Phylogeography of *Littorina brevicula* suggests post-glacial colonization from south to north along the Japanese Archipelago

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ABSTRACT

Littorina brevicula is one of the most common gastropods in the intertidal-supralittoral zone around Japan. The northernmost population of this species is around Hokkaido, and the determinant of this northern limit is likely seawater and air temperature. To reconstruct an evolutionary history of this species, we investigated genetic differentiation among 12 populations (three from Hokkaido, six from Honshu and three from Kyushu) using a mitochondrial DNA marker (partial sequence of the NADH dehydrogenase 6 gene). The haplotype network showed shallow genetic divergence within the species, suggesting a bottleneck followed by population expansion. One major haplotype that occurred in 70.5% of all individuals examined was the most frequent in every population sampled. A second major haplotype was abundant around Kyushu but not found in Hokkaido. This skewed haplotype distribution resulted in significant genetic differentiation along the north-south axis of Japan. The importance of the southern clade, which included the second major haplotype, was supported by population analyses of datasets that excluded either the southern clade or the northern clades. The north-south differentiation remained when datasets were analyzed that excluded the northern clades, but disappeared when datasets were analyzed that excluded the southern clade. The combined evidence of shallow divergence and the north-south population structure suggests that the *L. brevicula* population around Japan once declined and then expanded and colonized northward. Although the time of population reduction and re-colonization could not be precisely estimated, the observation that this species is absent further north in Japan suggests that it would have been unable to survive in northern Japan during the last glacial maximum (LGM) and therefore re-colonization likely occurred after the LGM, probably from south to north.

INTRODUCTION

Many species in the northern hemisphere probably underwent changes to their distribution as a result of strong influence from climate oscillation during glacial periods, particularly in the last glacial maximum (LGM) that lasted until around 20,000 years ago and in the subsequent rapid warming. Such distributional changes can be detected by phylogeographic investigations using suitable molecular markers (Hewitt, 2000). The coastal ecosystem was considerably influenced by the LGM and thus various species exhibit genetic evidence of rapid expansion and/or colonization, probably occurring in the post-glacial period. Examples include the near-shore fish *Syngnathus leptorhynchus* (Wilson, 2006) and *Xiphister mucosus* (Marko *et al*., 2010), seaweed *Pelvetia canaliculata* (Neiva *et al*., 2014), gastropods *Nassarius nitidus* (Albaina *et al*., 2012), *Littorina sitkana* and *L. scutulata* (Marko *et al.*, 2010), and starfish *Pisaster ochraceus* and *Evasterias troschelii* (Marko *et al*., 2010). Around Japan, glacial effects on intertidal molluscs have been suggested in *Batillaria cumingi* (Kojima *et al.*, 2004) and *Cellana nigrolineata* (Nakano, Sasaki & Kase, 2010). However, the more stable temperature of the ocean compared to that of the land allowed some coastal species to maintain their genetic diversity during glacial maxima. Furthermore, the glacial effects sometimes varied within a single species, resulting in a complex population genetic structure, as in *Littorina saxatilis*, in which some populations seemed to have colonized following climate change, while others maintained diversity during a long continuous history (Panova *et al*., 2011). Such a spatially variable effect within a single species has also been suggested for *Nucella ostrina* and *N. lamellosa* (Marko *et al*., 2010). The variable genetic consequences of the LGM between or within species are probably due to specific ecological characteristics of each species, i.e. distribution range, habitat, life cycle and abundance, and also to area-specific physical effects of climatic oscillation such as temperature and sea-level change.

In addition to the effects of climate oscillations over paleontological time scales, genetic population structure within a species is affected by contemporary gene flow. Populations of marine species are often weakly structured (i.e. show a low level of differentiation among local populations and/or weak correlation between geography and genetic differentiation) over the species' range (Palumbi, 1994), due to a high level of gene flow caused by active and passive dispersal. In invertebrates that generally showed poor active mobility,

gene flow has been thought to be largely affected by the developmental mode and the length of the planktonic larval period, and colonization and contemporary gene flow in marine species are generally mediated by larval dispersal along ocean currents (Palumbi, 1994; Tsang *et al.*, 2008). Recently, larval dispersal has been revealed to be less crucial for determining the genetic structure of marine species than previously supposed (Marko, 2004; Weersing & Toonen, 2009), although it remains an important factor. A comparative study of two planktonic- and two direct-developing *Littorina* species suggested that planktonic larval dispersal reduced genetic differentiation between local populations (Kyle & Boulding, 2000). In their analyses, planktonic-developing species (*Littorina scutulata* and *L. plena*) showed very low levels of genetic differentiation among local populations, while the direct-developing *L. subrotundata* showed higher structuring with larger differentiation between localities. Subsequent research revealed that direct-developing *Littorina* species showed spatial rather than temporal differentiation, and that planktonic-developing species showed the reverse, due to sweepstakes-like reproductive success (Lee & Boulding, 2009). These studies show that *Littorina* is a good model in which to investigate the relationships between genetic population structure, larval dispersal, and historical factors in marine coastal species.

Littorina brevicula occurs in the littoral fringe of the temperate coast of the northwestern Pacific. Its range extends from Hong Kong to Peter the Great Bay along the continental coast of Asia and from Okinawa to Hokkaido along the Japanese Archipelago (Reid, 1996; Okutani, 2000). On the central to northern Japanese coast, *L. brevicula* is one of the most common snails, occurring in dense aggregations on rocky and boulder shores and on artificial constructions such as breakwaters, tetrapods and slipways. However, it is scarce along the southeastern coast of Hokkaido, which is under the influence of a cold current (Ohgaki, 1983). The spawning season is January to April around Japan and Korea (Kojima, 1957; Son & Hong, 1998). The egg capsule is pelagic and the veligers hatch about 7 days after spawning (Son & Hong, 1998). The total length of the planktonic egg and larval period is several weeks (Golikov, 1976). Such a long planktotrophic period is expected to lower the genetic differentiation between local populations of *L. brevicula* (Reid, 1996). Nevertheless, a spatial genetic structure could potentially be found if samples from a wide geographic range were investigated using highly variable molecular markers (Palumbi, 1994). The fine details of genetic structure across a wide area may provide knowledge about the effects of environmental change on coastal

86 species on a paleontological scale.

87 Several studies of population genetics in *L. brevicula* using allozyme variation appeared in the 1990s (e.g.
88 Zaslavskaya, Sergievsky & Tatarenkov, 1992; Tatarenkov, 1995; Park *et al.*, 1999; Zaslavskaya & Takada,
89 1998), and some of these studies reported genetic differentiation among samples. This was variously attributed
90 to reduced gene flow between distant populations (the east and west coasts of the Sea of Japan; Zaslavskaya &
91 Takada, 1998), the effect of heavy metal pollution (Park *et al*., 1999), and possible local selection on specific
92 genes (Tatarenkov, 1995). Since these studies did not aim to clarify the total phylogeography of *L. brevicula*
93 throughout the distribution range, they provided little information about hierarchical genetic structure among
94 populations. Nevertheless, Zaslavskaya & Takada (1998) showed genetic differentiation between distant
95 populations, which suggested the possibility of some structuring throughout the species' range.

96 More recently, DNA markers were used to investigate population structure in *L. brevicula* around Korea
97 (Kim *et al*., 2003b; Kim, Rodriguez-Lanetty & Song, 2003a). Kim *et al*. (2003b) failed to detect genetic
98 differences among populations, even for populations 1,000 km distant from each other along the coastline.
99 Kim *et al.* (2003a) found significant population differentiation which they attributed to a loss of genetic
100 diversity caused by heavy metal pollution of some populations.

101 In this study, we investigated the genetic structure among populations of *L. brevicula* in its the main
102 distribution area around Japan, from Hokkaido to Kyushu (Fig. 1). These populations span approximately
103 2,500 km, and experience considerably different climatic conditions, therefore we expected to observe genetic
104 structure. We chose the mitochondrial NADH dehydrogenase 6 gene (ND6) as a genetic marker, because this
105 region has previously been shown to be more polymorphic than the commonly-used cytochrome-*b* in *L.*
106 *brevicula* (Kim *et al*., 2003a). We found population genetic structure that revealed the evolutionary history of
107 *L. brevicula* around Japan, and suggested patterns of population decline, expansion, and migration during
108 recent glacial and post-glacial periods.
109

MATERIAL AND METHODS

Sampling, DNA sequencing, and analysis of haplotype genealogy

We examined 540 individuals collected from 12 populations in three regions: three populations from Hokkaido (SSB, ABS, USJ), six from Honshu (FKU, HCH, OBM, CHO, URG, OSK) and three from Kyushu (SIK, AMK, MKR) (Table 1, Fig. 1). Genomic DNA was extracted from a piece of muscle (approximately 1 mm^3) of ethanol-fixed specimens using the Pure Gene Kit (Qiagen) according to the manufacturer's protocol and resuspended in 100 µl of Tris-EDTA buffer.

A partial sequence of ND6 was amplified by PCR in a 30-μl reaction mixture containing template DNA (approximately 200 pg), dNTPs, a pair of primers [Lbnd-F (5′ -AGG TAC ATA TTC CTG CGC TCT GAA A-3′) and Lbnd-R (5′ -GTG TGC GCA TGA AAT GTA T-3′)] (Kim *et al*., 2003) and ExTaq (Takara Bio Inc.), according to the manufacturer's instructions. The thermal cycling profile included precycling denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 20 s, and extension at 72°C for 45 s. The PCR products were then examined by electrophoresis on a 2% agarose gel, purified with magnetic beads (AMPure, Agencourt), cycle-sequenced using the above-mentioned forward primer and the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and loaded onto an automated sequencer, ABI PRISMTM 3130 (Applied Biosystems). The obtained sequences were aligned and edited to 450 bp using DNASIS-Mac v. 3.5 (Hitachi) and ClustalX v. 1.81 software (Thompson *et al*., 1997) for defining haplotypes and deposited in the DDBJ/Genbank database with accession nos. AB968435–AB968512.

The haplotype genealogy was resolved with a parsimony network using the TCS Network Program (Clement, Posada & Crandall, 2000) under a 95% connection limit. The age of lineage divergence was estimated using the divergence rate of 2.46% per million years (Myr). The rate was based on a calibration for mtDNA including the ND6 region in *L. saxatilis* (Quesada *et al*., 2007), which assumed a sequence divergence rate of 1.83 ± 0.21% per Myr. Since Quesada *et al*. applied this rate for the 1.8-kbp sequence including a not very variable region, the calibration may underestimate the evolutionary rate of our ND6 data. To correct the rate, we examined the GenBank data of *L. saxatilis* (accession nos. AM500948–500955,

deposited by Quesada *et al*.) and compared sequence divergence within 1.8 kbp of the full length of these data and 0.45 kbp of the ND6 region, orthologous to our sequence data. In the 1.8-kbp sequence, the number of different nucleotides per site was 0.145, while it was 0.195 in the ND6 region in the *L. saxatilis* data. We therefore employed the corrected divergence rate of 2.46% (1.83×0.195/0.145) per Myr.

Population genetic analyses

Arlequin v. 3.1 (Excoffier, Laval & Schneider, 2005) was used to estimate haplotype (h) and nucleotide diversity (π) in each population and to calculate pairwise F_{ST} (Weir & Cockerham, 1984). Genetic distance between populations based on the pairwise F_{ST} was visualized on a two-dimensional surface by nonmetric multidimensional scaling (nMDS) plotting using statistical software R v. 2.9.0 (R Development Core Team, 2005). To assess geographic-genetic correlation, the isolation-by-distance (IBD) model (Wright, 1943) was tested using Arlequin 3.1. For the IBD test, the matrix of geographic distances along the coastline between sampling sites was compared with the F_{ST} matrix, and the significance of the correlations was evaluated using the Mantel test with 10,000 permutations.

To test the significance of the hierarchical population structure, analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was conducted with Arlequin 3.1, comparing the three categories of clustering suggested by geography and/or nMDS plotting based on F_{ST} analysis: (1) three regional group clusterings—Hokkaido, Honshu and Kyushu, i.e. [SSB, ABS, USJ], [FKU, HCH, OBM, CHO, URG, OSK] and [SIK, AMK, MKR], respectively; (2) three groups suggested by nMDS plotting based on pairwise F_{ST} analysis—Hokkaido+HCH, Honshu (except HCH), and Kyushu, i.e. [SSB, ABS, USJ, HCH], [FKU, OBM, CHO, URG, OSK] and [SIK, AMK, MKR], respectively; and (3) two groups of clustering by coast—the Sea of Japan and the Pacific Ocean (including the Yellow Sea), i.e. [SSB, ABS, FKU, OBM] and [USJ, HCH, CHO, URG, OSK, SIK, AMK, MKR], respectively. Since Category (3) took into account ocean currents, ABS on the coast of the Sea of Okhotsk, where a branch of the Tsushima current flows in from the Sea of Japan (Fig. 1), was included in the Sea of Japan cluster.

When we detected significant genetic structure, we evaluated the effect of each clade (haplotype group derived from the most abundant haplotype shown in Figure 3) on population structure by preparing virtual

datasets for population analyses, performing an IBD test, estimating pairwise F_{ST}, and conducting AMOVA using these datasets. Datasets were individually modified by excluding an area-restricted clade (haplotype groups connected to each other in a haplotype network; Fig. 3) and then used to test whether or not the structure appeared. If we found that the structure was lost when a certain clade was excluded from the modified dataset, we would know that the excluded clade was essential for the structure.

To test recent population expansions, neutrality tests and mismatch distribution (MMD) analysis with Arlequin 3.1 were added. When our MMD analysis detected population and range expansion, the expansion age was estimated based on the τ value, assuming a sequence-divergence rate of 2.46% per Myr. Neutrality tests of Fu's F_S and Tajima's D were conducted to detect the possibility of population expansion.

Based on the genealogy and distribution of haplotypes, we detected the population structure and inferred the shift of the distribution area following a series of steps in the flow chart in Figure 2. The results of several analyses were employed to test the determinants in Figure 2: pairwise F_{ST} estimation for step 1; test of isolation by distance (IBD), nMDS plotting of populations based on pairwise F_{ST}, and AMOVA, for steps 2 and 4; construction of the haplotype network and survey of the genealogy for step 3; and estimation of indices of genetic diversity in each population and each area for step 5. The first step, checking heterogeneity between populations, is also informative to evaluate the level of contemporary gene flow, which causes panmictic populations all through the distribution area in extreme cases. The second step, checking the genetic-geographic association, can reveal genetic drift and is useful for detecting population structures skewed by human impact as in *L. brevicula* (Kim *et al.*, 2003b) and *Neptunea arthritica* around Japan (Azuma *et al.*, 2015).

RESULTS

Genealogy and distribution of haplotypes

A total of 78 haplotypes were detected. *LbN6AB03* was the most abundant haplotype, occurring in 381 individuals (70.5% of all individuals examined in the present study). This haplotype was also the most frequent in all populations, and was located at the centre of the haplotype network (Fig. 3). The second most

191 frequent haplotype, *LbND6OT02*, occurred in 44 individuals (8.1% of all individuals examined) from Honshu
192 and Kyushu, and the third, *LbND6AO10*, occurred in 10 individuals from Honshu and Hokkaido. Other
193 haplotypes occurred in less than 9 individuals, and 65 haplotypes were private, i.e. found exclusively in a
194 single population (see Supplementary Data). Given that nucleotide divergence between *LbN6AB0*3 and
195 *LbN6OT02* was 0.22% per site, the time of divergence was estimated as 89 thousand years ago (kya). The
196 largest number of nucleotide substitutions observed was six, 1.33% per site.
197 Several clades appeared in the haplotype network. Six of the clades were found only in Hokkaido and
198 Honshu (northern clades), and one only in Honshu and Kyushu (a southern clade). The rest of the clades
199 appeared in Hokkaido, Honshu and Kyushu, or only in Honshu (middle clades). Some of the following
200 analyses employed modified datasets that excluded the northern clades or the southern clade, to evaluate the
201 importance of these clades in creating the significant genetic structure that we detected.
202
203 *Genetic population structure*
204 As shown in Table 1, h in each population ranged from 0.199 for USJ to 0.677 for MKR, and π ranged from
205 0.0004 for USJ to 0.0025 for URG. The mean diversities of each population were 0.304 (h) and 0.0009 (π) in
206 Hokkaido, 0.546 (h) and 0.0018 (π) in Honshu, and 0.503 (h) and 0.0013 (π) in Kyushu. Although these
207 diversity indices seemed highest in Honshu followed by Kyushu and Hokkaido, no significant differentiation
208 between these three areas appeared in the Kruskal-Wallis test for both h ($\chi 2 = 4.5256$, df = 2, $P = 0.104$) and π
209 ($\chi 2 = 3.2488$, df = 2, $P = 0.197$).
210 The pairwise F_{ST} values between Hokkaido and Kyushu populations was always higher than 0.07 and
211 always was significantly different from 0, whereas none of the pairwise values from population comparisons
212 within Hokkaido and Kyushu were significant ($\alpha = 0.01$; Table 2). These results indicated a north-south
213 genetic differentiation in this species. The HCH population in Honshu, which did not include haplotypes from
214 the southern clade, showed a significant difference from populations in Kyushu and the URG population. The
215 nMDS plot based on F_{ST} indicated that genetic distance was correlated with a spatial distribution at the region
216 (island) level (Fig. 4 (A)). On the two-dimensional plot, Hokkaido populations were on the left, Kyushu on the
217 right, and Honshu in the middle. However, the relationship among populations within each region did not

218 always follow this genetic-geographic trend.

219 The DNA sequences of Honshu populations did not follow the spatial pattern. FKU, the northernmost
220 among Honshu populations, appeared closest to the Kyushu populations, while HCH, the second northernmost
221 population, was genetically closer to the Hokkaido populations than to the other Honshu populations. The
222 OSK population, the southernmost in Honshu, was genetically close to populations in Hokkaido and not to
223 those in Kyushu. The reason for this variance from an IBD model will be discussed later.

224 The Mantel test showed a significant correlation between genetic (F_{ST}) and geographic distance (P<0.01),
225 indicating that the set of study populations of *L. brevicula* followed an IBD model.

226 As shown in Table 3, AMOVA supported hierarchical population structure clustering (1) comprising
227 Hokkaido, Honshu and Kyushu, and (2) comprising Hokkaido+HCH, Honshu (except HCH) and Kyushu, (P
228 = 0.001 and 0.000, respectively). However, the AMOVA did not support the structure of clustering (3) that
229 comprised two groups—the Sea of Japan and the Pacific Ocean (P = 0.762)—indicating that the west and east
230 sides of the Japanese Archipelago could not be separated by their genetic profiles. In each clustering, the
231 variance component was the highest in the category of “within a population” and much higher than “among
232 groups” or “among populations within a group” because of the relatively high genetic diversity in each
233 population and the genetic similarity between populations due to the high frequency of *LbN6AB03* in every
234 population.

235 When the significance of the northern clades and the southern clade was tested using modified datasets, it
236 appeared that the southern clade was essential for population structure. While the unmodified original dataset,
237 the six modified datasets that each excluded one of the six northern clades, and a dataset that excluded all
238 northern clades all showed significant IBD correlations (P<0.01), the genetic-geographic correlation was not
239 significant (P=0.840) when a modified dataset that excluded the southern clade was used. Further the nMDS
240 plot based on pairwise F_{ST} values showed no genetic structure for the modified dataset that excluded the
241 southern clade (Fig. 4 (B)). The significance of the hierarchical structure of [Hokkaido+HCH], [Honshu
242 except HCH] and [Kyushu] was supported by the lowest P-value (0.000) in AMOVA with the unmodified
243 dataset. Table 3 shows the results of analyses on modified datasets testing this structure where all six northern
244 clades were excluded ((2a), significant with P = 0.000) and where only the southern clade was excluded ((2b),

not significant with $P = 0.679$), indicating the importance of the southern clade as the determinant of the north-south structure of *L. brevicula* populations around Japan.

Demographic history

The MMD analysis with pooled populations showed that the observed frequencies of haplotype difference did not significantly deviate from the sudden-expansion model when either the sum of squared deviation (SSD; $P = 0.995$) or Harpending's raggedness index ($P = 0.724$) was used. Additionally, the deviation from spatial expansion assuming a constant deme (local population) size model was not significant using either the SSD ($P = 0.990$) or Harpending's raggedness index ($P = 0.730$). The τ values corresponding to the sudden expansion of a population and spatial expansion were estimated to be 0.72 and 0.70, respectively. Given $\tau = 2ut$ ($2u$ = divergence rate, t = time from expansion) and $2u = 2.46\%$ per Myr, the population and spatial expansion dates were estimated to be 65.0 kya and 63.2 kya, respectively (i.e. Upper Pleistocene).

Both Fu's F_S (-29.709, P=0.0000) and Tajima's D (-2.653, $P = 0.0000$) indicated that the observed haplotype frequency deviated from the one expected in neutrality, suggesting that the population expanded.

DISCUSSION

Characterization of population genetic structure of Littorina brevicula *around Japan*

The haplotype distribution of *Littorina brevicula* showed a population genetic structure with north-south differentiation. As expected from the planktotrophic developmental mode of *L. brevicula* (Reid, 1996), genetic differentiation between geographically close populations is not significant, and significant genetic differentiation observed between distant populations agreed with the results of Zaslavskaya & Takada (1998). The correlation of geographic and genetic distance was statistically significant in the isolation-by-distance (IBD) test, and the north-south structure was supported by both AMOVA and F_{ST} analyses. The deviation from IBD within the Honshu group (Figure 4A), was probably caused by a stochastic balance of private haplotypes resulting from genetic drift and contemporary gene flow, similar to the lack of genetic structure in the Korean populations (Kim *et al*., 2003a). In the case of OSK, however, it was possible to attribute the

unreasonable situation to recent human disturbance. OSK showed an unexpected deviation from IBD (Fig. 4 A) and the lowest observed values of both haplotype and nucleotide diversity among all the Honshu populations that could be the result of high genetic drift. Its small effective population size could be attributed to the effects of human impacts such as pollution, as has been argued for the Korean populations (Kim *et al*., 2003b). OSK is in the Seto Inland Sea, where pollution likely accumulates more than in the open sea. Overall, genetic-geographic correlation and a fair level of genetic diversity within each population indicated that examined populations had not been as affected by recent human activity as it was in the case of *Neptunea arthritica* (Azuma *et al.*, 2015). The higher resistance of *L. brevicula* to anthropogenic effects compared to that of *N. arthritic*a is attributable to much larger population sizes and higher gene flow in *L. brevicula* than in *N. arthritica*.

Population genetic structure can be greatly affected by ocean currents. *Littorina brevicula* distributed around Japan currently experiences warm currents (the Kuroshio and Tsushima currents). Comparison of Figures 1 and 5 show that the proportion of the southern clade in each population declines as one travels along the direction of both these currents, until no haplotypes of this clade occur in Hokkaido and HCH. This correlation implies corresponding dispersal pathways for the *L. brevicula* veligers. The HCH population, which is on Honshu where it is less affected by these warm currents, did not include the haplotypes characteristic of the southern clade. We argue below that gene flow by passive dispersal along ocean currents was an important factor for the colonization of *L. brevicula* on a paleontological time scale. Surprisingly, contemporary gene flow mediated by currents has not been strong enough to establish uniform or panmictic populations throughout the distribution of *L. brevicula*; the southern clade has not reached Hokkaido from Kyushu even though it has been 20 ky since the LGM.

AMOVA failed to find east-west genetic differentiation, a result that may be attributable to the remarkable north-south differentiation; the high variation within the east and west groups, which corresponds to a north-south difference, hindered any statistical significance for east-west differentiation in AMOVA, based on the analysis of variance. Additionally, the frequency of the southern clade within each population was similar across Honshu Island, as shown in Figure 5, indicating that east-west differentiation was fundamentally small. In fact, pairwise F_{ST} was 0.000 between CHO and OBM, which are at a similar latitude

299 on the east and west coasts of Honshu Island respectively.

300

301 *A glacial effect on* Littorina brevicula *populations inferred from population genetics*

302 We inferred the glacial effect following the flow chart in Figure 2. First, the presence of a possible glacial
303 effect was evaluated in steps 1 to 3, and then, if a glacial effect was suggested, the position of glacial refugia
304 and post-glacial colonization was identified in steps 4 and 5. The determinants and resolutions in this chart are
305 based on empirical and theoretical knowledge accumulated in phylogeography as reviewed in Avise (2000),
306 Hewitt (2000) and Freeland (2005). The determinant keys of steps 1 and 2 correspond to gene flow and
307 genetic drift. The IBD model is a powerful tool for detecting continuous genetic-geographic structure based
308 on the balance of gene flow and genetic drift. The key of step 3 corresponds to coalescent theory and sequence
309 mismatch distribution (MMD) analysis detailed in Avise (2000). A comparison of genetic diversity in each
310 population can be a good way to detect the direction of colonization, as the sequential founder effect likely
311 caused lower diversity in newly established populations. The southern refugia is plausible for species in the
312 temperate zone of the northern hemisphere (Hewitt, 2000), and the southern refugia often caused post-glacial
313 colonization to trend northward and likely resulted in higher diversity in southern than in northern
314 populations.

315 Testing the determinants in the flow chart in Figure 2, the presence of step 1 (Genetic difference between
316 local populations) and step 2 (Populations with genetic-geographic correlation) were supported by pairwise
317 F_{ST}, IBD and AMOVA, and we assumed that the genetic-geographic pattern of *L. brevicula* was basically
318 shaped by natural evolution and distribution during the paleontological time scale. Since step 3 (Deep
319 genealogy of haplotypes) was absent, we assumed that recent population expansion probably occurred after
320 the LGM. Significant deviation from neutral evolution indicated by Fu's *F* and Tajima's *D* as well as MMD
321 analysis confirmed recent population expansion. Step 4 (North-south differentiation) was present, and step 5
322 (Higher diversity in the south) could not be proved by statistical analyses, although the mean of diversity
323 indices are lowest in Hokkaido compared to Honshu and Kyushu.

324 Though the glacial effect was highly possible, northward post-glacial colonization could not be directly
325 supported by genetic evidence. The skewed frequency of the southern clade, however, which appeared as a

key factor in the north-south structure, suggested northward colonization. The fact that the highest proportion of the southern clade was found at MKR (which faces the point of east-west separation) and that the proportion of the southern clade decreased in a similar pattern along both the east and west sides of the Japanese Archipelago (Fig. 5) indicated a gradual colonization of the southern clade to the north in a stepwise manner. Since the northern population of *L. brevicula* likely declined with a decline in air and seawater temperatures, it is possible to assume that a shift in the distribution of *L. brevicula* occurred during glacial and post-glacial periods. Following the decline in temperatures, the northern range of this species trended south, and it seems that a bottleneck effect left only a single haplotype, *LbN6AB03*, around Honshu. An additional haplotype, *LbN6OT02*, survived in the southern area of the Yellow Sea, where temperatures were higher than around Hokkaido or Honshu. In the present study, genetic diversities in Hokkaido populations tend to be lower than those in Honshu populations, though the differences cannot be statistically significant due to the low number of examined Hokkaido populations. This tendency, plus the direction of the ocean currents and the ecological character of *L. brevicula* as described below, implies that this species was absent around Hokkaido during the glacial period and recovered during the post-glacial colonization.

Time estimation corresponding to population expansion

Population and range expansion was estimated to have occurred at 65.0 kya and 63.2 kya, long before the end of the LGM at 20 kya. The estimated date might be earlier than the actual expansion time, however, as the divergence rate of 2.46% per Myr, a rate modified in this study from the molecular clock calibration by Quesada *et al* (2007), might overestimate the divergence time within *L. brevicula*. The calibration by Quesada *et al* (2007) may not be adequate for the time scale of the present study, as the evolutionary rate of 1.83 ± 0.21% per Myr was based on the split of the *Hydrobia*/*Peringia* 5.33 million years ago, far earlier than the age in the scope of our study, and such calibration based on speciation is known to overestimate divergence time at the population level within a species (Ho *et al*., 2005). Thus, for precise dating, molecular clock calibration based on a more recent event should be established for *L. brevicula* or closely-related *Littorina* species.

Ecological and paleontological perspectives supporting the hypothesis of post-glacial colonization

353 The hypothesis of glacial effects and post-glacial colonization inferred from phylogeography is also supported
354 by ecological and paleontological perspectives of *L. brevicula* around Japan. Fossil records suggest that the
355 speciation event of *L. brevicula* occurred no later than the upper Pliocene, and molecular phylogeny suggests
356 that the divergence between *L. brevicula* and *L. mandshurica* occurred by the lower Pleistocene at the latest
357 (Reid, Dyal & Williams, 2012). The species should therefore have a long history, but the genetic genealogy of
358 the extant population at present was shallow, indicating that the population declined in the upper Pleistocene.
359 Probably only two mitochondrial lineages (*LbN6AB03* and *LbN6OT02*) remained after the latest bottleneck
360 event, which was likely associated with glacial cooling. Generally, temperature is arguably the most critical
361 factor determining the geographical distribution of species (e.g., Raffaelli & Hawkins, 1996). Considering the
362 temperature range in which extant populations are currently found, the low temperatures during glacial
363 periods can be expected to have been severe for the survival and reproduction of *L. brevicula* around northern
364 Japan. Though the lower temperature limit for the reproduction of *L. brevicula* has not been reported, it is
365 probably 3 - 5 °C given that this species is rare along areas of the Pacific coast under the influence of the cold
366 Kuril Current but abundant on the Sea of Okhotsk coast of Hokkaido (Ohgaki, 1983; Azuma & Chiba personal
367 observation). Since low seawater temperature likely determines the present northern limit of *L. brevicula* in
368 Hokkaido, it is safe to assume that there were no *L. brevicula* around Hokkaido during glacial periods and that
369 post-glacial colonization formed the extant Hokkaido populations.
370 We could not find higher genetic diversity in Kyushu, the southern area, as mean diversity indices in each
371 population were estimated to be higher in Honshu than Kyushu (Table 1). This might be attributable to
372 population decline around Kyushu in recent years. Although both haplotype and nucleotide diversity at the
373 southernmost sampling point (MKR) was the highest among all examined populations, *L. brevicula* is not
374 common there at present, suggesting that conditions are no longer optimal and might possibly be too warm for
375 this species. The upper limit for spawning was reported to be 13 °C (Son & Hong, 1998), and high seawater
376 temperatures around Kyushu, ordinarily greater than 15 °C even in January, may also limit the reproduction of
377 *L. brevicula* and may have caused population decline and a reduction in genetic diversity in this area after
378 post-glacial colonization. Since genetic diversity in Kyushu was probably higher in the past (long after the
379 LGM) compared to the present, we assume a southern refugia existed around Kyushu or further south during

LGM and that subsequent northward post-glacial colonization resulted in higher genetic diversity in the south than in the north.

A glacial effect was also suggested in *Batillaria cumingi* (Kojima *et al*., 2004) and *Cellana nigrolineata* (Nakano *et al*., 2010) around Japan, although no evidence of post-glacial colonization was provided in those studies. The present study represents the first report of genetic evidence for such colonization in an intertidal mollusc species along the Japanese Archipelago.

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486

FIGURE CAPTIONS

Figure 1. Ocean currents around Japan, and sampling locations of *Littorina brevicula* in the present study: three in Hokkaido, six in Honshu, and three in Kyushu.

Figure 2. Flow chart to evaluate glacial effects on population structure and post-glacial colonization inferred from genetic data. The path of inference in the present study follows the circled determinations (present or absent).

Figure 3. Parsimony network of the mtDNA ND6 haplotypes of *Littorina brevicula*. A solid line between circles indicates a single nucleotide substitution. Circle size reflects haplotype abundance (number of individuals that had the haplotype), and the colors in the circles indicate the proportions of Hokkaido, Honshu, and Kyushu individuals. Dotted lines indicate the criteria of each clade (a monophyletic group of multiple haplotypes). Colors of dotted lines indicate clade categories (northern, southern, middle).

Figure 4. nMDS plot of *Littorina brevicula* with pairwise F_{ST} values based on a 450-bp sequence of partial mtDNA ND6 for unmodified dataset (A) and a dataset excluding the southern clade (B). While population structure along the Japanese Archipelago from north to south (from left to right in this plot) appeared in (A), no such structure was observed in (B), indicating the north-south structure was attributable to the existence of the southern clade.

Figure 5. Distribution of mtDNA ND6 haplotype groups in each sampling locality of *Littorina brevicula*. *LbND6AB03* was the most abundant in all populations, while the southern clade was abundant in southern but not in northern populations.

506 TABLES

Table 1. Geographical and genetic information for *Littorina brevicula* populations analyzed in the present study. Sampling locality, Collection date, Sample size (number of individuals), number of haplotype and diversity indices, haplotype richness (*HR*, number of haplotypes standardized by the smallest sample size), haplotype diversity (*h*) and nucleotide diversity (π) estimated in partial ND6 sequence. *Average for all samples. **Calculated pooling all samples as a single population.

Region	code. Sample name	latitude; longitude	year / month	Sample size	No. of haplotype	*HR*	*h*	π
Hokkaido	1. SSB	44°32'N; 141°45'E	2010/July	48	10	5.5	0.343	0.0011
	2. ABS	44°03'N; 144°15'E	2010/June	39	8	5.6	0.371	0.0012
	3. USJ	41°56'N; 140°56'E	2013/June	48	6	3.5	0.199	0.0004
	mean of Hokkaido					**4.8**	**0.304**	**0.0009**
Honshu	4. FKU	40°46'N; 140°03'E	2011/October	48	11	7.6	0.647	0.0018
	5. HCH	40°32'N; 141°33'E	2011/November	48	11	6.8	0.469	0.0015
	6. OBM	35°32'N; 135°42'E	2011/October	48	12	7.6	0.578	0.0018
	7. CHO	35°42'N; 140°52'E	2012/January	47	17	9.8	0.646	0.0021
	8. URG	35°15'N; 139°44'E	2013/August	48	14	8.4	0.629	0.0025
	9. OSK	34°17'N; 132°54'E	2011/November	47	7	4.4	0.311	0.0009
	mean of Honshu					**7.4**	**0.546**	**0.0018**
Kyushu	10. SIK	32°48'N; 131°53'E	2011/September	46	5	3.6	0.423	0.0011
	11. AMK	32°36'N; 130°29'E	2011/September	24	3	3	0.409	0.0009
	12. MKR	31°15'N; 130°18'E	2013/May	49	14	7.9	0.677	0.0020
	mean of Kyushu					**4.8**	**0.503**	**0.0013**
Total				**540**	**78**	**6.0***	**0.497****	**0.0015****

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Table 2. Left lower: Pairwise F_{ST} between *Littorina brevicula* populations based on partial sequence of ND6. Bold font indicates the P value < 0.05. Right upper: Significance level of F_{ST}. + indicates a P value < 0.01 after sequential Bonferroni correction.

		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Hokkaido	1. SSB		-	-	+	-	-	-	-	-	+	+	+
	2. ABS	0.010		-	-	-	-	-	+	-	+	+	+
	3. USJ	0.000	0.010		-	-	+	-	-	+	+	+	+
Honshu	4.FKU	0.033	0.016	0.034		-	-	-	-	-	-	-	-
	5. HCH	0.009	0.000	0.010	0.009		-	-	+	-	+	-	+
	6. OBM	0.014	0.017	0.021	0.005	0.014		-	-	-	-	-	-
	7. CHO	0.007	0.002	0.005	0.000	0.001	0.000		-	-	+	-	-
	8. URG	0.010	0.021	0.017	0.016	0.020	0.011	0.002		-	-	-	-
	9. OSK	0.006	0.008	0.009	0.011	0.006	0.006	0.000	0.010		-	-	-
Kyushu	10. SEK	**0.089**	**0.093**	**0.128**	0.017	**0.078**	0.027	0.030	0.027	0.041		-	-
	11. AMK	**0.073**	**0.077**	**0.138**	0.003	**0.060**	0.013	0.013	0.011	0.030	0.000		-
	12. MKR	**0.080**	**0.079**	**0.098**	0.019	**0.071**	0.032	0.034	0.030	0.042	0.000	0.000	

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Table 3. Results of five separate AMOVA using K2P distance. (1): 3 groups = [Hokkaido] [Honshu] [Kyushu]. (2): 3 groups = [Hokkaido+HCH] [Honshu (except HCH)] [Kyushu]. (3): 2 groups = [Pacific Ocean + Yellow Sea] [Sea of Japan + Sea of Okhotsk]. (2a): Population groups were similar to (2), and all northern clades were excluded before analysis. (2b): Population groups were similar to (2), and the southern clade was excluded before analysis.

	Source of variation	Variance component	% of variation	*P* value	Fixation indices
(1)	Among groups	0.00994	2.86	0.001	F_{CT}=0.028
	Among population within groups	0.00124	0.36	0.154	F_{SC}=0.003
	Within populations	0.33538	96.78	0.000	F_{ST}=0.032
(2)	Among groups	0.01035	2.99	0.000	F_{CT}=0.029
	Among population within groups	0.00059	0.17	0.260	F_{SC}=0.001
	Within populations	0.33538	96.84	0.000	F_{ST}=0.031
(3)	Among groups	-0.00128	-0.37	0.762	F_{CT}=-0.003
	Among population within groups	0.00852	2.48	0.000	F_{SC}=0.021
	Within populations	0.33538	97.89	0.000	F_{ST}=0.025
(2a)	Among groups	0.01111	5.02	0.000	F_{CT}=0.050
	Among population within groups	-0.00048	-0.21	0.570	F_{SC}=-0.002
	Within populations	0.21044	95.1	0.000	F_{ST}=0.048
(2b)	Among groups	-0.00015	-0.12	0.679	F_{CT}=-0.000
	Among population within groups	0.00219	0.81	0.000	F_{SC}=0.007
	Within populations	0.28352	99.31	0.000	F_{ST}=0.007

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Fig 1

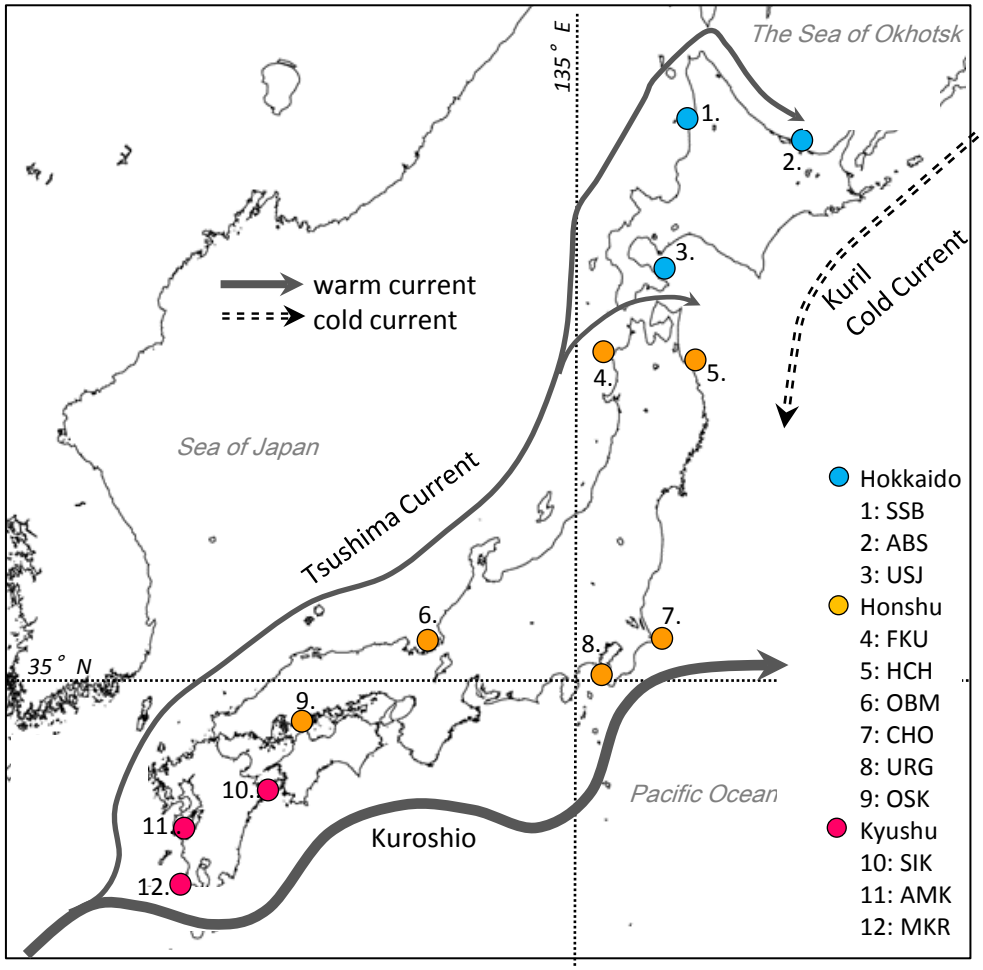

The Sea of Okhotsk
135° E
warm current
cold current
Kuril
Cold Current
Sea of Japan
Tsushima Current
35° N
Pacific Ocean
Kuroshio
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
Hokkaido
1: SSB
2: ABS
3: USJ
Honshu
4: FKU
5: HCH
6: OBM
7: CHO
8: URG
9: OSK
Kyushu
10: SIK
11: AMK
12: MKR

Fig 2

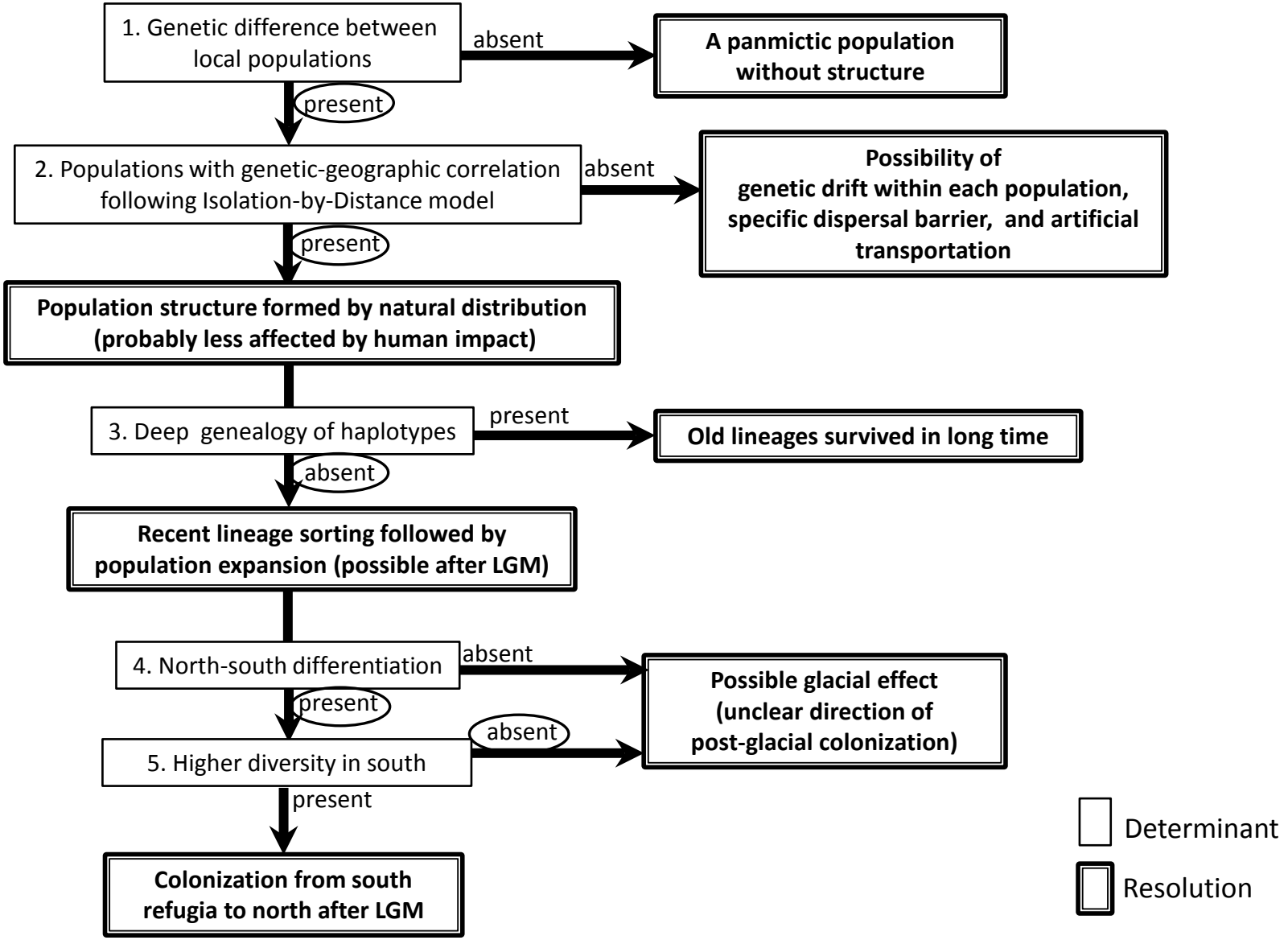
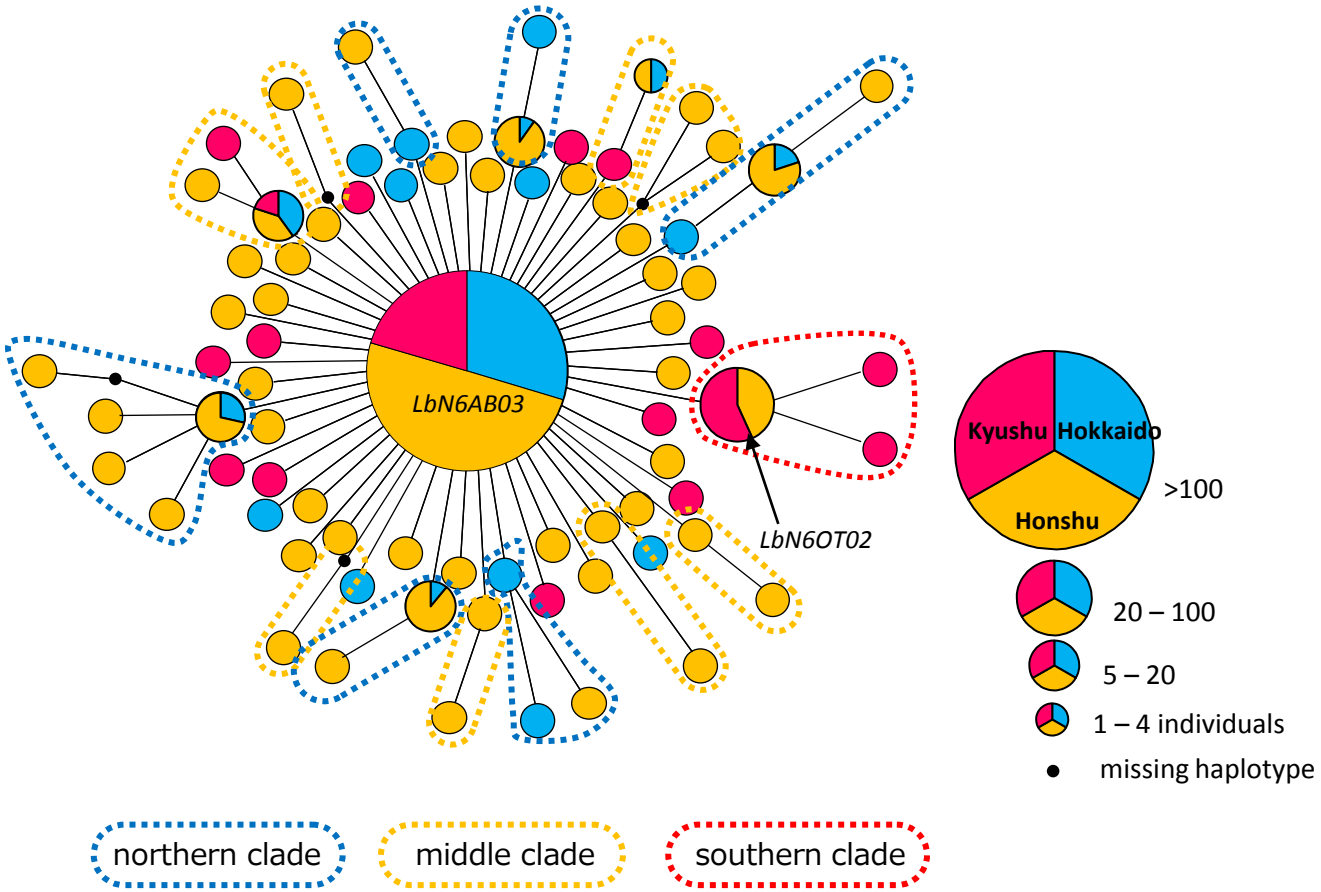

1. Genetic difference between local populations
absent
A panmictic population without structure
present
2. Populations with genetic-geographic correlation following Isolation-by-Distance model
absent
Possibility of genetic drift within each population, specific dispersal barrier, and artificial transportation
present
Population structure formed by natural distribution (probably less affected by human impact)
3. Deep genealogy of haplotypes
present
Old lineages survived in long time
absent
Recent lineage sorting followed by population expansion (possible after LGM)
4. North-south differentiation
absent
Possible glacial effect (unclear direction of post-glacial colonization)
present
5. Higher diversity in south
absent
present
Colonization from south refugia to north after LGM
Determinant
Resolution

Fig 3


LbN6AB03
LbN6OT02
Kyushu
Hokkaido
Honshu
>100
20 – 100
5 – 20
1 – 4 individuals
missing haplotype
northern clade
middle clade
southern clade

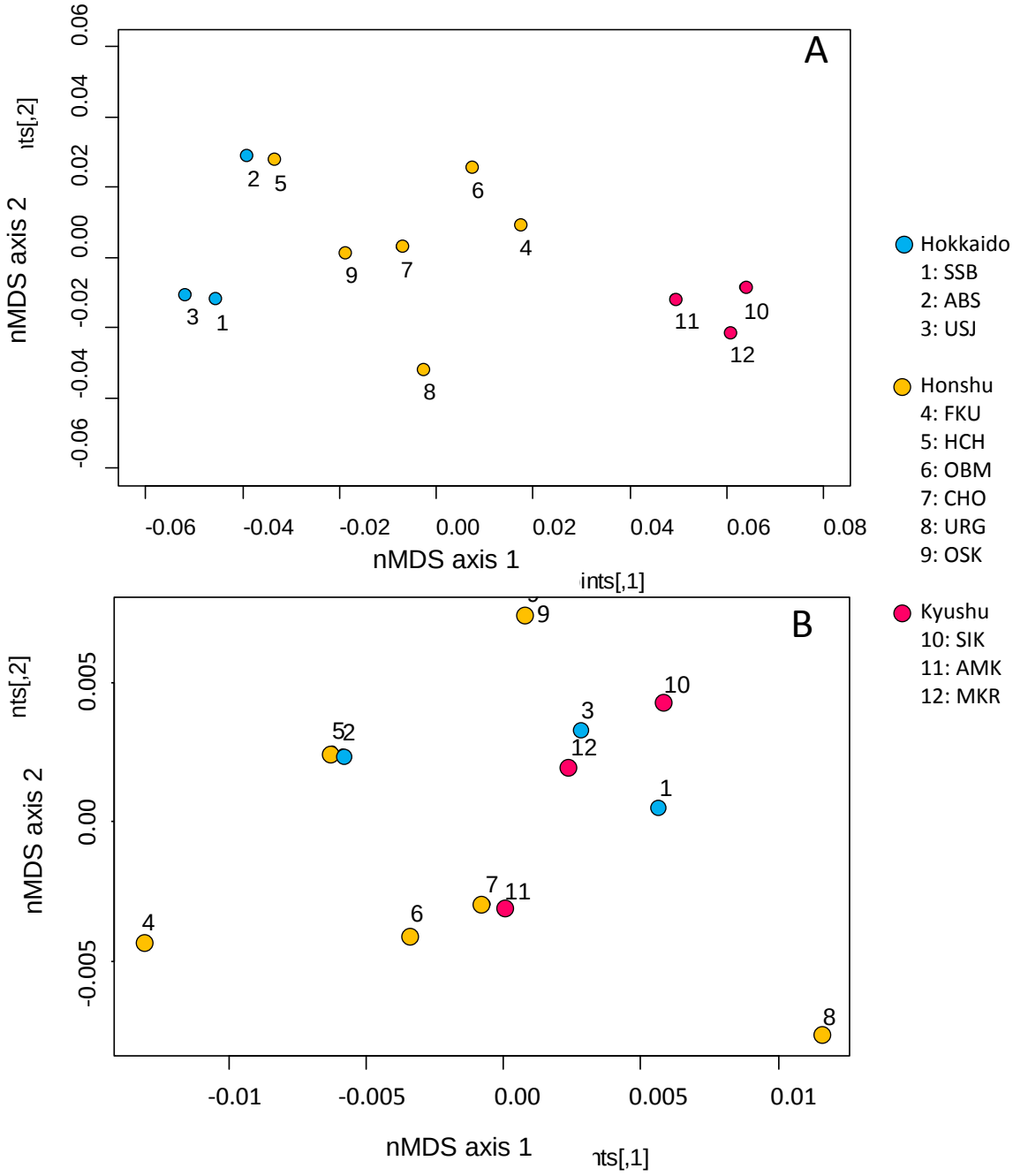
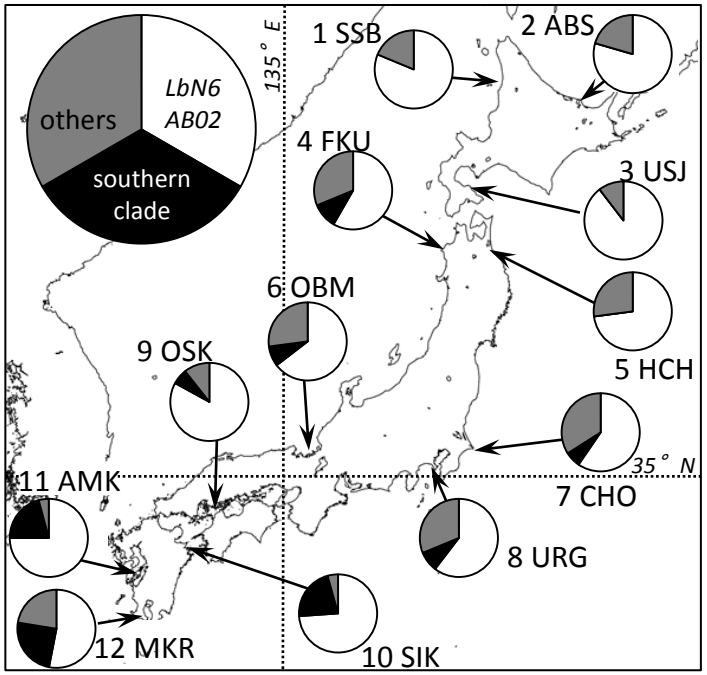

A
nMDS axis 2
nMDS axis 1
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.06
-0.04
-0.02
0.00
0.02
0.04
0.06
0.08
2
5
6
4
9
7
3
1
11
10
12
8
B
nMDS axis 2
nMDS axis 1
0.005
0.00
-0.005
-0.01
-0.005
0.00
0.005
0.01
9
10
3
5
2
12
1
7
11
6
4
8
Hokkaido
1: SSB
2: ABS
3: USJ
Honshu
4: FKU
5: HCH
6: OBM
7: CHO
8: URG
9: OSK
Kyushu
10: SIK
11: AMK
12: MKR

Fig 5


others
LbN6
AB02
southern
clade
135° E
1 SSB
2 ABS
4 FKU
3 USJ
6 OBM
5 HCH
9 OSK
35° N
11 AMK
7 CHO
8 URG
12 MKR
10 SIK

Supplementary Data

Haplotype frequency (number of individuals which showed the haplotype) of partial ND6 in each populations. Haplotype name is abbreviated as *LbN6SS18* into *SS18*.

	SS18	*SS25*	*SS26*	*SS28*	*SS37*	*SS38*	*SS44*	*SS47*	*AB03*	*AB13*	*AB15*	*AB21*	*AB23*	*AB25*	*AB29*	*AB32*
1. SSB	1	1	1	1	1	1	1	1	39	-	-	-	-	-	-	-
2. ABS	-	-	-	-	-	-	-	-	31	1	2	1	1	1	1	1
3. USJ	-	-	-	-	-	-	-	-	43	-	-	-	-	-	-	-
4. FKU	-	-	-	-	-	-	-	-	28	-	2	3	-	-	-	-
5. HCH	-	-	-	-	-	-	-	-	35	-	2	2	-	-	-	-
6. OBM	-	-	-	-	-	-	-	1	31	-	-	2	-	-	-	-
7. CHO	-	-	-	-	-	-	-	-	28	-	1	1	-	-	-	-
8. URG	-	4	-	-	-	-	-	-	29	-	-	-	-	-	-	-
9. OSK	-	-	-	-	-	-	-	-	39	-	-	-	-	-	-	-
10. SIK	-	-	-	-	-	-	-	-	34	-	-	-	-	-	-	-
11. AMK	1	-	-	-	-	-	-	-	18	-	-	-	-	-	-	-
12. MKR	-	-	-	-	-	-	-	-	26	-	-	-	-	-	-	-
Total	2	5	1	1	1	1	1	2	381	1	7	9	1	1	1	1

	UJ03	*UJ33*	*UJ36*	*AO09*	*AO10*	*AO11*	*AO17*	*AO22*	*AO41*	*AO46*	*HH08*	*HH18*	*HH22*	*HH24*	*HH41*	*HH47*
1. SSB	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
2. ABS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3. USJ	1	1	1	-	1	-	-	-	1	-	-	-	-	-	-	-
4. FKU	-	-	-	1	4	1	1	1	1	1	-	-	-	-	-	-
5. HCH	-	-	-	-	2	-	-	-	1	-	1	1	1	1	1	1
6. OBM	-	-	-	-	1	-	-	-	-	-	1	-	-	-	-	-
7. CHO	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
8. URG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9. OSK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
10. SIK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11. AMK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12. MKR	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Total	1	1	1	1	10	1	1	1	5	1	2	1	1	1	2	1

	OB05	*OB11*	*OB12*	*OB26*	*OB27*	*OB35*	*CH07*	*CH09*	*CH10*	*CH20*	*CH25*	*CH26*	*CH28*	*CH29*	*CH34*	*CH46*
1. SSB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. ABS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3. USJ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. FKU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5. HCH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6. OBM	1	1	1	3	1	1	-	-	-	-	-	-	-	-	-	-
7. CHO	-	-	1	-	-	-	1	1	1	1	1	1	1	1	1	1
8. URG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

9. OSK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10. SIK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11. AMK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12. MKR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	1	1	2	3	1	1	1	1	1	1	1	1	1	1	1	1

	CH47	*KG08*	*KG09*	*KG11*	*KG14*	*KG19*	*KG22*	*KG32*	*KG33*	*KG42*	*KG48*	*HT09*	*HT18*	*HT41*	*HT46*	*OT02*
1. SSB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. ABS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3. USJ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. FKU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
5. HCH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
6. OBM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
7. CHO	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
8. URG	-	1	1	1	1	1	1	1	1	1	1	-	-	-	-	4
9. OSK	-	-	-	-	-	-	-	-	-	-	-	1	1	1	1	3
10. SIK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9
11. AMK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
12. MKR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11

Total	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	44

	OT14	*OT26*	*OT43*	*MK03*	*MK04*	*MK09*	*MK10*	*MK12*	*MK18*	*MK19*	*MK21*	*MK33*	*MK44*	*MK47*	Total
1. SSB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	48
2. ABS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39
3. USJ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	48
4. FKU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	48
5. HCH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	48
6. OBM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	48
7. CHO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	47
8. URG	-	1	-	-	-	-	-	-	-	-	-	-	-	-	48
9. OSK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	47
10. SIK	1	1	1	-	-	-	-	-	-	-	-	-	-	-	46
11. AMK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	24
12. MKR	-	-	-	1	1	1	1	1	1	1	1	1	1	1	49
Total	1	2	1	1	1	1	1	1	1	1	1	1	1	1	540