced by the Quaternary climate (#70165)

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Genetic diversity and demography of *Bufo japonicus* and *B. torrenticola* (Amphibia: Anura: Bufonidae) influenced by the Quaternary climate

Kazumi Fukutani [Corresp., 1], **Masafumi Matsui** [1], **Dung Van Tran** [2, 3], **Kanto Nishikawa** [1, 2]

[1] Graduate School of Human and Environmental Studies, Kyoto University, Kyoto, Japan

[2] Graduate School of Global Environmental Studies, Kyoto University, Kyoto, Japan

[3] Wildlife Department, Vietnam National University of Forestry, Ha Noi, Vietnam

Corresponding Author: Kazumi Fukutani
Email address: fukutani.kazumi.55a@st.kyoto-u.ac.jp

The glacial climate in the Quaternary period affected the present species richness of amphibians, limiting their activities and restoring diversity. In this study, we examined the phylogenetic relationships of the Japanese toads (*Bufo japonicus* and *B. torrenticola*) and the demography of each lineage from past to present based on the mitochondrial sequences and the ecological niche models. The Japanese toads were a monophyletic group with two main clades, one of which contained *B. torrenticola.* The main two clades diverged at the Early Pliocene, and the genetic divergences within each main clade occurred from the Late Pliocene to the Middle Pleistocene. Especially, the northernmost clades in the Tohoku region were identified to be genetically diverged affected by the glacial climate. Each lineage retreated to each refugium in low-elevation along the coastal area in the glacial period, and effective population sizes have increased to construct the current populations after the glacial period. The climate stability from the last glacial maximum to the present likely affected the distribution of each lineage of the Japanese toads.

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Kazumi Fukutani[1], Masafumi Matsui[1], Dung Van Tran[2,3], and Kanto Nishikawa[1,2]

[1]Graduate School of Human and Environmental Studies, Kyoto University, Kyoto, Japan

[2]Graduate School of Global Environmental Studies, Kyoto University, Kyoto, Japan

[3]Wildlife Department, Vietnam National University of Forestry, Ha Noi, Vietnam

Corresponding Author:

Kazumi Fukutani[1]

Email address: fukutani.kazumi.55a@st.kyoto-u.ac.jp

Abstract

The glacial climate in the Quaternary period affected the present species richness of amphibians, limiting their activities and restoring diversity. In this study, we examined the phylogenetic relationships of the Japanese toads (*Bufo japonicus* and *B. torrenticola*) and the demography of each lineage from past to present based on the mitochondrial sequences and the ecological niche models. The Japanese toads were a monophyletic group with two main clades, one of which contained *B. torrenticola.* The main two clades diverged at the Early Pliocene, and the genetic divergences within each main clade occurred from the Late Pliocene to the Middle Pleistocene. Especially, the northernmost clades in the Tohoku region were identified to be genetically diverged affected by the glacial climate. Each lineage retreated to each refugium in low-elevation along the coastal area in the glacial period, and effective population sizes have increased to construct the current populations after the glacial period. The climate stability from the last glacial maximum to the present likely affected the distribution of each lineage of the Japanese toads.

Introduction

One of the main aims of biogeography is understanding the biological and physical processes that lead to species evolution and distribution (*Lomolino et al., 2010*). Biogeographic studies have often emphasized the effects of Quaternary climate because glacial-interglacial repeated cycles have led to distribution changes in many species, affecting the present distribution (e.g. *Taberlet* et al*., 1998; Hewitt, 2004*). Amphibians are particularly vulnerable to climate change because of their limited migration capacity, ectotherms, and the strong influence of climate factors on reproduction (e.g. *Carey & Alexander, 2003*; *Blaustein et al., 2010*; *LI, COHEN &*

47 *ROHR, 2013*; *Ficetola & Maiorano, 2016*). As a result, the glacial climate strongly affected the
48 present species richness of amphibians, limiting their activities, followed by restoration of
49 diversity in herpetofauna after the Last Glacial Maximum (LGM; *Araújo et al., 2008*; *Zeisset &*
50 *Beebee, 2008*; *Martínez-Monzón et al., 2021*). Japan is endowed with rich amphibian faunas,
51 with many taxa and high endemism (*Nishikawa, 2017*). The areas with high species richness are
52 likely to function as refugia at the glacial period due to the climate stability (*Sandel et al., 2011*).
53 In addition, the high endemism may have resulted from in situ diversification affected by the
54 island-specific environment (*Kubota, Shiono & Kusumoto, 2015; Kubota et al., 2017*). In the
55 Japanese archipelago, multiple refugia in glacial periods were formed along the latitude, mainly
56 in low elevation areas such as coastal areas influenced by Quaternary climate (e. g., *Tomaru et*
57 *al., 1998; Nunome et al., 2010; Aoki, Kato & Murakami, 2011*). Furthermore, amphibians widely
58 distributed on the Japanese mainland (Hokkaido, Honshu, Shikoku, and Kyushu) are genetically
59 more diverse in multiple refugia than previously thought (*Tominaga et al., 2013; Dufresnes et al.,*
60 *2016; Matsui et al., 2019*).

61 In this study, we focus on the Japanese toads (Genus *Bufo*, Bufonidae). There are two
62 endemic *Bufo* species on the Japanese mainland, *B. japonicus* and *B. torrenticola* (*Matsui &*
63 *Maeda, 2018*). Although the effects of Quaternary climate on the European toads have been well
64 studied (e.g., *Garcia-Porta et al., 2012; Arntzen et al., 2018; Chiocchio et al., 2021*), the effects
65 on *B. japonicus* and *B. torrenticola* have not been studied. *Bufo japonicus* is widely distributed in
66 Honshu, Shikoku, Kyushu, and some adjacent islands and has a habit of lentic breeding as most
67 other congeneric species. The species is divided into two subspecies, *B. j. japonicus from*
68 western and *B. j. formosus* from eastern Japan. In contrast to *B. japonicus*, the range of *B.*
69 *torrenticola* is limited to the mountainous area of the central Honshu, with the lotic breeding

habits exceptional for *Bufo*. Two subspecies of *B. japonicus* are distributed parapatrically. *B. torrenticola* is distributed sympatrically with *B. j. formosus* in several areas of the central Honshu (*Matsui & Maeda, 2018*). *Igawa et al. (2006)* suggested that the geological events during the formation of the Japanese archipelago had led to the genetic diversification in the Japanese toads.

Studies combining ecological niche models (ENM) with phylogeography have become mainstream in biogeography. Combining the gene-based estimates and analyses of the environmental effects allows for more robust results (*Waltari et al., 2007; Hickerson et al., 2010; Alvarado-Serrano & Knowles, 2014*). Few quaternary fossils of amphibians have been found on the Japanese mainland, so combined genetic analyses with environmental analysis will provide more powerful insight into the Quaternary biogeography of the Japanese amphibians. Furthermore, these analyses will contribute to clarifying the factors that maintain the high endemism of Japanese amphibians. Here, we present the biogeographic processes for the diversification of the Japanese toads based on the mitochondrial sequences and explain the effects of the glacial climate on them by genetic analyses and ENM.

Materials & Methods

DNA sampling and sequencing

A total of 213 samples from 191 localities of *B. japonicus* and 27 samples from 25 localities of *B. torrenticola* were collected, covering each distribution range (Fig. 1). According to the manufacturer's instruction, we extracted DNA from frozen or ethanol-preserved tissue samples (e.g., muscles, livers, or skin) with Qiagen DNeasy Blood and Tissue Kit (Qiagen). Animal

Experimentation Ethics Committee in Graduate School of Human and Environmental Studies, Kyoto University provided full approval for this research (20-A-5, 20-A-7).

We amplified the mitochondrial DNA from the 3' region in *tRNA-Glu* to *cytochrome b*. We used the newly designed primer set (5'-TTCCTACAAGGACTTTAACCTAGAC-3'; 5'-GTTGGGCTAGTTTGTTCTCTG-3') for PCR, with the length of the products being 1208 bp. The PCR protocol followed 2 min soak at 94°C, followed by 33 cycles with 15 s at 94°C, 15 s at 53°C, and 90 s at 72°C, and final extension of 4 min at 72°C. Primers, dNTPs, and polymerase were separated from the successful PCR amplification products by precipitation with polyethylene glycol. We performed cycle sequencing reactions (CSR) by BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). The same primers used for PCR and two more newly designed internal primers (5'-CGAACTTGTTCAATGAATCTGAG-3', 5'-CTTGTCGAAGTTGGGGTTAAG-3') were used for CSR and then purified the products by ethanol precipitation. Amplified fragments were sequenced on ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), assembled with ChromasPro v.1.34 (Technelysium Pty Ltd.), and aligned with MAFFT v7.222 (default parameters: *Katoh & Standley, 2013*). We got aligned 1071 bp *cytochrome b* sequences and submitted the haplotypes determined in this study to DNA Data Bank of Japan (DDBJ; accession numbers. LC581513–LC581757: Table S1). The *cytochrome b* regions were usually used in the previous studies on the Japanese toads and are known to have enough variation to evaluate the population genetics (e.g., *Hase, Shimada & Nikoh, 2012; Iwaoka et al., 2021*), so we used only the region to allow comparison with the previous studies.

Phylogenetic analyses

We first built phylogenetic trees by maximum likelihood (ML) and Bayesian inference (BI) methods. We selected the optimum substitution models for each partition by Kakusan4 (*Tanabe, 2011*) based on the Akaike information criterion (*Akaike, 1974*) for ML analysis and Schwarz's Bayesian information criterion (*Schwarz, 1978*) for BI analyses. The best-fit substitution models chosen for ML and BI analyses were GTR+G models. We performed the ML analyses with estimation node supports by 1,000 bootstrapping replications using RAxML v.8.2 (*Stamatakis, 2014*). In the BI analyses, we conducted two independent runs of three million generations, each with four Markov chains, and sampled the resulting trees every 100 generations by MrBayes v3.2.6 (*Ronquist et al., 2012*). We checked the parameter estimates and convergence using Tracer v.1.7 (*Rambaut et al., 2018*). The initial 10% of trees were discarded as burn-in. Sequences from *B. g. gargarizans*, *B. g. miyakonis*, and *B. bankorensis* were used as outgroup as these sister lineages are the closest relatives of the Japanese toads (*Matsui, 1984, 1986; Igawa et al., 2006*; Table S1).

Divergence dates for the Japanese toads were estimated using BEAST v.2.6 (*Bouckaert et al., 2019*). We reduced our dataset, maintaining solely one representative of each clade as appeared in our ML phylogeny. To introduce the calibration points, we added the sequences of four *Bufo* species and one species belonging to the family Bufonidae as outgroups (Genbank Accession numbers: *B. gargarizans*: NC_008410, *B. stejnegeri*: NC_027686, *B. bufo*: MN432913, *B. verrucosissimus*: MN432915, *B. eichwaldi*: JN647474, *Epidalea calamita*: MT483697). Two external nodes of the Japanese toads were calibrated: (1) the split between *B. bufo* and *B. gargarizans* species complexes as 12.33 million years ago (Mya; 95% highest posterior density [HPD], 8.81–16.36 Mya) according to the timetree of *Garcia-Porta et al. (2012)*; (2) the oldest fossil record attributable to the *B. verrucosissimus* (1.81–2.59 Mya), setting

a lognormal prior with an offset of 1.81 Mya and 95% of the values between 2.0 and 4.5 Mya followed by *Recuero et al. (2012)*. The analysis was run for 50 million generations, sampling every 100,000 using the HKY+G model with the strict clock model. Tracer v.1.7 (*Rambaut et al., 2018*) was used to assess the stationarity and effective samples of parameters. Finally, we generated the maximum clade credibility consensus tree with median node heights using the TreeAnnotator program, discarding the first 10% of the tree as burn-in.

Demographic analyses

Haplotype (*Hd*) and nucleotide diversity (π) within each main clade were calculated in DnaSP v.6 (*Rozas et al., 2017*). To examine deviations from neutrality, which would be expected with population expansion, we calculated Fu's F_S (*Fu, 1997*) with 10,000 permutations for significances using Arlequin ver 3.5 (*Excoffier & Lischer, 2010*). The mismatch distributions analyses were performed by computing observed pairwise differences to distributions simulated under demographic (*Rogers & Harpending, 1992*) and range expansions models (*Ray, Currat & Excoffier, 2003; Excoffier, 2004*) implemented in Arlequin. The observations were compared to model predictions based on 10,000 permutations of the data. We also tested the goodness-of-fit of simulated distribution with the expected distributions using a population expansion model by calculating the sum of square deviation (SSD). Genetic Landscape Shape interpolation analyses were performed using Alleles In Space (AIS; *Miller, 2005*; *Miller et al., 2006*) to obtain spatial patterns in genetic diversity. The analysis produces three-dimensional surface plots of interpolated genetic distances where X and Y coordinates correspond to geographical locations on the rectangular grid, and surface plot heights (Z) reflect genetic distances. We performed the analysis for each group with a grid of 150 × 150 and a distance weighting value of 1.0. All

analyses implemented in AIS used sequences as the input matrix (raw genetic distances) and UTM coordinates. We expected that areas shown by warm color in this analysis indicate high genetic diversity and thus represent refugia.

We estimated the shifts of the effective population size of each lineage in the Japanese toads based on Bayesian skyline plots (BSP: *Drummond et al., 2005*) by BEAST v.2.6 (*Bouckaert et al., 2019*). We applied the HKY+G model of molecular evolution and a strict molecular clock model for BSP analyses. The analyses consisted of one Markov chain Monte Carlo analysis with the chain runs for 50 million generations, sampling every 100,000 generations with discarding 10% as burn-in. We verified the effective sample sizes for each parameter and convergence of chains in Tracer v.1.7 (*Rambaut et al., 2018*). For BSP, we employed a rate calibration based on the calibration of the demographic transition method (CDT; *Hoareau, 2016*), which uses the timing of climatic changes over the late glacial warming period to calibrate expansions and provides a robust clock. The CDT is advanced the expansion dating (*Crandall et al., 2012*) based on the two-epoch demographic model (*Shapiro et al., 2004*) and enables us to overcome the problem that using older (< 1 Mya) or interspecific phylogenetic calibration leads to incorrect estimates for intraspecific demographic parameters (*Ho & Larson, 2006; Grant, 2015*). We performed the rate calibration following default CDT procedures (*Hoareau, 2016*) using Beast v1.8.4 (*Drummond et al., 2012*). We considered no problem with the low sample size of the northernmost lineage for inferring past population size because we collected samples to cover their distribution range. BSP analyses were constructed for each lineage of the Japanese toads by using the calibrated rate.

Ecological niche models

To trace the location of glacial refuge, we constructed ENM for each lineage of the Japanese toads and predicted their ranges under the present and LGM conditions. We gathered distribution localities with the known occurrence of *B. japonicus* and *B. torrenticola*, respectively, combining our sampling localities used for the phylogenetic analyses in this study and our records. This initial dataset was filtered to avoid spatial autocorrelation and duplication by randomly selected occurrence points more than 1 km apart from each other in 10 replicates using the R package spThin (*Aiello-Lammens et al., 2015*). The final dataset comprised 422 and 26 records for *B. japonicus* and *B. torrenticola*, respectively (Table S2). We regarded the lineages of the record for *B. japonicus* based on their distribution.

We extracted 19 bioclimatic layers representative of the climatic date over 1970–2000 from the WorldClim v.2.1 (*Fick & Hijmans, 2017*), featuring 30 arc seconds of spatial resolutions: 11 layers related to temperature and eight layers related to precipitation. First, Pearson correlation coefficients for all pairs of bioclimatic variables were calculated using ENMTools v.1.4.4 (*Warren, Glor & Turelli, 2010*) to eliminate predictor collinearity before generating the model. Then, variables of correlated pairs with $|r| > 0.85$ were excluded, considering that they were biologically less important based on known preferences of the Japanese toads. The resulting data set contained eight bioclimatic variables: BIO 2 (mean diurnal range), BIO 3 (isothermality; BIO 2/BIO 7), BIO 8 (mean temperature of the wettest quarter), BIO 10 (mean temperature of the warmest quarter), BIO 11 (mean temperature of the coldest quarter), BIO 15 (precipitation seasonality; CV), BIO 18 (precipitation of warmest quarter), and BIO 19 (precipitation of coldest quarter).

Distribution models were built with ten replicates using the default setting in Maxent v.3.4.4 (*Phillips, Anderson & Schapire, 2006*). We used the areas under the receiving operator

characteristics curve (AUC) to evaluate the models' performances. Ecological niche models were constructed according to current environmental factors and projected for present and LGM. To project the current ecological niches of the Japanese toads on climate conditions during LGM (21,000 years ago), we applied two widely-used general circulation climate models with species-specific mask and 2.5 arc minutes spatial resolutions: the Community Climate System (CCSM4; *Gent et al., 2011*), and the Model for Interdisciplinary Research on Climate (MIROC-ESM 2010; *Watanabe et al., 2011*) from the WorldClim version 1.4 (https://www.worldclim.org/data/v1.4/worldclim14.html). Logistic thresholds of 10 percentile training presence generated in the Maxent output were used to define the minimum probability of suitable habitat.

ENM were constructed for each lineage, considering the possibility of niche divergence between populations, and thus we tested niche overlap among the lineages. We used Schoener's *D* (*Schoener, 1968*) and Hellinger's *I* metric (*Warren, Glor & Turelli, 2008*) to test for niche conservatism and divergence. These metrics were computed from climatic variation under present conditions in ENMTools. We build niche models of identity and background tests based on 100 pseudoreplicates generated from a random sampling of data points pooled for each pair of groups. The Schoener's *D* and Hellinger's *I* of the true calculated niche between groups were compared with the null distribution by two-tailed *t*-tests.

We included the putative populations of introduced origin (see below in result) for the phylogenetic analysis to identify their haplotypes but excluded them for the demographic analysis and ENM because they might hinder estimating actual demography and suitable distribution area.

Results

Phylogeny and divergence time

Our phylogenetic analyses of mitochondrial *cytochrome b* (1,071 bp) identified old but monophyletic radiation of the Japanese toads, including *B. torrenticola* and five mitochondrial lineages of *B. japonicus*, with varying degrees of divergence. ML and BI phylogenetic trees showed congruent topologies (Fig. 1), as those previously reported (*Igawa et al., 2006; Hase, Shimada & Nikoh, 2012*). We specified the possible phylogenetic boundaries between lineages with higher resolution than the previous studies. The distribution of each lineage overlapped at the boundary. The boundaries between the major clades, clade A and B, diverged 5.15 Mya (HPD: 6.68–3.50 Mya), located on the west side of Lake Biwa in the Kinki region.

The first clade (A) has a wide distribution across the eastern parts of the Japanese mainland and corresponds to *B. j. formosus.* This clade is further subdivided into three lineages distributed on the northern Tohoku region (clade A1), from the southern Tohoku to northern Kanto regions (clade A2), and from the southern Tohoku to Kinki regions (clade A3). The common ancestor of clade A1 and A2 diverged from clade A3 at 1.48 Mya (HPD: 2.13–0.94 Mya), and clade A1 and A2 diverged 0.68 Mya (HPD: 1.01–0.40 Mya). In addition, two samples, which were identified as *B. torrenticola* morphologically, from Toyama and Ishikawa Prefectures (locality 81, 84) had the haplotype of clade A2, indicating that genetic introgression of *B. j. formosus* mtDNA may occur at the boundary between *B. j. formosus* and *B. torrenticola* as suggested in the previous studies (*Yamazaki et al., 2008; Iwaoka et al., 2021*).

The second clade (B) is distributed widely across the western parts of the Japanese mainland. This clade is further subdivided into three lineages: two lineages of *B. j. japonicus*, corresponding to *B. j. japonicus*, and one of *B. torrenticola.* Of *B. japonicus*, one lineage is

253 distributed in the Kinki, Chugoku, and Shikoku regions (clade B1), and another from the western
254 end of Honshu to Kyushu (clade B2). The lineage of *B. torrenticola* is distributed along the
255 mountain range northwest of Lake Biwa and from Hokuriku to the Kii Peninsula. *Bufo j.*
256 *japonicus* was paraphyletic because clade B2 and *B. torrenticola* made a sister group. Clade B1
257 diverged 2.84 Mya (HPD: 3.94–2.12 Mya), and clade B2 and *B. torrenticola* diverged 2.11 Mya
258 (HPD: 2.94–1.38 Mya).
259 Our phylogenetic analysis reconfirmed previously suggested artificially introduced
260 populations in Hokkaido, Izu Islands, and the Kanto region (*Matsui, 1984; Kawamura et al.,*
261 *1990; Igawa et al., 2006; Hase, Shimada & Nikoh, 2012; Matsui & Maeda, 2018; Suzuki et al.,*
262 *2020*; Fig. 1).
263
264 **Demographic analyses**
265 The high genetic diversities (*Hd* = 0.967–0.995), low nucleotide diversities (π = 0.00486–
266 0.00805), and significantly negative Fu's F_S values for all clades of *B. japonicus* and *B.*
267 *torrenticola* indicated the experience of the historical demographic expansion (*Fu, 1997; Grant*
268 *& Bowen, 1998*; Table 1).
269 MtDNAs from clade B1 showed a ragged mismatch distribution suggesting demographic
270 equilibrium, whereas the unimodal distribution of the A1, A2, and B2 clearly indicated recent
271 population expansions (*Harpending, 1994*; Fig. 2). Clade A3 and *B. torrenticola* had two peaks,
272 suggesting the inclusion of multiple populations, each undergoing bottlenecks followed by
273 expansion (*Hayes et al., 2008*). Based on the SSD, the fits to the demographic expansion models
274 could never be rejected for clades A1 and A3 (Fig. 2). The mismatch distributions simulated
275 under the models of spatial expansion were matched for clade A1, A3, and *B. torrenticola.*

276 Genetic Landscape Shape interpolation analyses revealed the geographic gradient of
277 genetic variation in each group (Fig. 3). The high genetic diversity areas for clade A1 were
278 distributed in the southern and western range, while those for clade A2 had higher genetic
279 diversity in the south of the range. The areas with high genetic diversity in clade A3 were
280 distributed in the low elevations areas on both sides of the Japan Alps (Hida, Kiso, and Akaishi
281 Mountains) at the center of Honshu. Clade B1 had high genetic diversity in the western area,
282 some parts of Chugoku and Kinki districts, and clade B2 had high genetic diversity in the
283 northern region. *Bufo torrenticola* had high genetic diversity, mainly in the southern area and
284 scattered northwestern, northwestern, and central distribution regions. Because populations that
285 remain in refugia during the glacial period have a longer dynamic history and greater genetic
286 diversity than that have expanded during post-glacial age (*Comes & Kadereit, 1998; Taberlet et*
287 *al., 1998*), it is possible to consider regions with high genetic diversities within clades as refugia.
288 The calibrated divergence rate by CDT was very large, 0.166 changes/site/million years,
289 which could be considered reasonable compared with the result in *Hoareau (2016)*, and other
290 evolutionary rates estimated for recent time scale for mitochondrial *cytochrome b* (*Ho et al.,*
291 *2005; Suzuki et al., 2015*). BSP reconstructed the demographic histories of mtDNA lineages of
292 the Japanese toads since the last glacial maximum (Fig. 2). All of the lineages presented signals
293 of recent population expansions. The expansion was much stronger (more than 10-fold increase)
294 for *B. japonicus* than *B. torrenticola* (less than 10-fold increase).
295
296 **Ecological niche models**

297 Each ENM estimated under current climate conditions had mean test AUC values ≥ 0.9,
298 indicating a better than random prediction. The predicted potential niche models under the
299 current climate conditions for each lineage of the Japanese toads are shown in Fig. 4.
300 The extent of range shrinkage varies depending on the global circulation model for each
301 clade in clade A. The predicted distributions showed that the suitable range for clade A1 almost
302 vanished from all areas based on MIROC, while some small parts of the Japan Sea coastal area
303 were left based on CCSM during the LGM. According to the CCSM model, the suitable
304 environmental conditions for clade A2 during the LGM contracted to some areas along the coast
305 of the Sea of Japan and the Pacific Ocean, to the contrary, based on MIROC, the suitable
306 conditions were distributed along the Pacific coast from southern Tohoku to Shikoku. For clade
307 A3, the predicted distribution range expanded mainly from Chubu and Kinki by CCSM and
308 MIROC during both LGM and present. On the other hand, both the CCSM and MIROC models
309 for each clade in clade B suggested that the projected potential niche models for the LGM were
310 limited significantly southward of their ranges.
311 Niche overlap under present climate conditions between lineages ranged between 0.04
312 and 0.59 for Schoener's *D* and between 0.18 and 0.85 for Hellinger's *I* metrics, respectively
313 (Table 2). The null hypotheses of the niche identity test were rejected for all pairs of lineages (*p*
314 < 2.2e-16), indicating the environmental niches of all adjacent phylogroup were not equivalent.
315 The null hypotheses of the similarity test could not be rejected between clade A1 and A2
316 based on the direction test of known localities of clade A2 to background range of clade A1 for
317 Schoener's *D* and based on both directions for Hellinger's *I.* Additionally, the null hypotheses of
318 the similarity test could not be rejected between clade A2 and A3 based on the direction test of
319 known localities of clade A2 to the background range of clade A3 for Hellinger's *I* (Table 3).

(~~Table 3~~). The observed niche overlaps were significantly higher than expected under the null hypotheses between each pair of *B. japonicus* (except between clade A3 and B2 and not rejected pairs of clades described above) and between clade A1 and *B torrenticola,* indicating that each lineage was more similar than expected (Table 3). This opposite result between the identity test and similarity test is a false positive; that is, the identity test tends to unduly reject the null hypothesis of niche identity (*Peterson, 2011*). In addition, the background test is known to be more suitable for understanding speciation than the identity test (*Smith & Donoghue, 2010*). Therefore, because the null hypotheses of the identity tests were rejected for all of the lineages in this study, we focused on the similarity test like *Collart et al. (2021)*.

The environmental niche of *B. japonicus* (except clade A1) and *B. torrenticola* was also more similar than expected based on the habitat available to *B. japonicus* but more diverged than expected based on the habitat available to *B. torrenticola* (Table 3). These opposite results were also confirmed between clade A3 and B2. This counterintuitive result is likely to be driven by the differences in the heterogeneity of the environmental background for the two species (*Nakazato, Warren & Moyle, 2010*), and their overall similarity is low.

Discussion

Phylogeography of the Japanese toads

The divergence time between clade A and B (c.a. 5.0 Mya) fell within the timeframe reported for the other Japanese frogs (5–7 Mya; *Nishizawa et al., 2011; Dufresnes et al., 2016*). The ancient basins, described as a divergence factor in the previous study (Igawa et al., 2006), were dammed in the Middle Miocene under warm and humid climates by the strength of the East Asia summer monsoon (*Hatano & Yoshida, 2017*). These dammed ancient basins were likely to limit the route

between eastern and western Japan. In addition to the ancient basins, the late Miocene global cooling related to an intensified East Asian winter monsoon (*Herbert et al., 2016; Matsuzaki, Suzuki & Tada, 2020*) may also restrict the activities of the frogs. The Japanese frogs may have diverged into eastern and western populations by being divided into allopatric refugia.

The divergence pattern and time in clade A are similar to those of the northern lineages of *Cynops pyrrhogaster*, a lentic breeder like *B. japonicus,* which diverged with the glacial cycles (*Tominaga et al., 2013*). The dry climate at LGM might have affected the lentic-breeding amphibians by limiting the breeding place. We could consider that the glacial cycles might affect the divergence between clade A1 and A2 in clade A. Assuming that the refugia at glacial age before LGM during Quaternary consisted with that at LGM, clade A1 and A2 could be diverged by utilizing the different refugia along the coastal areas of the Japan Sea and the Pacific Ocean, respectively. Clade A1 and A2 might diverge by genetic drift followed by isolation into different refugia (*Provan & Bennett, 2008*) just after the middle Pleistocene transition when the glacial cooling became severer (*Lisiecki & Raymo, 2005; Clark et al., 2006*) and the significant flora change also occurred on the Japanese mainland (*Momohara, 2016*). Our result of ENM recognized the suitable area in CCSM for clade A1 at LGM along the Japan Sea coast on the northern Tohoku, consisting of the region with high genetic diversity. We also found the high genetic diversity area of clade A1 in the southern part of the distribution, but this southern area was not suitable at LGM. The southeastern area of the present distribution on the Pacific Ocean side was also an unsuitable area despite the actual distribution. There might be areas that are not suitable based on the climate factors but can be inhabited. The refugia for clade A2 were along the Pacific coast on the southern Tohoku with high genetic diversity, indicating the reasonability of MIROC for clade A2. The refugia might be located slightly different enough to diverge, but

366 we cannot conclude that the common ancestor of clade A1 and A2 diverged from clade A3 in the
367 different refugia because the refugia of clade A2 and A3 were located close together.
368 We got high support for the divergence between clade B2 and *B. torrenticola* and the monoph
369 of *B. torrenticola* for the first time by increasing the number of samples. Clade B1 and B2 are
370 paraphyletic, which may be due to incomplete lineage sorting caused by the recent speciation or
371 ancient hybridization (*Maddison, 1997; Funk & Omland, 2003; McKay & Zink, 2010; Toews &*
372 *Brelsford, 2012*). In the case of incomplete lineage sorting, speciation of *B. torrenticola* with
373 morphological and ecological divergence occurred so recently that there might be no time for
374 genetical divergence (*McGuire et al., 2007*). The divergence within clade B is estimated to have
375 occurred from the Late Pliocene to the Early Pleistocene. We might overestimate this divergence
376 time if there was incomplete lineage sorting (*Angelis & Reis, 2015*) and underestimate if there
377 was gene flow on the contrary (*Leaché et al., 2014*), although the divergence times were similar
378 to those for the other amphibians distributed in western Japan (*Tominaga et al., 2006, 2013;*
379 *Nishizawa et al., 2011*). Indeed, it is also a problem that we set the calibration on the only
380 external nodes of the Japanese toads (*Ho et al., 2008*) and used only a single mitochondrial
381 marker.
382 The niche similarities between clades of *B. japonicus* likely indicate their allopatric
383 speciation. In contrast, the dissimilarities between *B. japonicus* and *B. torrenticola* indicate their
384 sympatric speciation (*Wiens & Graham, 2005*), although, of course, it is not perfect to judge only
385 by the niche similarity because ENM alone do not capture the local-scale niche differences
386 between different lineages (*McCormack, Zellmer & Knowles, 2010*).
387
388 **Demography from last glacial maximum to present**

The narrow suitable habitats at LGM for each clade in clade B were estimated based on only western Japan's current temperature and precipitation. If the present habitats are more limited by some factors such as interaction with other populations than solely by climate factors, then the suitable habitats area at LGM may be underestimated. We, therefore, adjusted the estimated area of suitable habitats at LGM for discussion based on the results of the niche similarity. Specifically, it is considered that the suitable habitat of one population can be applied to another population with a similar present niche. This idea could also apply to adjust the suitable habitat at LGM under the assumption that the niche has not changed from LGM to the present.

Clade A1 and A2, distributed in the Tohoku region, shrank their ranges into refugia and expanded after the glacial period. Some amphibians sympatric with toads also diverged in the Tohoku region (*Sumida & Ogata, 1998*; *Yoshikawa et al., 2008; Aoki, Matsui & Nishikawa, 2013; Tominaga et al., 2013; Yoshikawa & Matsui, 2014; Matsui et al., 2020*). Although the divergence times do not coincide, the maintenance of genetic structures within the Tohoku region suggests the presence of multiple refugia in this region. The amphibians diverged in the Tohoku region have cold tolerance and may have survived in multiple refugia by moving to lower elevation areas during the glacial periods. On the other hand, some sympatric amphibians do not diverge genetically in the region (*Nishizawa et al., 2011; Matsui et al., 2019*). They may have been unable to live in harshly cold and dry environments at the glacial, and they could only have one refugium in the south region even if there were refugia in the Tohoku region. These differences may reflect current ecological characteristics such as habitat elevation and breeding season. The divergence between clade A1 and A2, between Tohoku region clades and clade A3, and between clade B2 and *B. torrenticola* occurred in the Quaternary.

411 The effective population sizes of clade A3 and *B. torrenticola* increased around 10
412 thousand years ago (Kya), sometime after the last glacial period. The region with high genetic
413 diversity for clade A3 could be found on both sides of the high elevation area in central Japan,
414 especially the eastern side, which is also demonstrated by the bimodal mismatch distribution,
415 indicating a contemporary geographic barrier to gene flow (*Bremer et al., 2005*). The high
416 elevation areas in central Japan were covered with glaciers followed by volcanic activities (*Ono*
417 *et al., 2005; Shiba, 2021*), which may have prevented clade A3 from expanding the distribution
418 soon after LGM. Even if the population of clade A3 was fragmented, we could not find any
419 phylogroup in the clade, which may be due to the high mobility when their spatial and
420 population expansion, as suggested by many other *Bufo* species (*Yu, Lin & Weng, 2014; Borzée*
421 *et al., 2017; Dufresnes et al., 2020*).
422 The suitable area for *B. torrenticola* at LGM vanished except the southern end of their
423 distribution, but we could consider that the coastal area of the Sea of Japan on the Hokuriku
424 region also was the suitable habitat, applying the similar niche with clade A1. Genetic Landscape
425 Shape interpolation analysis suggested that the highest genetic diversity area was a southern area,
426 and the northern area also has high genetic diversity, and these regions may have become refugia.
427 The expansion degree of the effective population size for *B. torrenticola* was lower than for *B.*
428 *japonicus*, probably because the lotic environments were more available than the lentic
429 environments under the dry climate in the glacial period, and *B. torrenticola* may be less affected
430 by the glacial climate than *B. japonicus*. Considering that the northern and southern ends of the
431 distribution have become refugia, the undistributed central region with high genetic diversity
432 may indicate the separation between north and southern populations. There may have been some
433 factors preventing the expansion to the present overlapped area until 10 Kya because *B.*

434 *torrenticola* increased their effective population size not immediately after LGM, as was the case
435 with clade A3, and their distribution overlap now. Therefore, it would be appropriate to adopt
436 MIROC as a suitable habit for clade A3 in LGM, which included the unsuitable areas from
437 Hokuriku to the Kii Peninsula.

438 The suitable area for clade B1 at LGM in CCSM and MIROC has almost vanished from
439 their present distribution area. We identified the high genetic diversity for clade B1 on the
440 western side of their distribution and central area of the Kinki region, which were shown to be
441 the suitable area for clade A3 with a similar niche to clade B1. The low-elevation areas in the
442 central Chugoku region (*Sonehara et al., 2020*) also had high genetic diversity, although this
443 area was not identified as suitable habitat for any clade with a niche similar to clade B1. These
444 areas with high genetic diversity coincide with the region of the paleo-rivers (*Sakaguchi et al.,*
445 *2021*), indicating that clade B1 could keep their population along the paleo-rivers.

446 For clade B2, the estimated area at LGM in MIROC is more suitable than CCSM because
447 the suitable habitat in the region connected by land with Kyushu in CCSM vanished. The
448 suitable area may be in the central and southern regions in Kyushu, considering the suitable
449 habitat of clade B1, which had a similar niche. Contrary to the result of ENM, the areas with
450 high genetic diversity were distributed in the northern and southern areas in Kyushu. The
451 volcanic activities in the central area of Kyushu (*Mahony et al., 2011*) might prevent clade B2
452 from inhabiting, which was also suggested by the increase of the effective population size after
453 LGM. The vegetation at central Kyushu was also affected by the volcanic activity around LGM,
454 adding to the cool climate (*Miyabuchi et al., 2012*; *2020*). *Hynobius yatsui* distributed in Kyushu
455 diverged into the northern and southern population by the central Kyushu (*Sakamoto et al., 2009*),
456 indicating the volcanic activity has long restricted the amphibian migration.

457 The refugia are often consistent with the stable climate areas since LGM, and they
458 frequently harbor highly endemic faunas (*Sandel et al., 2011*). The climate stability between the
459 LGM and the present day is indicated to be a better predictor of species richness of the European
460 amphibian species (*Araújo et al., 2008*). However, *Lehtomäki et al. (2018)* suggested that the
461 historical climate stability was of relatively minor importance for the Japanese amphibians,
462 though this previous study could not reflect the characteristics of each species. They also
463 suggested that historical climate stability was predominantly important for plants species
464 richness. Our identified refugia of the Japanese toads tended to coincide with the areas with plant
465 species richness in *Lehtomäki et al. (2018)*. Accordingly, the distributions of each lineage of the
466 Japanese toads are likely to be affected by the climate stability after expansion from the refugia.
467

468 **Conclusions**

469 Our phylogeography of the Japanese toads provided insight into the diverged process of their
470 lineages. Most of the divergence data and patterns between the lineages were similar to those of
471 other amphibians. The tectonic events during the construction of the Japanese Archipelago and
472 the glacial-interglacial cycle on the Quaternary may have diverged the lineages in each region.
473 Furthermore, demographic analyses and ENM revealed the localities of refugia. Except for a
474 clade influenced by volcanic activities, refugia were constructed in the areas with climate
475 stability. The present distributions of genetic diversities have been formed by expansion from the
476 refugia after LGM. The interactions between clades after expansion may also influence the
477 current distribution, which will be revealed by examining the effects of gene flow on the
478 secondary contact zones between clades.
479

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References

Aiello-Lammens ME, Boria RA, Radosavljevic A, Vilela B, Anderson RP. 2015. spThin: an R package for spatial thinning of species occurrence records for use in ecological niche models. *Ecography* **38**:541–545 DOI: 10.1111/ecog.01132.

Akaike H. 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19**:716–723 DOI: 10.1109/tac.1974.1100705.

Alvarado-Serrano DF, Knowles LL. 2014. Ecological niche models in phylogeographic studies: applications, advances and precautions. *Molecular Ecology Resources* **14**:233–248 DOI: 10.1111/1755-0998.12184.

Angelis K, Reis MD. 2015. The impact of ancestral population size and incomplete lineage sorting on Bayesian estimation of species divergence times. *Current Zoology* **61**:874–885 DOI: 10.1093/czoolo/61.5.874.

Aoki K, Kato M, Murakami N. 2011. Phylogeography of phytophagous weevils and plant species in broadleaved evergreen forests: A congruent genetic gap between western and eastern parts of Japan. *Insects* **2**:128–150 DOI: 10.3390/insects2020128.

Aoki G, Matsui M, Nishikawa K. 2013. Mitochondrial cytochrome b phylogeny and historical biogeography of the Tohoku Salamander, *Hynobius Lichenatus* (Amphibia, Caudata). *Zoological Science* **30**:167–173 DOI: 10.2108/zsj.30.167.

Araújo MB, Nogués-Bravo D, Diniz-Filho JAF, Haywood AM, Valdes PJ, Rahbek C. 2008. Quaternary climate changes explain diversity among reptiles and amphibians. *Ecography* **31**:8–15 DOI: 10.1111/j.2007.0906-7590.05318.x.

Arntzen JW, McAtear J, Butôt R, Martínez-Solano I. 2018. A common toad hybrid zone that runs from the Atlantic to the Mediterranean. *Amphibia-Reptilia* **39**:41–50 DOI: 10.1163/15685381-00003145.

Becker RA, Wilks AR, Brownrigg R. 2018. *mapdata: Extra Map Databases. R Package Version 2.3.0.*

Blaustein AR, Walls SC, Bancroft BA, Lawler JJ, Searle CL, Gervasi SS. 2010. Direct and indirect effects of climate change on amphibian populations. *Diversity* **2**:281–313 DOI: 10.3390/d2020281.

Borzée A, Santos JL, Sánchez-RamÍrez S, Bae Y, Heo K, Jang Y, Jowers MJ. 2017. Phylogeographic and population insights of the Asian common toad (*Bufo gargarizans*) in Korea and China: population isolation and expansions as response to the ice ages. *PeerJ* **5**:e4044 DOI: 10.7717/peerj.4044.

Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, Heled J, Jones G, Kühnert D, Maio ND, Matschiner M, Mendes FK, Müller NF, Ogilvie HA, Plessis L du, Popinga A, Rambaut A, Rasmussen D, Siveroni I, Suchard MA, Wu C-H, Xie D, Zhang C, Stadler T, Drummond AJ. 2019. BEAST 2.5: An advanced

software platform for Bayesian evolutionary analysis. *PLoS Computational Biology* **15**:e1006650 DOI: 10.1371/journal.pcbi.1006650.

Bremer JRA, Viñas J, Mejuto J, Ely B, Pla C. 2005. Comparative phylogeography of Atlantic bluefin tuna and swordfish: the combined effects of vicariance, secondary contact, introgression, and population expansion on the regional phylogenies of two highly migratory pelagic fishes. *Molecular Phylogenetics and Evolution* **36**:169–187 DOI: 10.1016/j.ympev.2004.12.011.

Carey C, Alexander MA. 2003. Climate change and amphibian declines: is there a link? *Diversity and Distributions* **9**:111–121 DOI: 10.1046/j.1472-4642.2003.00011.x.

Chiocchio A, Arntzen JanW, Martínez-Solano I, Vries W de, Bisconti R, Pezzarossa A, Maiorano L, Canestrelli D. 2021. Reconstructing hotspots of genetic diversity from glacial refugia and subsequent dispersal in Italian common toads (*Bufo bufo*). *Scientific Reports* **11**:260 DOI: 10.1038/s41598-020-79046-y.

Clark PU, Archer D, Pollard D, Blum JD, Rial JA, Brovkin V, Mix AC, Pisias NG, Roy M. 2006. The middle Pleistocene transition: characteristics, mechanisms, and implications for long-term changes in atmospheric pCO_2. *Quaternary Science Reviews* **25**:3150–3184 DOI: 10.1016/j.quascirev.2006.07.008.

Collart F, Hedenäs L, Broennimann O, Guisan A, Vanderpoorten A. 2021. Intraspecific differentiation: Implications for niche and distribution modelling. *Journal of Biogeography* **48**:415–426 DOI: 10.1111/jbi.14009.

Comes HP, Kadereit JW. 1998. The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**:432–438 DOI: 10.1016/s1360-1385(98)01327-2.

546 **Crandall ED, Sbrocco EJ, DeBoer TS, Barber PH, Carpenter KE. 2012.** Expansion dating:
547 calibrating molecular clocks in marine species from expansions onto the Sunda Shelf
548 following the Last Glacial Maximum. *Molecular Biology and Evolution* **29**:707–719 DOI:
549 10.1093/molbev/msr227.
550 **Drummond AJ, Rambaut A, Shapiro B, Pybus OG. 2005.** Bayesian coalescent inference of
551 past population dynamics from molecular sequences. *Molecular Biology and Evolution*
552 **22**:1185–1192 DOI: 10.1093/molbev/msi103.
553 **Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012.** Bayesian phylogenetics with BEAUti
554 and the BEAST 1.7. *Molecular Biology and Evolution* **29**:1969–1973 DOI:
555 10.1093/molbev/mss075.
556 **Dufresnes C, Litvinchuk SN, Borzée A, Jang Y, Li J-T, Miura I, Perrin N, Stöck M. 2016.**
557 Phylogeography reveals an ancient cryptic radiation in East-Asian tree frogs (*Hyla japonica*
558 group) and complex relationships between continental and island lineages. *BMC*
559 *Evolutionary Biology* **16**:253 DOI: 10.1186/s12862-016-0814-x.
560 **Dufresnes C, Litvinchuk SN, Rozenblut-Kościsty B, Rodrigues N, Perrin N, Crochet P,**
561 **Jeffries DL. 2020.** Hybridization and introgression between toads with different sex
562 chromosome systems. *Evolution Letters* **4**:444–456 DOI: 10.1002/evl3.191.
563 **Excoffier L. 2004.** Patterns of DNA sequence diversity and genetic structure after a range
564 expansion: Lessons from the infinite-island model. *Molecular Ecology* **13**:853–864 DOI:
565 10.1046/j.1365-294x.2003.02004.x.
566 **Excoffier L, Lischer HEL. 2010.** Arlequin suite ver 3.5: a new series of programs to perform
567 population genetics analyses under Linux and Windows. *Molecular Ecology Resources*
568 **10**:564–567 DOI: 10.1111/j.1755-0998.2010.02847.x.

569 **Fick SE, Hijmans RJ. 2017.** WorldClim 2: new 1-km spatial resolution climate surfaces for
570 global land areas. *International Journal of Climatology* **37**:4302–4315 DOI:
571 10.1002/joc.5086.
572 **Fu Y-X. 1997.** Statistical tests of neutrality of mutations against population growth, hitchhiking
573 and background selection. *Genetics* **147**:915–925 DOI: 10.1093/genetics/147.2.915.
574 **Funk DJ, Omland KE. 2003.** Species-level paraphyly and polyphyly: frequency, causes, and
575 consequences, with insights from animal mitochondrial DNA. *Ecology, Evolution, and*
576 *Systematics* **34**:397–423 DOI: 10.1146/annurev.ecolsys.34.011802.132421.
577 **Garcia-Porta J, Litvinchuk SN, Crochet PA, Romano A, Geniez PH, Lo-Valvo M,**
578 **Lymberakis P, Carranza S. 2012.** Molecular phylogenetics and historical biogeography of
579 the west-palearctic common toads (*Bufo bufo* species complex). *Molecular Phylogenetics and*
580 *Evolution* **63**:113–130 DOI: 10.1016/j.ympev.2011.12.019.
581 **Gent PR, Danabasoglu G, Donner LJ, Holland MM, Hunke EC, Jayne SR, Lawrence DM,**
582 **Neale RB, Rasch PJ, Vertenstein M, Worley PH, Yang Z-L, Zhang M. 2011.** The
583 community climate system model version 4. *Journal of Climate* **24**:4973–4991 DOI:
584 10.1175/2011jcli4083.1.
585 **Grant WS. 2015.** Problems and cautions with sequence mismatch analysis and Bayesian skyline
586 plots to infer historical demography. *Journal of Heredity* **106**:333–346 DOI:
587 10.1093/jhered/esv020.
588 **Grant W, Bowen B. 1998.** Shallow population histories in deep evolutionary lineages of marine
589 fishes: Insights from sardines and anchovies and lessons for conservation. *Journal of*
590 *Heredity* **89**:415–426 DOI: 10.1093/jhered/89.5.415.

Harpending HC. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human biology* **66**:591–600.

Hase K, Shimada M, Nikoh N. 2012. High degree of mitochondrial haplotype diversity in the Japanese common toad *Bufo japonicus* in urban Tokyo. *Zoological Science* **31**:702–708 DOI: 10.2108/zsj.29.702.

Hatano N, Yoshida K. 2017. Sedimentary environment and paleosols of middle Miocene fluvial and lacustrine sediments in central Japan: Implications for paleoclimate interpretations. *Sedimentary Geology* **347**:117–129 DOI: 10.1016/j.sedgeo.2016.11.004.

Hayes KA, Joshi RC, Thiengo SC, Cowie RH. 2008. Out of South America: multiple origins of non-native apple snails in Asia. *Diversity and Distributions* **14**:701–712 DOI: 10.1111/j.1472-4642.2008.00483.x.

Herbert TD, Lawrence KT, Tzanova A, Peterson LC, Caballero-Gill R, Kelly CS. 2016. Late Miocene global cooling and the rise of modern ecosystems. *Nature Geoscience* **9**:843–847 DOI: 10.1038/ngeo2813.

Hewitt GM. 2004. Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **359**:183–195 DOI: 10.1098/rstb.2003.1388.

Hickerson MJ, Carstens BC, Cavender-Bares J, Crandall KA, Graham CH, Johnson JB, Rissler L, Victoriano PF, Yoder AD. 2010. Phylogeography's past, present, and future: 10 years after Avise, 2000. *Molecular Phylogenetics and Evolution* **54**:291–301 DOI: 10.1016/j.ympev.2009.09.016.

Ho SYW, Larson G. 2006. Molecular clocks: when times are a-changin'. *Trends in Genetics* **22**:79–83 DOI: 10.1016/j.tig.2005.11.006.

Ho SYW, Phillips MJ, Cooper A, Drummond AJ. 2005. Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. *Molecular Biology and Evolution* **22**:1561–1568 DOI: 10.1093/molbev/msi145.

Ho SYW, Saarma U, Barnett R, Haile J, Shapiro B. 2008. The effect of inappropriate calibration: Three case studies in molecular ecology. *PLoS ONE* **3**:e1615 DOI: 10.1371/journal.pone.0001615.

Hoareau TB. 2016. Late glacial demographic expansion motivates a clock overhaul for population genetics. *Systematic Biology* **65**:449–464 DOI: 10.1093/sysbio/syv120.

Igawa T, Kurabayashi A, Nishioka M, Sumida M. 2006. Molecular phylogenetic relationship of toads distributed in the Far East and Europe inferred from the nucleotide sequences of mitochondrial DNA genes. *Molecular Phylogenetics and Evolution* **38**:250–260 DOI: 10.1016/j.ympev.2005.09.003.

Iwaoka Y, Watanabe T, Satoh SS, Nambu H, Yamazaki Y. 2021. Hybridization of two species of Japanese toads, *Bufo torrenticola* and *Bufo japonicus formosus*, in the central part of Japan. *Zoological Science* **38**:506–512 DOI: 10.2108/zs210023.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* **30**:772–780 DOI: 10.1093/molbev/mst010.

Kawamura T, Nishioka M, Sumida M, Ryuzaki M. 1990. An electrophoretic study of genetic differentiation in 40 populations of *Bufo japonicus* distributed in Japan. *Scientific report of the Laboratory for Amphibian Biology, Hiroshima University* **10**:1–51 DOI: 10.15027/291.

Kubota Y, Kusumoto B, Shiono T, Tanaka T. 2017. Phylogenetic properties of Tertiary relict flora in the east Asian continental islands: imprint of climatic niche conservatism and in situ diversification. *Ecography* **40**:436–447 DOI: 10.1111/ecog.02033.

Kubota Y, Shiono T, Kusumoto B. 2015. Role of climate and geohistorical factors in driving plant richness patterns and endemicity on the east Asian continental islands. *Ecography* **38**:639–648 DOI: 10.1111/ecog.00981.

Leaché AD, Harris RB, Rannala B, Yang Z. 2014. The influence of gene flow on species tree estimation: A simulation study. *Systematic Biology* **63**:17–30 DOI: 10.1093/sysbio/syt049.

Lehtomäki J, Kusumoto B, Shiono T, Tanaka T, Kubota Y, Moilanen A. 2018. Spatial conservation prioritization for the East Asian islands: A balanced representation of multitaxon biogeography in a protected area network. *Diversity and Distributions* **25**:414–429 DOI: 10.1111/ddi.12869.

Lisiecki LE, Raymo ME. 2005. A Pliocene-Pleistocene stack of 57 globally distributed benthic $\delta^{18}O$ records. *Paleoceanography* **20**:PA1003 DOI: 10.1029/2004pa001071.

Lomolino MV, Riddle BR, Whittaker RJ, Brown JH. 2010. *Biogeography*. Sunderland, Massachusetts: Sinauer Associates, Inc.

Maddison WP. 1997. Gene trees in species trees. *Systematic Biology* **46**:523–536 DOI: 10.1093/sysbio/46.3.523.

Mahony SH, Wallace LM, Miyoshi M, Villamor P, Sparks RSJ, Hasenaka T. 2011. Volcano-tectonic interactions during rapid plate-boundary evolution in the Kyushu region, SW Japan. *Geological Society of America bulletin* **123**:2201–2223 DOI: 10.1130/b30408.1.

656 **Martínez-Monzón A, Cuenca-Bescós G, Bisbal-Chinesta J, Blain H. 2021.** One million years
657 of diversity shifts in amphibians and reptiles in a Mediterranean landscape: Resilience rules
658 the Quaternary. *Palaeontology* **64**:673–686 DOI: 10.1111/pala.12547.
659 **Matsui M. 1984.** Morphometric variation analyses and revision of the Japanese toads (Genus
660 *Bufo*, Bufonidae). *Contributions from the Biological Laboratory, Kyoto University* **26**:209–
661 428.
662 **Matsui, M. 1986.** Geographic variation in toads of the *Bufo bufo* complex from the Far East,
663 with a description of a new subspecies. *Copeia* **1986**: 561–579 DOI: 10.2307/1444939.
664 **Matsui M, Kawahara Y, Nishikawa K, Ikeda S, Eto K, Mizuno Y. 2019.** Molecular
665 phylogeny and evolution of two *Rhacophorus* species endemic to mainland Japan. *Asian*
666 *Herpetological Research* **10**:86–104 DOI: 10.16373/j.cnki.ahr.190015.
667 **Matsui M, Maeda N. 2018.** *Encyclopedia of Japanese frogs*. Tokyo: Bun-ichi Sogo Shuppan.
668 **Matsui M, Yoshikawa N, Aoki G, Sasamori K, Matsui M, Tanabe S, Misawa Y, Nishikawa**
669 **K. 2020.** Wide distribution but low differentiation: Phylogenetic relationships and
670 phylogeography of *Hynobius nigrescens* (Amphibia: Caudata). *Zoological Science* **37**:529–
671 537 DOI: 10.2108/zs200099.
672 **McCormack JE, Zellmer AJ, Knowles LL. 2010.** Does niche divergence accompany allopatric
673 divergence in *Aphelocoma* Jays as predicted under ecological speciation?: Insights from tests
674 with niche models. *Evolution* **64**:1231–1244 DOI: 10.1111/j.1558-5646.2009.00900.x.
675 **McGuire JA, Linkem CW, Koo MS, Hutchison DW, Lappin AK, Orange DI,**
676 **Lemos-Espinal J, Riddle BR, Jaeger JR. 2007.** Mitochondrial introgression and incomplete
677 lineage sorting through space and time: phylogenetics of crotaphytid lizards. *Evolution*
678 **61**:2879–2897 DOI: 10.1111/j.1558-5646.2007.00239.x.

679 **McKay BD, Zink RM. 2010.** The causes of mitochondrial DNA gene tree paraphyly in birds.
680 *Molecular Phylogenetics and Evolution* **54**:647–650 DOI: 10.1016/j.ympev.2009.08.024.
681 **Miller MP. 2005.** Allele In Space (AIS): Computer software for the joint analysis of
682 interindividual spatial and genetic information. *Journal of Heredity* **96**:722–724 DOI:
683 10.1093/jhered/esi119.
684 **Miller MP, Bellinger MR, Forsman ED, Haig SM. 2006.** Effects of historical climate change,
685 habitat connectivity, and vicariance on genetic structure and diversity across the range of the
686 red tree vole (*Phenacomys longicaudus*) in the Pacific Northwestern United States.
687 *Molecular Ecology* **15**:145–159 DOI: 10.1111/j.1365-294x.2005.02765.x.
688 **Miyabuchi Y, Sugiyama S. 2020.** Vegetation history after the late period of the Last Glacial
689 Age based on phytolith records in Nangodani Valley basin, southern part of the Aso caldera,
690 Japan. *Journal of Quaternary Science* **35**:304–315 DOI: 10.1002/jqs.3153.
691 **Miyabuchi Y, Sugiyama S, Nagaoka Y. 2012.** Vegetation and fire history during the last
692 30,000 years based on phytolith and macroscopic charcoal records in the eastern and western
693 areas of Aso Volcano, Japan. *Quaternary International* **254**:28–35 DOI:
694 10.1016/j.quaint.2010.11.019.
695 **Momohara A. 2016.** Stages of major floral change in Japan based on macrofossil evidence and
696 their connection to climate and geomorphological changes since the Pliocene. *Quaternary*
697 *International* **397**:93–105 DOI: 10.1016/j.quaint.2015.03.008.
698 **Nakazato T, Warren DL, Moyle LC. 2010.** Ecological and geographic modes of species
699 divergence in wild tomatoes. *American Journal of Botany* **97**:680–693 DOI:
700 10.3732/ajb.0900216.

Nishikawa K. 2017. Species diversity of Japanese amphibians: Recent progress and future prospects of systematic studies. In: Motokawa M, Kajihara H eds. *Species Diversity of Animals in Japan. Diversity and Commonality in Animals.* Tokyo: Springer, 165–181 DOI: 10.1007/978-4-431-56432-4_6.

Nishizawa T, Kurabayashi A, Kunihara T, Sano N, Fujii T, Sumida M. 2011. Mitochondrial DNA diversification, molecular phylogeny, and biogeography of the primitive rhacophorid genus *Buergeria* in East Asia. *Molecular Phylogenetics and Evolution* **59**:139–147 DOI: 10.1016/j.ympev.2011.01.015.

Nunome M, Torii H, Matsuki R, Kinoshita G, Suzuki H. 2010. The Influence of Pleistocene refugia on the evolutionary history of the Japanese hare, *Lepus brachyurus*. *Zoological Science* **27**:746–754 DOI: 10.2108/zsj.27.746.

Ono Y, Aoki T, Hasegawa H, Dali L. 2005. Mountain glaciation in Japan and Taiwan at the global Last Glacial Maximum. *Quaternary International* **138–139**:79–92 DOI: 10.1016/j.quaint.2005.02.007.

Peterson AT. 2011. Ecological niche conservatism: a time-structured review of evidence. *Journal of Biogeography* **38**:817–827 DOI: 10.1111/j.1365-2699.2010.02456.x.

Phillips SJ, Anderson RP, Schapire RE. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* **190**:231–259 DOI: 10.1016/j.ecolmodel.2005.03.026.

Provan J, Bennett KD. 2008. Phylogeographic insights into cryptic glacial refugia. *Trends in Ecology & Evolution* **23**:564–571 DOI: 10.1016/j.tree.2008.06.010.

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* **67**:901–904 DOI: 10.1093/sysbio/syy032.

Ray N, Currat M, Excoffier L. 2003. Intra-deme molecular diversity in spatially expanding populations. *Molecular Biology and Evolution* **20**:76–86 DOI: 10.1093/molbev/msg009.

Recuero E, Canestrelli D, Vörös J, Szabó K, Poyarkov NA, Arntzen JW, Crnobrnja-Isailovic J, Kidov AA, Cogălniceanu D, Caputo FP, Nascetti G, Martínez-Solano I. 2012. Multilocus species tree analyses resolve the radiation of the widespread *Bufo bufo* species group (Anura, Bufonidae). *Molecular Phylogenetics and Evolution* **62**:71–86 DOI: 10.1016/j.ympev.2011.09.008.

Rogers AR, Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* **9**:552–69 DOI: 10.1093/oxfordjournals.molbev.a040727.

Ronquist F, Teslenko M, Mark P van der, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**:539–542 DOI: 10.1093/sysbio/sys029.

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A. 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular biology and evolution* **34**:3299–3302 DOI: 10.1093/molbev/msx248.

Sakaguchi S, Asaoka Y, Takahashi D, Isagi Y, Imai R, Nagano AJ, Qiu Y-X, Li P, Lu R, Setoguchi H. 2021. Inferring historical survivals of climate relicts: the effects of climate

744 changes, geography, and population-specific factors on herbaceous hydrangeas. *Heredity*
745 **126**:615–629 DOI: 10.1038/s41437-020-00396-4.
746 **Sandel B, Arge L, Dalsgaard B, Davies RG, Gaston KJ, Sutherland WJ, Svenning J-C.**
747 **2011.** The influence of Late Quaternary climate-change velocity on species endemism.
748 *Science* **334**:660–664 DOI: 10.1126/science.1210173.
749 **Sawaura R, Sawada J, Sato T, Suzuki T, Sasaki K. 2018.** Late Pleistocene hares of the
750 Japanese archipelago: Paleobiogeographic implication at the Last Glacial Maximum.
751 *International Journal of Osteoarchaeology* **28**:179–187 DOI: 10.1002/oa.2645.
752 **Schoener TW. 1968.** The Anolis Lizards of Bimini: Resource partitioning in a complex fauna.
753 *Ecology* **49**:704–726 DOI: 10.2307/1935534.
754 **Schwarz G. 1978.** Estimating the dimension of a model. *The Annals of Statistics* **6**:461–464
755 DOI: 10.1214/aos/1176344136.
756 **Shapiro B, Drummond AJ, Rambaut A, Wilson MC, Matheus PE, Sher AV, Pybus OG,**
757 **Gilbert MTP, Barnes I, Binladen J, Willerslev E, Hansen AJ, Baryshnikov GF, Burns**
758 **JA, Davydov S, Driver JC, Froese DG, Harington CR, Keddie G, Kosintsev P, Kunz**
759 **ML, Martin LD, Stephenson RO, Storer J, Tedford R, Zimov S, Cooper A. 2004.** Rise
760 and fall of the Beringian steppe bison. *Science* **306**:1561–1565 DOI:
761 10.1126/science.1101074.
762 **Shiba M. 2021.** Characteristics of crustal uplift since the Pliocene in central Honshu, Japan, and
763 sea level rise. *Earth Science (Chikyu Kagaku)* **75**:37–55 DOI:
764 10.15080/agcjchikyukagaku.75.1_37.

765 **Smith SA, Donoghue MJ. 2010.** Combining historical biogeography with niche modeling in the
766 *Caprifolium* clade of *Lonicera* (Caprifoliaceae, Dipsacales). *Systematic Biology* **59**:322–341
767 DOI: 10.1093/sysbio/syq011.
768 **Sonehara T, Yagi K, Takeshita H, Aoki K, Aoki S, Otofuji Y, Itaya T. 2020.** Kibi Plateau: A
769 stable-coherent tectonic unit in the active Japanese Islands. *Scientific Reports* **10**:3786 DOI:
770 10.1038/s41598-020-60448-x.
771 **Stamatakis A. 2014.** RAxML version 8: A tool for phylogenetic analysis and post-analysis of
772 large phylogenies. *Bioinformatics* **30**:1312–1313 DOI: 10.1093/bioinformatics/btu033.
773 **Sumida M, Ogata M. 1998.** Intraspecific differentiation in the Japanese brown frog *Rana*
774 *japonica* inferred from mitochondrial DNA sequences of the cytochrome *b* gene. *Zoological*
775 *Science* **15**:989–1000 DOI: 10.2108/zsj.15.989.
776 **Suzuki D, Kawase T, Hoshina T, Tokuda T. 2020.** Origins of nonnative populations of *Bufo*
777 *japonicus formosus* (Amphibia: Bufonidae) in Hokkaido, Japan, as inferred by a molecular
778 approach. *Current Herpetology* **39**:47–54 DOI: 10.5358/hsj.39.47.
779 **Suzuki Y, Tomozawa M, Koizumi Y, Tsuchiya K, Suzuki H. 2015.** Estimating the molecular
780 evolutionary rates of mitochondrial genes referring to Quaternary ice age events with inferred
781 population expansions and dispersals in Japanese Apodemus. *BMC Evolutionary Biology*
782 **15**:187 DOI: 10.1186/s12862-015-0463-5.
783 **Taberlet P, Fumagalli L, Wust-Saucy A-G, Cosson J-F. 1998.** Comparative phylogeography
784 and postglacial colonization routes in Europe. *Molecular Ecology* **7**:453–464 DOI:
785 10.1046/j.1365-294x.1998.00289.x.
786 **Tanabe AS. 2011.** Kakusan4 and Aminosan: two programs for comparing nonpartitioned,
787 proportional and separate models for combined molecular phylogenetic analyses of

788 multilocus sequence data. *Molecular Ecology Resources* **11**:914–921 DOI: 10.1111/j.1755-
789 0998.2011.03021.x.
790 **Toews DPL, Brelsford A. 2012.** The biogeography of mitochondrial and nuclear discordance in
791 animals. *Molecular Ecology* **21**:3907–3930 DOI: 10.1111/j.1365-294x.2012.05664.x.
792 **Tomaru N, Takahashi M, Tsumura Y, Takahashi M, Ohba K. 1998.** Intraspecific variation
793 and phylogeographic patterns of *Fagus crenata* (Fagaceae) mitochondrial DNA. *American*
794 *Journal of Botany* **85**:629–636 DOI: 10.2307/2446531.
795 **Tominaga A, Matsui M, Nishikawa K, Tanabe S. 2006.** Phylogenetic relationships of
796 *Hynobius naevius* (Amphibia: Caudata) as revealed by mitochondrial 12S and 16S rRNA
797 genes. *Molecular Phylogenetics and Evolution* **38**:677–684 DOI:
798 10.1016/j.ympev.2005.10.014.
799 **Tominaga A, Matsui M, Yoshikawa N, Nishikawa K, Hayashi T, Misawa Y, Tanabe S, Ota**
800 **H. 2013.** Phylogeny and historical demography of *Cynops pyrrhogaster* (Amphibia:
801 Urodela): Taxonomic relationships and distributional changes associated with climatic
802 oscillations. *Molecular Phylogenetics and Evolution* **66**:654–667 DOI:
803 10.1016/j.ympev.2012.10.015.
804 **Waltari E, Hijmans RJ, Peterson AT, Nyári ÁS, Perkins SL, Guralnick RP. 2007.** Locating
805 Pleistocene refugia: Comparing phylogeographic and ecological niche model predictions.
806 *PLoS ONE* **2**:e563 DOI: 10.1371/journal.pone.0000563.
807 **Warren DL, Glor RE, Turelli M. 2008.** Environmental niche equivalency versus conservatism:
808 Quantitative approaches to niche evolution. *Evolution* **62**:2868–2883 DOI: 10.1111/j.1558-
809 5646.2008.00482.x.

Warren DL, Glor RE, Turelli M. 2010. ENMTools: a toolbox for comparative studies of environmental niche models. *Ecography* **33**:607–611 DOI: 10.1111/j.1600-0587.2009.06142.x.

Watanabe S, Hajima T, Sudo K, Nagashima T, Takemura T, Okajima H, Nozawa T, Kawase H, Abe M, Yokohata T, Ise T, Sato H, Kato E, Takata K, Emori S, Kawamiya M. 2011. MIROC-ESM 2010: model description and basic results of CMIP5-20c3m experiments. *Geoscientific Model Development* **4**:845–872 DOI: 10.5194/gmd-4-845-2011.

Wiens JJ, Graham CH. 2005. Niche conservatism: integrating evolution, ecology, and conservation biology. *Annual Review of Ecology, Evolution, and Systematics* **36**:519–539 DOI: 10.1146/annurev.ecolsys.36.102803.095431.

Yamazaki Y, Kouketsu S, Fukuda T, Araki Y, Nambu H. 2008. Natural hybridization and directional introgression of two species of Japanese toads *Bufo japonicus formosus* and *Bufo torrenticola* (Anura: Bufonidae) resulting from changes in their spawning habitat. *Journal of Herpetology* **48**:427–436 DOI: 10.1670/07-186.1.

Yoshikawa N, Matsui M. 2014. Two new Salamanders of the genus *Onychodactylus* from Eastern Honshu, Japan (Amphibia, Caudata, Hynobiidae). *Zootaxa* **3866**:53–78 DOI: 10.11646/zootaxa.3866.1.3.

Yoshikawa N, Matsui M, Nishikawa K, Kim J-B, Kryukov A. 2008. Phylogenetic relationships and biogeography of the Japanese clawed salamander, *Onychodactylus japonicus* (Amphibia: Caudata: Hynobiidae), and its congener inferred from the mitochondrial cytochrome b gene. *Molecular Phylogenetics and Evolution* **49**:249–259 DOI: 10.1016/j.ympev.2008.07.016.

832 **Yu T-L, Lin H-D, Weng C-F. 2014.** A new phylogeographic pattern of endemic *Bufo*
833 *bankorensis* in Taiwan Island is attributed to the genetic variation of populations. *PLoS ONE*
834 **9**:e98029 DOI: 10.1371/journal.pone.0098029.
835 **Zeisset I, Beebee TJC. 2008.** Amphibian phylogeography: a model for understanding historical
836 aspects of species distributions. *Heredity* **101**:109–19 DOI: 10.1038/hdy.2008.30.

Figure 1

Phylogenetic relationships and distribution of *Bufo japonicus* and *B. torrenticola* based on mitochondrial *cytochrome b* haplotypes.

Bootstrap supports (maximum-likelihood)/posterior probabilities (Bayesian inference) are provided for major nodes. Arrows indicate estimated divergence times and 95% HPD (Mya). Enlarged maps with locality numbers are available in Figure S1, and full haplotype names on the phylogenetic trees are available in Figures S2, respectively. The map was created in QGIS 3.16 (https://qgis.org). The map layer was extracted from GADM database (www.gadm.org, version 3.4). The source of the layer of inland water area was Digital Chart of the World. The elevation layer was created by editing the source data from Geospatial Information Authority of Japan (https://fgd.gsi.go.jp/download/mapGis.php?tab=dem).

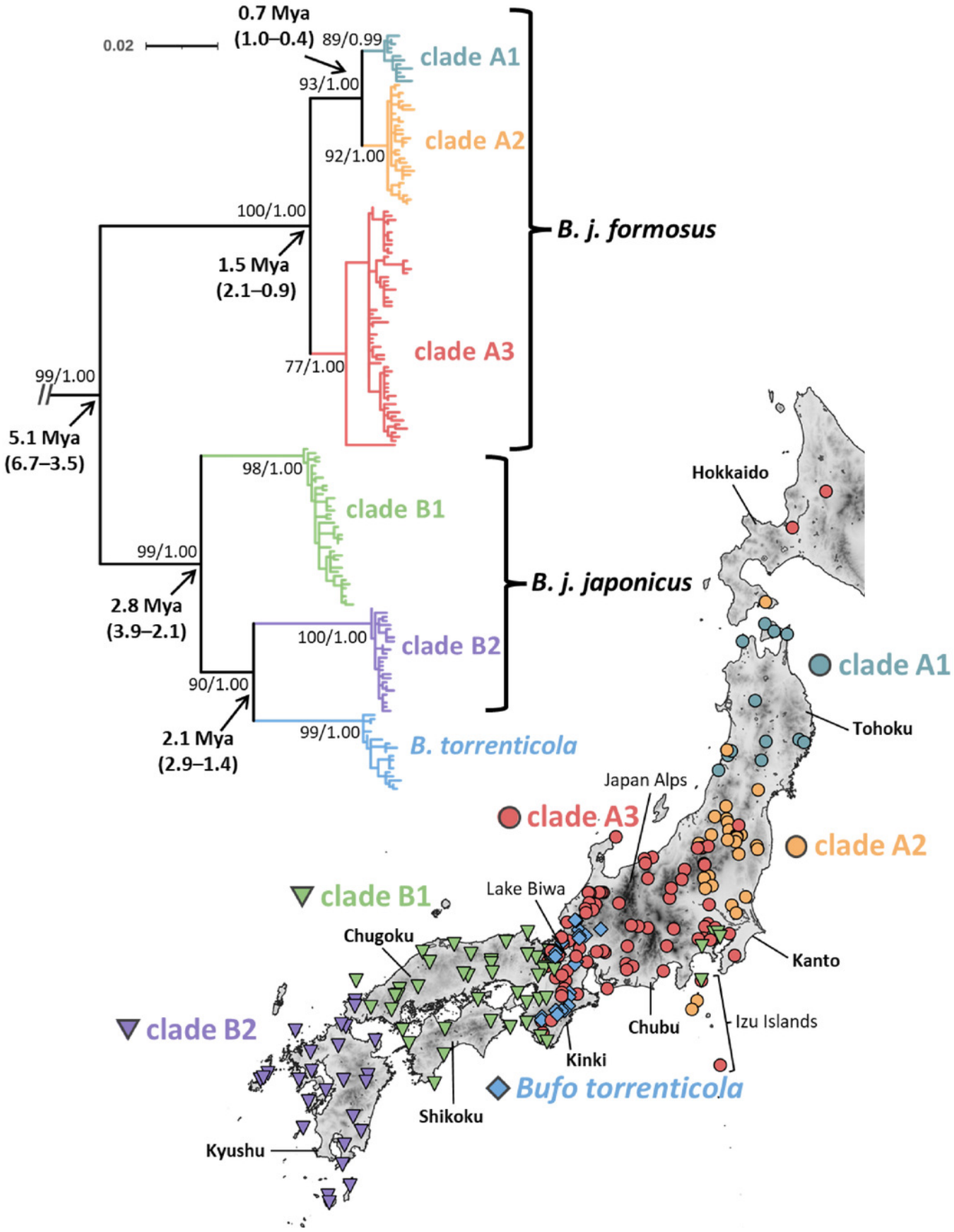
0.02
0.7 Mya
(1.0–0.4)
89/0.99
clade A1
93/1.00
clade A2
92/1.00
100/1.00
1.5 Mya
(2.1–0.9)
B. j. formosus
clade A3
77/1.00
99/1.00
5.1 Mya
(6.7–3.5)
98/1.00
clade B1
99/1.00
2.8 Mya
(3.9–2.1)
B. j. japonicus
100/1.00
clade B2
90/1.00
2.1 Mya
(2.9–1.4)
99/1.00
B. torrenticola
Hokkaido
clade A1
Tohoku
Japan Alps
clade A3
clade A2
clade B1
Lake Biwa
Chugoku
Kanto
clade B2
Chubu
Izu Islands
Kinki
Bufo torrenticola
Shikoku
Kyushu

Figure 2

Demographic analyses of each clade of *Bufo japonicus* and *B. torrenticola* defined from mitochondrial sequences data.

Left charts display the distribution of observed (histograms) and expected (bold dash lines: under demographic expansion, and thin dash lines: under spatial expansion models) pairwise nucleotide differences. The sums of squared deviations (SSD) and *p*-values are shown for demographic and spatial expansion models. Asterisks indicate significant *p*-values ($p < 0.05$). Right charts display Bayesian skyline plots (BSP) showing the evolution of effective population size (N_e) over time (colored solid lines: median estimates, and colored dash lines: 95% confidence intervals of highest posterior densities). The vertical lines show the time to the most recent common ancestor (solid lines: median, and dotted lines: lower estimates).

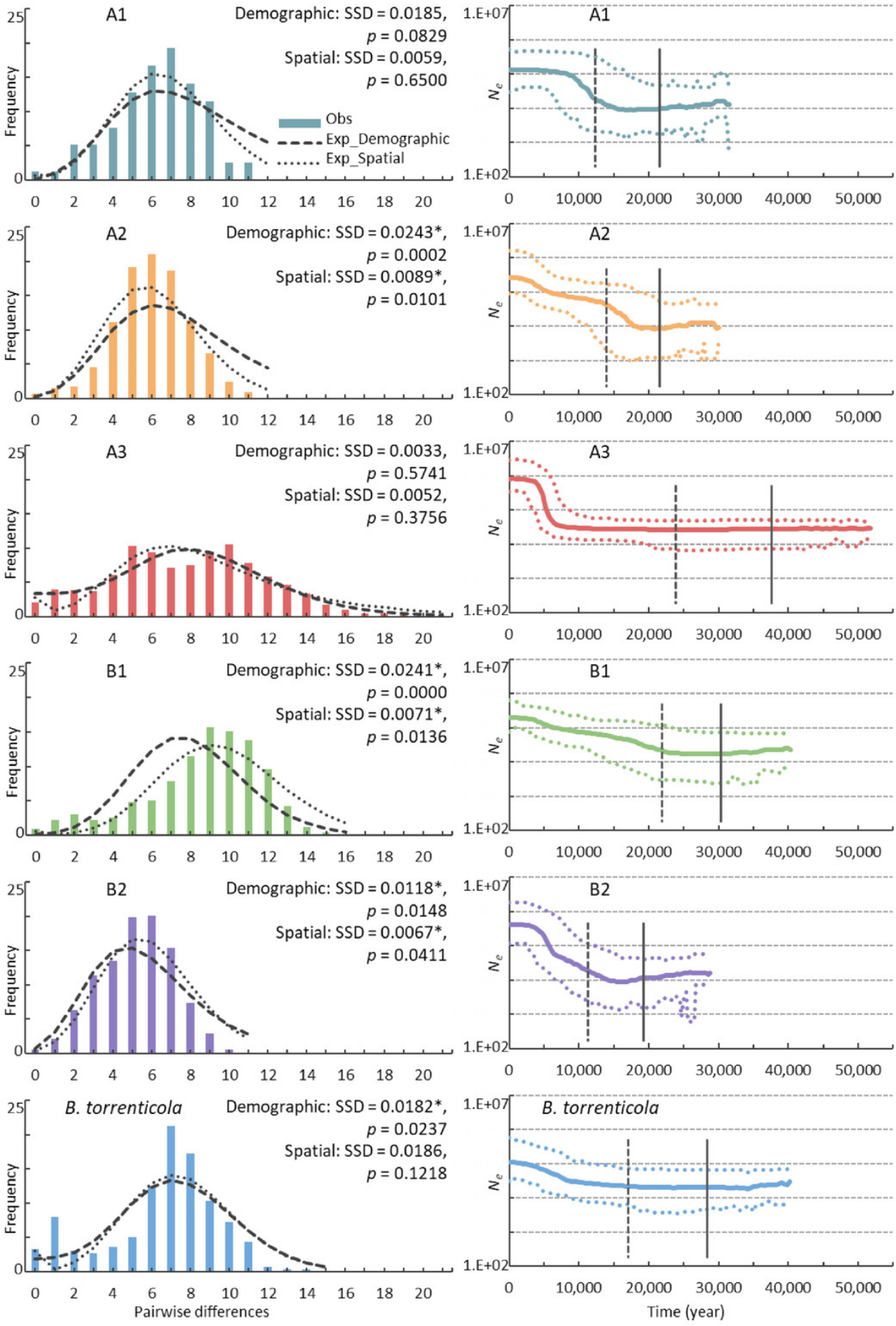
A1
Demographic: SSD = 0.0185, p = 0.0829
Spatial: SSD = 0.0059, p = 0.6500
Obs
Exp_Demographic
Exp_Spatial
A2
Demographic: SSD = 0.0243*, p = 0.0002
Spatial: SSD = 0.0089*, p = 0.0101
A3
Demographic: SSD = 0.0033, p = 0.5741
Spatial: SSD = 0.0052, p = 0.3756
B1
Demographic: SSD = 0.0241*, p = 0.0000
Spatial: SSD = 0.0071*, p = 0.0136
B2
Demographic: SSD = 0.0118*, p = 0.0148
Spatial: SSD = 0.0067*, p = 0.0411
B. torrenticola
Demographic: SSD = 0.0182*, p = 0.0237
Spatial: SSD = 0.0186, p = 0.1218
Frequency
Pairwise differences
Ne
Time (year)

Figure 3

Results of Genetic Landscape Shape interpolation analyses of each clade of *Bufo japonicus* and *B. torrenticola*.

Warmer colors indicate higher genetic diversities between individuals. White circles indicate the localities of samples used for Genetic Landscape Shape interpolation analyses. The maps were created in QGIS 3.16 (https://qgis.org).

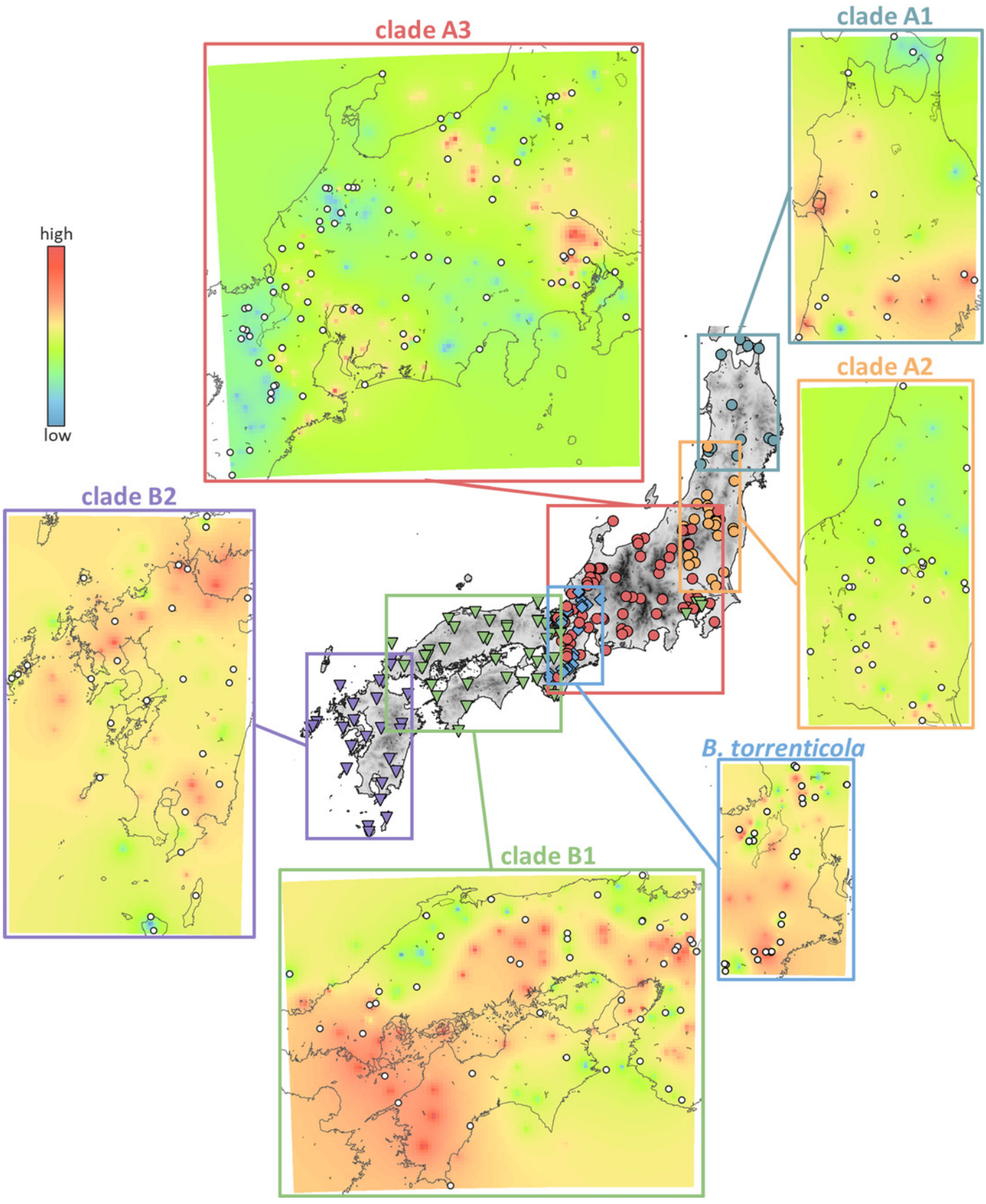
clade A3
clade A1
high
low
clade A2
clade B2
B. torrenticola
clade B1

Figure 4

Predicted suitable distributions under the last glacial maximum (LGM; CCSM and MIROC scenarios) and present conditions for *Bufo japonicus* and *B. torrenticola*.

Warmer colors indicate higher probabilities of occurrence. The maps were created using R package mapdata version 2.3.0 (Becker, Wilks & Brownrigg, 2018).

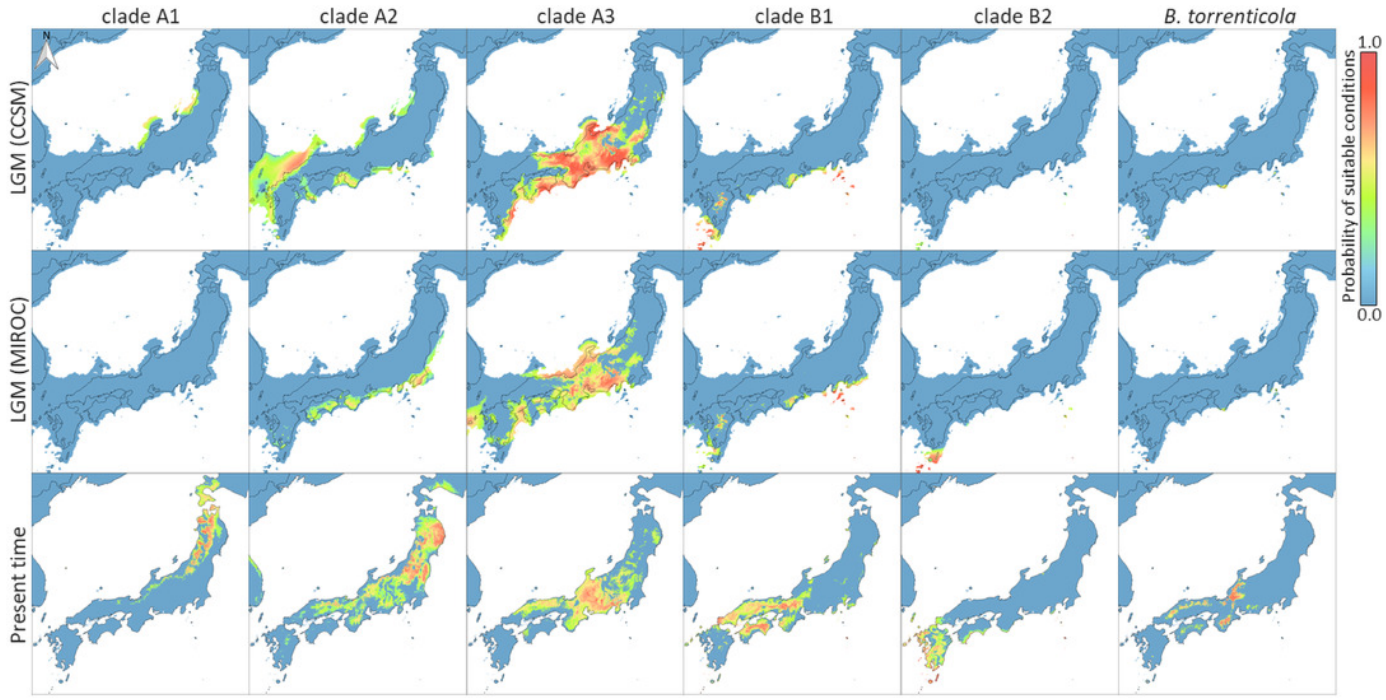

Table 1(on next page)

Genetic diversity indices and neutrality tests for all populations of the five clades of *Bufo japonicus* and *Bufo torrenticola* based on the *cytochrome b* genes.

		N	N_a	$H_d \pm$ SD	$\pi \pm$ SD	Fu's F_S	
						F_S	p-value
Bufo japonicus	clade A1	13	12	0.99 ± 0.04	0.006 ± 0.003	-5.46*	0.0061
	clade A2	33	29	0.99 ± 0.01	0.006 ± 0.003	-24.10*	0
	clade A3	83	57	0.98 ± 0.01	0.007 ± 0.004	-24.83*	0
	clade B1	45	36	0.99 ± 0.01	0.008 ± 0.004	-23.06*	0
	clade B2	28	26	0.99 ± 0.01	0.005 ± 0.003	-24.34*	0
Bufo torrenticola		25	19	0.97 ± 0.02	0.006 ± 0.003	-8.15*	0.0018

N, number of individuals; N_a, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity; SD, standard deviation. Asterisks indicate significant p-values ($p < 0.01$).

Table 2(on next page)

Niche similarity score of Schoener's *D* (above diagonal) and Hellinger's *I* (below diagonal) obtained from known occurrences between lineages of *B. japonicus* and *B. torrenticola*.

	A1	A2	A3	B1	B2	*torrenticola*
A1		0.45	0.26	0.22	0.04	0.20
A2	0.72		0.57	0.47	0.16	0.37
A3	0.52	0.83		0.59	0.21	0.48
B1	0.48	0.77	0.85		0.37	0.51
B2	0.18	0.41	0.49	0.68		0.25
torrenticola	0.46	0.69	0.76	0.78	0.53	

1

Table 3(on next page)

Results of background similarity tests.

The *t*- and *p*-values in two-tailed *t*-tests, and whether the observed niche similarities are more or less similar than expected by chance ($p < 0.01$) are shown.

lineage for the observed distribution			lineage for the background distribution								
			A1			A2			A3		
			t-value	*p*-value	similarity	*t*-value	*p*-value	similarity	*t*-value	*p*-value	similarity
	Schoener's *D*	A1				-4.06	1.E-04	more	-8.48	2.E-13	more
		A2	0.36	0.72	NS				-3.43	9.E-04	more
		A3	-20.32	2.E-16	more	-8.69	8.E-14	more			
		B1	-15.20	2.E-16	more	-837.44	2.E-16	more	-29.88	2.E-16	more
		B2	-32.92	2.E-16	more	-29.73	2.E-16	more	-13.25	2.E-16	more
		torrenticola	-26.40	2.E-16	more	-23.64	2.E-16	more	-69.19	2.E-16	more
	Hellinger's *I*	A1				-1.10	0.27	NS	-3.20	2.E-03	more
		A2	-1.44	0.15	NS				1.52	0.13	NS
		A3	-16.50	2.E-16	more	-5.79	8.E-08	more			
		B1	-15.08	2.E-16	more	-434.87	2.E-16	more	-25.06	2.E-16	more
		B2	-37.04	2.E-16	more	-27.28	2.E-16	more	-10.02	2.E-16	more
		torrenticola	-22.78	2.E-16	more	-20.86	2.E-16	more	-56.47	2.E-16	more

lineage for the observed distribution			lineage for the background distribution								
			B1			B2			*B. torrenticola*		
			t-value	*p*-value	similarity	*t*-value	*p*-value	similarity	*t*-value	*p*-value	similarity
	Schoener's *D*	A1	-43.23	2.E-16	more	32.67	2.E-16	more	-17.43	2.E-16	more
		A2	-37.68	2.E-16	more	-5.29	7.E-07	more	15.41	2.E-16	less
		A3	-20.50	2.E-16	more	13.92	2.E-16	less	31.92	2.E-16	less
		B1				-15.64	2.E-16	more	53.89	2.E-16	less
		B2	-7.63	1.E-11	more				4.45	2.E-05	less
		torrenticola	-36.69	2.E-16	more	-24.64	2.E-16	more			
	Hellinger's *I*	A1	-38.55	2.E-16	more	-19.32	2.E-16	more	-20.14	2.E-16	more
		A2	-30.82	2.E-16	more	-7.38	5.E-11	more	7.02	3.E-10	less
		A3	-19.02	2.E-16	more	12.98	2.E-16	less	35.61	2.E-16	less
		B1				-18.25	2.E-16	more	63.10	2.E-16	less
		B2	-4.01	1.E-04	more				10.53	2.E-16	less
		torrenticola	-30.27	2.E-16	more	-25.15	2.E-16	more			

1