Running title : Phylogeography of a Japanese stonefly

Title: Phylogeography of the stonefly *Kamimuria tibialis*: multiple glacial refugia and sympatric occurrence of different lineages in the southern islands of Japan

Authors: Akiko Otsuki, Kazunori Yoshizawa and Shin-ichi Akimoto

Postal address: Systematic Entomology, Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Corresponding author:
Akiko OTSUKI
Systematic Entomology, Department of Ecology and Systematics, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
Phone +81-(0)11-706-2486
Fax +81-(0)11-706-4939
otsuki@res.agr.hokudai.ac.jp

Abstract

To elucidate the effect of Pleistocene climatic fluctuations on the historical distribution and geographic genetic structure of temperate Japanese species, we performed phylogeographical and demographic analyses using mitochondrial gene sequences obtained from the stonefly species *Kamimuria tibialis*, sampled from four main islands of the Japanese Archipelago (i.e., Hokkaido, Honshu, Shikoku, and Kyushu) and Tsushima Island. We detected three main clades with distinct geographical distributions, including the Tsushima, Kyushu, and Hokkaido-Honshu-Shikoku phylogroups. These groups were estimated to have diverged from one another 0.54-2.02 Mya, suggesting they have undergone several glacial cycles in different refugia. Our results showed that during the glacial epochs and with a fall in sea-level, gene flow was limited among Tsushima and Kyushu, and among Hokkaido and Honshu, probably because the straits between these islands are deep. The population in Kyushu and Shikoku, the southernmost islands, exhibited high genetic diversity, with two distinct haplotype lineages occurring sympatrically. These results suggest that the population division into multiple refugia and the existence of stable southern refugia have contributed to the high genetic diversity of the species in this region.

Key words: phylogeography - Japan - multiple glacial refugia - mtDNA - Plecoptera - population demographic analysis

Introduction

The role of Pleistocene glacial cycles on the distribution and geographical genetic structure of current plant and animal species has been intensively studied over the last two decades, in an attempt to understand the biodiversity of temperate and subarctic regions (Hewitt, 1996, 1999, 2011; Avise, 2000; Willis & Whittaker, 2000; Knowles, 2001b; Tautz, 2004; Weiss & Ferrand, 2007; Wallis *et al*, 2016; Lucati *et al*., 2020). During glacial periods, high latitudes in the Northern Hemisphere were covered with ice sheets and permafrost (Williams *et al*., 1998), which led species to extinct over large part in these regions (Hewitt, 2000). Phylogeographical evidence suggests that there was limited distribution of most temperate species during glacial periods, surviving in southern refugia, and subsequently expanding northward during interglacial periods (Avise, 2000; Hewitt, 2000, 2004; Weiss & Ferrand, 2007). Furthermore, it has been postulated that the isolation of populations within separated refugia during glacial periods promoted genetic differentiation among populations (Hewitt, 1988, 2000; Knowles, 2001b, 2005; Weng *et al*., 2020), and sometimes led to speciation, possibly driven by sexual selection or local adaptations (Hewitt, 1988; Butlin, 1998; Knowles, 2000, 2001a; Tautz, 2004; Carstens & Knowles, 2007, Wallis *et al*, 2016).

The Japanese islands have a diverse array of endemic species, and are recognized as a biodiversity hotspot (Haffer, 1985; Contreras-Medina *et al*., 2001; Tojo *et al*., 2017). This is partly due to the lack of ice sheet development in this region, with the exception of small parts of the alpine region, during glacial periods (Ono & Igarashi, 1991; Yonekura *et al*., 2001). The unglaciated region could have enabled subalpine stonefly species to survive the glacial epochs (McCulloch, 2010). If the area was not

glaciated, cold-tolerant species could have survived the glacial periods, when the surrounding forests and food insects remained there. In addition, the Japanese Archipelago has a complex geography and topography with various climates, which could have enabled many organisms to survive the Pleistocene epoch by latitudinal and altitudinal shifts in their distribution range. Evidence obtained from the analysis of pollen has shown that southern regions along the Pacific coast (Fig. 1b) were mild in climate even during the glacial periods because of the influence of a warm current (Ohba 1993). Phylogeographical analysis of six species of broadleaved evergreen plants revealed that rare haplotypes and large numbers of common haplotypes were observed in southern regions along the Pacific coast (Aoki *et al*. 2004). This result led the authors to postulate that these regions served as glacial refugia for temperate and subtropical species, and that there were multiple glacial refugia in the Japanese Archipelago. Another significant factor that may have promoted high biological diversity in this region is that the four main islands of the Japanese Archipelago (Hokkaido, Honshu, Shikoku, and Kyushu) were connected during the glacial periods and separated during the interglacial periods in conjunction with sea-level fluctuations (Fig. 1). This may have caused repeated cycles of migration of populations between the four main Japanese islands and population fragmentation.

Tojo *et al*. (2017) reviewed phylogeographical studies on insect species distributed throughout the Japanese Islands and indicated several patterns of speciation and genetic differentiation in relation to geological events. Regarding the factors of genetic differentiation or secondary contacts, they emphasized vicariance events such as separation of the Proto-Japanese Islands from the Asian continent, the formation of the Fossa Magna and the Median Tectonic Line (see Supporting Information Fig. S1), and

the formation of northern and southern land-bridges during the Pleistocene (Fig. S2). Tojo *et al*. (2017) also discusseed climatic changes in glacial and interglacial cycles as a factor of latitudinal shifts in the distribution ranges of the Japanese insects. Many studies have addressed the phylogeography of Japanese insects; however, the migration patterns of insect species between the four main Japanese islands during the Pleistocene are not fully understood.

This study attempts to elucidate the detailed process behind distributional changes in temperate Japanese species during the Pleistocene, specifically in relation to the formation of glacial refugia, and to the connection and the separation of the Japanese islands, using the stream-dwelling stonefly *Kamimuria tibialis* (Plecoptera, Perlidae). *Kamimuria tibialis* is endemic to the Japanese Archipelago and is distributed throughout the four main islands: Hokkaido, Honshu, Shikoku, and Kyushu (Uchida & Isobe, 1991). This species is quite common in south-western Japan, which has a mild climate (Shimizu *et al*., 2005), whereas in north-western Honshu and Hokkaido, with low average temperatures, it is found in a few streams of relatively warm waters, such as the lower reaches of rivers and the downstream areas of lakes or dams (A. O., pers. ovserv.). Cold climatic changes could have lead to a reduction in the distribution of *K. tibialis*. *Kamimuria tibialis* utilizes many kinds of aquatic insects as food sources (Otsuki & Iwakuma, 2008). Given these facts, it is expected that the distribution of this species has been affected by the glacial cycles of the Pleistocene, and may not be limited by the species composition of local prey. Furthermore, stoneflies generally have low dispersal ability (Schultheis *et al*. 2002; Yasick *et al.* 2007), so that past genetic features could have been conserved up to the present. Consequently, the wide distribution throughout the Japanese Archipelago, ecological features (i.e., preference

for warm water and euryphagous dietary habit), and the limited dispersal ability of *K. tibialis* make this species appropriate for the investigation of past population divisions, migrations, and fusions of temperate Japanese species. The specific objectives of the present study were to (1) reconstruct the intraspecific phylogeny of mitochondrial haplotypes collected throughout the Japanese islands, (2) infer the times of divergence between geographical lineages, and (3) estimate the genetic population structure and demographic history of local populations.

Materials and Methods

Sampling, DNA extraction, PCR amplification and sequencing

Mature nymphs of *K. tibialis* were collected in spring from streams across the Japanese Archipelago. A total of 53 streams belonging to different stream systems were selected, and samples were collected from one point per stream. One to ten individuals were collected at each point and used for analysis (a total of 197 individuals; Supporting Information Table S1; Fig. 2). Six samples of *Kamimuria uenoi* Kohno, 1947, the sister species of *K. tibialis*, were collected from six distinct sites (Fig. 2) to be used as an outgroup. DNA was extracted from the hind legs of each individual, using a DNeasy Tissue Extraction Kit (QIAGEN). Partial sequences of mitochondrial NADH dehydrogenase subunit 5 region (ND5) and cytochrome oxidase subunit I (COI) were amplified with primer sets F7081 + R7495 (Yoshizawa, 2004) and F1859 (5′-GGA ACA GGA TGA ACA GTT TAC CCT CC-3′) + R2740 (Kanbe & Akimoto, 2009),

respectively. The reaction cycle was 94˚C for 1 min, followed by 35 cycles of 94˚C for 30 s, 42˚C (ND5) or 44˚C (COI) for 30 s, and 65˚C for 45 s. Amplified products were purified with a QIA quick purification kit (QIAGEN), and were then sequenced with a CEQ2000 DNA Analysis System (Beckman Coulter) following the manufacturer's protocols. Sequences were aligned visually in MacClade 4.03 (Maddison & Maddison, 2001). The alignment was unequivocal because the sequences included no indels or repeats. Sequences were trimmed to 413 bp for ND5 and 853 bp for COI, giving a total of 1266 bp.

Phylogenetic analysis and estimation of divergence times

Phylogenetic analysis of mitochondrial haplotypes from all the samples was conducted by the maximum-parsimony (MP) and maximum-likelihood (ML) methods, using PAUP 4.0b10 PPC (Swofford, 2002), and by the Bayesian method using MrBayes 3.1 (Huelsenbeck *et al.*, 2001). For MP analysis, all characters were equally weighted, and heuristic searches with 100 random stepwise additions of taxa using TBR branch swapping were performed. ML analysis was conducted using the TrN + I + G model of evolution (unequal base frequencies: A = 0.3332, C = 0.1930, G = 0.1373, T = 0.3365; six substitution categories: A-C = 1, A-G = 20.6145, A-T = 1, C-G = 1, C-T = 14.6008, G-T = 1; gamma distribution shape parameter = 0.8423; proportion of invariant sites = 0.6719), which was indicated as the best fitting model, according to Akaike's information criterion (AIC) values, in Modeltest 3.7 (Posada & Crandall, 1998). Branch support was evaluated using 1000 and 100 replicate bootstrap analyses for MP and ML

analysis respectively. Bayesian analysis was conducted using two independent Markov Chain Monte Carlo (MCMC) runs, each with four simultaneous chains, under the HKY + I + G model that was selected according to AIC values in MrModeltest 2.2 (Nylander, 2004). From each chain of 1 million generations, trees were sampled every 100 generations. After discarding the first 2500 trees as a burn-in, a 50% majority consensus tree was constructed. When a well-supported clade, with a broad distributional area, was identified in the phylogenetic analysis, we constructed a median joining network for that clade using NETWORK 4.5 (Bandelt *et al*., 1999) to explore the geographical relationships among haplotypes within the clade.

We estimated the divergence times of the major clades based on a Bayesian coalescent approach using BEAST 1.4 (Drummond & Rambaut, 2007). These analyses were performed under the HKY + I + G model, assuming 0.0075–0.0177 substitutions per site per Myr. These rates were simply postulated from the mitochondrial sequence divergence rates of 1.5%–3.54% per Myr in arthropods, which were derived from comparisons among the geological, fossil and molecular data (Brower, 1994; Quek *et al.*, 2004; Papadopoulou *et al*., 2010). We used a strict clock model in the analysis because almost clock-like evolution was suggested for our data by the value of ucld.stdev (the standard deviation of the branch rates under the uncorrelated lognormal relaxed molecular clock), 0.007. The ucld.stdev value was yielded from a tentative analysis assuming an uncorrelated relaxed clock model. In the relaxed-clock analysis, the ucld.stdev parameter is close to 0.0 if the sequences are evolving in an almost clock-like manner, whereas substantial rate heterogeneity among lineages is expected if the ucld.stdev value is much higher than 1.0 (Drummond *et al.*, 2007). After the MCMC runs, the traces of each parameter had checked whether they had converged on a

stationary distribution, and the effective sample size (ESS) in each analysis was estimated from the MCMC samples, using Tracer 1.3 (Rambaut & Drummond, 2005).

Population genetic structure and demographic history

Pairwise F_{ST} values (Slatkin, 1995) were calculated between all pairs of populations with more than four individuals (two populations from Tsushima, two populations from Kyushu, two populations from Shikoku, ten populations from Honshu and one population from Hokkaido; Supporting Information, Table S1). The calculations were performed using ARLEQUIN 2.0 (Schneider *et al*., 2000).

We performed several population demographic analyses for regional groups of populations. For the phylogroup with a wide distribution (Hokkaido–Honshu–Shikoku; see Results), we used the program SAMOVA (spatial analysis of molecular variance; Dupanloup *et al*., 2002) in order to estimate the population structure and to define groups of populations without a priori hypotheses of the population structure. SAMOVA aims to cluster geographically homogeneous populations into a user-defined number of groups (K), such that the proportion of total genetic variance observed between groups (F_{CT} index) is maximized. For SAMOVA analysis of the Hokkaido-Honshu-Shikoku phylogroup, we used populations with more than four sequences. The analysis was run from $K = 2$ to $K = 10$, with 100 simulated annealing processes for each value of K, and the significance of fixation indices was tested by 1000 permutations.

Demographic analyses were applied to six regional populations that consisted of four population groups recognized by SAMOVA in the Hokkaido–Honshu–Shikoku

phylogroup (see Results) and two other main phylogroups (Tsushima and Kyushu; see Results). To determine whether a regional population has recently undergone an expansion or not, we calculated mismatch distributions of substitution differences (Roger & Harpending, 1992) between any two different samples from the same regional population. The mismatch distribution is usually unimodal in populations that have passed through a recent demographic expansion, whereas the distribution is multimodal in populations at a demographic equilibrium (Slatkin & Hudson, 1991; Roger & Harpending, 1992). The observed values were first compared to those expected from a sudden expansion demographic model that was estimated using the generalized least-square procedure (Schneider & Excoffier, 1999). Second, Tajima's *D* (Tajima, 1989) and Fu's *F*s (Fu, 1997) were calculated to test whether each regional population is in stationary equilibrium or has experienced a recent demographic expansion. These values tend to be negative for recently expanded populations (Tajima, 1989; Aris-Brosou & Excoffier, 1996; Fu, 1997). The significance of Tajima's *D* and Fu's *F*s was tested by generating random samples under the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation algorithm (Hudson, 1990). Additionally, the demographic history of each regional population was inferred from a comparison between the values of haplotype diversity and nucleotide diversity (Grant & Bowen, 1998; Avise, 2000). Haplotype diversity was calculated using ARLEQUIN 2.0 (Schneider *et al.*, 2000), in which the equation for 'gene diversity' can be used to calculate the haplotype diversity (Nei, 1987). All of these calculations were performed using ARLEQUIN 2.0 (Schneider *et al.*, 2000).

To estimate the temporal dynamics of population size fluctuation, we used the Bayesian Skyline Plot (BSP, Drummond *et al.*, 2005) method, implemented in BEAST

1.4 (Drummond & Rambaut, 2007), for the Tsushima, Kyushu and Hokkaido-Honshu-Shikoku phylogroups. The analyses were performed under the HKY + I + G model, assuming 0.0075–0.0115 substitutions per site per Myr. MCMC was run for 10 million generations, with sampling for every 1000 generations. After discarding the first 10% of the chain, we determined whether the chains had converged by monitoring the traces of sampled parameters, and whether the condition for the ESS was satisfied for each parameter, using TRACER 1.3 (Rambaut & Drummond, 2005).

Data availability

The obtained sequences have been deposited in the GenBank database [*K. uenoi*: LC620871–LC620876, Tsushima phylogroup: LC620877–LC620890, Kyushu phylogroup: LC620891–LC620908, Hokkaido-Honshu-Shikoku phylogroup excluding Hokkaido lineage: LC620909–LC620978, Hokkaido lineage: LC620979–LC620981 (COI); *K. uenoi*: LC621736–LC621740, Tsushima phylogroup: LC621741, Kyushu phylogroup: LC621742–LC621750, Hokkaido-Honshu-Shikoku phylogroup excluding Hokkaido lineage: LC621751–LC621755 and LC621758–LC621796, Hokkaido lineage: LC621756–LC621757 (ND5)].

Results

Haplotype phylogeny, distribution and divergence

A total of 131 unique haplotypes with 151 variable sites (including 84 parsimoniously informative sites) were identified across 197 *K. tibialis* individuals (GenBank, see Data Availability). When an outgroup was included, 150 characters were found to be parsimoniously informative.

MP analysis generated 69 equally parsimonious trees [length = 361, consistency index (CI) = 0.66, retention index (RI) = 0.92], which were consistent in topology, except for some weakly supported tip branches. Each of the trees constructed by ML and Bayesian analyses were consistent with the MP trees, except for some tip branches. All the trees revealed a clear phylogeographical structure of *K. tibialis* across the Japanese Archipelago (Fig. 3). Three lineages, Tsushima, Kyushu and Hokkaido–Honshu–Shikoku, were identified, which were confirmed by relatively high bootstrap and posterior probability values. These lineages were clustered geographically without overlaps in geographic distributions; hence, they constitute discrete phylogroups. In the Hokkaido–Honshu–Shikoku phylogroup, all four haplotypes from Hokkaido formed a strongly supported clade. The Kyushu phylogroup was further divided into two clades, Kyushu-1 and Kyushu-2 within Kyushu Island. Individuals of the two clades coexisted in the K1 and K2 populations (Supporting Information, Table S1: haplotypes of the Kyushu-2 clade are shown in bold).

A haplotype network depicted for the Hokkaido–Honshu–Shikoku phylogroup demonstrated that Shikoku Island harboured two divergent haplotype groups; one haplotype group was widely distributed in East Honshu and West Honshu, whereas another group was specific to Shikoku (Fig. 4). The latter Shikoku-specific group consisted of the deeply divergent haplotypes (haplotypes: 36, 43, 44, 45, 46, 47, and

68). This pattern was also detected in the phylogenetic trees, although the branch support of these haplotype groups was weak (Fig. 3).

Many haplotypes were population-specific but some were found extensively across populations. For instance, haplotype 37 was observed in sampling sites S1, S3, S5, S6, H1, H2, H4, H5, H8, H11 and H22, and haplotype 100 was observed in sampling sites H3, H5, H6, H24 and H28 (Supporting Information, Table S1).

Bayesian coalescent analyses estimated that the Tsushima phylogroup was separated from the remaining phylogroups 0.90–2.02 Mya (Table 1). The Kyushu phylogroup was presumed to have diverged from the Hokkaido–Honshu–Shikoku phylogroup 0.54–1.28 Mya, and the Kyushu phylogroup bifurcated into the Kyushu-1 and Kyushu-2 lineages 0.35–0.77 Mya. The Hokkaido lineage in the Hokkaido–Honshu–Shikoku phylogroup was estimated to have coalesced 0.07–0.16 Mya.

Population genetic structure and demographic history

Pairwise population F_{ST} values showed significant differentiation for all of the population pairs from the different phylogroups (range: 0.48–0.95; $P < 0.05$, permutation tests with 100 permutations), while F_{ST} values were not significant for 32 of the 68 population pairs within the same phylogroups (range: –0.09 to 0.16, $P = 0.072$–0.955) (Supporting Information, Table S2). These results were congruent with the geographical clustering of the haplotype lineages and the overlapping of some haplotype distributions within phylogroups.

SAMOVA for the 14 populations of the Hokkaido–Honshu–Shikoku phylogroup showed that the smallest number of 'groups of populations' (K) having a significant F_{CT}

value was four, and that F_{CT} values did not increase with increasing K values. Thus, we have parsimoniously selected four regional groups within the Hokkaido–Honshu–Shikoku phylogroup, including Hokkaido, Shikoku, the eastern region of Honshu containing sampling sites H3–H9, and the western region of Honshu containing sampling sites H1, H2 and H10. Demographic analyses were applied to these four regional populations, along with the insular populations of Tsushima and Kyushu.

In the Kyushu population, the mismatch distribution pattern was clearly bimodal (Fig. 5; significance of the mismatch distribution, P, and sum of squared deviation, SSD, were 0.05 and 0.036, respectively). This bimodal distribution pattern suggests the coexistence of genetically divergent groups; this result was consistent with the results obtained from the phylogenetic analysis (Fig. 3). A bimodal pattern was also found in Shikoku Island (P = 0.67 and SSD = 0.016), and this result agreed with that of the haplotype network (Fig. 4). In the Kyushu and Shikoku populations, neither Tajima's D nor Fu's Fs differed significantly from the values estimated under the population equilibrium model. In particular, the Kyushu population exhibited high gene diversity (0.97) and conspicuously high nucleotide diversity (0.0077) (Table 2). The eastern Honshu population also had a nearly bimodal mismatch distribution (Fig.5; P = 0.35 and SSD = 0.004), high gene diversity (0.98), and relatively high nucleotide diversity (0.0046) (Table 2); both Tajima's D and Fu's Fs were significantly negative, suggesting recent population expansion (Table 2).

In contrast, the mismatch distribution patterns in the Tsushima and western Honshu populations were unimodal, similar to the distribution expected during sudden range expansion (Fig. 5; P = 0.58 and SSD = 0.005 for the Tsushima population; P = 0.26 and SSD = 0.011 for the western Honshu population). Both Tajima's D and Fu's Fs

were significantly negative in the western Honshu population, and Fu's *F*s was significantly negative in the Tsushima population (Table 2). The Tsushima and western Honshu populations exhibited high gene diversity (0.93 and 0.98) but relatively low nucleotide diversity (0.0022 and 0.0029). In the Hokkaido population, we observed a markedly unimodal pattern in the mismatch distribution ($P = 0.17$ and SSD = 0.016) and a very low mean value in the distribution (0.58). In addition, the Hokkaido population exhibited very low values of gene diversity (0.52) and nucleotide diversity (0.0005). Neither Tajima's *D* and Fu's *F*s were significantly negative.

The BSP predicted a population expansion that began ~0.10–0.15 Mya in the Kyushu and Hokkaido–Honshu–Shikoku phylogroups (Fig. 6). The extent of the estimated population expansion differed greatly among the phylogroups, ranging from a 10-fold increase in the Kyushu phylogroup to a 100-fold increase in the Hokkaido-Honshu-Shikoku phylogroup. The Tsushima phylogroup was characterized by constant population growth for the last 50 000 years, with a 50-fold increase.

Discussion

Japanese insects considered to have established their distribution through the following main processes: 1) when part of the Asian Continent separated to form proto-Japanese Islands (70-6 Mya), some insects that had inhabited the Asian Continent survived on the separated landmasses and evolved into new species, and 2) migration from the Asian Continent and its peninsula via land-bridges during the glacial periods (reviewed by Tojo *et al.*, 2017). In the present study, divergence times of the three main phylogroups

of *K. tibialis* (Tsushima, Kyushu, and Hokkaido–Honshu–Shikoku) were estimated to be about 0.54–2.02 Mya. Adding to this result, both *K. tibialis* and its sister species *K. uenoi* are endemic in Japan, implying that *K. tibialis* originated in Japan after the Japanese Archipelago had been established, and its intraspecific divergence has occurred during the Pleistocene glacial epoch. Considerable genetic distances among the Tsushima, Kyushu, and Hokkaido–Honshu–Shikoku phylogroups, and a lack of geographic overlap in their distribution indicate long-term geographical isolation of each phylogroups that may have persisted during several glacial cycles in different refugia.

The Kyushu population had high genetic diversity and two distinct haplotype lineages. The estimated coalescent time of 0.35–0.77 Mya for the Kyushu phylogroup indicates that two lineages have survived several glacial epochs. Two groups of haplotype lineages also occur sympatrically in Shikoku Island. Although two haplotype groups in the Shikoku Island were not strongly supported by the bootstrap values, the genetic divergence between them is quite large (Figs 4, 5). The coexistence of two haplotype lineages observed in the Kyushu and Shikoku Islands can be explained primarily by the recent secondary contact between two populations that had persisted in different regions. This scenario implies that at least two glacial refugia had existed in each island for the several glacial cycles, and that populations from these different refugia were recently mixed. An alternative scenario for the coexistence of two lineages within the Kyushu and Shikoku Islands postulates that large population sizes had been maintained throughout the climatic fluctuation during the Pleistocene epochs on these islands, so that haplotype lineages that had branched off long ago persisted within each population. Avise (2000) indicated that the probability of survival of two or more

matrilineal lineages reduces dramatically with a declining population size. Therefore, if the second scenario was true, reduction in population size during the glacial periods should have been quite low in these populations, suggesting the presence of one large refugium on each island. The result of the Bayesian simulation and the non-significant Tajima's *D* and Fu's *F*s of the Kyushu and Shikoku populations imply that population expansion after the last glacial period was not prominent in these islands, thus supporting the second scenario. In either case, there were certainly climatic and environmental factors that allowed insect populations found on the Kyushu and Shikoku Islands to maintain high genetic diversity during the climatic fluctuations of the Pleistocene.

In the Hokkaido–Honshu–Shikoku phylogroup, haplotype distribution was not geographically structured, except for the Hokkaido lineage. *Kamimuria tibialis* is a relatively good flyer (discussed below), as some haplotypes found across populations (e.g., haplotypes 37 and 100; Supporting Information, Table S1). In studies of freshwater phylogeography, species with terrestrial dispersal ability often exhibit genetic isolation by distance (Hughes *et a*l., 2009). In *K. tibialis*, F_{ST} values were not significant between many pairs of sampling sites of East Honshu (Table S2). For example, non-significant F_{ST} values were found between the sampling site H3 and H4, H5, H6, H8 and H9, and between H7 and H5, H6, H8 and H9. It appears that there are some neighbouring groups of sampling sites with recent gene flow. On the other hand, in West Honshu, most pairs between three sampling sites showed significant F_{ST} values, although the distances among sampling sites were smaller than those of East Honshu. Furthermore, Shikoku Island harboured divergent haplotypes and high nucleotide diversity ( ) compared with the West and East Honshu populations and the Hokkaido population (Table 2). These genetic features are not explained just by the distances among sampling sites.

The Tsushima Island and western Honshu populations have demographic characteristics that suggest a recent rapid expansion of a single lineage group (Fig. 5, Table 2). These results suggest that these regions were rapidly colonized by *K. tibialis* populations that may have migrated from the putative refugia. Although Tsushima Island and western Honshu are located in the southern part of the Japanese Archipelago and currently have warm climates, these regions would have been dry and cold during the glacial periods, because the warm current did not flow into the Sea of Japan (Fig. 1b).

On Kyushu Island, a rare haplotype highly divergent from other Kyushu haplotypes, 17, was detected at sampling site K1 (Fig. 3; Supporting Information, Table S1), located on the southern edge of the island (Fig. 2). We were unable to determine the precise locality of the glacial refugia from our results, but the demographic features of Kyushu, Shikoku and Tsushima Islands, and western Honshu as a whole corroborate the hypothesis of refugia along the south-western Pacific coast, which was theorized based on fossil records (Ono & Igarashi, 1991) and phylogeographical analyses of evergreen plants (Aoki *et al*., 2004).

The separation of the Tsushima phylogroup from other phylogroups was estimated to be 0.90–2.02 Mya, which was consistent with the estimated age (1.55 Mya) of the Tsushima Strait formation (Kitamura & Kimoto, 2006; Osozawa *et al*., 2013). The long-term independent history of the Tsushima phylogroup may be due to the presence of a deep strait between Tsushima and Kyushu (currently about 130 m deep: Tada, 1998), which would have prevented the formation of a land-bridge between these islands even during the glacial maxima (Ohshima 1990). The fauna of Tsushima Island is rich in endemic species and subspecies, in contrast to the poor level of endemicity of other

islands near Kyushu (Shirouzu, 1976; Miyata, 1986). This indicates that the environment in Tsushima may have allowed many temperate organisms to survive the glacial cycles. However, the recent population expansion estimated by the demographic analyses suggests that a refugium of *K. tibialis* was restricted to a narrow area on Tsushima Island. Conversely, on Hokkaido, specific and quite low divergent haplotypes were detected. The estimated coalescent time of these haplotypes (0.07-0.16 Mya; Table 1) indicates that migration from Honshu to Hokkaido occurred before the last glacial maximum (~0.02 Mya). This hypothesis is also congruent with the geological evidence for the presence of a strait between Hokkaido and Honshu during the last glacial period (Ohshima, 1990). On Hokkaido, it is possible that the last glacial refugium was restricted to a very narrow area, and that postglacial population expansion may have been suppressed by the subarctic climate in this northern island.

The straits between Shikoku and Honshu and between Kyushu and Honshu are currently about 15–30 m in depth (Wadachi, 1972), and it is suggested that these islands were connected to form a large land during each glacial epoch (Yonekura *et al*, 2001; Fig. 1b). The connections of islands could have resulted in the frequent migrations between Shikoku and Honshu, which have led to an unclear geographical structure within the haplotypes identified in these islands. Nevertheless, a profound divergence was evident between the Kyushu and Hokkaido–Honshu–Shikoku phylogroups. Most phylogeographical studies on Japanese organisms have not detected a distinct divergence between the Kyushu and Honshu–Shikoku populations (e.g. for Fagaceae, Fujii *et al*., 2002; for dormice, Yasuda *et al*., 2007; for weevils, Aoki *et al*., 2008; for several stream-dwelling insects, Tojo *et al*., 2017). There should be some significant factors that have prevented gene flow between the Kyushu and Honshu–Shikoku

populations of *K. tibialis* since 0.54–1.28 Mya (estimated coalescent times of node 3 in Figure 3; Table 1), which will be an issue in the future.

Studies on the genetic structures of stonefly species have recently been successively reported. It appears to be clear that for the stoneflies, genetic differentiation correlated with flight ability: the clear phylogeographical structures are shown in *Zelandoperla fenestrata* (Gripopterygidae) species group with low flying ability, whereas in the very same region, the significant geographical structures were not detected for *Z. decorata* having high flying ability (McCulloch *et al*., 2009); intraspecific divergences of COI were 0.7–1.3% for the three apterous genera and 0.6–0.7% for the three macropterous genera in the New Zealand stoneflies (McCulloch *et al*., 2010). McCulloch *et al*. (2010) also showed the impact of glaciations on the distribution and genetic structures of these stoneflies: the centre of the glaciated region in the South Island of New Zealand has become the current "biotic gap", a low diversity region, and on either side of this gap there were obvious intrageneric divergences for all six genera. For the Nemouridae species inhabiting the highest reaches of Rocky Mountain alpine streams, nucleotide diversity (π) were 0.0035 for *Lednia tumana*, 0.0013 for *L. tetonica*, and 0.0696 for *Zapada glacier*, three populations in isolated regions (Hotaling *et al*., 2019), showing population divisions based on distance would have resulted in genetic divergence for these short-winged meltwater depending species. For the Perlidae to which *K.tibialis* belongs, nucleotide diversity (π) of the cytochrome b (*cytb*) region was 0.00166 for *Hesperoperla pacifica* populations from across the Great Basin of western North America (Schultheis *et al.*, 2014), a ubiquitous species found in a wide variety of streams of western North America (Stewart & Stark 1988). *Kamimuria tibialis* has a good flying ability, and females usually fly long distances

upstream to oviposit (Nishimura, 1962; Ichikawa et al., 2020). Nevertheless its good flying ability, the degree of intraspecific divergence seems high in *K. tibialis* as compared with other stoneflies: mitochondrial nucleotide diversity were 0.0077 for the Kyushu population, 0.0052 for the Hokkaido–Honshu–Shikoku population, and 0.0022 for the Tsushima population; pairwise distances of COI+ND5 of *K. tibialis* were about 2.5% for the Tsushima and Kyushu populations, about 2.1% for the Tsushima and Hokkaido–Honshu–Shikoku populations, and about 1.6% for the Kyushu and Honshu populations. This intraspecific genetic diversity is mainly due to the population division between the Japanese islands and high genetic diversity in the Kyushu and Shikoku Islands, putative glacial refugia. Our study indicates that for the stoneflies with relatively high flying ability, population division (e.g., by deep straits between islands) and the existence of stable glacial refugia could have contributed to current intraspecific genetic diversity.

Acknowledgements

We express our sincere thanks to Shigekazu Uchida, Yu Isobe, Zhang Yuping, Kazuhisa Inada, Kaori Nio and Tsutomu Miyashita for information regarding stonefly distribution. Gyo Yoshinari, Fumiko Tamura and Noriko Matsumoto kindly provided specimens from their personal collections. Izumi Yao, Tsuyoshi Urata and Ichii assisted the first author with specimen collection. The first author would like to thank Izumi Yao and Takashi Kanbe for their assistance with laboratory work. This study was supported in part by a 21st Century Center of Excellence (COE) Program on the 'Neoscience of Natural History' (Program Leader: Hisatake Okada) at Hokkaido University, financed

by the Ministry of Education, Culture, Sports, Science, and Technology, Japan (ID: 964644); and in part by the NOASTEC foundation (H17-Y-106). We sincerely thank three anonymous reviewers for their helpful comments, which greatly improved an earlier draft of this manuscript. The authors declare no conflicts of interests.

References

Aoki K, Suzuki T, Hsu T-W, Murakami N. 2004. Phylogeography of the component species of broad-leaved evergreen forests in Japan, based on chloroplast DNA. *Journal of Plant Research* 117: 77-94.

Aoki K, Kato M, Murakami N. 2008. Glacial bottleneck and postglacial recolonization of a seed parasitic weevil, *Curculio hilgendorfi*, inferred from mitochondrial DNA variation. *Molecular Ecology* 17: 3276-3289.

Aris-Brosou S, Excoffier L. 1996. The impact of population expansion and mutation rate heterogeneity on DNA sequence polymorphism. *Molecular Biology and Evolution* 13: 494-504.

Avise JC. 2000. *Phylogeography: the history and formation of species*. Cambridge: Harvard University Press.

Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring

intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37-48.

Brower AVZ. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proceedings of the National Academy of Sciences, USA* 91: 6491-6495.

Butlin RK. 1998. What do hybrid zones in general, and the *Chorthippus parallelus* zone in particular, tell us about speciation? In: Howard DJ, Berlocher SH, eds. *Endless Forms: Species and Speciation*. New York: Oxford University Press, 367-378.

Carstens BC, Knowles LL. 2007. Shifting distributions and speciation: species divergence during rapid climate change. *Molecular Ecology* 16: 619-627.

Contreras-Medina R, Morrone JJ, Luna I. 2001. Biogeographic methods identify gymnosperm biodiversity hotspots. *Naturwissenschaften* 88: 427-430.

Drummond AJ, Rambaut A, Shapiro B, Pybus OG. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution* 22: 1185-1192.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7: 214.

Drummond AJ, Ho SYW, Rawlence N, Rambaut A. 2007. *A Rough Guide to BEAST*

1.4 (manual of BEAST 1.4). Available at: https://www.researchgate.net/profile/Nicolas-Rawlence/publication/228988177_A_Rough_Guide_to_beast_14/links/0a85e532a8d141a0e2000000/A-Rough-Guide-to-beast-14.pdf.

Dupanloup I, Schneider S, Excoffier L. 2002. A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology* 11: 2571-2581.

Excoffier L. 2001. Analysis of population subdivision, pp. 271–307 in *Handbook of Statistical Genetics*, edited by D. BALDING, M. BISHOP and C. CANNINGS. John Wiley & Sons, New York.

Fu Y-X. 1997. Statistical tests of neutrality against population growth, hitchhiking and background selection. *Genetics* 147: 915-925.

Fujii N, Tomaru N, Okuyama K, Mikami T, Ueda K. 2002. Chloroplast DNA phylogeography of *Fagus crenata* (Fagaceae) in Japan. *Plant Systematics and Evolution* 232: 21-33.

Grant WS, Bowen BW. 1998. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. *The Journal of Heredity* 89: 415-426.

Haffer J. 1985. Avian zoogeography of the neotropical lowlands. In: Buckley PA, Forster MS, Morton ES, Ridgely RS, Buckley FG eds. *Neotropical ornithology.*

Ornithological monographs 36. Washington: American Ornithologists Union, 113-146.

Hewitt GM. 1988. Hybrid zones - natural laboratories for evolutionary studies. *Trends in Ecology & Evolution* 7: 158-167.

Hewitt GM. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* 58: 247-276.

Hewitt GM. 1999. Post-glacial re-colonization of European Biota. *Biological Journal of the Linnean Society* 68: 87-112.

Hewitt GM. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405: 907-913.

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.

Hewitt GM. 2011. Quaternary phylogeography: the roots of hybrid zones. *Genetica* 139: 617-638.

Hotaling S, Giersch JJ, Finn DS, Tronstad LM, Jordan S, Serpa LE, Call RG, Muhlfeld CC, Weisrock DW. 2019. Congruent population genetic structure but differing depths of divergence for three alpine stoneflies with similar ecology and geographic distributions. *Freshwater Biology* 64: 335-347.

Hudson RR. 1990. Gene genealogies and the coalescent process. *Oxford Survey in Evolutionary Biology* 7: 1-44.

Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Science* 294: 2310-2314.

Hughes JM, Schmidt DJ, Finn DS. 2009. Genes in streams: using DNA to understand the movement of freshwater fauna and their riverine habitat. *BioScience* 59: 573-583.

Ichikawa S, Uchida S, Itō M. 2020. Distribution and life cycle of stoneflies (Insecta, Plecoptera) (especially *Xanthoneuria*) in the Yahagi River system and surrounding rivers, central Honshu, Japan. *Bulletin of Aichi Institute of Technology* 55: 60-82.(in Japanese)

Kanbe T, Akimoto S. 2009. Allelic and genotypic diversity in long-term asexual populations of the pea aphid, Acyrthosiphon pisum in comparison with sexual populations. *Molecular Ecology* 18: 801-816.

Kitamura A, Kimoto K. 2006. History of the inflow of the warm Tsushima Current into the sea of Japan between 3.5 and 0.8 Ma. *Palaeogeography, Palaeoclimatology, Palaeoecology* 236: 355-366.

Knowles LL. 2000. Tests of Pleistocene speciation in montane grasshoppers (genus

Melanoplus) from the sky islands of western North America. *Evolution* 54: 1337-1348.

Knowles LL. 2001a. Genealogical portraits of speciation in montane grasshoppers (genus *Melanoplus*) from the sky island of the Rocky Mountains. *Proceedings of the Royal Society of London. Series B, Biological Sciences* 268: 319-324.

Knowles LL. 2001b. Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. *Molecular Ecology* 10: 691-701.

Knowles LL. 2005. Importance of genetic drift during Pleistocene divergence as revealed by analyses of genomic variation. *Molecular Ecology* 14: 4023-4032.

Lucati F, Poignet M, Miró A, Trochet A, Aubret F, Barthe L, Bertrand R, Buchaca T, Calvez O, Caner J, Darnet E, Denoël M, Guillaume O, Chevalier HL, Martínez-Silvestre A, Mossoll-Torres M, O'Brien D, Osorio V, Pottier G, Richard M, Sabás I, Souchet J, Tomàs J, Ventura M. 2020. Multiple glacial refugia and contemporary dispersal shape the genetic structure of an endemic amphibian from the Pyrenees. *Molecular Ecology* 29: 2904-2921.

Maddison DR, Maddison WP. 2001. *MacClade 4: analysis of phylogeny and character evolution. Version 4.03*. Sinauer Associates, Sunderland, Massachusetts.

McCulloch GA, Wallis GP, Waters JM. 2009. Do insects lose flight before they lose their wings? Population genetic structure in subalpine stoneflies. *Molecular Ecology* 18:

4073-4087.

McCulloch GA, Wallis GP, Waters JM. 2010. Onset of glaciation drove simultaneous vicariant isolation of alpine insects in New Zealand. *Evolution* 64(7): 2033-2043.

Miyata A. 1986. An insect fauna of Tsushima. In: Kimoto S ed. *Insect biogeography of Japan*. Tokyo: Tokai University Press, 77-84.

Nei M. 1987. *Molecular Evolutionary Genetics*. New York: Columbia University Press.

Nishimura N. 1962. On the flighting of a stonefly, *Kamimuria tibialis* Pictet (Plecoptera). *Hyogo Seibutsu* 4: 1-3. (in Japanese)

Nylander JAA. 2004. *MrModeltest v2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Ohba T. 1993. Change of the currents around the Japanese Archipelago after the last glacial. *Gakujyutsu Geppo* 46: 36-40.

Ohshima K. 1990. The history of straits around the Japanese Islands in the late-Quaternary. *Quaternary Research* 29: 193-208. *(in Japanese with English summary)*

Ono Y, Igarashi Y. 1991 *Natural history of Hokkaido*. Sapporo: Hokkaido University Press.

Osozawa S, Su ZH, Oba Y, Yagi T, Watanabe Y, Wakabayahi J. 2013. Vicariant speciation due to 1.55 Ma isolation of the Ryukyu islands, Japan, based on geological and GenBank data. *Entomological Science* 16: 267-277.

Otsuki A, Iwakuma T. 2008. Life history, growth patterns and feeding habits of two predatory stoneflies, *Skwala pusilla* (Perlodidae) and *Kamimuria tibialis* (Perlidae) in northern Japan. *Aquatic Insects* 30: 29-41.

Papadopoulou A, Anastasiou I, Vogler AP. Revisiting the insect mitochondrial molecular clock: the mid-Aegean trench calibration. *Molecular Biology and Evolution* 27(7): 1659-1672.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14, 817-818.

Quek SP, Davies SJ, Itino T, Pierce NE. 2004. Codiversification in an ant-plant mutualism: stem texture and the evolution of host use in Crematogaster (Formicidae: Myrmicinae) inhabitants of Macaranga (Euphorbiacecae). *Evolution* 58: 554-570.

Rambaut A, Drummond AJ. 2005. *TRACER version 1.3: MCMC trace file analyser*. University of Oxford. Available at http://evolve.zoo.ox.ac.uk/software.html.

Rogers AR, Harpending HC. 1992. Population growth makes waves in the distribution

of pairwise genetic differences. *Molecular Biology and Evolution* 9: 552-569.

Schneider S, Excoffier L. 1999. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics* 152: 1079-1089.

Schneider S, Roessli D, Excoffier L. 2000. *ARLEQUIN, Version 2.000: Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva.

Schultheis AS, Weigt LA, Hendricks AC. 2002. Gene flow, dispersal, and nested clade analysis among populations of the stonefly Peltoperla tarteri in the southern Appalachians. *Molecular Ecology* 11(3): 317-327.

Schultheis AS, Davis N, Page JT, Fenwick AM, Bond JE, Shiozawa DK. 2014. Comparative transcriptomics allows for rapid development of population-level nuclear markers in *Hesperoperla pacifica* (Plecoptera: Perlodae). *Freshwater Science* 33(1): 364-373.

Shimizu T, Inada K, Uchida S. 2005. Plecoptera. In: Kawai T, Tanida K, eds. *Aquatic Insects of Japan: Manual with keys and illustrations*. Kanagawa, Japan: Tokai University Press, 237-290.

Shirouzu T. 1976. Speciality of the insect fauna of Tsushima. In: *Fauna of Tsushima*.

Nagasaki: Nagasaki Biological Society, 815-822.

Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139: 457-462.

Slatkin M, Hudson RR. 1991. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics* 129: 555-562.

Stewart KW, Stark BP. 1988. *Nymphs of North American stonefly genera (Plecoptera).* 2nd ed. Columbus; The caddis press.

Swofford DL. 2002. PAUP*. *Phylogenetic Analysis Using Parsimony (and Other Methods). Version4.* Sinauer and Associates, Sunderland, Massachusetts.

Tada R. 1998. Japanese Archipelago during last glacial period. *Iden* 52: 10-15.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123: 585-595.

Tautz D. 2004. Phylogeography and patterns of incipient speciation. In: Dieckmann U, Doebeli M, Metz JAJ, Tautz D, eds. *Adaptive speciation*. Cambridge: Cambridge University Press, 305-321.

Tojo K, Sekine K, Takenaka M, Isaka Y, Komaki S, Suzuki T, Schoville SD..2017.

Species diversity of insects in Japan: Their origins and diversification process. *Entomological Science* 20: 357-381.

Uchida S, Isobe Y. 1991. Designation of a neotype for *Kamimuria tibialis* (Pictet, 1841), and *K. uenoi* Kohno, 1947, spec. propr., stat. n. (Plecoptera, Perlidae). *Aquatic Insects* 13: 65-77.

Wadachi K. 1972. *Encyclopedia of oceans*. Tokyo: Tokyo-do Press. [in Japanese]

Wallis GP, Waters JM, Upton P, Craw D. 2016. Transverse alpine speciation driven by glaciation. *Trends in Ecology & Evolution* 31(12): 916-926.

Weiss S, Ferrand N (eds.). 2007. *Phylogeography of southern European refugia*. The Netherlands: Springer.

Weng YM, Kavanaugh DH, Schoville SD. 2020. Drainage basins serve as multiple glacial refugia for alpine habitats in the Sierra Nevada Mountains, California. *Molecular Ecology* 30: 826-843.

Williams D, Dunkerley D, DeDeckker P, Kershaw P, Chappell M. 1998. *Quaternary Environments*. London: Arnold.

Willis KJ, Whittaker RJ. 2000. The refugial debate. *Scinece* 287: 1406-1407.

Yasick AL, Krebs RA, Wolin JA. 2007. The effect of dispersal ability in winter and summer stoneflies on their genetic differentiation. *Ecological Entomology* 32(4): 399-404.

Yasuda SP, Minato S, Tsuchiya K, Suzuki H. 2007. Onset of cryptic vicariance in the Japanese dormouse *Glirulus japonicus* (Mammalia, Rodentia) in the Late Tertiary, inferred from mitochondrial and nuclear DNA analysis. *Journal of Zoological Systematics and Evolutionary Research* 45: 155-162.

Yonekura N, Kaizuka S, Nogami M, Chinzei K (eds). 2001. *Regional Geomorphology of the Japanese Islands. 1. Introduction to Japanese Geomorphology*. Tokyo: University of Tokyo Press.

Yoshioka K. 1973. *Phytogeography*. Tokyo: Kyoritsu Press.

Yoshizawa K. 2004. Molecular phylogeny of major lineages of *Trichadenotecnum* and a review of diagnostic morphological characters (Psocoptera: Psocidae). *Systematic Entomology* 29: 383-394.

Figure 1. Distribution of the broad-leaved evergreen forest in Japan in currently (a) and during the last glacial (b) (solid area; modified from Yoshioka, 1973 and Ono & Igarashi, 1991). Bold and dotted arrows indicate warm and cold currents, respectively (according to Ohba 1993). The current coastline is shown by the dashed line in b.

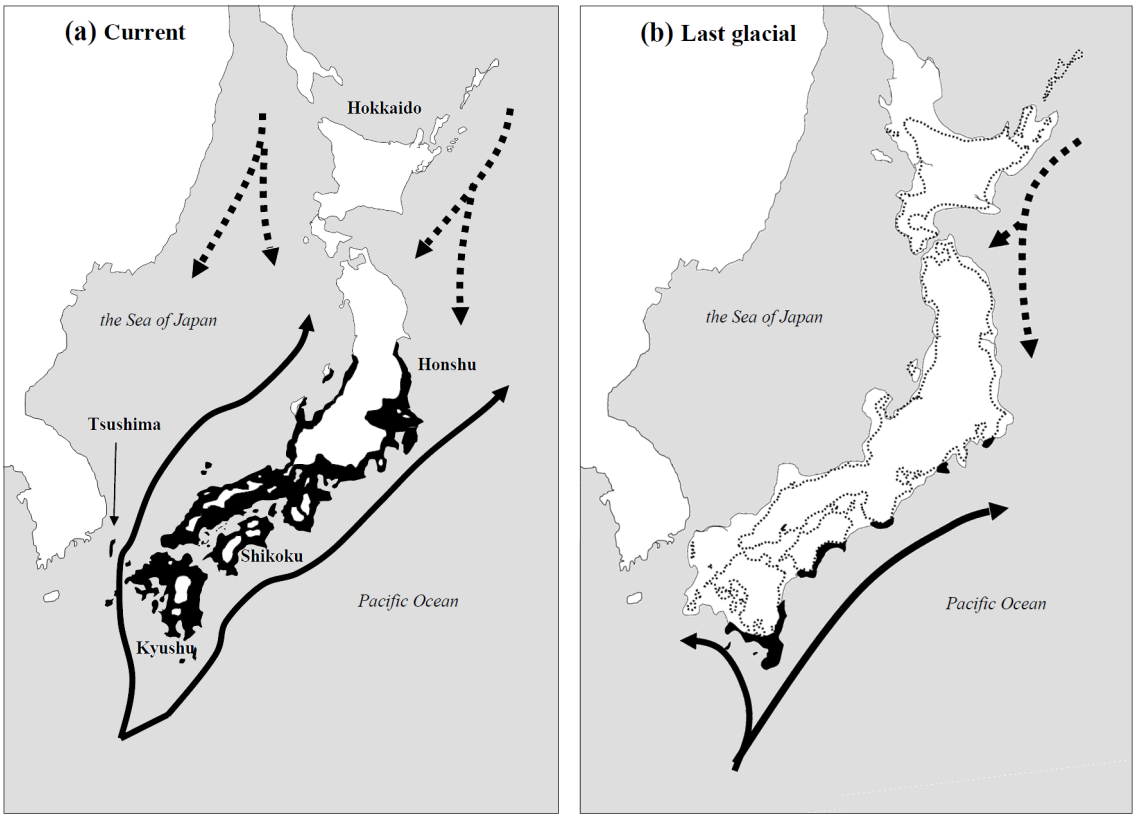


Figure 2. Sampling localities (circles) and sampling site ID (Supporting Information, Table S1). Sample sizes (numbers in the circles) are shown for populations with more than one sample. Six sampling localities of *Kamimuria uenoi*, an outgroup species, are shown by squares.

Fig. 2

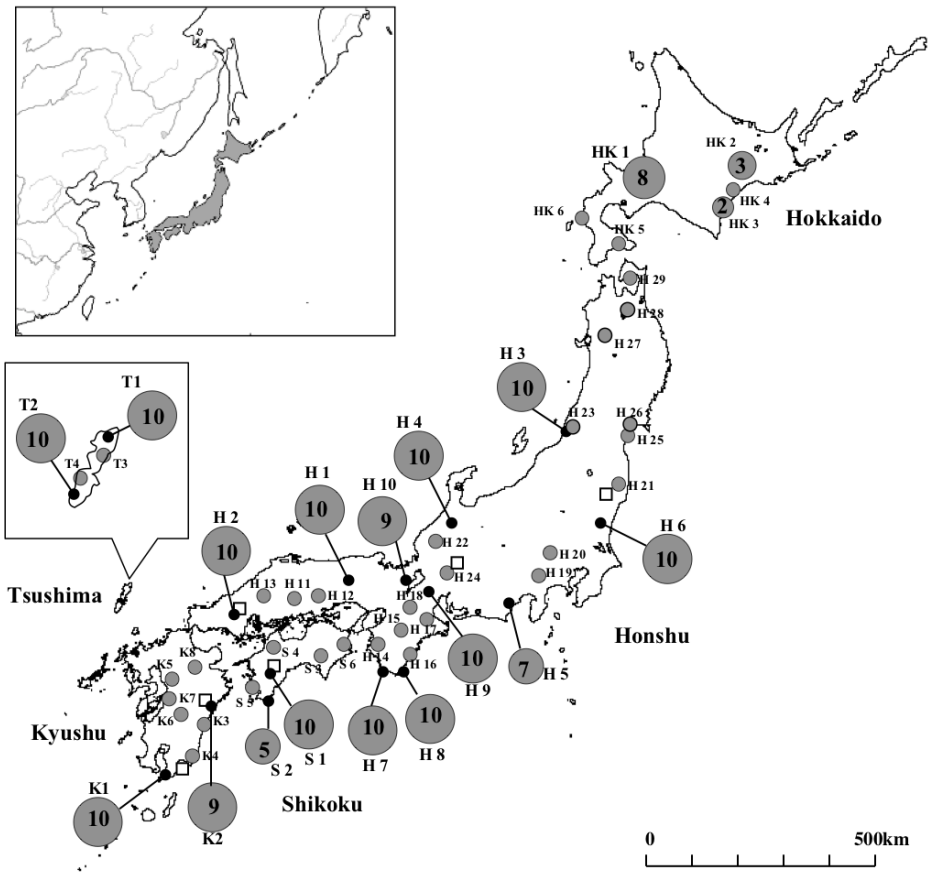


Figure 3. One of the 69 most-parsimonious topologies of the haplotype phylogeny recovered in MP analysis. The topology is consistent with that from ML and Bayesian analysis, except for some tip branches. The number in each operational taxonomic unit, haplotype ID; vertical lines, the main subclades in the text; numbers at nodes, MP bootstrap value / ML bootstrap value / posterior probabilities from the Bayesian analysis. Bootstrap values and posterior probabilities are shown only for the branches with >50% MP bootstrap value. Estimated coalescent times (Mya) for the numbered nodes are shown in Table 1.

Fig. 3

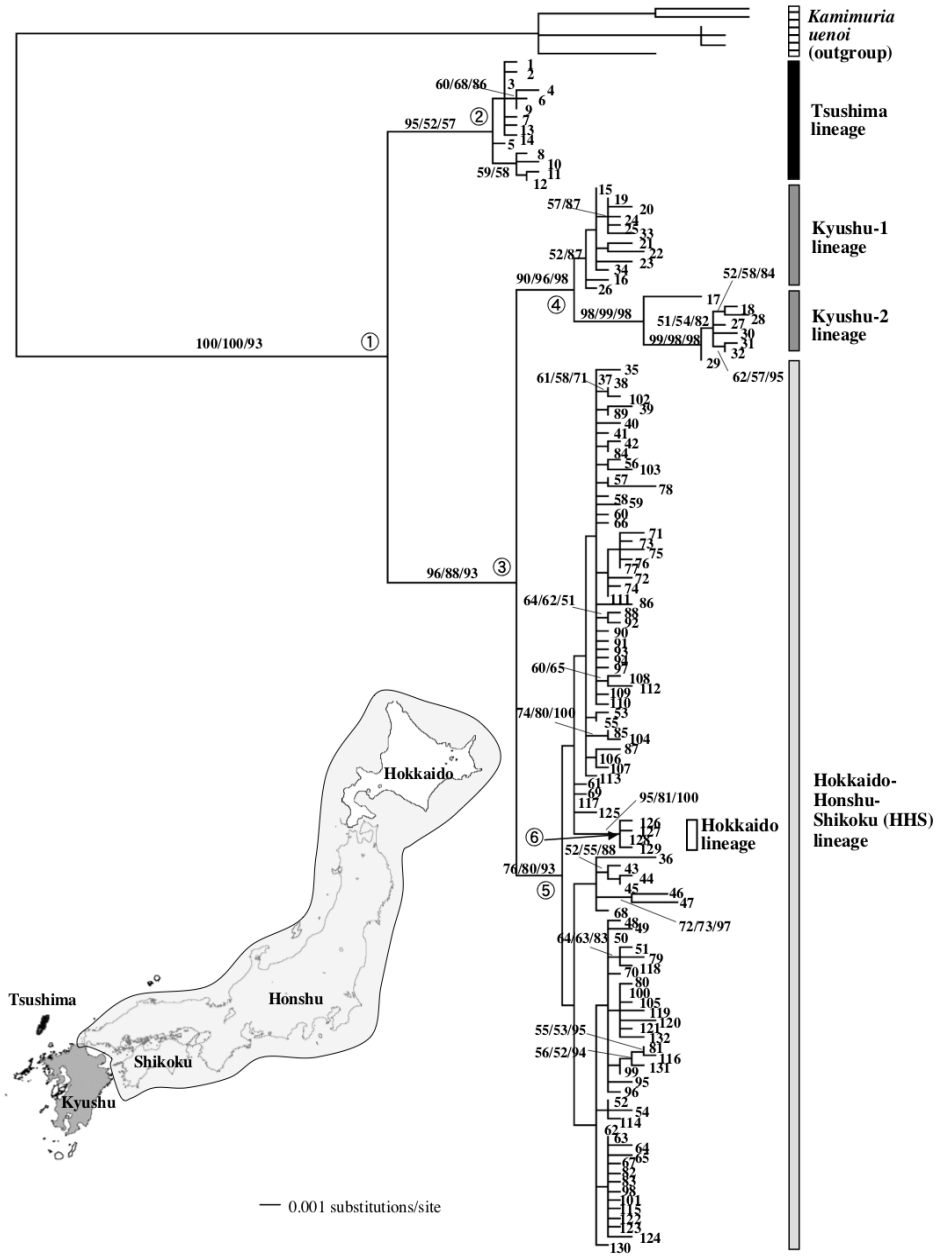


Figure 4. The median joining network of haplotypes in the HHS clade and the map of their distribution. The size of each circle corresponds to the number of haplotypes. Lines connecting circles are proportional to the number of mutations. Small dots indicate median vectors (i.e. possibly extant unsampled haplotypes or extinct ancestral haplotypes). The regional populations (Hokkaido, East Honshu, West Honshu and Shikoku) in the map are recognized by SAMOVA. Haplotype numbers are shown for seven Shikoku-specific haplotypes.

Fig. 4

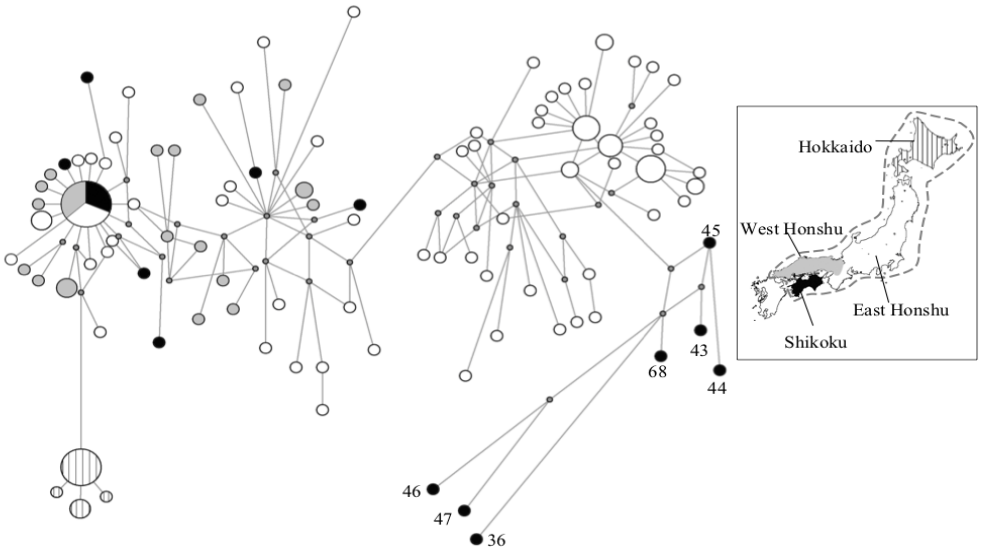


Figure 5. Mismatch distributions among haplotypes from six regional populations defined by SAMOVA. The histogram shows the observed distribution of pairwise differences between the individuals of analyzed populations. The line shows the expectations from the sudden expansion model (Schneider & Excoffier 1999). The circles indicate the sampling localities and the bold line in Honshu Island is a border dividing West and East Honshu regional populations. Unimodal distributions (e.g. Tsushima, West Honshu, and Hokkaido) are usually shown when populations have passed through a recent demographic expansion, whereas multimodal distributions (e.g. East Honshu, Shikoku, and Kyushu) are shown in the populations at a demographic equilibrium (Slatkin & Hudson, 1991; Roger & Harpending, 1992).

Fig. 5

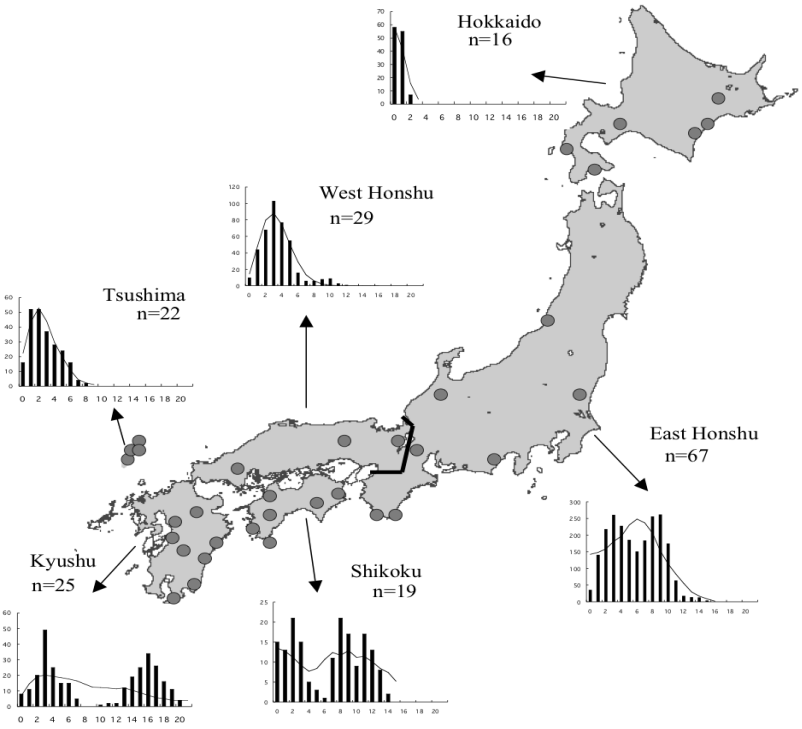
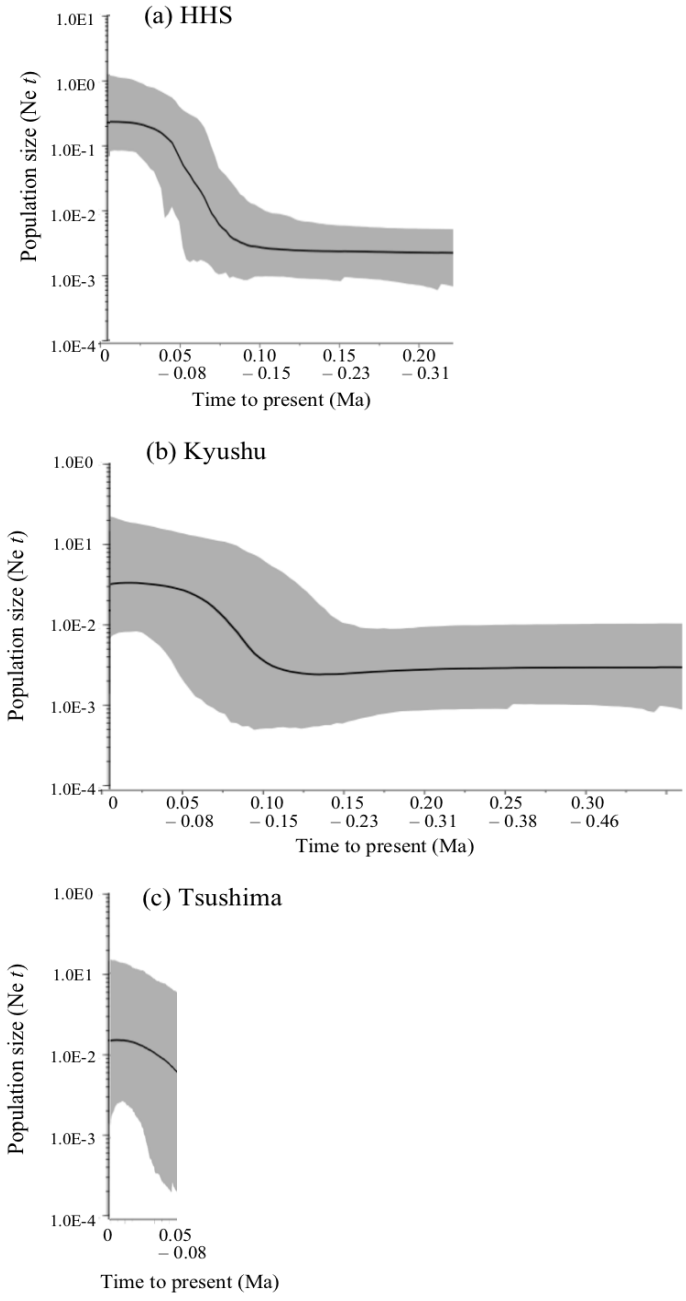


Figure 6. Bayesian Skyline Plots (BSP) showing the effective population size expansion throughout time. (a), Whole Hokkaido–Honshu–Shikoku (HHS) populations; (b), whole Kyushu populations; (c), whole Tsushima populations. The median estimation (solid line) and 95% confidence interval (grey area) are indicated.

Fig. 6



Supporting Information

Figure S1.
Figure S2.
Table S1-1.
Table S1-2.
Table S2.

Table 1. Coalescent time estimates (Myr) in mitochondrial lineages within *Kamimuria tibialis* for nodes in the phylogenetic tree (Fig. 3). Values in parentheses are the 95% confidence intervals for each estimate.

Node number in Fig. 3	Molecular clock		
	0.75 % / Myr	1.15 % / Myr	1.77 % / Myr
1	2.02 (1.37-2.71)	1.33 (0.92-1.79)	0.90 (0.64-1.17)
2	0.28 (0.11-0.49)	0.21 (0.07-0.43)	0.16 (0.08-0.25)
3	1.28 (0.83-1.74)	0.86 (0.60-1.58)	0.54 (0.38-0.72)
4	0.77(0.49-1.11)	0.62(0.37-0.92)	0.35(0.22-0.48)
5	0.61 (0.38-0.86)	0.42 (0.27-0.57)	0.30 (0.20-0.40)
6	0.16(0.11-0.23)	0.11 (0.07-0.15)	0.07 (0.05-0.10)

Table 2. Summary statistics of mitochondrial variation observed in each regional population. *N*, number of sequences; *h*, number of haplotypes; *Gd*, gene diversity; π, nucleotide diversity; *D*, Tajima's *D*; *Fs*, Fu's *Fs*. Asterisks indicate statistical significance ($P < 0.05$ for Tajima's *D* and $P < 0.02$ for Fu's *F*s) (Excoffier 2001). Standard deviations are in parentheses.

Regional population	*N*	*h*	*Gd*	π	Tajima's *D*	*p*	Fu's F_s	*p*
Tsushima	22	14	0.93 (0.04)	0.0022 (0.0013)	-1.18	0.115	-8.20	< 0.001*
Kyushu	25	20	0.97 (0.02)	0.0077 (0.0041)	-0.40	0.395	-6.57	0.140
Shikoku	19	14	0.91 (0.06)	0.0051 (0.0028)	-1.08	0.126	-4.14	0.036
West Honshu	29	25	0.98 (0.02)	0.0029 (0.0017)	-2.32	0.002*	-12.40	< 0.001*
East Honshu	67	48	0.98 (0.01)	0.0046 (0.0025)	-1.68	0.020*	-11.72	< 0.001*
Hokkaido	16	4	0.52 (0.13)	0.0005 (0.0004)	-1.06	0.160	-1.48	0.047