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Phylogeography of *Littorina sitkana* in the northwestern Pacific Ocean: evidence of eastward trans-Pacific colonization after the Last Glacial Maximum

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Running title: Phylogeography of Sitka Periwinkle

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Abstract

We investigated genetic diversity and population structure of the Sitka periwinkle *Littorina sitkana* along the coastlines of the northwestern Pacific (NWP) to evaluate the possibility of trans-Pacific colonization of this species from the NWP to the northeastern Pacific (NEP) after the Last Glacial Maximum. We sampled *L. sitkana* from 32 populations in the NWP, and sequenced a region of the mitochondrial cytochrome *b* oxidase gene for population genetic analyses. The results were compared with those of previous reports from the NEP. The genetic diversity of *L. sitkana* was much higher in the NWP than in the NEP. Genetic connectivity between the NWP and NEP populations was indicated by an extremely abundant haplotype in the NEP that was also present in eastern Hokkaido and the Kuril Islands. To confirm these results, we compared sequences of the longest intron of the aminopeptidase N gene (APN54) in the nuclear genome in four populations of *L. sitkana* in the NWP with previous results from the NEP. Again, much higher genetic diversity was found in the NWP than in the NEP and genetic connectivity was supported between the Kuril Islands and the NEP. These results imply postglacial colonization of this species from the NWP to the NEP, probably along the Kuril and Aleutian Island chains. This study is the first report of possible trans-Pacific postglacial colonization of a direct-developing gastropod, inferred from genetic data.

Keywords: Trans-Pacific, gastropod, postglacial colonization, Sitka periwinkle

Introduction

The Last Glacial Maximum (LGM) is believed to have had a strong influence on the demography and distribution of numerous species (reviewed by Hewitt 2000). In the Northern Hemisphere, many species are thought to have survived in southern refugia during the LGM and then recolonized from south to north after the ice receded (Hewitt 2004), in line with the 'expansion-contraction' (EC) model proposed by Provan and Bennett (2008). According to this model, southern populations maintained higher genetic diversity than northern populations (Taberlet et al. 1998; Hewitt 1999). On the other hand, several amphi-Pacific marine species, distributed throughout the North Pacific Ocean, show greater genetic diversity in the northwestern Pacific (NWP) than in the northeastern Pacific (NEP) (Sato et al. 2004; Cassone and Boulding 2006; Liu et al. 2007; Canino et al. 2010; Cox et al. 2014). Such species appear to have the axis of the EC model orientated not north–south, but east–west. This implies that transoceanic colonization occurred across the North Pacific, comparable to that suggested for some amphi-Atlantic species (e.g. Wares and Cunningham 2001).

We selected an amphi-Pacific gastropod, the Sitka periwinkle *Littorina sitkana* (Philippi 1846), as a model species to investigate trans-Pacific colonization after the LGM. *Littorina sitkana* is distributed in the North Pacific from Oregon to the Bering Sea, the Sea of Okhotsk and the northern Sea of Japan, typically on rocky and boulder shores between the mean low water and the littoral fringe (Behrens Yamada 1977; Ohgaki 1983; Reid 1996; Hasegawa 2000). It is a direct developer, lacking a planktonic larval stage (Buckland-Nicks et al. 1973; Buck land-Nicks and Chia 1990; Reid 1996) and its populations are therefore likely structured by limited gene flow. In the NWP, allozyme analyses have detected regional differentiation in Russia (Zaslavskaya and Pudovkin 2005) and northern Japan (Nohara 1999). Nevertheless, when using the mitochondrial DNA (mtDNA) cytochrome *b* oxidase (Cytb) gene as a genetic marker, the species exhibited no population structure and low genetic diversity in the NEP (Kyle and Boulding 2000; Lee and Boulding 2009; Marko et al. 2010). Some studies on the population genetics of this species in the NEP have revealed genetic variation in nuclear DNA markers (Sokolova and Boulding 2003; Lee and Boulding 2009; Botta et al. 2014). However,

sampling has been limited to a narrow area of Vancouver Island, Canada, which is not sufficient to investigate genetic structure on a geographical scale. So far, no studies have examined phylogeography using common markers for *L. sitkana* in both the NEP and the NWP, in order to deduce population structure throughout its amphi-Pacific distribution.

The aim of the present study is to investigate the genetic structure of *L. sitkana* populations in the NWP and to evaluate the effects of environmental change during glacial-interglacial periods, especially the LGM and post-LGM, on this species. We used Cytb as a marker, which had previously been used to analyse a wide range of NEP populations of *L. sitkana*, in order to compare population structure and genetic diversity in the NWP and the NEP. Additionally, partial sequence of the longest intron of the aminopeptidase N gene (APN54), was employed as a nuclear DNA (nDNA) marker. Previous data on the APN54 in the NEP are limited to populations from the southwestern coast of Vancouver Island (Lee and Boulding 2009; Botta et al. 2014) and thus permit only limited phylogenetic comparison between NEP and NWP populations. The results of a previous study based on mtDNA analyses (Marko et al. 2010) were suggestive of post-LGM recolonization and expansion of the *L. sitkana* population in the NEP, but no plausible location of an ice age refugium in the NEP was found. We identified genetic connectivity between the NEP and the NWP and high diversity in the NWP from the results of both mtDNA and nDNA markers, suggesting that the ice age refugium of the present NEP population was in the NWP. The present study represents, to our knowledge, the first report identifying a possible location of the ice age refugium of an amphi-Pacific gastropod. We further discuss the evolution of this species in relation to environmental factors, such as water temperature, topography and ocean currents, which limited or promoted gene flow and determined its genetic structure.

Materials and methods

Sampling, DNA sequencing and analysis of haplotype genealogy

99 Samples of *L. sitkana* were picked by hand from 32 populations: two in Primorye, one in Sakhalin,
100 eight in the Kuril Islands, 19 in Hokkaido and two in Honshu (Table 1, Fig. 1). Following the
101 elimination of cryptic species (see Azuma et al. 2011), we used 1022 individuals of *L. sitkana sensu*
102 *stricto* for analyses. Genomic DNA was extracted from a piece of muscle (approximately 1 mm^3) of
103 ethanol-fixed specimens using the Pure Gene Kit (Qiagen, Valencia, CA, USA) according to the
104 manufacturer's protocol and then resuspended in 100 µL Tris-EDTA buffer.
105 A partial sequence of the Cytb gene was amplified by PCR in a 30-µl reaction mixture containing
106 template DNA (approximately 5 ng), dNTPs, a pair of primers (F:
107 CCTTCCCGCACCTTCAAATCTTTC, R: GCAAAGAAGCGA GTGAGGGTAGC; Lee & Boulding,
108 2009) and ExTaq (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. The
109 thermal-cycling profile included precycling denaturation at 95 °C for 1 min, followed by 35 cycles of
110 denaturation at 95 °C for 30 s, annealing at 50 °C for 20 s and extension at 72 °C for 25 s. The PCR
111 products were then examined by electrophoresis on a 2% agarose gel, purified with magnetic beads
112 (AMPure Agencourt; Beckman-Coulter, Brea, CA, USA), cycle-sequenced using the
113 above-mentioned forward primer and the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied
114 Biosystems, Foster City, CA, USA) and loaded onto an automated sequencer, ABI PRISM® 3130
115 (Applied Biosystems). The obtained sequences were aligned and edited to 428 bp using Bioedit
116 software (Hall 1999) to define the haplotypes and subsequently deposited in the DDBJ/GenBank
117 database under the accession nos AB665092, AB665093, AB665096–AB665098, AB691553–
118 AB691573 and LC146771–LC146792. The haplotype genealogy was resolved as a parsimony
119 network using the TCS Network Program (Clement et al. 2000) under a 95% connection limit.
120 For comparison with the results of the mtDNA marker, sequence variation of the APN54 intron in
121 nDNA was examined. Alleles from samples of four populations in Hokkaido and the Kuril Islands
122 were sequenced by PCR and cloning methods. The target DNA region was amplified by PCR in a
123 20-µl reaction mixture containing template DNA (approximately 20 ng), dNTPs, a pair of primers
124 (AP54F2 and AP54R2, Sokolova and Boulding 2004) and DNA polymerase (Amplitaq Gold, Thermo
125 Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. The thermal cycling

126 profile included precycling denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at
127 95 °C for 30 s, annealing at 58 °C for 15 s and extension at 72 °C for 25 s, then final extension at
128 72 °C for 25 min. PCR products were ligated in a vector using Takara Mighty Cloning Kit and cloned
129 with TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Then, eight to 16 clones were selected for
130 each individual and sequenced using primers similar to the PCR primers and the BigDye® Terminator
131 v. 3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM® 3130 (Applied Biosystems).
132 Collected sequences were edited and aligned using Bioedit software (Hall 1999) and deposited in
133 DDBJ/GenBank with accession nos LC189242–LC189260. The sequence genealogy, including alleles
134 from the NEP (Lee and Boulding 2009; Botta et al. 2014), was resolved as a parsimony network using
135 the TCS Network Program (Clement et al. 2000).
136
137 *Population genetic analyses using mtDNA marker*
138 The haplotype (h) and nucleotide (π) diversities of the total sample and of each local population were
139 estimated using Arlequin v. 3.5 (Excoffier and Lischer 2010). The population genetic structure was
140 analysed by estimating pairwise F_{ST} between populations (Weir and Cockerham 1984), evaluating the
141 isolation-by-distance (IBD) model (Wright, 1943) and conducting analysis of molecular variance
142 (AMOVA; Excoffier et al. 1992) using Arlequin v. 3.5 (Excoffier and Lischer 2010). The genetic
143 distance between populations based on the pairwise F_{ST} was visualized on a two-dimensional surface
144 by nonmetric multidimensional scaling (nMDS) plots using statistical software R v. 2.9.0 (R
145 Development Core Team 2005). For the IBD test, the matrix of geographic distances between
146 sampling sites and the F_{ST} matrix were compared, and the significance of correlations was evaluated
147 using the Mantel test with 10,000 permutations. In the AMOVA, the hierarchical population structure
148 indicated by nMDS plotting based on F_{ST} was tested in the following two partitions: (1) three regional
149 groups suggested by F_{ST}: Region 1 (populations 6–10; see Table 1 for codes), Region 2 (4, 11, 26–31),
150 Region 3 (1–3, 5, 12–25, 32); (2) two regional groups: Region 1 (6–10), Region 2 (4, 11, 26–31). The
151 second partition was used to confirm independence between Regions 1 and 2, which are
152 geographically close but separate according to the results of the F_{ST} analysis.

153 To infer the demographic history of *L. sitkana*, mismatch distribution (MMD) analysis and the
154 neutrality test of Fu's F_S and Tajima's *D* were conducted using Arlequin v. 3.5 (Excoffier and Lischer
155 2010). The times since population expansion and range expansion were estimated based on the results
156 of the MMD analysis, assuming a sequence-divergence rate of 0.5–1.01% per million years (Myr).
157 This rate was estimated based on a comparison of *L. sitkana* with its congeners *L. saxatilis* (Olivi
158 1792), *L. arcana* (Hannaford-Ellis 1978) and *L. fabalis* (Turton 1825). The average sequence
159 divergence between the most frequent haplotype observed in the present study and the homologous
160 region in *L. saxatilis*, *L. arcana* and *L. fabalis* (GenBank accession nos AJ237711, AJ237712,
161 AJ237714, AJ237715, AJ237717, AJ237720 and AJ237721) was 8.08%, and the divergence time
162 between *L. sitkana* and the lineage including these three species was 8–16 million years ago (Mya)
163 (Fig. 2 in Reid et al. 2012), giving the rate of 0.5–1.01% per Myr.
164
165 *Analyses using nDNA marker*
166 Alleles of APN54 were assigned for each individual and expected heterozygosity was calculated in
167 Arlequin v. 3.5 (Excoffier and Lischer, 2010). Genetic diversities in the NWP and NEP were
168 compared at population level.
169
170
171 **Results**
172
173 *Genealogy and spatial distribution of Cytb haplotypes*
174 A total of 48 Cytb haplotypes were detected (Fig. 2A). Among them, K2 was the most abundant,
175 occurring in 35.0% of all individuals examined, followed by U16 in 26.8%. Haplotype U9 had an
176 identical sequence to those that were most abundant in NEP population (GenBank accession nos
177 GQ2686–2751). Haplotypes were categorized into four hap-groups, each consisting of one abundant
178 core haplotype (K2, U16, U9 or U2) and less common haplotypes, probably derived from core
179 haplotypes by one or a few nucleotide substitutions. The regional distribution indicated

region-specific abundance of the hap-groups (Fig. 2B, Fig S1). The U16 group was abundant along coasts of the Sea of Japan and the Sea of Okhotsk, the K2 group along the Pacific Ocean, the U9 group in the Kuril Islands, and the U2 group along the Hokkaido coast of the Sea of Okhotsk.

Spatial population structure based on distribution of Cytb haplotypes

As shown in Table 1, *h* in each population ranged from 0.000 (populations 4, 8, 10, 11) to 0.742 (16), π ranged from 0.000 (4, 8, 10, 11) to 0.0049 (32) and the mean values of *h* and π were 0.354 and 0.0017, respectively. The low *h* and π values in populations in the Kuril Islands may be attributable to the small sample sizes rather than actual low diversity. When samples with fewer than 20 individuals were eliminated, the lowest *h* and π were observed in populations 30 and 31. The *h* and π values of the total sample were estimated at 0.979 and 0.0038, respectively, suggesting that the examined samples contained various haplotypes of shallow divergence.

Pairwise F_{ST} values were significantly different from 0 in 83.2% of pairwise comparisons, even after Bonferroni correction, and F_{ST} values were higher than 0.5 in 37% of pairs, indicating that there was genetic differentiation probably due to limitation of gene flow among populations (Table S1). The nMDS plots based on F_{ST} showed that populations could be roughly clustered into three regions (Figs 1 and 3): Region 1, consisting of the islands of Iturup and Simushir in the Kuril Islands; Region 2, consisting of the Pacific coasts of Hokkaido, Honshu, Kunashir Island and Zeleny Island; and Region 3, consisting of other areas, i.e. the coasts of the Sea of Japan and the Sea of Okhotsk, with the exception of the islands of Iturup and Simushir. It should be noted that although Regions 1 and 2 were geographically contiguous, genetically they were very different. When visualized based on genetic distance, these two regions were discrete, with Region 3 between them.

The Mantel test for the IBD model showed a significant correlation (P=0.002) between genetic (F_{ST}) and geographic distance.

As shown in Table 2, AMOVA supported a hierarchically clustered population structure in the three regional groups suggested by F_{ST}, and it strongly supported isolation between Regions 1 and 2.

Demographic history based on Cytb variation

Results of the MMD analysis for all samples showed that the observed frequencies of haplotype differences deviated significantly from the sudden-expansion model in both the sum of squared deviation (SSD; P=0.03) and Harpending's raggedness index (P=0.02) at the P<0.05 level. However, deviation from spatial expansion assuming a constant deme (local population)-size model was not significant, using either the SSD (P=0.05) or Harpending's raggedness index (P=0.11). The τ values corresponding to the spatial expansion were estimated to be 1.95 (0.4% of 428 bp). Given that $\tau = 2ut$ (where $2u$ = divergence rate, t = time from expansion) and $2u$ = 0.5–1.01% per Myr, the spatial expansion dates were estimated to be 0.81–0.37 Mya (i.e. Upper Pleistocene).

Both Fu's F_S (-26.25, P=0.0000) and Tajima's D (-1.71, P = 0.0130) indicated that the observed haplotype frequency deviated significantly from that expected under neutral molecular evolution.

Genetic analyses using nDNA markers

Although sample size and population numbers were limited, 19 unique sequences were detected in the NWP populations. Table 3 shows that genetic diversity of APN54 in each local population was higher in the NWP than in the NEP, even when ignoring sequence diversity. Furthermore, the haplotype network shown in Figure 4 including NEP alleles (FJ53656–FJ53661 and KM592256) showed that sequence diversity was much higher in the NWP than in the NEP. Two alleles found in the Kunashir Islands could not be included in this network, because they were too separated from the others with more than 25 mutation steps (number of nucleotide sites of indel/substitution). Nevertheless, these alleles were included in the *L. sitkana* clade when a phylogenetic tree was constructed using neighbour-joining or maximum parsimony methods including alleles from other species (result not shown). This indicated that there was a extremely high level of sequence diversity in APN54 in the NWP population. Sequences IP16 and KN3, closely related to NEP alleles, were found in the Kuril Islands.

Discussion

Comparison of genetic diversity and observed haplotypes/alleles in Littorina sitkana *between the northwestern and northeastern Pacific*

The haplotype and nucleotide diversity indices of the mtDNA gene Cytb for all NWP samples examined in the present study were 0.979 and 0.0038, respectively, and are markedly higher than the indices for the total NEP sample, which were 0.141 and 0.0003, respectively (Marko et al. 2010). Moreover, the mean diversity indices h and π within each population in the NWP (mean sample size = 31.94; Table 1) were 0.354 and 0.0017, respectively, which were higher than those for the total NEP sample (n=96; Marko et al. 2010). The higher genetic diversity in the NWP than in the NEP indicates that, on a palaeontological time scale, *L. sitkana* has a longer history in the NWP than in the NEP.

The sequence of the most abundant Cytb haplotype in the NEP (GenBank accession nos GQ2686–2751) is identical to haplotype U9 in the present study. The U9 hap-group was abundant in the Kuril Islands, suggesting that this island chain acted as a migration route for *L. sitkana*, connecting both sides of the North Pacific. The NWP and NEP populations were therefore genetically continuous, probably through the Kuril and Aleutian Islands.

Results of the analysis of the nDNA marker are consistent with this hypothesis. Genetic diversity was again much higher in the NWP than in the NEP, and genetic connectivity between NWP and NEP via the Kuril Islands was supported by the finding of IP16 and K3 alleles, which connect NWP and NEP alleles in the network (Fig. 4), in the Kuril Islands.

Spatial population structure within the northwestern Pacific inferred from mtDNA marker analysis

In the NWP, genetic diversity of mtDNA was sufficient to detect a spatial population structure, unlike the NEP, where diversity is extremely low. The genetic structure of the *L. sitkana* population in the NWP is correlated with geography. This is indicated by the significant F_{ST} value observed between populations, the region-specific distribution of hap-groups, support for the IBD model and the significant hierarchical population structure revealed by AMOVA.These results are consistent with

previous reports on genetics of *L. sitkana* from parts of the NWP (Nohara 1999; Zaslavskaya and Pudovkin 2005), which have suggested geographic structure. As shown in the results of AMOVA and in Figure 3, the present study categorized NWP populations into three regional groups: Region 1 (Iturup and Simushir in the Kuril Islands), Region 2 (Pacific coasts of Hokkaido, Honshu and islands of Kunashir and Zeleny) and Region 3 (coasts of Sea of Japan and Sea of Okhotsk, except for islands of Iturup and Simushir). Regions 1 and 3 were genetically continuous, as were Regions 2 and 3. However, Regions 1 and 2, though spatially adjacent, were clearly separate. The deep separation between Regions 1 and 2 corresponds with the pattern of haplotype distribution. In Region 1, the U9 hap-group was dominant, while the K2 hap-group was dominant in Region 2 (where it occupied every population except the SB population; Fig S1). The genetic similarity within Region 3 along the Hokkaido coast of the Sea of Japan and the Sea of Okhotsk, similar to the results of the allozyme study of Nohara (1999), is likely a consequence of connection via the Tsushima, Tsugaru and Soya currents (Fig. 5).

We noted an exception to the regional distribution of haplotypes, in population 19 from northeastern Hokkaido. Figure 3 shows that samples from population 19, belonging to Region 3 based on its spatial position, clustered in Region 2 in the nMDS plot. This could be attributed to extremely low genetic diversity in this population. Since *L. sitkana* is easily dislodged by waves (Reid, 1996), population 19 on an exposed rocky promontory might have been small and vulnerable, leading to strong bottlenecks and genetic drift. Thus this case hints at an influence of fine-scale topology on genetic diversity.

Evolutionary history of Littorina sitkana

The ancestral lineage of *L. sitkana* is thought to have diverged from other northern Pacific *Littorina* species in the Middle Miocene; subsequently, *L. horikawai* (Matsubayashi et al. 1979) separated from *L. sitkana* in the Pliocene (Reid et al. 2012). The results of MMD analysis suggest that range expansion of *L. sitkana*, coupled with diversification, was likely associated with climatic oscillation in the Upper Pleistocene. Populations in the NWP partially persisted through the severe climatic changes

of several glacial-interglacial cycles, including the LGM, when the NEP populations almost became extinct. In contrast to the low levels of genetic diversity in NEP populations, the present study reveals greater diversity within the NWP. This is consistent with reports of some other marine species with amphi-Pacific distributions (e.g. Sato et al. 2004; Cassone and Boulding 2006; Cox et al. 2014). *Littorina sitkana* populations in NEP exhibited the patterns of genetic variation consistent with post-LGM recolonization and expansion (Marko et al. 2010). The connectivity between the NWP and NEP populations observed in the present study indicates that the origin of the NEP populations was postglacial colonization from the NWP, achieved through migration along the Kuril Islands, the Kamchatka Peninsula and the Aleutian Islands. Zaslavskaya and Pudovkin (2005) found genetic similarity on the Kuril and Commander Islands (at the western end of the Aleutians) and revealed lower genetic diversity within the Kuril-Commander group, which supports our hypothesis of the eastward migration along these islands from the NWP. Westward migration along this route from the NEP to the NWP, while consistent with the flow of the Kuril Current, is unlikely to have occurred, because the Cytb haplotype U9, occupying NEP and Iturup and Simushir Islands, was never found in Region 2 in Hokkaido, which is the destination of the Kuril Current. Although *L. sitkana* lacks a planktonic dispersal stage, colonization from the NWP to the NEP after the LGM could have been achieved by occasional rafting on seaweed, as this herbivorous gastropod is often found on 'wracks' of floating seaweed. Several genetic studies have supported dispersal of direct-developing benthos over a wide range by rafting (e.g. Thiel and Gutow 2005; Nikula et al. 2010; Gutow et al. 2015). Furthermore, Gordillo and Nielsen (2013) provided strong evidence of long-distance rafting of gastropods on kelp carried by surface currents.

While occasional long-distance dispersal probably promoted transoceanic colonization of the NEP, gene flow seems to have been restricted between established populations in the NWP. Hierarchical genetic structure within the NWP suggests the possibility of isolation between populations, local extinction and subsequent recovery with limited gene flow, perhaps corresponding with the climatic oscillation. Around Hokkaido, populations on the southeast coast (Region 2) are thought to have been damaged at some time, because they show extremely low levels of genetic

315 diversity. The warmer waters of the Sea of Japan, with a large and stable population of *L. sitkana*,
316 probably acted as an ice age refugium, where all the Cytb hap-groups persisted. Populations on the
317 Hokkaido coast of the Sea of Okhotsk might have been damaged during the LGM and subsequently
318 recovered under the influence of the warm Soya current, which promoted migration from the Sea of
319 Japan and reduced genetic evidence of LGM damage. Regions 1 and 2, however, did not seem to
320 benefit much from gene flow from the Sea of Japan. Bottleneck effects during the LGM and/or
321 founder effects in subsequent colonization might have resulted in the observed low level of genetic
322 diversity in these two regions.
323 The isolation of Region 2 from other regions revealed by the Cytb analyses serves as a case
324 study from which to infer the origin and maintenance of genetic isolation by topography and ocean
325 currents. Our results indicate the presence of a genetic barrier in the Nemuro Strait, between
326 Hokkaido and Kunashir, which separates Region 2 and Region 3. At its shallowest the Nemuro Strait
327 is around 10 m deep and therefore formed land bridge during the LGM (when sea level was at least
328 100 m lower than at present; Lambeck et al. 2014), thus isolating the northern (along Sea of Okhotsk)
329 and southern (along Pacific Ocean) coastal populations of eastern Hokkaido and Kunashir. The Pacific
330 coast of this area is washed by a cold current at the present day (Fig. 5) and it is likely that the water
331 temperature was even lower in this region than in other coastal areas of Hokkaido during the LGM,
332 owing to the land bridge across the Nemuro Strait, which prevented the flow of warmer water. This
333 might have caused the extinction or decline of *L. sitkana*, leaving only the K2 haplotype of Cytb in
334 this area. Subsequently, postglacial colonization from the Sea of Japan to the Pacific coast of
335 Hokkaido did not occur, because the Soya Current promoted migration only from the Sea of Japan to
336 the Sea of Okhotsk, but was unable to pass the Nemuro Strait (Fig. 5). Therefore, the isolation of
337 populations along these coasts probably originated with the formation of the land bridge in the
338 Nemuro Strait during the LGM, and has been maintained since by ocean currents (Fig. 5).
339
340 *Conclusion*
341 This is the first report to suggest trans-Pacific postglacial colonization by a coastal gastropod, inferred

from phylogeographic studies using both mtDNA and nDNA markers. In addition, our results indicate limited gene flow between local populations in a direct-developing coastal species. The discovery of isolated populations along the Pacific coast of Hokkaido should be considered in the conservation and management of marine species in this area. .

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

References

Behrens Yamada S (1977) Geographic range limitation of the intertidal gastropods *Littorina sitkana* and *L. planaxis*. Mar Biol 39: 61–65

Botta AM, Rochette R, Saunders GW, Addison JA, Barbeau MA (2016) Evidence for genotypic differentiation between marine snails (*Littorina sitkana*) from the upper- and lower-intertidal zone in

Bamfield Inlet (British Columbia, Canada). J Exp Mar Biol Ecol 461:389–396

Buckland-Nicks JA, Chia F-S (1990) Egg Capsule Formation and Hatching in the Marine Snail *Littorina sitkana*. Philos T Roy Soc B 326:159–176

Buckland-Nicks JA, Chia F-S, Behrens S (1973) Oviposition and development of two intertidal snails, *Littorina sitkana* and *Littorina scutulata*. Can J Zoolog 51:359–365

Canino MF, Spies IB, Cunningham KM, Hauser L, Grant WS (2010) Multiple ice-age refugia in Pacific cod, *Gadus macrocephalus*. Mol Ecol 19:4339–4351

Cassone BJ, Boulding EG (2006) Genetic structure and phylogeography of lined shore crab, *Pachygrapsus crassipes*, along the northeastern and western Pacific coasts. Mar Biol 149:213–226. doi:10.1007/s00227-005-0197-9

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1659

Cox LN, Zaslavskaya NI, Marko PB (2014) Phylogeography and trans-Pacific divergence of the rocky shore gastropod *Nucella lima*. J Biogeogr 41:615–627. doi: 10.1111/jbi.12217

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131:479–491 doi: 10.1111/j.1755-0998.2010.02847.x

Excoffier, L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10:564–567

Favorite F, Dodimead AJ, Nasu K. (1976) Oceanography of the Subartic Pacific Region, 1960–71. International North Pacific Fisheries Commission, Vancouver, Cana doi:10.1007/s00227-005-0197-9da. https://ecos.fws.gov/ServCat/Reference/Profile/49463

Gibson RN, Atkinson RJA, Gordon JDM (2005) The ecology of rafting in the marine environment. II. The rafting organisms and community. Oceanogr Mar Biol 43:279–418

Gordillo S, Nielsen SN (2013) The Australasian muricid gastropod *Lepsiella* as Pleistocene visitor to southernmost South America. Acta Palaeontol Pol 58:777–783. doi: http://dx.doi.org/10.4202/app.2011.0186

396 Gutow L, Beermann J, Buschbaum C, Rivadeneira MM, Thiel M (2015) Castaways can't be choosers
397 — Homogenization of rafting assemblages on floating seaweeds. J Sea Res 95:161–171. doi:
398 10.1016/j.seares.2014.07.005
399 Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for
400 Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98
401 Hasegawa K (2000) Family Littorinidae. In: Okutani T (ed) Marine Mollusks in Japan. Tokai
402 University Press, Tokyo, pp 137-143.
403 Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol Jour Linn Soc 68:87–112
404 Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913. doi:
405 10.1111/j.1095-8312.1999.tb01160.x
406 Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Phil Trans Biol
407 Sci 359:183–195. doi: 10.1098/rstb.2003.1388
408 Kitamura A, Kimoto K (2006) History of the inflow of the warm Tsushima Current into the Sea of
409 Japan between 3.5 and 0.8 Ma. Palaeo3 236:355–366
410 Kyle CJ, Boulding EG (2000) Comparative population genetic structure of marine gastropods
411 (*Littorina* spp.) with and without pelagic larval dispersal. Mar Biol 137:835–845. doi:
412 10.1007/s002270000412
413 Lambeck K, Rouby H, Purcell A, Sun Y, Sambridge M (2014) Sea level and global ice volumes from
414 the Last Glacial Maximum to the Holocene. Proc Nat Acad Sci 111:15296–15303
415 Lee HJ, Boulding EG (2009) Spatial and temporal population genetic structure of four northeastern
416 Pacific littorinid gastropods: the effect of mode of larval development on variation at one
417 mitochondrial and two nuclear DNA markers. Mol Ecol 18:2165–2184. doi:
418 10.1111/j.1365-294X.2009.04169.x
419 Liu JX, Gao TX, Wu SF, Zhang YP (2007) Pleistocene isolation in the Northwestern Pacific marginal
420 seas and limited dispersal in a marine fish, *Chelon haematocheilus* (Temminck & Schlegel, 1845).
421 Mol Ecol 16:275–288. doi: 10.1111/j.1365-294X.2006.03140.x

422 Marko PB, Hoffman JM, Emme SA, Mcgovern TM, Keever C, Cox LM (2010) The
423 expansion-contraction model of Pleistocene demography: rocky shores suffer a sea change? Mol
424 Ecol 19:146–169. doi: 10.1111/j.1365-294X.2009.04417.x
425 Nikula R, Fraser CI, Spencer HG, Waters JM (2010) Circumpolar dispersal by rafting in two
426 subantarctic kelp-dwelling crustaceans. Mar Ecol Prog Ser 40:221-230
427 Nohara M (1999) Genetic and conchologicl variation in *Littorina sitkana* Philippi (Mollusca,
428 Gastropoda) on Northern Japanese Coasts. Zool Sci 16:309-317
429 Ohgaki S (1983) Distribution of the family Littorinidae (Gastropoda) in Hokkaido, with special
430 emphasis on the distribution in Akkeshi Bay. Nankiseibutu 25:173–180 (in Japanese)
431 Ohshima KI, Wakatsuchi M, Fukamachi Y (2002) Near-surface circulation and tidal currents of the
432 Okhotsk Sea observed with satellite-tracked drifters. J Geophys Res 107:3195.
433 doi:10.1029/2001JC001005.
434 Provan J, Bennett KD (2008) Phylogeographic insights into cryptic glacial refugia. Trends Ecol
435 Evol 23: 564–571. doi: 10.1016/j.tree.2008.06.010
436 R Development Core Team (2005) R: A language and environment for statistical computing. R
437 Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0
438 Reid DG (1996) Systematics and evolution of *Littorina.* The Ray Society, London.
439 Reid DG, Dyal P, Williams ST (2012) A global molecular phylogeny of 147 periwinkle species
440 (Gastropoda Littorininae). Zool Scr 41:125–136. doi: 10.1111/j.1463-6409.2011.00505.x
441 Sato S, Kojima H, Ando J, Ando H, Wilmot RL, Seeb LW et al. (2004) Genetic population structure of
442 chum salmon in the Pacific Rim inferred from mitochondrial DNA sequence variation. Environ Biol
443 Fish 69:37–50. doi: 10.1023/B:EBFI.0000022881.90237.aa
444 Sokolova IM, Boulding EG (2004) Length polymorphisms in an intron of Aminopeptidase N provide
445 a useful nuclear DNA marker for *Littorina* species (Caenogastropoda). J Moll Stud 70:165–172
446 Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF (1998) Comparative phylogeography and
447 postglacial colonization routes in Europe. Mol Ecol 7:453–464

448 Thiel M, Gutow L (2005) The ecology of rafting in the marine environment. II. The rafting organisms
449 and community. Oceanogr Mar Biol 43:279–418
450 Wares JP, Cunningham CW (2001) Phylogeography and historical ecology of the North Atlantic
451 intertidal. Evolution 55:2455–2469
452 Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure.
453 Evolution 38:1358–1370. doi: 10.2307/2408641
454 Wright S (1943) Isolation by distance. Genetics 28:114–138
455 Zaslavskaya NI, Pudovkin (2005) Macrogeographic genetic variability in the gastropod mollusk
456 *Littorina sitkana* from Northewest Pacific. Rus J Genet 41:291–300
457
458 **Figure legends**
459 Figure 1
460 Map of sampling sites in the northwestern Pacific Ocean. Codes of localities correspond to those in
461 Table 1. Closed rhombuses, triangles and circles indicate populations belonging to Regions 1, 2 and 3,
462 respectively, which were categorized based on geographic position and genetic similarity (see Fig. 3).
463
464 Figure 2
465 A. Haplotype network of partial cytochrome-*b* gene sequence in *Littorina sitkana* and sister species *L.*
466 *horikawai*, including haplotypes observed in the present study and retrieved from GenBank. Solid
467 lines indicate single-nucleotide substitutions. Circle size indicates haplotype frequency (number of
468 individuals). Shading of circles indicate four hap-groups.
469 B. Geographical distribution of hap-groups. Closed circle indicate places where members of each
470 hap-group were found.
471
472 Figure 3
473 Two-dimensional nonmetric multidimensional scaling (nMDS) plot based on genetic distance (F_{ST}
474 estimated from Cytb haplotypes). Three clusters are associated with geographical distribution: Region

1, Kuril Islands (islands of Iturup and Simushir); Region 2, Pacific Ocean coasts of Hokkaido and Honshu, Japan, and islands of Kunashir and Zeleny; Region 3, other areas (along Sea of Japan and Sea of Okhotsk except Iturup and Simushir Islands). *As an exception, population 19, Sea of Okhotsk coast of Hokkaido, appears in Region 2 cluster (see Discussion).

Figure 4

Network of the partial sequences of the APN54 gene intron in *Littorina sitkana*, including sequences observed in the present study and those retrieved from GenBank. Sequences indicated with a star, FJ53656–FJ53661 and KM594456, were found in the NEP (Botta et al. 2014; Lee and Boulding 2009), while the others were found in the NWP in the present study. Numbers on solid lines indicate the number of mutation steps (nucleotide substitutions and indels) and lines without a number indicate a single mutation step.

Figure 5

Schematic diagram of ocean currents in the sampling areas of the NWP (based on Fig. 29 in Favorite, et al. 1976; Fig. 18 in Oshima et al.2002; and Fig. 1 in Kitamura and Kimoto 2006).

Table 1. List of *Littorina sitkana* samples examined in the present study. *h* and π indicate haplotype and nucleotide diversity, respectively.

Area	Sample code	Locality	year/month	Sample size	*h*	π
Primorye	1	Vostok Bay	2012/08	39	0.230	0.0030
	2	Ussurisky Bay	2012/08	37	0.156	0.0006
Sakhalin	3	Sakhalin Island	2012/08	19	0.205	0.0004
Kuril Islands	4	Kunashir Is., Pacific coast	2012/08	13	0.000	0.0000
	5	Kunashir Is., Okhotsk Sea coast	2012/08	39	0.345	0.0021
	6	Iturup Is., Pacific coast	2012/08	17	0.309	0.0014
	7	Iturup Is., Okhotsk Sea coast (east)	2012/08	38	0.448	0.0021
	8	Iturup Is., Okhotsk Sea coast (middle)	2010/08	2	0.000	0.0000
	9	Iturup Is., Okhotsk Sea coast (west)	2010/08	8	0.678	0.0019
	10	Simushir Island	2010/08	3	0.000	0.0000
	11	Zeleny Island	2010/08	5	0.000	0.0000
Hokkaido	12	Muroran	2012/06	40	0.561	0.0023
	13	Esan	2012/06	42	0.093	0.0004
	14	Shirakami	2012/06	39	0.681	0.0040
	15	Setana	2012/05	40	0.391	0.0027
	16	Rankoshi	2013/04	24	0.742	0.0038
	17	Ofuyu	2013/04	33	0.670	0.0022
	18	Soya straight	2012/07	47	0.268	0.0010
	19	Kamui misaki (Kitamiesashi)	2013/04	36	0.208	0.0007
	20	Saruru (Okoppe)	2011/03	38	0.506	0.0030
	21	Masuura (Abashiri)	2011/08	41	0.435	0.0026
	22	Shari	2011/06	41	0.425	0.0026
	23	Utoro	2011/07	45	0.578	0.0034
	24	Aburako bay	2011/08	44	0.659	0.0032
	25	Sakimui	2011/07	41	0.518	0.0019
	26	Shibetsu	2011/07	48	0.445	0.0014
	27	Horomoshirii	2011/06	46	0.240	0.0005
	28	Akkeshi	2011/11	42	0.172	0.0004
	29	Kushiro	2010/10	42	0.528	0.0012
	30	Erimo	2012/06	32	0.062	0.0001
Honshu	31	Hachinohe	2012/06	25	0.080	0.0001
	32	Oma	2012/07	16	0.691	0.0049
Mean				31.94	0.354	0.0017
Total				1022	0.979	0.0038

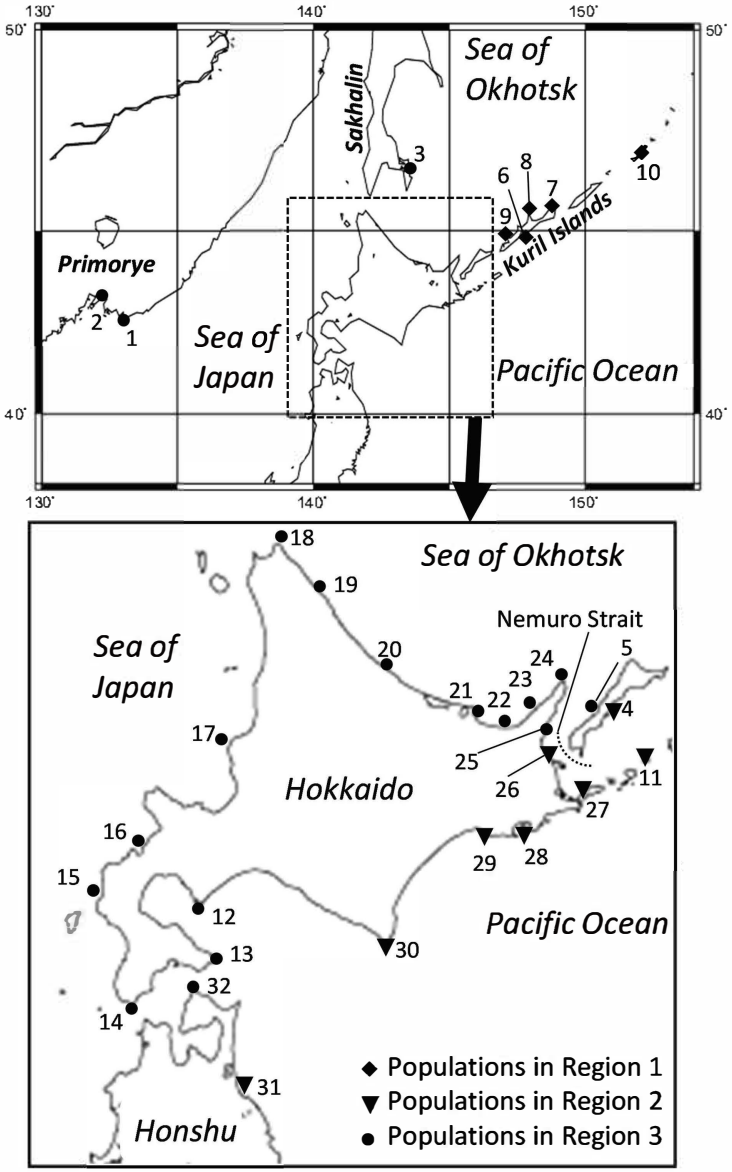
Table 2. Results of AMOVA. Category: (1) 3 groups suggested by F_{ST}, 1. Kuril Islands (Iturup and Simushir Is.), 2. Pacific Ocean along Hokkaido and Honshu, Japan, and Kunashir and Zeleny Islands, 3. others. (2) 2groups of 1. and 2..

Category	Source of variation	Variance component	% of variation	*P* value	Fixation indices
(1)	Among groups	0.25424	26.18	0.000	F_{CT}=0.262
	Among populations	0.30984	31.90	0.000	F_{SC}=0.432
	Within populations	0.40713	41.92	0.000	F_{ST}=0.580
(2)	Among groups	0.67364	74.34	0.000	F_{CT}=0.743
	Among populations within groups	0.03144	3.47	0.000	F_{SC}=0.135
	Within populations	0.20108	22.19	0.000	F_{ST}=0.778

Table 3. Levels of genetic diversity in a nDNA marker (APN54) in *L. sitkana* populations in Vancouver Island, Northeastern Pacific, and Hokkaido, Northwestern Pacific.

Area	Population	*N*	*A*	H_E	Reference
Northeastern Pacific	Vancouver-A	17	3	0.219	1.
	Vancouver-B	14	2	0.071	1.
	Vancouver-C	17	2	0.258	1.
	CB07	36	4	0.349	2.
	PP97	24	4	0.548	2.
	PP07	30	3	0.213	2.
	PR97	25	2	0.411	2.
	PP07	24	3	0.493	2.
Northwestern Pacific	19 (Kitamiesashi)	6	3	0.667	3.
	16 (Rankoshi)	12	8	0.826	3.
	6 (Itrup Is.)	12	6	0.771	3.
	5 (Kunashir Is.)	15	8	0.837	3.

N: sample size (number of individuals), *A*: number of observed alleles, H_E: expected heterozygosity. Reference column indicates 1. calculated from the data in Table S2.1 in Botta et al. 2014, 2. from Table 1 in Lee & Boulding 2009, and 3. from the observations of the present study.


130°
140°
150°
50°
40°
Sea of Okhotsk
Sakhalin
Primorye
Kuril Islands
Sea of Japan
Pacific Ocean
1
2
3
4
5
6
7
8
9
10
11
Nemuro Strait
Hokkaido
Honshu
12
13
14
15
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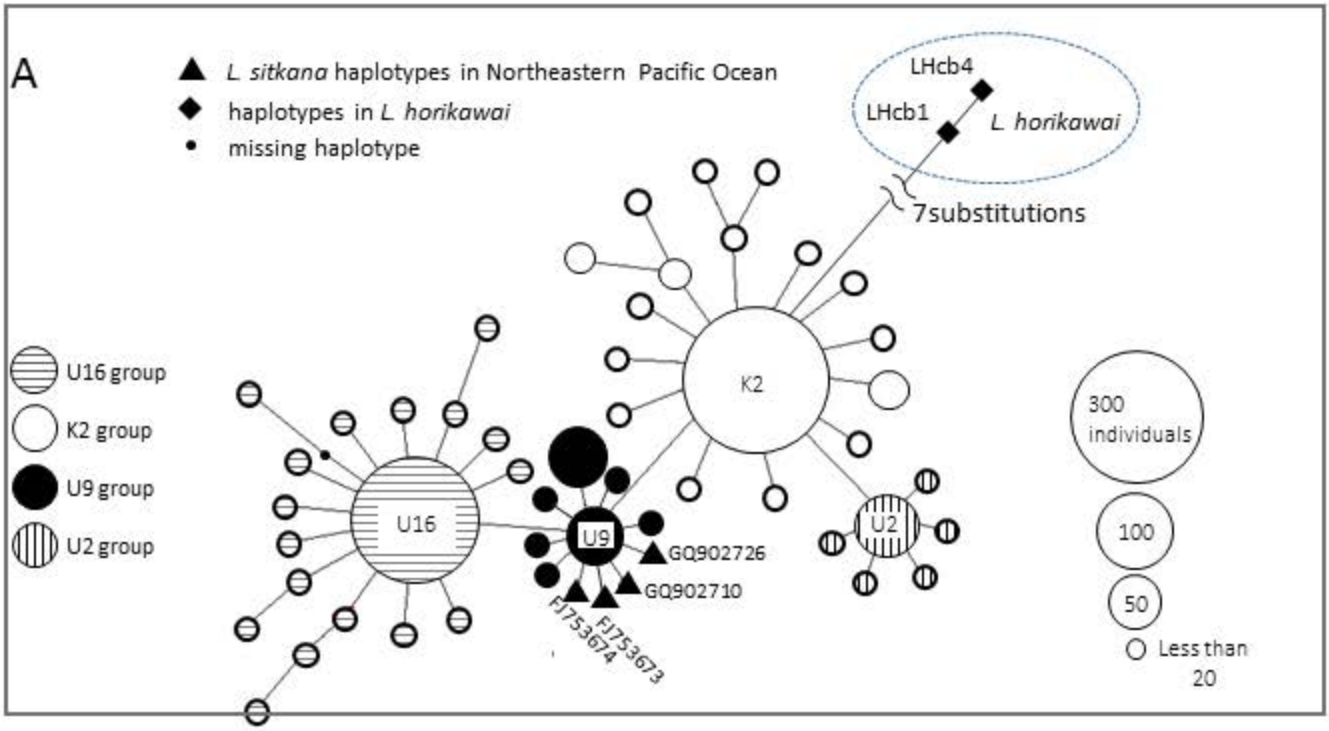
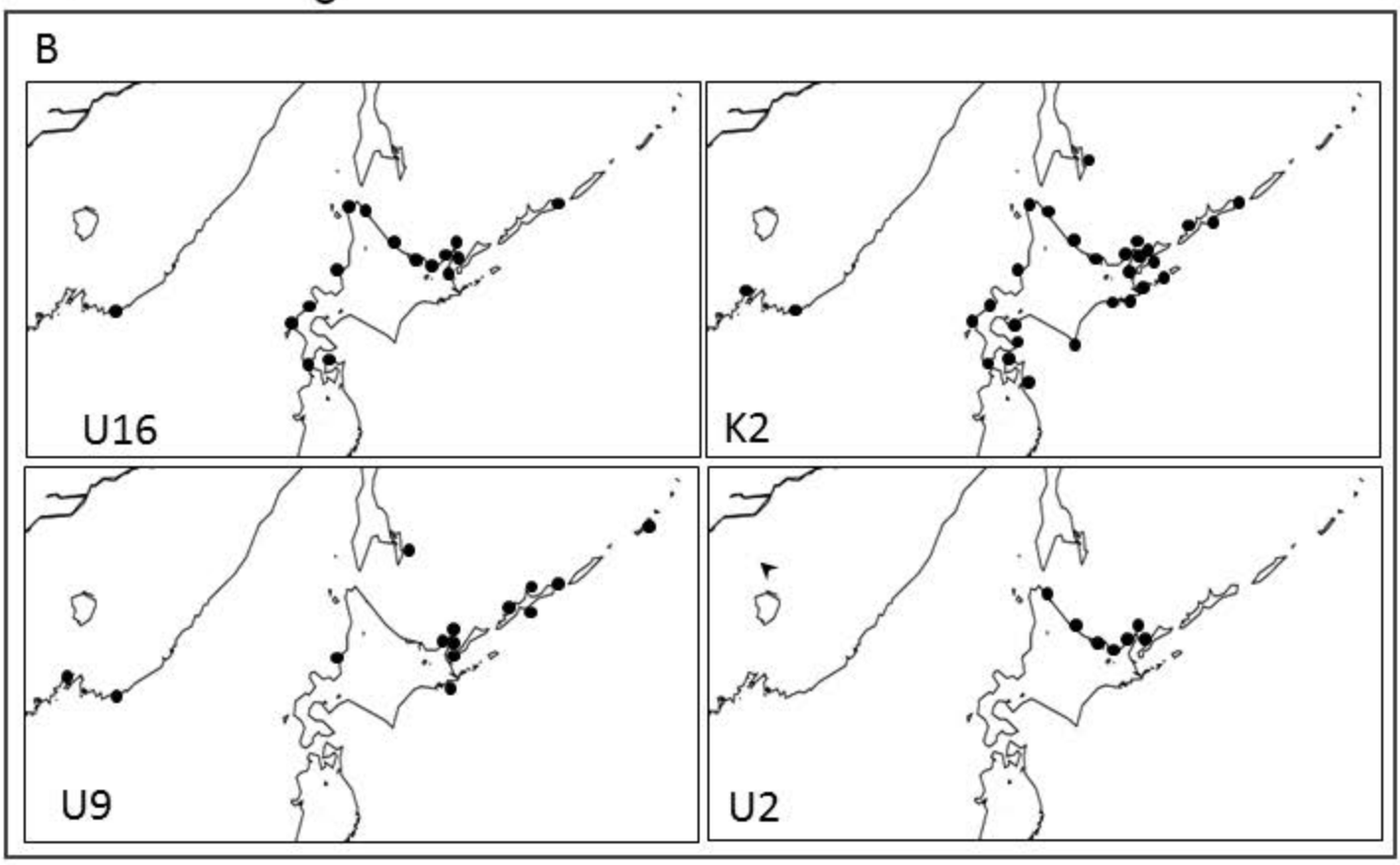
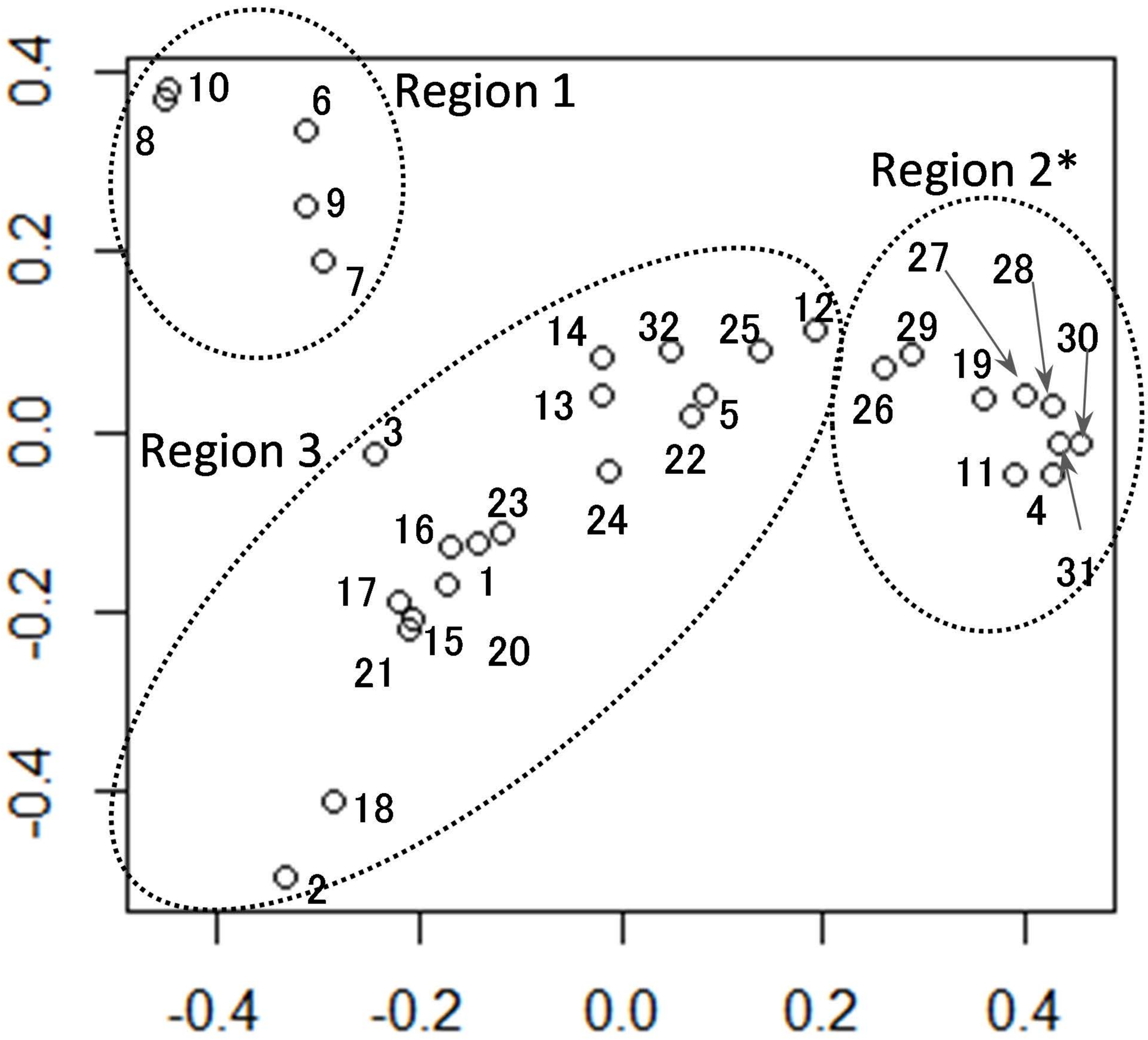
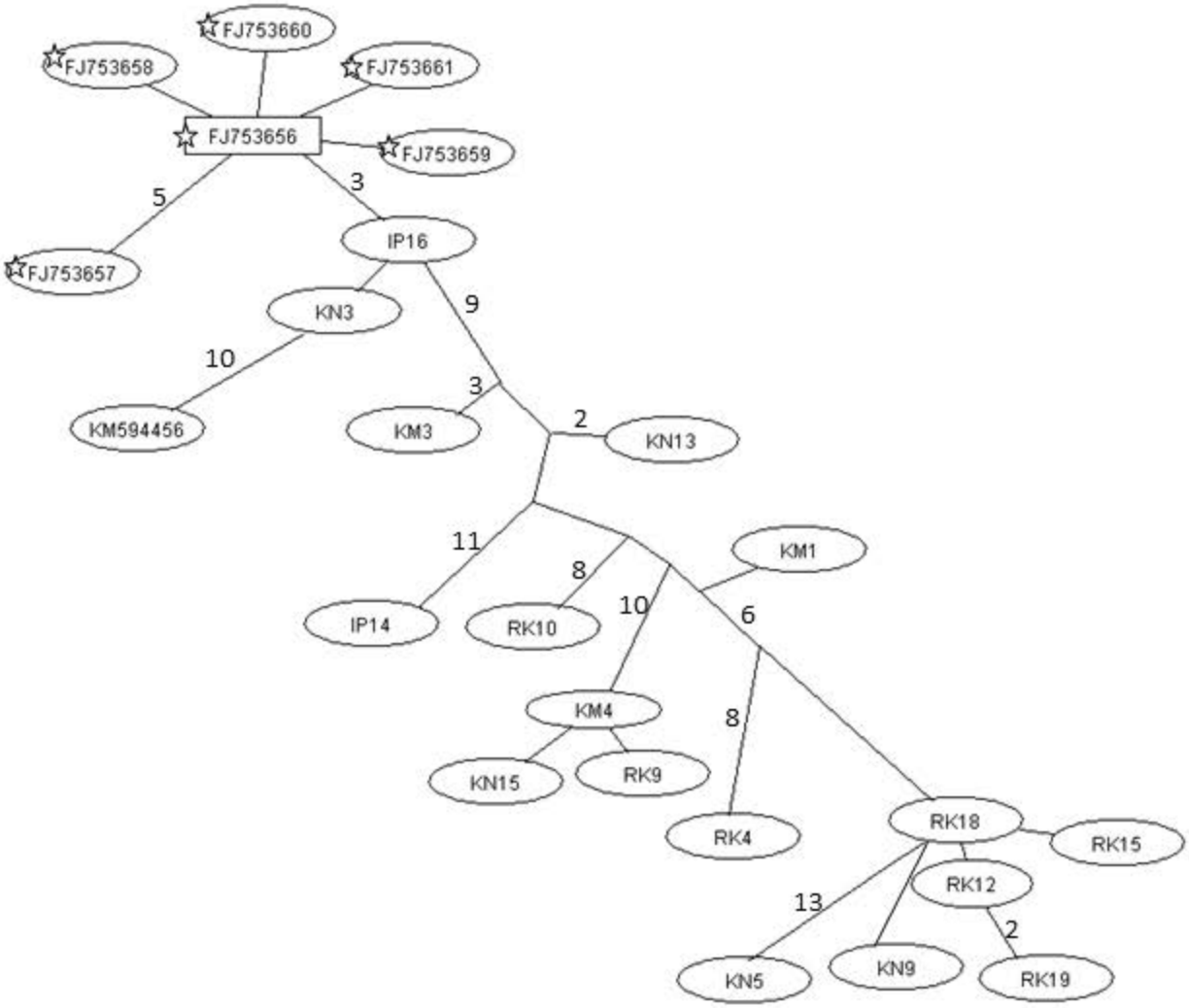
Populations in Region 1
Populations in Region 2
Populations in Region 3


A
L. sitkana haplotypes in Northeastern Pacific Ocean
haplotypes in L. horikawai
missing haplotype
LHcb4
LHcb1
L. horikawai
7substitutions
U16 group
K2 group
U9 group
U2 group
U16
K2
U9
U2
GQ902726
GQ902710
FJ753674
FJ753673
300 individuals
100
50
Less than 20


B
U16
K2
U9
U2


Region 1
Region 2*
Region 3
10
6
8
9
7
12
14
32
25
29
27
28
30
19
13
5
26
3
22
11
23
24
4
16
1
31
17
15
20
21
18
2
0.4
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
0.4


FJ753660
FJ753658
FJ753661
FJ753656
FJ753659
5
3
FJ753657
IP16
KN3
9
10
3
KM594456
KM3
2
KN13
11
8
10
6
KM1
IP14
RK10
KM4
8
KN15
RK9
RK4
RK18
RK15
RK12
13
2
KN5
KN9
RK19

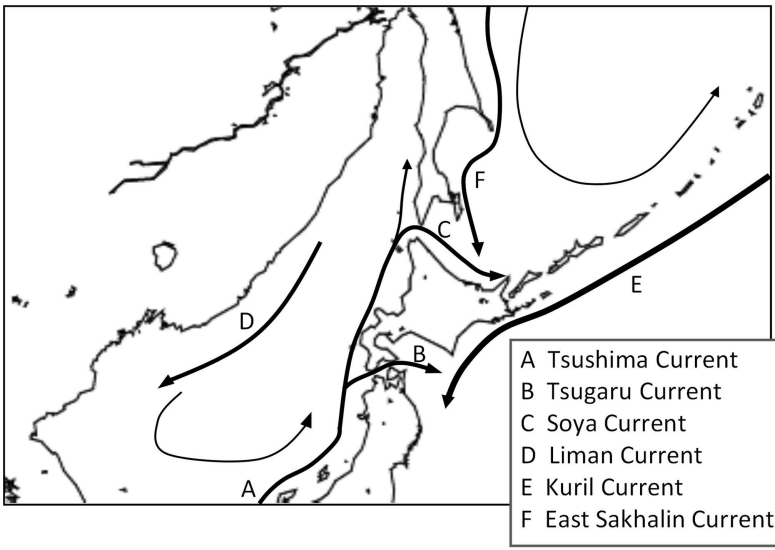
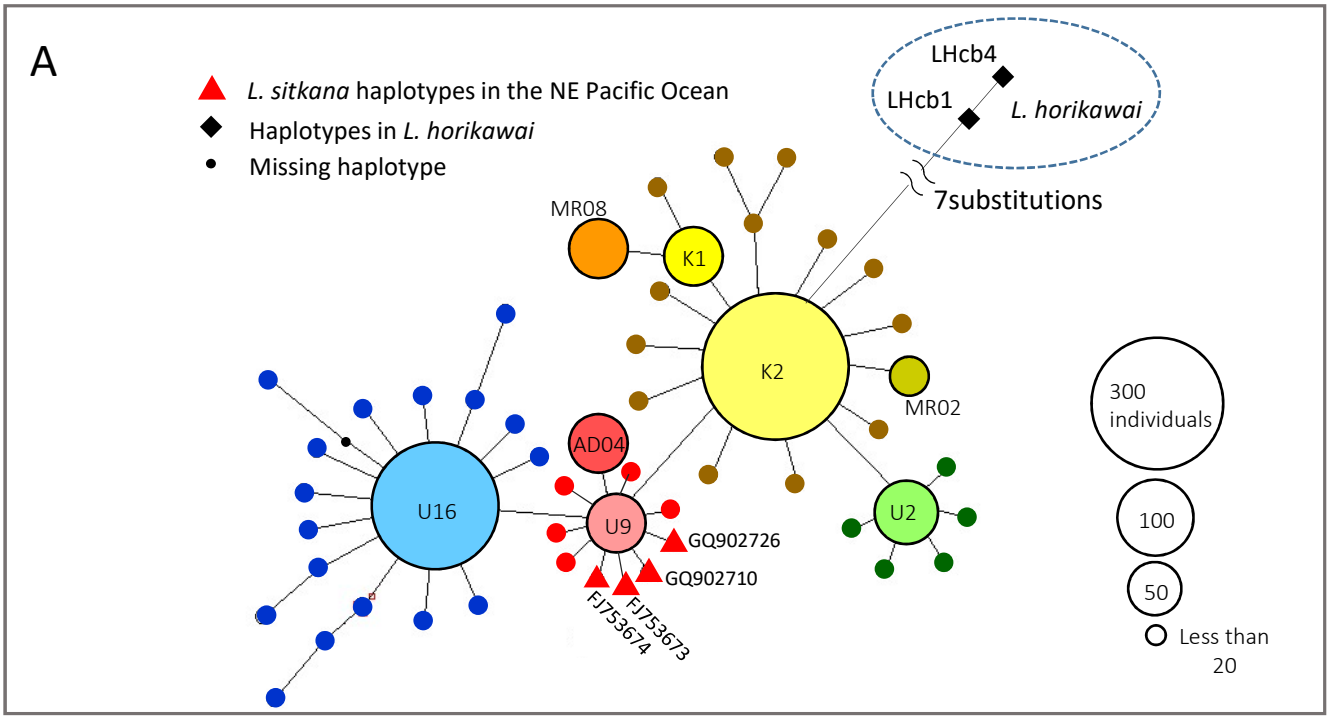

A
B
C
D
E
F
A Tsushima Current
B Tsugaru Current
C Soya Current
D Liman Current
E Kuril Current
F East Sakhalin Current

Table S 1. Below diagonal: F_{ST} value between samples Bold letters indicate F_{ST}>0.5. Above diagonal: + indicates the pair in which F_{ST} value was significantly deviated from 0 (P<0.01) after sequencial Bonferroni correction.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	0.000	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
2	0.214	0.000	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
3	0.294	**0.784**	0.000	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	0.462	**0.905**	**0.876**	0.000	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	+	-	-	+
5	0.230	**0.636**	0.280	0.208	0.000	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
6	0.484	**0.811**	**0.593**	**0.791**	**0.504**	0.000	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	0.384	**0.631**	0.406	**0.677**	0.464	0.058	0.000	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	0.474	**0.877**	**0.809**	**1.000**	**0.564**	-0.208	-0.111	0.000	-	-	-	-	+	-	-	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-
9	0.315	**0.763**	0.310	**0.765**	0.313	0.062	0.041	0.054	0.000	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
10	**0.504**	**0.881**	**0.820**	**1.000**	**0.586**	-0.090	-0.012	0.000	0.157	0.000	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
11	0.394	**0.887**	**0.836**	0.000	0.131	**0.723**	**0.627**	**1.000**	**0.640**	**1.000**	0.000	-	+	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-
12	**0.527**	**0.780**	**0.630**	0.326	0.388	**0.661**	**0.660**	**0.688**	**0.581**	**0.702**	0.253	0.000	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
13	**0.797**	**0.945**	**0.935**	**0.930**	**0.802**	**0.914**	**0.866**	**0.955**	**0.913**	**0.956**	0.918	**0.767**	0.000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	0.293	**0.555**	0.263	0.329	0.214	0.427	0.428	0.396	0.267	0.432	0.254	0.433	**0.696**	0.000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	0.060	0.098	0.436	**0.589**	0.384	**0.568**	0.453	**0.558**	0.434	**0.581**	0.532	**0.610**	**0.830**	0.391	0.000	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
16	0.051	0.201	0.371	0.487	0.299	**0.500**	0.413	0.442	0.339	0.480	0.396	**0.540**	**0.808**	0.321	0.034	0.000	-	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+
17	0.012	0.180	0.344	**0.607**	0.312	**0.550**	0.407	**0.559**	0.375	**0.583**	**0.544**	**0.603**	**0.853**	0.329	0.042	0.045	0.000	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+
18	0.123	0.051	**0.674**	**0.807**	**0.550**	**0.747**	**0.589**	**0.794**	**0.669**	**0.801**	**0.780**	**0.731**	**0.913**	**0.511**	0.033	0.117	0.095	0.000	+	-	-	+	+	+	+	+	+	+	+	+	+	+
19	0.493	**0.859**	**0.748**	-0.014	0.203	**0.758**	**0.691**	**0.856**	**0.699**	**0.861**	-0.090	0.370	**0.885**	0.383	**0.614**	**0.535**	**0.618**	0.782	0.000	+	+	+	+	+	+	+	-	-	+	-	-	+
20	0.044	0.132	0.389	**0.524**	0.305	**0.530**	0.427	**0.513**	0.386	**0.539**	0.460	**0.569**	**0.810**	0.352	0.044	0.056	0.040	0.072	**0.548**	0.000	-	+	-	-	+	+	+	+	+	+	+	+
21	0.069	0.080	0.449	**0.600**	0.380	**0.578**	0.461	**0.571**	0.447	**0.592**	**0.546**	**0.615**	**0.834**	0.400	0.031	0.067	0.051	0.041	0.617	-0.014	0.000	+	-	-	+	+	+	+	+	+	+	+
22	0.428	**0.682**	**0.565**	0.406	0.302	**0.619**	0.603	**0.634**	**0.524**	**0.651**	0.337	0.490	**0.795**	0.419	**0.510**	0.440	0.499	**0.636**	0.390	0.384	0.451	0.000	+	+	+	+	+	+	+	+	+	+
23	0.063	0.220	0.323	0.430	0.220	0.479	0.400	0.461	0.330	0.491	0.366	**0.512**	**0.769**	0.312	0.101	0.080	0.075	0.163	0.451	-0.002	0.029	0.273	0.000	-	+	+	+	+	+	+	+	+
24	0.079	0.389	0.288	0.253	0.069	0.447	0.395	0.454	0.287	0.484	0.181	0.401	**0.745**	0.241	0.183	0.123	0.134	0.296	0.267	0.098	0.159	0.237	0.050	0.000	+	+	+	+	+	+	+	+
25	0.307	**0.696**	0.377	0.115	0.019	**0.511**	0.489	**0.585**	0.349	**0.605**	0.039	0.356	**0.802**	0.244	0.453	0.366	0.401	**0.615**	0.130	0.393	0.464	0.369	0.315	0.135	0.000	-	+	+	+	+	+	+
26	0.434	**0.775**	**0.583**	0.033	0.154	**0.655**	**0.627**	**0.728**	**0.556**	**0.738**	-0.040	0.351	**0.820**	0.346	**0.558**	0.480	**0.538**	**0.704**	0.058	**0.502**	**0.567**	0.409	0.421	0.238	0.081	0.000	+	+	+	-	+	+
27	**0.549**	**0.881**	**0.791**	-0.006	0.277	**0.796**	**0.728**	**0.889**	**0.754**	**0.892**	-0.080	0.404	**0.898**	0.429	**0.661**	**0.599**	**0.670**	**0.812**	0.037	**0.608**	**0.669**	0.488	**0.519**	0.339	0.176	0.071	0.000	-	+	-	-	+
28	**0.558**	**0.898**	**0.829**	-0.012	0.286	**0.821**	**0.740**	**0.920**	**0.790**	**0.922**	-0.086	0.406	**0.908**	0.428	**0.670**	**0.610**	**0.684**	**0.827**	0.033	**0.617**	**0.678**	0.498	**0.524**	0.344	0.182	0.084	0.040	0.000	+	-	-	+
29	**0.538**	**0.833**	**0.705**	0.277	0.347	**0.728**	**0.695**	**0.793**	**0.663**	**0.800**	0.204	0.390	**0.770**	0.404	**0.636**	**0.569**	**0.636**	**0.773**	0.290	**0.589**	**0.644**	0.490	**0.515**	0.374	0.292	0.264	0.319	0.255	0.000	+	+	+
30	**0.541**	**0.917**	**0.885**	-0.034	0.277	**0.843**	**0.736**	**0.970**	**0.832**	**0.971**	-0.109	0.402	**0.935**	0.411	**0.658**	**0.591**	**0.679**	**0.837**	0.021	**0.601**	**0.667**	0.483	**0.505**	0.325	0.172	0.051	0.020	0.021	0.341	0.000	-	+
31	**0.513**	**0.909**	**0.868**	-0.029	0.253	**0.820**	**0.714**	**0.963**	**0.801**	**0.964**	-0.107	0.375	**0.929**	0.383	**0.633**	**0.556**	**0.653**	**0.823**	0.014	**0.574**	**0.643**	0.456	0.479	0.301	0.154	0.063	0.022	0.016	0.316	0.001	0.000	+
32	0.266	**0.611**	0.408	0.203	0.179	0.481	0.488	0.399	0.319	0.447	0.088	0.319	**0.597**	0.220	0.370	0.229	0.337	**0.536**	0.265	0.316	0.381	0.344	0.265	0.158	0.183	0.238	0.335	0.318	0.209	0.322	0.282	0.000



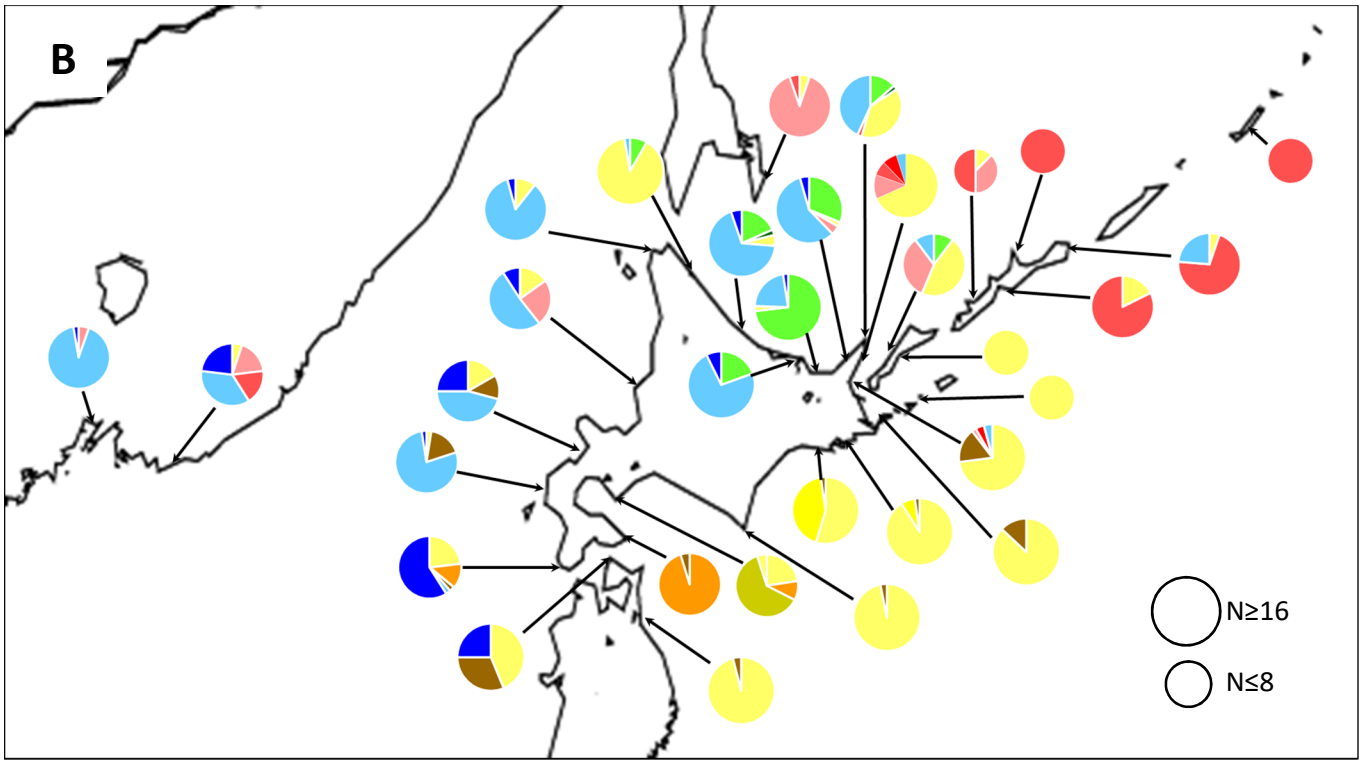


Fig. S1

A. Haplotype network of partial cytochrome-*b* gene in *Littorina sitkana* and allied species *L. horikawai* including haplotypes observed in the present study and retrieved from GenBank. Solid lines indicate single-nucleotide substitutions. Circle size indicates haplotype frequency (number of individuals). Circle colors indicate hap-group: yellow, hap-group K2; blue, hap-group U16; pink, hap-group U9; green, hap-group U2.

B. Geographical distribution of haplotypes. Pie graphs indicate the haplotype ratio in each sample. Colors in the pie graphs represent haplotypes shown in Fig. S1A.