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Title	Phylogeography of the stonefly <i>Kamimuria tibialis</i> : multiple glacial refugia and sympatric occurrence of different lineages in the southern islands of Japan
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Citation	Biological journal of the Linnean Society, 134(2), 316-330 https://doi.org/10.1093/biolinnean/blab066
Issue Date	2021-10
Doc URL	https://hdl.handle.net/2115/86855
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Type	journal article
File Information	manuscript_Otsuki.pdf



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1 Running title : Phylogeography of a Japanese stonefly

2

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

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18

1 Abstract

2

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

19

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

1 Introduction

2

3 The role of Pleistocene glacial cycles on the distribution and geographical genetic
4 structure of current plant and animal species has been intensively studied over the last
5 two decades, in an attempt to understand the biodiversity of temperate and subarctic
6 regions (Hewitt, 1996, 1999, 2011; Avise, 2000; Willis & Whittaker, 2000; Knowles,
7 2001b; Tautz, 2004; Weiss & Ferrand, 2007; Wallis *et al.*, 2016; Lucati *et al.*, 2020).
8 During glacial periods, high latitudes in the Northern Hemisphere were covered with ice
9 sheets and permafrost (Williams *et al.*, 1998), which led species to extinct over large
10 part in these regions (Hewitt, 2000). Phylogeographical evidence suggests that there
11 was limited distribution of most temperate species during glacial periods, surviving in
12 southern refugia, and subsequently expanding northward during interglacial periods
13 (Avise, 2000; Hewitt, 2000, 2004; Weiss & Ferrand, 2007). Furthermore, it has been
14 postulated that the isolation of populations within separated refugia during glacial
15 periods promoted genetic differentiation among populations (Hewitt, 1988, 2000;
16 Knowles, 2001b, 2005; Weng *et al.*, 2020), and sometimes led to speciation, possibly
17 driven by sexual selection or local adaptations (Hewitt, 1988; Butlin, 1998; Knowles,
18 2000, 2001a; Tautz, 2004; Carstens & Knowles, 2007, Wallis *et al.*, 2016).

19 The Japanese islands have a diverse array of endemic species, and are recognized
20 as a biodiversity hotspot (Haffer, 1985; Contreras-Medina *et al.*, 2001; Tojo *et al.*,
21 2017). This is partly due to the lack of ice sheet development in this region, with the
22 exception of small parts of the alpine region, during glacial periods (Ono & Igarashi,
23 1991; Yonekura *et al.*, 2001). The unglaciated region could have enabled subalpine
24 stonefly species to survive the glacial epochs (McCulloch, 2010). If the area was not

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

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

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

7 This study attempts to elucidate the detailed process behind distributional changes
8 in temperate Japanese species during the Pleistocene, specifically in relation to the
9 formation of glacial refugia, and to the connection and the separation of the Japanese
10 islands, using the stream-dwelling stonefly *Kamimuria tibialis* (Plecoptera, Perlidae).
11 *Kamimuria tibialis* is endemic to the Japanese Archipelago and is distributed throughout
12 the four main islands: Hokkaido, Honshu, Shikoku, and Kyushu (Uchida & Isobe,
13 1991). This species is quite common in south-western Japan, which has a mild climate
14 (Shimizu *et al.*, 2005), whereas in north-western Honshu and Hokkaido, with low
15 average temperatures, it is found in a few streams of relatively warm waters, such as the
16 lower reaches of rivers and the downstream areas of lakes or dams (A. O., pers.
17 observ.). Cold climatic changes could have led to a reduction in the distribution of *K.*
18 *tibialis*. *Kamimuria tibialis* utilizes many kinds of aquatic insects as food sources
19 (Otsuki & Iwakuma, 2008). Given these facts, it is expected that the distribution of this
20 species has been affected by the glacial cycles of the Pleistocene, and may not be
21 limited by the species composition of local prey. Furthermore, stoneflies generally have
22 low dispersal ability (Schultheis *et al.* 2002; Yasick *et al.* 2007), so that past genetic
23 features could have been conserved up to the present. Consequently, the wide
24 distribution throughout the Japanese Archipelago, ecological features (i.e., preference

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

8

9

10 Materials and Methods

11

12 *Sampling, DNA extraction, PCR amplification and sequencing*

13

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

1 respectively. The reaction cycle was 94°C for 1 min, followed by 35 cycles of 94°C for
2 30 s, 42°C (ND5) or 44°C (COI) for 30 s, and 65°C for 45 s. Amplified products were
3 purified with a QIA quick purification kit (QIAGEN), and were then sequenced with a
4 CEQ2000 DNA Analysis System (Beckman Coulter) following the manufacturer's
5 protocols. Sequences were aligned visually in MacClade 4.03 (Maddison & Maddison,
6 2001). The alignment was unequivocal because the sequences included no indels or
7 repeats. Sequences were trimmed to 413 bp for ND5 and 853 bp for COI, giving a total
8 of 1266 bp.

9

10

11 *Phylogenetic analysis and estimation of divergence times*

12

13 Phylogenetic analysis of mitochondrial haplotypes from all the samples was conducted
14 by the maximum-parsimony (MP) and maximum-likelihood (ML) methods, using
15 PAUP 4.0b10 PPC (Swofford, 2002), and by the Bayesian method using MrBayes 3.1
16 (Huelsenbeck *et al.*, 2001). For MP analysis, all characters were equally weighted, and
17 heuristic searches with 100 random stepwise additions of taxa using TBR branch
18 swapping were performed. ML analysis was conducted using the TrN + I + G model of
19 evolution (unequal base frequencies: A = 0.3332, C = 0.1930, G = 0.1373, T = 0.3365;
20 six substitution categories: A-C = 1, A-G = 20.6145, A-T = 1, C-G = 1, C-T = 14.6008,
21 G-T = 1; gamma distribution shape parameter = 0.8423; proportion of invariant sites =
22 0.6719), which was indicated as the best fitting model, according to Akaike's
23 information criterion (AIC) values, in Modeltest 3.7 (Posada & Crandall, 1998). Branch
24 support was evaluated using 1000 and 100 replicate bootstrap analyses for MP and ML

1 analysis respectively. Bayesian analysis was conducted using two independent Markov
2 Chain Monte Carlo (MCMC) runs, each with four simultaneous chains, under the HKY
3 + I + G model that was selected according to AIC values in MrModeltest 2.2 (Nylander,
4 2004). From each chain of 1 million generations, trees were sampled every 100
5 generations. After discarding the first 2500 trees as a burn-in, a 50% majority consensus
6 tree was constructed. When a well-supported clade, with a broad distributional area, was
7 identified in the phylogenetic analysis, we constructed a median joining network for that
8 clade using NETWORK 4.5 (Bandelt *et al.*, 1999) to explore the geographical
9 relationships among haplotypes within the clade.

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

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

3

4

5 *Population genetic structure and demographic history*

6

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

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

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

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

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

1

1 1.4 (Drummond & Rambaut, 2007), for the Tsushima, Kyushu and Hokkaido-Honshu-
2 Shikoku phylogroups. The analyses were performed under the HKY + I + G model,
3 assuming 0.0075–0.0115 substitutions per site per Myr. MCMC was run for 10 million
4 generations, with sampling for every 1000 generations. After discarding the first 10% of
5 the chain, we determined whether the chains had converged by monitoring the traces of
6 sampled parameters, and whether the condition for the ESS was satisfied for each
7 parameter, using TRACER 1.3 (Rambaut & Drummond, 2005).

8

9

10 *Data availability*

11

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

20

21

22 Results

23

24 *Haplotype phylogeny, distribution and divergence*

1

1

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

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

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

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

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

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

13

14 *Population genetic structure and demographic history*

15

16 Pairwise population F_{ST} values showed significant differentiation for all of the
17 population pairs from the different phylogroups (range: 0.48–0.95; $P < 0.05$,
18 permutation tests with 100 permutations), while F_{ST} values were not significant for 32 of
19 the 68 population pairs within the same phylogroups (range: –0.09 to 0.16, $P = 0.072$ –
20 0.955) (Supporting Information, Table S2). These results were congruent with the
21 geographical clustering of the haplotype lineages and the overlapping of some
22 haplotype distributions within phylogroups.

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

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

7 In the Kyushu population, the mismatch distribution pattern was clearly bimodal
8 (Fig. 5; significance of the mismatch distribution, P , and sum of squared deviation,
9 SSD, were 0.05 and 0.036, respectively). This bimodal distribution pattern suggests the
10 coexistence of genetically divergent groups; this result was consistent with the results
11 obtained from the phylogenetic analysis (Fig. 3). A bimodal pattern was also found in
12 Shikoku Island ($P = 0.67$ and $SSD = 0.016$), and this result agreed with that of the
13 haplotype network (Fig. 4). In the Kyushu and Shikoku populations, neither Tajima's D
14 nor Fu's F_s differed significantly from the values estimated under the population
15 equilibrium model. In particular, the Kyushu population exhibited high gene diversity
16 (0.97) and conspicuously high nucleotide diversity (0.0077) (Table 2). The eastern
17 Honshu population also had a nearly bimodal mismatch distribution (Fig.5; $P = 0.35$
18 and $SSD = 0.004$), high gene diversity (0.98), and relatively high nucleotide diversity
19 (0.0046) (Table 2); both Tajima's D and Fu's F_s were significantly negative, suggesting
20 recent population expansion (Table 2).

21 In contrast, the mismatch distribution patterns in the Tsushima and western
22 Honshu populations were unimodal, similar to the distribution expected during sudden
23 range expansion (Fig. 5; $P = 0.58$ and $SSD = 0.005$ for the Tsushima population; $P =$
24 0.26 and $SSD = 0.011$ for the western Honshu population). Both Tajima's D and Fu's F_s

1 were significantly negative in the western Honshu population, and Fu's F_s was
2 significantly negative in the Tsushima population (Table 2). The Tsushima and western
3 Honshu populations exhibited high gene diversity (0.93 and 0.98) but relatively low
4 nucleotide diversity (0.0022 and 0.0029). In the Hokkaido population, we observed a
5 markedly unimodal pattern in the mismatch distribution ($P = 0.17$ and $SSD = 0.016$)
6 and a very low mean value in the distribution (0.58). In addition, the Hokkaido
7 population exhibited very low values of gene diversity (0.52) and nucleotide diversity
8 (0.0005). Neither Tajima's D and Fu's F_s were significantly negative.

9 The BSP predicted a population expansion that began ~0.10–0.15 Mya in the
10 Kyushu and Hokkaido–Honshu–Shikoku phylogroups (Fig. 6). The extent of the
11 estimated population expansion differed greatly among the phylogroups, ranging from a
12 10-fold increase in the Kyushu phylogroup to a 100-fold increase in the Hokkaido-
13 Honshu-Shikoku phylogroup. The Tsushima phylogroup was characterized by constant
14 population growth for the last 50 000 years, with a 50-fold increase.

15

16

17 Discussion

18

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

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

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

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

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

1 The Tsushima Island and western Honshu populations have demographic
2 characteristics that suggest a recent rapid expansion of a single lineage group (Fig. 5,
3 Table 2). These results suggest that these regions were rapidly colonized by *K. tibialis*
4 populations that may have migrated from the putative refugia. Although Tsushima
5 Island and western Honshu are located in the southern part of the Japanese Archipelago
6 and currently have warm climates, these regions would have been dry and cold during
7 the glacial periods, because the warm current did not flow into the Sea of Japan (Fig.
8 1b).

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

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

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

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

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

3 Studies on the genetic structures of stonefly species have recently been
4 successively reported. It appears to be clear that for the stoneflies, genetic
5 differentiation correlated with flight ability: the clear phylogeographical structures are
6 shown in *Zelandoperla fenestrata* (Gripopterygidae) species group with low flying
7 ability, whereas in the very same region, the significant geographical structures were not
8 detected for *Z. decorata* having high flying ability (McCulloch *et al.*, 2009);
9 intraspecific divergences of COI were 0.7–1.3% for the three apterous genera and 0.6–
10 0.7% for the three macropterous genera in the New Zealand stoneflies (McCulloch *et*
11 *al.*, 2010). McCulloch *et al.* (2010) also showed the impact of glaciations on the
12 distribution and genetic structures of these stoneflies: the centre of the glaciated region
13 in the South Island of New Zealand has become the current “biotic gap”, a low diversity
14 region, and on either side of this gap there were obvious intrageneric divergences for all
15 six genera. For the Nemouridae species inhabiting the highest reaches of Rocky
16 Mountain alpine streams, nucleotide diversity (π) were 0.0035 for *Lednia tumana*,
17 0.0013 for *L. tetonica*, and 0.0696 for *Zapada glacier*, three populations in isolated
18 regions (Hotaling *et al.*, 2019), showing population divisions based on distance would
19 have resulted in genetic divergence for these short-winged meltwater depending species.
20 For the Perlidae to which *K. tibialis* belongs, nucleotide diversity (π) of the cytochrome
21 b (*cytb*) region was 0.00166 for *Hesperoperla pacifica* populations from across the
22 Great Basin of western North America (Schultheis *et al.*, 2014), a ubiquitous species
23 found in a wide variety of streams of western North America (Stewart & Stark 1988).
24 *Kamimuria tibialis* has a good flying ability, and females usually fly long distances

1 upstream to oviposit (Nishimura, 1962; Ichikawa et al., 2020). Nevertheless its good
2 flying ability, the degree of intraspecific divergence seems high in *K. tibialis* as
3 compared with other stoneflies: mitochondrial nucleotide diversity were 0.0077 for the
4 Kyushu population, 0.0052 for the Hokkaido–Honshu–Shikoku population, and 0.0022
5 for the Tsushima population; pairwise distances of COI+ND5 of *K. tibialis* were about
6 2.5% for the Tsushima and Kyushu populations, about 2.1% for the Tsushima and
7 Hokkaido–Honshu–Shikoku populations, and about 1.6% for the Kyushu and Honshu
8 populations. This intraspecific genetic diversity is mainly due to the population division
9 between the Japanese islands and high genetic diversity in the Kyushu and Shikoku
10 Islands, putative glacial refugia. Our study indicates that for the stoneflies with
11 relatively high flying ability, population division (e.g., by deep straits between islands)
12 and the existence of stable glacial refugia could have contributed to current intraspecific
13 genetic diversity.

14

15

16 Acknowledgements

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

1

1 by the Ministry of Education, Culture, Sports, Science, and Technology, Japan (ID:
2 964644); and in part by the NOASTEC foundation (H17-Y-106). We sincerely thank
3 three anonymous reviewers for their helpful comments, which greatly improved an
4 earlier draft of this manuscript. The authors declare no conflicts of interests.

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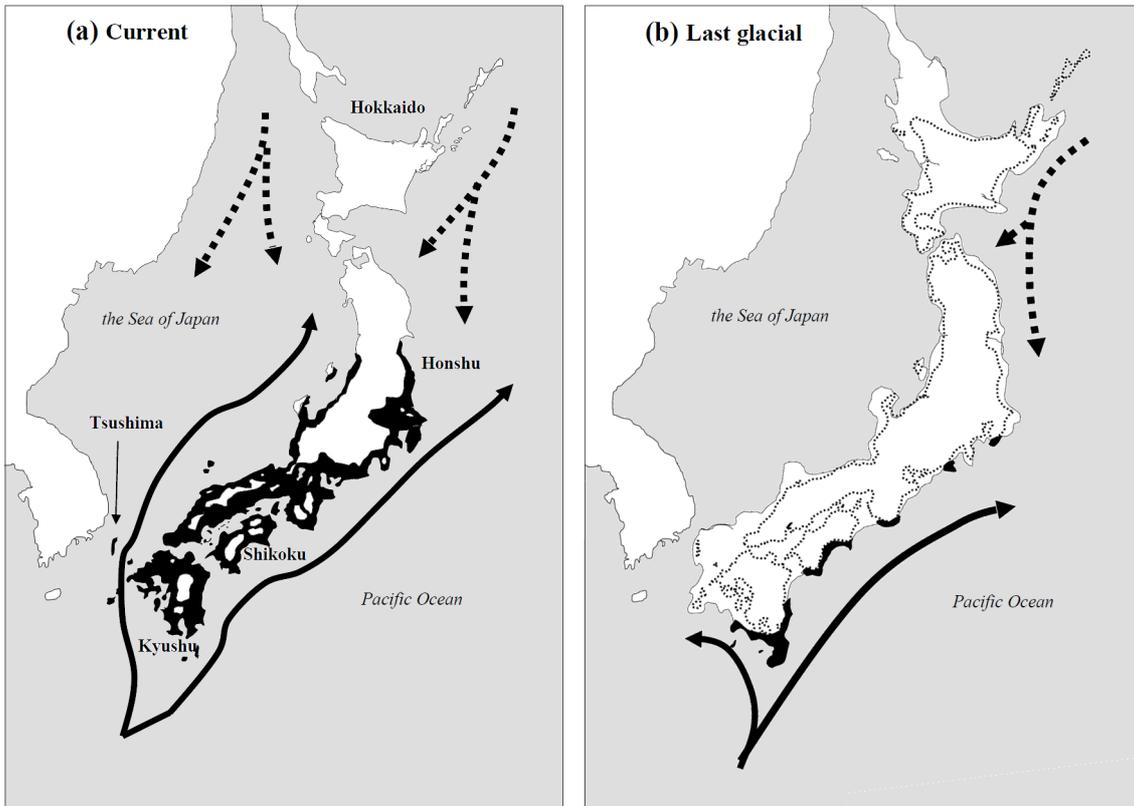
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2 Figure 1. Distribution of the broad-leaved evergreen forest in Japan in currently (a) and
3 during the last glacial (b) (solid area; modified from Yoshioka, 1973 and Ono &
4 Igarashi, 1991). Bold and dotted arrows indicate warm and cold currents, respectively
5 (according to Ohba 1993). The current coastline is shown by the dashed line in b.
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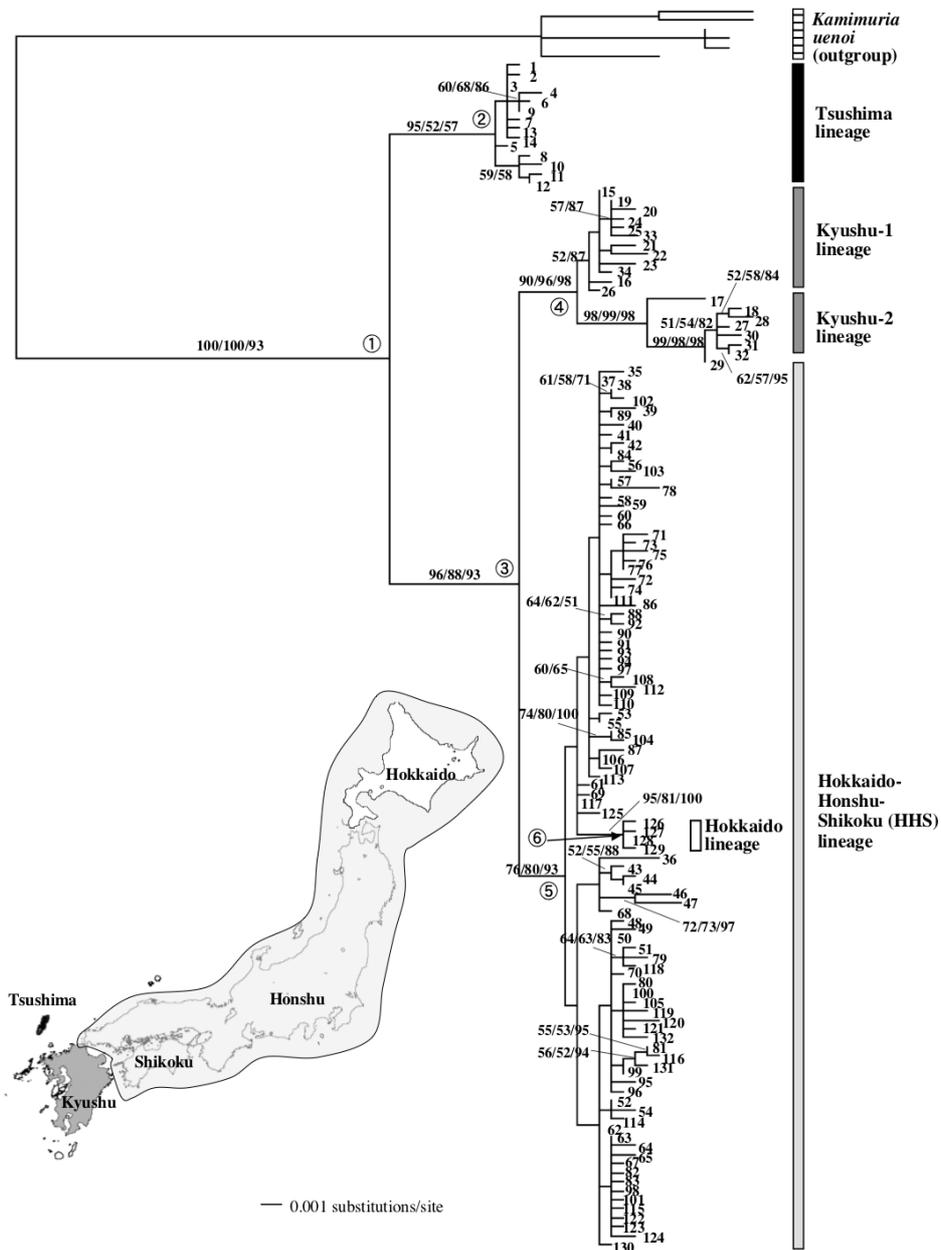
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1 Figure 3. One of the 69 most-parsimonious topologies of the haplotype phylogeny
 2 recovered in MP analysis. The topology is consistent with that from ML and Bayesian
 3 analysis, except for some tip branches. The number in each operational taxonomic unit,
 4 haplotype ID; vertical lines, the main subclades in the text; numbers at nodes, MP
 5 bootstrap value / ML bootstrap value / posterior probabilities from the Bayesian
 6 analysis. Bootstrap values and posterior probabilities are shown only for the branches
 7 with >50% MP bootstrap value. Estimated coalescent times (Mya) for the numbered
 8 nodes are shown in Table 1.
 9

Fig. 3



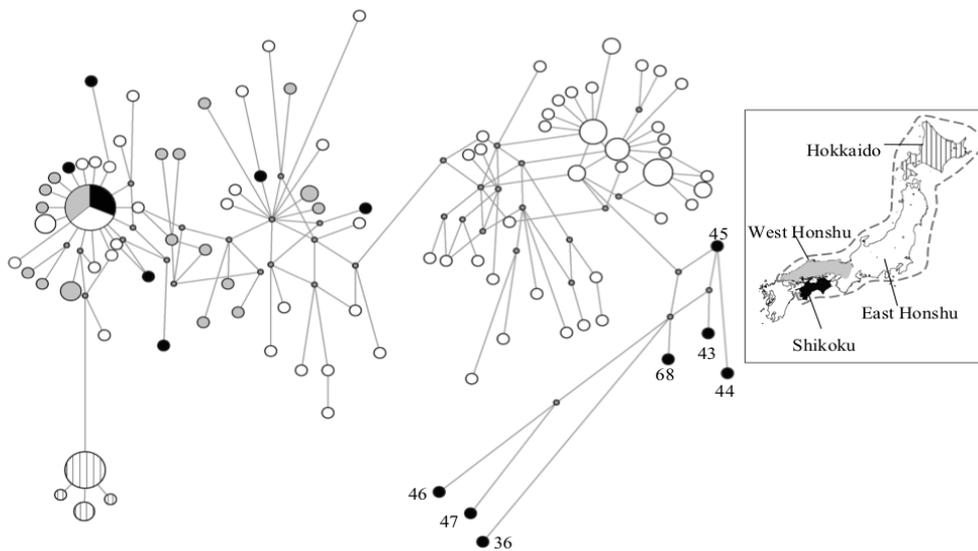
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3 Figure 4. The median joining network of haplotypes in the HHS clade and the map of
4 their distribution. The size of each circle corresponds to the number of haplotypes.
5 Lines connecting circles are proportional to the number of mutations. Small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

Fig. 4



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

Fig. 5

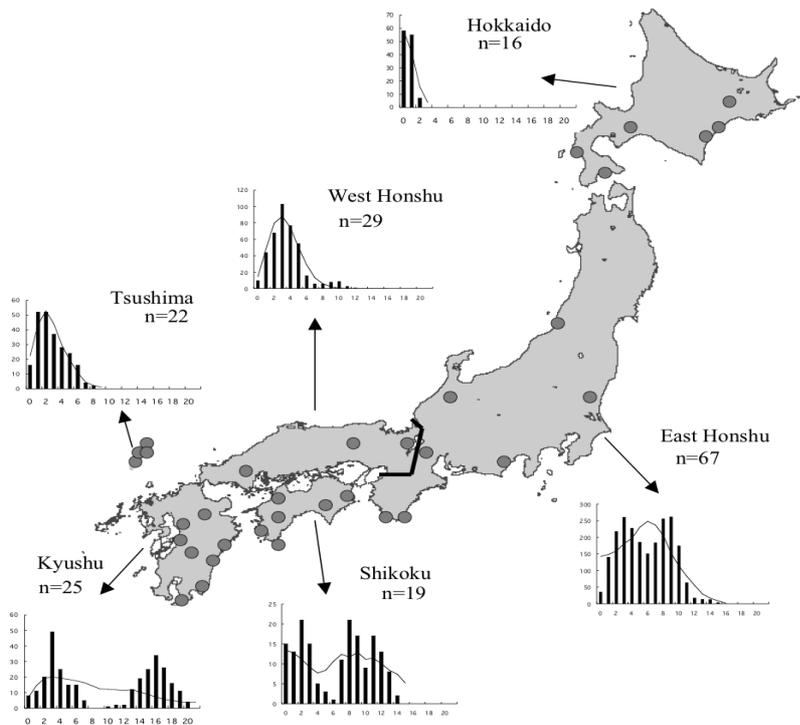
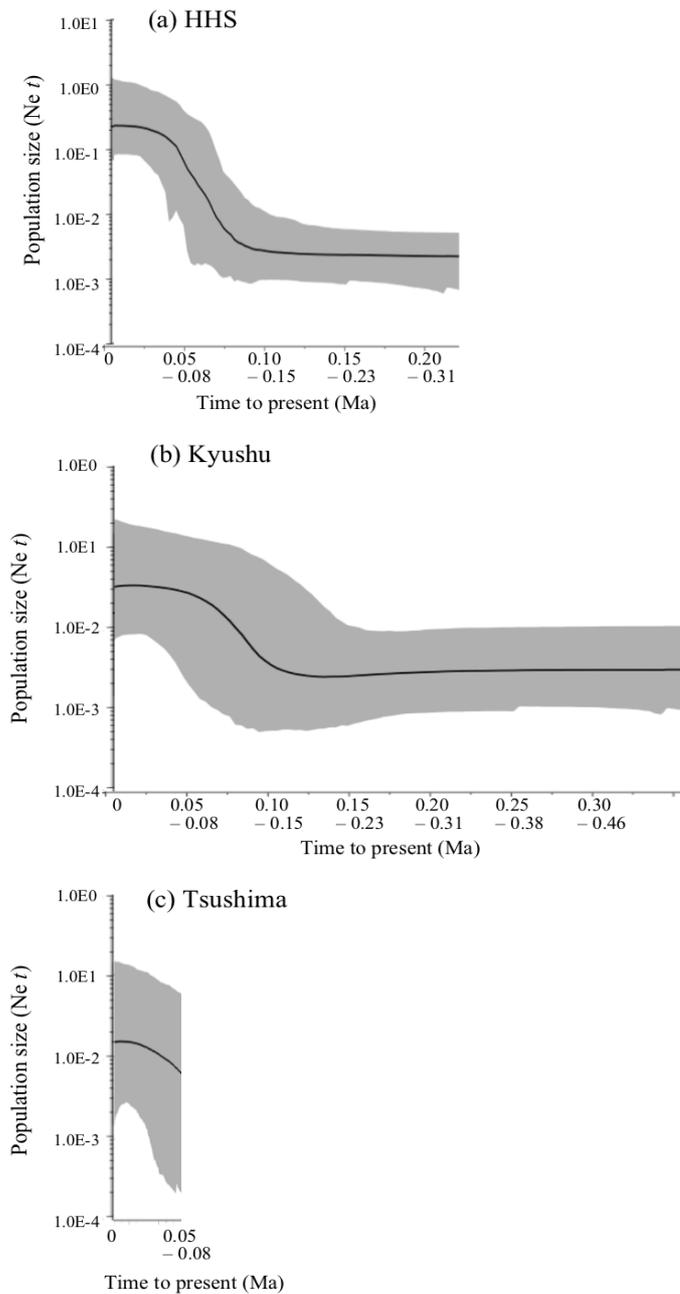


Fig. 6



1 **Supporting Information**

2

3 Figure S1.

4 Figure S2.

5 Table S1-1.

6 Table S1-2.

7 Table S2.

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12 Table 1. Coalescent time estimates (Myr) in mitochondrial lineages within *Kamimuria*
 13 *tibialis* for nodes in the phylogenetic tree (Fig. 3). Values in parentheses are the 95%
 14 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)

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

6 Standard deviations are in parentheses.

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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