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**Mitochondrial and nuclear DNA analysis revealed a cryptic species and genetic
introgression in *Littorina sitkana* (Mollusca, Gastropoda)**

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Abstract

We investigated mitochondrial and nuclear DNA genotypes in nominal *Littorina sitkana* samples from 2 localities in Eastern Hokkaido, northern Japan. Our results indicated the existence of cryptic species. In the analysis of partial mitochondrial Cytochrome *b* gene sequences, haplotypes of *L. sitkana* samples were monophyletic in a phylogenetic tree with orthologous sequences from other *Littorina* species, but were apparently separated in 2 clades. One included typical *L. sitkana* (CBa clade) samples, which formed a clade with an allopatric species, *L. horikawai*. The other, CBb, was independent from CBa and *L. horikawai*. Haplotypes of the mitochondrial 16S rRNA gene also separated into 2 clades. We additionally examined intron sequence of the heat shock cognate 70 (HSC70) nuclear gene and identified 17 haplotypes. These were also separated into 2 clades, HSCa and HSCb. Among the examined Hokkaido samples, 60% of individuals were heterozygotes. However, each heterozygote consisted of haplotypes from the same clade, HSCa or HSCb, and no admixture of HSCa and HSCb haplotypes was observed. These results indicate reproductive isolation between the 2 clades. Among the genotyped Hokkaido samples, 93% of individuals had CBa + HSCa or CBb + HSCb genotypes, and 7% had CBb + HSCa genotypes. The discrepancy between the mtDNA and nuclear DNA haplotypes in a few individuals may have been caused by genetic introgression due to past hybridization.

Key words; periwinkle, speciation, reproductive isolation, Cytb, 16S, HSC70

38 INTRODUCTION

39

40 Recent applications of molecular genetic approaches for reconstructing phylogenies
41 have successfully uncovered reproductive isolation and speciation among closely
42 related species within the same locality (e.g., Avise 2000; Barraclough and Vogler 2000;
43 Templeton 2001). In shell-bearing mollusks, shell characters have contributed to
44 taxonomy and are regarded as useful tools for species identification. However, in some
45 cases, molecular evidence has revealed the existence of cryptic species that cannot be
46 distinguished by shell morphologies (e.g., Liu et al. 2011; Puillandre et al. 2009). There
47 are several examples of gastropod phenotypes, which can become genetically adapted
48 or plastically changed in response to environmental factors (Dewitt and Scheiner 2004;
49 Piersma and Gils 2010). Therefore, species identification using shell characters may be
50 misleading if within species variation is high and/or a high level of similarity in shell
51 morphology can be observed among species.

52 High levels of intraspecific shell variation are common in the genus *Littorina*
53 (Mollusca, Gastropoda), and convergent evolution and/or ancestral effect can lead to
54 shell character similarities in different *Littorina* species (Reid 1996; Johannesson 2003).
55 Such variable shell characters have attracted many researchers, and *Littorina* species
56 have been used as models for evolutionary studies, not only for morphological, but also
57 for evolution, phylogeny and genetic research (e.g., Galindo et al. 2010; Graham et al.
58 2006; Hollander et al. 2006; Johannesson 2003; Johannesson et al. 1995; Kyle and
59 Boulding 2000, Lee and Boulding 2009 and 2010; Panova et al. 2011; Reid 1996; Reid
60 et al. 1996; Rolán-Alvarez 2007; Sokolova and Portner 2003; Sokolova et al. 2000;
61 Williams et al. 2003).

In Sitka Periwinkles, *Littorina sitkana* Philippi, the main focus of the present study, extreme variability in shell form has been observed (reviewed by Reid 1996). The phenotypic characters of the shell surface, such as smooth or riblet surface phenotypes, are thought to be plastic as Boulding et al. (1993) observed and related to environment gradients, especially wave action (Yamazaki and Goshima 2012). *L. sitkana* develops without a pelagic larval stage, and is common in intertidal zones on rocky and boulder shores throughout the northern Pacific, from Oregon to the Bering Sea, the Okhotsk Sea, and the northern Sea of Japan (Reid 1996). The species was first described by Philippi (1846) based on a ribbed shell from Sitka, Alaska (lectotype figured by Reid, 1996: fig. 56A).

The smooth-shelled type, which is abundant on the eastern Hokkaido coast, has been considered as one of the intraspecific variants (e.g., Reid 1996; Zaslavskaya 2006). Revealing the taxonomy of *L. sitkana* has been complicated due to the variability in shell characters and the wide distribution. Many phenotypes have been reported, but these have been classified as intraspecific variants as reviewed by Reid (1996). Some authors have considered smooth-shelled forms (named as *L. kurila*, *L. subtenebrosa*, *L. atkana* and others) to represent distinct species, and their synonymy with *L. sitkana* (see Reid 1996) needs to be tested with molecular data.

Genetic variation within *L. sitkana* has been investigated for Russian (Zaslavskaya 1995 and 2006; Zaslavskaya and Pudovkin 2005), North American (Boulding et al. 1993; Kyle and Boulding 2000, Lee and Boulding 2009, Marko et al. 2010), and Japanese (Nohara 1999) populations. Zaslavskaya (1995 and 2006) reported 3 unknown species, including *L. sp1*, *sp2*, and *sp3*, based on allozyme studies. The collected specimens of these unidentified species were mixed with *L. sitkana*, or assumed *L.*

sitkana, in the Russian Far East. The findings were unique and informative; however, allozyme data cannot be compared with the sequence data available in databases such as GenBank. Sequence data can be used to investigate the status of newly found species relative to previously reported ones.

The objective of the present study was to evaluate the status of cryptic *Littorina sitkana* species, found on the eastern Hokkaido coast, using molecular genetic markers. To clarify relationships among typical *L. sitkana* and potential cryptic species, we used 3 molecular markers, including partial sequences of the cytochrome *b* (Cytb) gene and the 16S rRNA (16S) gene in mitochondrial DNA, and intron sequences of the heat shock cognate 70 (HSC70) gene in nuclear DNA. Sequences of Cytb and 16S have previously been used to reconstruct intraspecific and interspecific *Littorina* phylogenies (Kyle and Boulding 2000; Kim et al. 2003; Lee and Boulding 2009; Marko et al. 2010; Reid et al. 1996). Consequently, many orthologous sequences from other *Littorina* species are available in the GenBank database. The HSC70 region has recently been acknowledged as an effective marker for population genetic studies of *Littorina* species with intraspecific variation (Lee and Boulding 2009 and 2010). We employed this marker to examine the existence of reproductive barriers, as well as to confirm genetic clustering indicated by the mtDNA analysis. We discuss the speciation and hybridization between the 2 clade found in the present study and the discrepancies between our results and the new species suggested by Zaslavskaya (1995 and 2006).

MATERIALS AND METHODS

Sample collection and DNA extraction

Samples regarded as *Littorina sitkana* were collected from 2 localities of Eastern Hokkaido, Utoro, and Kushiro (Table 1, Fig. 1). The species was identified based on habitat (upper intertidal to supralittoral zone on rocky shores) and shell character observations. We selected individuals with blackish or purple-brown smooth shells (Fig. 2, A-C), the most common shell forms of *L. sitkana* on the east coast of Hokkaido (Reid 1996) and identical to Type C in Nohara (1999), described as having a smooth surface without sculptures. The smooth-shelled *L. sitkana* strongly resemble 4 other Pacific species, *L. subrotundata*, *L. aleutica*, *L. natica*, and *L. kasatka* (Reid 1996; Reid and Golikov 1991; Reid et al. 1991; Reid et al. 1996; Zaslavskaya, 1995 and 2006). However, there are no records of *L. subrotundata*, *L. aleutica*, or *L. natica* from the Japanese coast. *L. kasatka* is observed less frequently than *L. sitkana*, *L. subrotundata*, *L. aleutica*, and *L. natica* in collections (Reid 1996), and is probably less abundant than *L. sitkana* around Hokkaido. Three additional *Littorina* species, *L. brevicula*, *L. mandshurica*, and *L. squalida*, occur around Hokkaido, and all of these have ribs on their shells. Considering the above, most of the smooth-shelled periwinkles found on the eastern Hokkaido coast are likely to be *L. sitkana*. We also collected *L. horikawai* from the coast of Nejiko, Hirado Island off Kyushu (Table 1, Fig. 1). This species is regarded as a sister species of *L. sitkana* (Reid 1996; Reid et al. 1996), and is expected to be useful for comparisons in phylogenetic analyses. All collected samples were fixed in 99% ethanol.

We extracted genomic DNA from ca. 20 mg tissue of foot muscle from each of the

84 Hokkaido samples and 8 *L. horikawai* samples using either a PUREGENE™ kit (Qiagen) or a DNeasy™ Blood & Tissue kit (Qiagen), following the manufacture's protocols. Extracted DNA was dissolved in 150–250 µL elution buffer.

Mitochondrial DNA analysis

Polymerase chain reactions (PCRs) for partial mtDNA Cytb sequences from all samples were carried out in separate 40 µL reactions, containing ca. 10 ng genomic DNA, 5 pmol each of the forward and reverse primers (F: CCTTCCCGCACCTTCAAATCTTTC R: GCAAAGAAGCGAGTGAGGGTAGC; Lee and Boulding 2009) and 0.3 U TaKaRa Ex Taq (TaKaRa) following the manufacturers' protocols. Thermal-cycle profiles consisted of an initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 45 s, 50°C for 30 s, and 72°C for 60 s, with a post-cycle extension at 72°C for 5 min. The same thermal conditions were also used for the 16S and HSC70 PCRs. The PCR products were purified with magnetic beads (AMPure, Agencourt, Beverly, MA), and were sequenced by MacroGen Japan Inc. (Tokyo, Japan) using the forward and reverse primers noted above. Sequences were aligned and edited to 428 bp using DNASIS-Mac v.3.5 (Hitachi) and ClustalX 1.81 (Thompson et al. 1997) software, and were deposited in the DDBJ database (Accession nos. AB665092–AB665101). Previously reported orthologous sequences for the *Littorina* species *L. horikawai* (GenBank accession no. U46797), *L. kasatka* (U46800), *L. fabalis* (U46808), *L. compressa* (U46811), *L. obtusata* (U46813), *L. aleutica* (U46789), *L. saxatilis* (U46817), *L. subrotundata* (U46828), and *L. sitkana* (GQ902751), and an *L. sitkana* cytochrome *b* pseudogene sequence (U46821) identified by Reid et al. (1996), were aligned and added to the data set for subsequent phylogenetic analysis.

A phylogenetic tree was reconstructed using Bayesian methods in MrBayes 3.12 (Ronquist and Huelsenbeck 2003). We applied the substitution model GTR + G + I, which was recommended as the best fitting substitution model for our data set by jModelTest 0.1.1 (Posada 2008; Guindon and Gascuel 2003). In the Bayesian analysis, the posterior probability distribution of trees was approximated by drawing a sample every 100 steps over 1,000,000 MCMC cycles, after discarding a burn-in of 250,000 cycles.

We used an additional mtDNA marker, 16S, and a nuclear DNA marker, HSC70, to resolve the following issues. Usage of multiple markers was expected to confirm and compliment the results of the Cytb analysis.

1. In the Cytb analysis, we could not compare our samples to *Littrina natica*, which is distributed in the neighboring area, the Kurile Islands, and sometimes has a similar shell shape to *L. sitkana*, because no Cytb sequence for this species was available in the database. Therefore, the possibility that samples of the newly found lineage were members of *L. natica* could not be rejected.
2. The observed haplotypes might have included pseudogene sequences. As our data did not include the entire full-length Cytb gene, the possibility that our sequences represented a pseudogene remained, even after we found no stop codon in the observed sequences.
3. The observed genetic divergence should be regarded as intraspecific variation if there was no reproductive barrier between the 2 lineages.

L. natica 16S sequences were available from the database, and examining linkage between 16S and Cytb types could be used as an approach to distinguish pseudogene sequences. Because the mitochondrial genome is circular and non-recombining,

sequence variation of functional genes on mtDNA must link between different gene regions. However, pseudogene in the nuclear genome evolves randomly and do not appear to be linked to functional mitochondrial genes. Thus, 16S was employed to resolve the former 2 issues, and HSC70 in nuclear DNA was analyzed to resolve the latter issue.

PCR and sequencing for partial 16S was carried out in the same manner as for Cytb using the primers 16Sar: CGCCTGTTTATCAAAAACAT and 16Sbr: CCGGTCTGAACTCAGATCACGT (Simon et al. 1991). For comparative analysis, we added reported *Littorina* species 16S rRNA sequences, including *L. kasatka* (GenBank accession no. U46799), *L. fabalis* (U46807), *L. natica* (U46809), *L. compressa* (U46810), *L. obtusata* (U46812), *L. saxatilis* (U46816), *L. subrotundata* (U46827), *L. aleutica* (U46788), *L. arcane* (U46790), *L. horikawai* (U46796), and *L. sitkana* (U46820). All sequences were aligned and edited for 434 bp, and newly identified haplotypes were deposited in DDBJ (Accession nos. AB665316–665319). For phylogenetic analysis, we used Bayesian methods with the GTR + I + G model of nucleotide substitution. In this region, some insertion/deletion sites (indels) were observed that could not be used as informative sites using a Bayesian approach. To consider all of the sequence variations, including indels, maximum parsimony (MP) methods were also employed, and deletions were treated as a fifth element in addition to nucleotide substitutions, using PAUP* 4.0 (Swofford 2002). For the MP method, 100 MP trees with equal total branch lengths were chosen and reconstructed into a >50% strict consensus tree. Bootstrap values were estimated to provide measures of relative support for each branch using 1,000 replications.

Nuclear DNA analysis of HSC70 intron variation

The most important aim of nuclear DNA analysis was to test for reproductive isolation between 2 clades that were identified in the mtDNA analysis. Because samples from Kushiro included only 1 clade of Cytb, CBa (see Results), we used 6 representative individuals with different Cytb haplotypes. However, as samples from Utoro included members of both clades, CBa and CBb (see Results), all samples from this area were examined, with the exception of 3 individuals that were assumed to be *L. kasatka* following the mtDNA analysis. A partial fragment (*ca.* 650 bp) of HSC70 was amplified by PCR using the primers F: GGCACCTTTGACGTGTCAGTCC and R: TCCACAGGCTCCAGTGTGCCAC (Lee and Boulding 2009). Amplification by PCR, purification of the products, and direct sequencing were carried out in the same manner as for Cytb. Some samples showed heterozygosity; therefore, the corresponding PCR products were cloned with a TOPO TA Cloning[®] Kit (Invitrogen) and were sequenced using universal M13 primers. Sequence variation was observed only in the intron region, so we trimmed both ends of each sequence to remove the exons, and edited the sequences to 287 bp. Unique sequences were deposited in DDBJ/GenBank with accession nos. AB665223–665239. Expected and observed heterozygosities for 32 individuals from Utoro, for which haplotypes were successfully identified, were calculated using Genepop (Raymond and Rousset 1995; Rousset 2008). Hardy-Weinberg equilibrium (HWE) was also tested using Genepop. Phylogenetic trees for the haplotypes were reconstructed in 2 ways, using both Bayesian and MP methods, because, similar to the 16S region, indels were observed within the HSC70 introns. For the Bayesian method, we selected the GTR + G + I model by using jModelTest 0.1.1. Indels were treated as a fifth element of nucleotide variation in the MP method, and reconstruction

of the consensus tree and estimation of bootstrap values for each branch were carried out in the same manner as for the 16S region. For the HSC70 region, we could not correctly align sequences from other *Littorina* species in Genbank due to the large number of indels observed among species, and because the *L. sitkana* HSC70 sequence in GenBank included only the exon region in which no variety was observed in our data. Therefore, we used only the newly identified sequences in the present study to reconstruct the phylogeny.

RESULTS

Mitochondrial DNA analysis

We found 13 unique Cytb *L. sitkana* haplotypes among the Hokkaido samples and 2 *L. horikawai* haplotypes (Fig. 3). Nucleotide variations were observed at 39 sites, and 33 of these were parsimoniously informative. One *L. horikawai* haplotype was identical to a previously reported sequence for this species (GenBank accession no. U46797), and the other has been newly identified in this study (LhCB4). The phylogeny of haplotypes is shown in Fig. 3. Haplotype names in Italics represent sequences previously reported in GenBank, and all others indicate newly identified haplotypes from the present study. Among the Utoro samples, 3 individuals had apparently distant haplotypes, LkCBU18, LkCBU42, and LkCBU36, which form a monophyletic clade with the *L. kasatka* sequence from GenBank (U46799). The Bayesian posterior probability value for this clade was 100%, indicating that the 3 individuals were either members of *L. kasatka* or of an unknown sister species. The remaining 10 haplotypes were monophyletic, but were separated into 2 clades. One clade was the CBa cluster, which included a

previously reported *L. sitkana* sequence assumed to be typical *L. sitkana*, and the other was the CBb cluster, potentially a different species. Genetic diversity between the CBa and CBb clades was estimated, using the uncorrected *p*-distance as 5.8%, and was considerably higher than observed levels of interspecific differentiation among other *Littorina* species, such as 1.2% between *L. saxatilis* and *L. compressa*, 2.1% between *L. fabalis* and *L. obtusata*, and 2.6% between *L. sitkana* and *L. horikawai* following our calculation from previously reported sequences. Interestingly, *L. horikawai*, which is considered as a different species in the current taxonomy and is endemic to southern Japan, was included in the CBa cluster.

In the analysis of partial 16S sequences, 4 haplotypes were found among the Hokkaido samples (Fig.4). Haplotype Lk16SU36 occurred in individuals that were regarded as *L. kasatka* following the Cytb analysis, and again formed a monophyletic cluster with a previously reported *L. kasatka* sequence. The 8 *L. horikawai* individuals had a single haplotype that was similar to the reported sequence for this species (GenBank accession no. U46796). Both the Bayesian and MP trees for the 16S region indicated an apparent separation of the 16Sa and 16Sb clades, and these corresponded to CBa and CBb, respectively (Fig. 4). The posterior probability and bootstrap support for clade 16Sb in the tree was in fact below significance level; however, non-monophyly of *L. sitkana* was confirmed, because *L. horikawai* was included. At the individual level, all of the individuals with CBa haplotypes showed 16Sa haplotypes, and all of the CBb individuals had 16Sb haplotypes. The 16Sb clade appeared to be independent from *L. natica*. These results indicated that members of the CBb clade were not *L. natica*, and that the Cytb region that we used in this study was not part of a pseudogene. The branching topology is different between the Cytb

and 16S trees, probably due to complex variations among 16S sequences, including indels. Nevertheless, we successfully confirmed that there were 2 mitochondrial lineages in the nominal species of *L. sitkana*.

Nuclear DNA Analysis of HSC70 intron

Among 6 and 39 individuals from Kushiro and Utoro, respectively, 27 individuals were heterozygotes, indicating an observed heterozygosity of 60% for the examined Hokkaido samples. Among the heterozygotes, 8 individuals (1 from Kushiro and 7 from Utoro) failed to be genotyped during sequencing via subcloning. These were excluded from the HWE analysis, but were assigned to the HSCa or HSCb clades as described below. Thirty-two individuals from Utoro were successfully sequenced and were assigned to 17 haplotypes. Among them, expected and observed heterozygosities were estimated as 0.75 and 0.56, respectively, and deviation from HWE was significant ($p < 0.0001$), suggesting subdivision within the population. Both the Bayesian and MP trees for the haplotypes revealed 2 distinctive clades, HSCa and HSCb, as displayed in Fig. 5. We found that 8 *L. horikawai* individuals had only a single haplotype that was first identified in the present study, and all of these were homozygotes. Similar to the mtDNA analysis, this haplotype comprised a monophyletic clade with HSCa members. Comparing the sequences of HSCa and HSCb, we identified key variations between 2 clades, including 4 nucleotide substitutions and 2 indels (see Table 2). The 8 individuals that could not be successfully genotyped were assigned to either HSCa or HSCb using these key variations. Admixture of 2 clades within a single individual (i.e., heterozygotes with HSCa and HSCb haplotypes) was not observed among the samples.

Linkage of mtDNA and HSC70 haplotypes

Linkage between Cytb, 16S, and HSC70 is shown in Table 3. This table exclusively includes individuals that were examined for all 3 markers and excludes some others that were assigned to Clade A or B using only one or two markers. In total, 93% of the genotyped samples from Hokkaido had CBa + 16Sa + HSCa or CBb + 16Sb + HSCb, and 7% had CBb + 16Sb + HSCa. Among the examined samples, Cytb and 16S haplotypes were completely linked. None of individuals showed a combination of CBa + HSCb.

DISCUSSION

Molecular evidence revealed separation of 2 clades of nominal *Littorina sitkana* in the east coast of Hokkaido. The sequence from GenBank with the accession number GQ902751 was reported in Marko et al. (2010) as the most abundant haplotype along the northeastern Pacific coast, including Sitka in Alaska, the type locality from where Philippi's specimens were collected. The sequence appeared in the CBa group in the phylogenetic tree, as shown in Fig. 3, thus we believe that CladeA with the CBa and HSCa haplotypes contains typical *L. sitkana* specimens. The other, CladeB with the CBb and HSCb haplotype, may be a sister taxon of *L. sitkana*. We are aware that the phylogenetic trees based on 2 mtDNA markers, Cytb and 16S, were inconsistent with each other in some points, and that they failed to reconstruct precise phylogenies among the species. Other markers, such as mtDNA 12S rRNA or nuclear DNA 18S or 28SrRNA, may supply more accurate results. However, for the present study, we required molecular markers that exhibit variations within the species of interest. We detected high levels of genetic differentiation and reproductive isolation between *L.*

324 *sitkana* and CladeB, and our results implied that CladeB may be independent at the full
325 species level. Therefore, we tentatively treated *L. sitkana* and CladeB as different
326 species in the following discussion.

327 In the Utoro population, from which both *L. sitkana sensu stricto* (CladeA) and
328 CladeB specimens were collected, 25 individuals (64.1% of genotyped samples) were
329 heterozygous in the HSC70 region. This indicates that HSC70 alleles have been well
330 shuffled through mating and recombination. However, no heterozygotes were observed
331 with a combination of HSCa and HSCb, which correspond to *L. sitkana* and CladeB,
332 suggesting that a genetic barrier may exist between the 2 species. The exceptional
333 associations between HSC70 and mtDNA haplotypes (7% of genotyped samples from
334 Hokkaido had CBb + HSCa, see Table 3) can probably be attributed to genetic
335 introgression of mitochondrial DNA caused by occasional hybridization after separation
336 between *L. sitkana* and CladeB, as observed in many other species (e.g., Lamb and
337 Avise 1986; Mikhailova et al. 2009; Reid et al. 2006; Shimizu and Ueshima 2000;
338 Yamazaki et al. 2008; Wiwegweaw et al. 2009). Nevertheless, such hybridization events
339 found in the present study are unlikely to have occurred recently because no HSCa and
340 HSCb heterozygotes were observed in our investigation. Also, considering that no CBa
341 + HSCb individuals were observed, it is assumed that directional hybridization has
342 occurred. For instance, CladeB females and *L. sitkana* males may have mated, and
343 offspring may have backcrossed with *L. sitkana*.

344 Among 3 of the new species suggested by Zaslavskaya (2006), *Littorina* sp1 is most
345 likely identical to CladeB. *Littorina* sp1 was genetically close to *L. sitkana*, but was
346 independent from it, like as CladeB. *Littorina* sp1 was collected from Kasatka Bay,
347 Itrup Island, which is the closest area to Utoro among the sampling sites of her study.

Zaslavskaya (2006) suggested that *L. sitkana* and *L. sp1* could be distinguished from one another based on shell morphology. The difference was shown on a photograph, but quantitative information and statistical analyses of morphometric data may be preferable to distinguish the new species from *L. sitkana*, which is a hyper-variable species in shell morphology. Based on the results of Zaslavskaya (2006) and this study, we recommend a revision of the current taxonomy and would like to encourage extensive work on morphology to facilitate description and nomenclature of this new species.

The results of the present study highlight the potential utility of molecular markers to elucidate the phylogeny and taxonomy of shell-bearing mollusks, which can be confused due to intraspecific variation and interspecific similarities in shell morphology. The combination of mitochondrial and nuclear DNA markers provided us with a multidimensional perspective for species identification, and such a method is expected to resolve taxonomic and phylogenetic problems in other species complexes in addition to *Littorina sitkana*.

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370 REFERENCES

- 371 Avise JC (2000). *Phylogeography: The History and Formation of Species*, Harvard
372 University Press, Cambridge, MA.
- 373 Barraclough TG, Vogler AP (2000) Detecting the geographical pattern of speciation
374 from species-level phylogenies. *Am Nat* 155:419–434
- 375 Boulding EG, J. Buckland-Nicks J, Van Alstyne KL (1993) Morphological and
376 allozyme variation in *Littorina sitkana* and related *Littorina* species from the
377 northeastern Pacific. *Veliger* 36:43-68
- 378 Dewitt TJ, Scheiner SM (2004) *Phenotypic Plasticity: Functional and Conceptual*
379 *Approaches*. Oxford University Press, New York
- 380 Galindo J, Grahame JW, Butlin RK (2010) An EST-based genome scan using
381 sequencing in the marine snail *Littorina saxatilis*. *J Evolution Biol*
382 23:2004-2016
- 383 Grahame JW, Wilding CS, Butlin RK (2006) Adaptation to a steep environmental
384 gradient and an associated barrier to gene exchange in *Littorina saxatilis*
385 *Evolution* 60:268-278
- 386 Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large
387 phylogenies by maximum likelihood. *Syst Biol* 52:696–704
- 388 Hollander J, Collyer ML, Adams DC, Johannesson K (2006) Phenotypic plasticity in
389 two marine snails: constraints superseding life history. *J Evolution Biol*
390 19:1861-1872
- 391 Johannesson K (2003) Evolution in *Littorina*: ecology matters. *J Sea Res* 49:107–117

392 Johannesson K, Rolán-Alvarez E, Ekendahl A (1995) Incipient reproductive isolation
393 between two sympatric morphs of the intertidal snail *L. saxatilis*. *Evolution*
394 49:1180-1190.

395 Kim SJ, Rodriguez-Lanetty M, Song JI (2003) Genetic population structure of *Littorina*
396 *brevicula* around Korean waters. *Hydrobiologia* 505:41–48

397 Kyle CJ, Boulding EG (2000) Comparative population genetic structure of marine
398 gastropods (*Littorina* spp.) with and without pelagic larval dispersal. *Mar Biol*
399 137:835–845

400 Lamb T, Avise JC (1986) Directional introgression of mitochondrial DNA in a hybrid
401 population of tree frogs: The influence of mating behavior. *Proc Natl Acad Sci*
402 USA 83:2526–2530

403 Lee HJ, Boulding EG (2009) Spatial and temporal population genetic structure of four
404 northeastern Pacific littorinid gastropods: the effect of mode of larval development
405 on variation at one mitochondrial and two nuclear DNA markers. *Mol Ecol*
406 18:2165–2184

407 Lee HJ, Boulding EG (2010) Latitudinal clines in body size, but not in thermal
408 tolerance or heat shock cognate 70 gene (HSC70) in the highly-dispersing
409 intertidal gastropod, *Littorina keenae* (Gastropoda: Littorinidae). *Biol J Linn Soc*
410 100:494–505

411 Liu J, Li Q, Kong L, Zheng X (2011) Cryptic diversity in the pen shell *Atrina pectinata*
412 (Bivalvia: Pinnidae): high divergence and hybridization revealed by molecular and
413 morphological data. *Mol Ecol* 20:4332–4345

414 Marko PB, Hoffman JM, Emme SA, McGovern TM, Keever CC, Cox LN (2010) The
415 'Expansion-Contraction' model of Pleistocene biogeography: rocky shores suffer a

416 sea change? Mol Ecol 19:146–169

417 Mikhailova NA, Gracheva YA, Backeljau T, Granovitch AI (2009) A potential species-
 418 specific molecular marker suggests interspecific hybridization between sibling
 419 species *Littorina arcana* and *L. saxatilis* (Mollusca, Caenogastropoda) in natural
 420 populations. Genetica 137:333-340

421 Nohara M (1999) Genetic and Conchological Variation in *Littorina sitkana* Philippi
 422 (Mollusca, Gastropoda) on Northern Japanese Coasts. Zool Sci 16:309–317

423 Panova M, Blakeslee AMH, Miller AW, Mäkinen T, Ruiz GM, Johannesson K, Andre C
 424 (2011) Glacial History of the North Atlantic Marine Snail, *Littorina saxatilis*,
 425 Inferred from Distribution of Mitochondrial DNA Lineages. PLoS ONE 6:
 426 e17511.

427 Philippi RA (1846) Descriptions of a new species of *Trochus*, and of eighteen new
 428 species of *Littorina*, in the collection of H. Cuming, Esq. P Zool Soc Lond
 429 13:138–143

430 Piersma T, Gils JA (2010) The Flexible Phenotype. Oxford University Press, New York

431 Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol
 432 25:1253–1256

433 Puillandre N, Cruaud C, Kantor YI (2009) Cryptic species in *Gemmuloborsonia*
 434 (Gastropoda: Conoidea). J Mollus Stud 76:11–23

435 Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests
 436 and ecumenism, Ver. 1.2. J Hered 86:248–249

437 Reid DG (1996) Systematics and Evolution of *Littorina*. The Ray Society, London

438 Reid DG, Golikov AN (1991) *Littorina naticoides*, new species, with notes on the
 439 other smooth-shelled *Littorina* species from the northwestern Pacific. Nautilus

440 105:7–15

441 Reid DG, Rumbak E, Thomas RH (1996) DNA, morphology and fossils: phylogeny
 442 and evolutionary rates of the gastropod genus *Littorina*. Philos T Roy Soc B
 443 351: 877–895

444 Reid DG, Zaslavskaya NI, Sergievsky SO (1991) *Littorina kasatka*, a new species from
 445 the Kuril Islands and Okhotsk Sea. Nautilus 105:1–6

446 Reid DG, Lal K, Mackenzie-Dodds J, Kaligis F, Littlewood D, Williams S (2006)
 447 Comparative phylogeography and species boundaries in *Echinolittorina* snails in
 448 the central Indo-West Pacific. J Biogeogr 33:990–1006

449 Rolán-Alvarez, E (2007) Sympatric speciation as a by-product of ecological adaptation
 450 in the Galicia *Littorina saxatilis* hybrid zone. J Mollus Stud 73:1-10

451 Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference
 452 under mixed models. Bioinformatics 19:1572–1574

453 Rouset F (2008) Genepop'007: a complete reimplementation of the Genepop software
 454 for Windows and Linux. Mol Ecol Res 8:103–106

455 Shimizu Y, Ueshima R (2000) Historical biogeography and interspecific mtDNA
 456 introgression in *Euhadra peliomphala* (the Japanese land snail). Heredity
 457 85:84–96

458 Simon C, Franke A, Martin A (1991) The polymerase chain reaction: DNA extraction
 459 and amplication. In: Hewitt GM, Johnston AWB, Younr JPW (eds) Molecular
 460 techniques in taxonomy. NATO ASI Series, Springer-Verlag, Berlin Heidelberg,
 461 pp 329–355

462 Sokolova IM, Pörtner HO (2003) Metabolic plasticity and critical temperatures for
 463 aerobic scope in a eurythermal marine invertebrate (*Littorina saxatilis*,

464 Gastropoda: Littorinidae) from different latitudes. J Exp Biol 206:195–207
 465 Sokolova IM, Granovitch AI, Berger VJ, Johannesson K (2000) Intraspecific
 466 physiological variability of the gastropod *Littorina saxatilis* related to the vertical
 467 shore gradient in the White and North Seas. Mar Biol 137:297–308
 468 Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (and other
 469 methods). Version 4. Sinauer, Sunderland, MA.
 470 Templeton AR (2001) Using phylogeographic analyses of gene trees to test species
 471 status and processes. Mol Ecol 10:779–791
 472 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal
 473 X windows interface: flexible strategies for multiple sequence alignment aided by
 474 quality analysis tools. Nucleic Acids Res 25:4876–4882
 475 Williams ST, Reid DG, Littlewood DTJ (2003) A molecular phylogeny of the
 476 Littorininae (Gastropoda: Littorinidae): unequal evolutionary rates, morphological
 477 parallelism and biogeography of the Southern Ocean. Mol Phylogenet Evol
 478 28:60-86
 479 Wiwegweaw A, Seki K, Utsuno H, Asami T (2009) Fitness Consequences of
 480 Reciprocally Asymmetric Hybridization between Simultaneous Hermaphrodites.
 481 Zool Sci 26:191–196
 482 Yamazaki T, Goshima S (2012) Adaptive differences in morphology of the periwinkle
 483 *Littorina sitkana* between two habitats separated by an artificial structure. Venus
 484 (in press)
 485 Yamazaki Y, Koketsu S, Fukuda T, Araki Y, Nambu H (2008) Natural hybridization and
 486 directional introgression of two species of Japanese toads *Bufo japonicus*
 487 *formosus* and *Bufo torrenticola* (Anura: Bufonidae) resulting from changes in

488 their spawning habitat. J Herpetol 42:427–436

489 Zaslavskaya NI (1995) Allozyme comparison of four littorinid species morphologically

490 similar to *Littorina sitkana*. Hydrobiologia 309:123–128

491 Zaslavskaya NI (2006) Allozyme comparison of *Littorina* species from the northwestern

492 Pacific. J Mollus Stud 72:163–166

493 Zaslavskaya NI, Pudovkin AI (2005) Macrogeographic genetic variability in the

494 gastropod mollusk *Littorina sitkana* from the northwest Pacific. Rus J Genet

495 41:291–300

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Figure legends

Fig. 1

Map of Japan displaying the 2 sampling sites in Hokkaido (inset A, *Littorina sitkana*) and 1 in Kyushu (inset B, *L. horikawai*).

Fig. 2

Shells of the examined *Littorina* samples. A: *L. sitkana* from Utoro, Hokkaido. B: *L. sp.* from Utoro, Hokkaido. C: *L. sitkana* from Kushiro, Hokkaido. D: *L. horikawai* from Hirado, Kyushu. Sample B was potentially new species, CladeB, having CBb + 16Sb + HSCb genotype.

Fig.3

Fifty-percent majority-rule Bayesian tree inferred from partial mitochondrial DNA Cytb sequences (428bp) using the GTR + G + I model. The tree was rooted using *L. kasatka* as the outgroup. Numbers above the branches represent Bayesian posterior probability values (%). Species names in italics correspond to sequences of related *Littorina* species previously reported in GenBank, and other names indicate haplotypes that were identified in the present study.

Fig. 4

Fifty-percent majority-rule Bayesian tree inferred from partial mitochondrial DNA 16S sequences (434 bp) using the GTR + G + I model. The tree was rooted using *L. kasatka* as the outgroup. Numbers above the branches represent Bayesian posterior probability values (%) and bootstrap values (%) from 1000 replications. The latter were calculated

after reconstruction of a maximum parsimony consensus tree, treating gaps as a fifth element. Species names in italics correspond to sequences of related *Littorina* species previously reported in GenBank, and other names indicate haplotypes that were identified in the present study.

Fig. 5

Fifty-percent majority-rule Bayesian tree (unrooted) inferred from partial nuclear DNA HSC70 intron sequences (287bp) using the GTR + G + I model. Numbers above the branches represent Bayesian posterior probability values (%) and bootstrap values (%) from 1000 replications, which were calculated after reconstruction of a maximum parsimony consensus tree treating gaps as a fifth element. Haplotypes were separated into 2 of the diverged clade, HSCa (CladeA, typical *L. sitkana*) and HSCb (CladeB, *L. sp.*).

Fig. 1

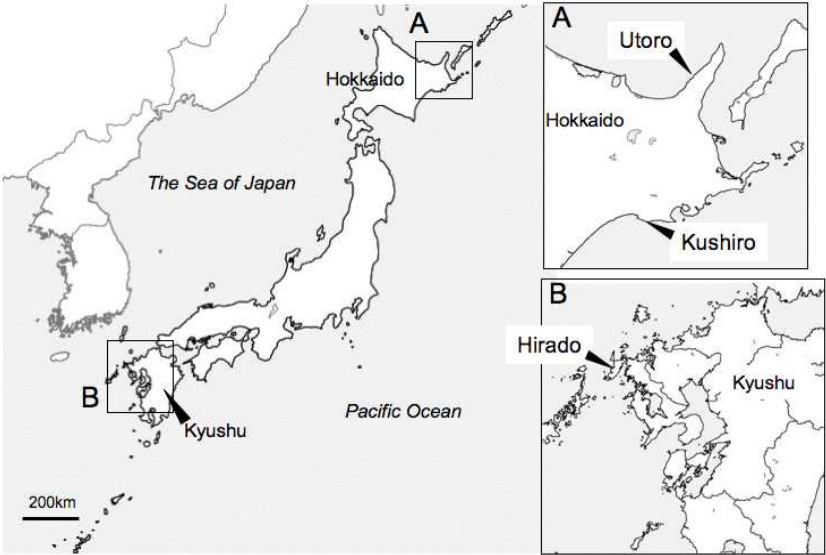
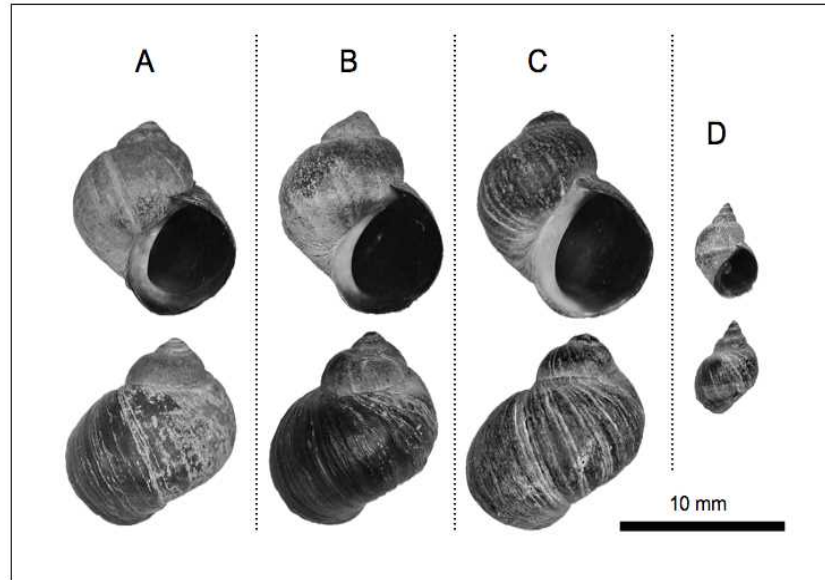


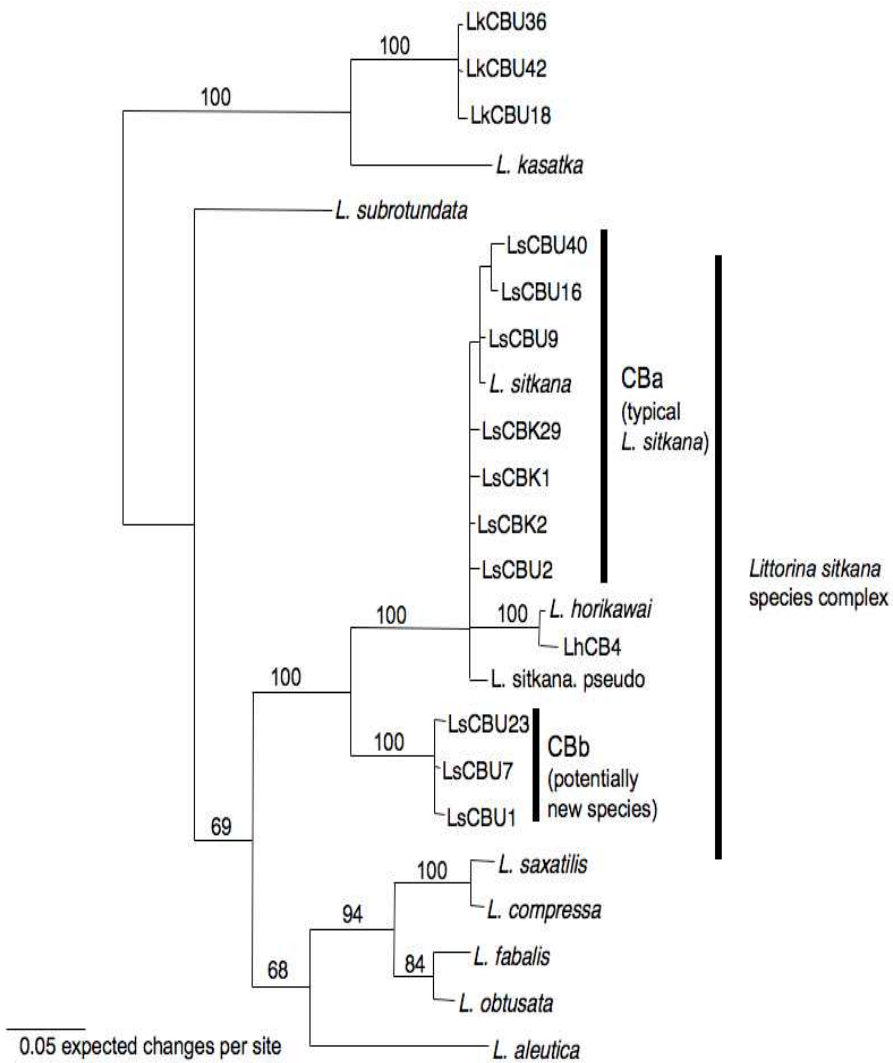
Fig. 2



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Fig. 3



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Fig. 4

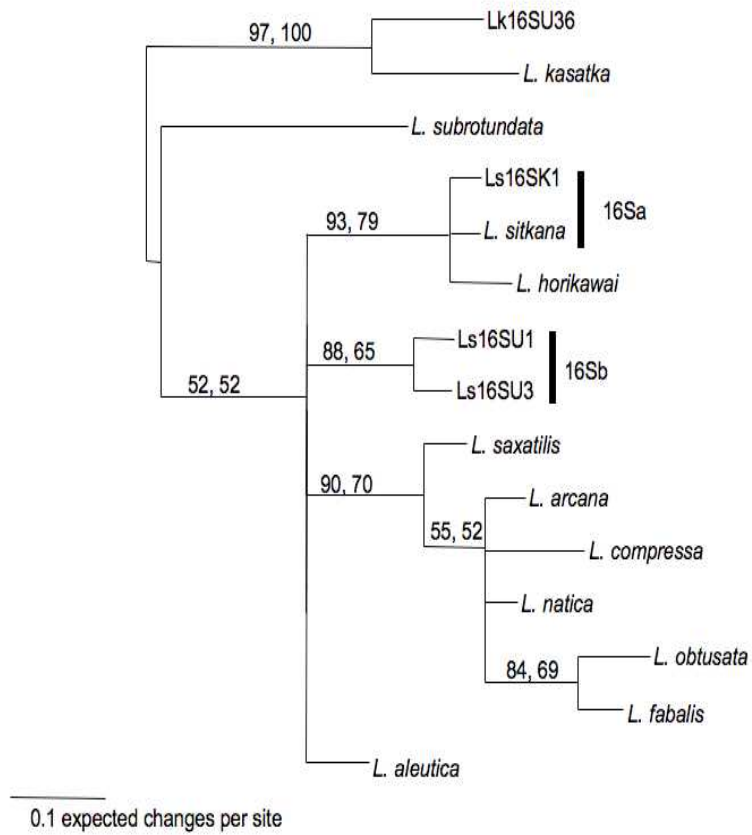
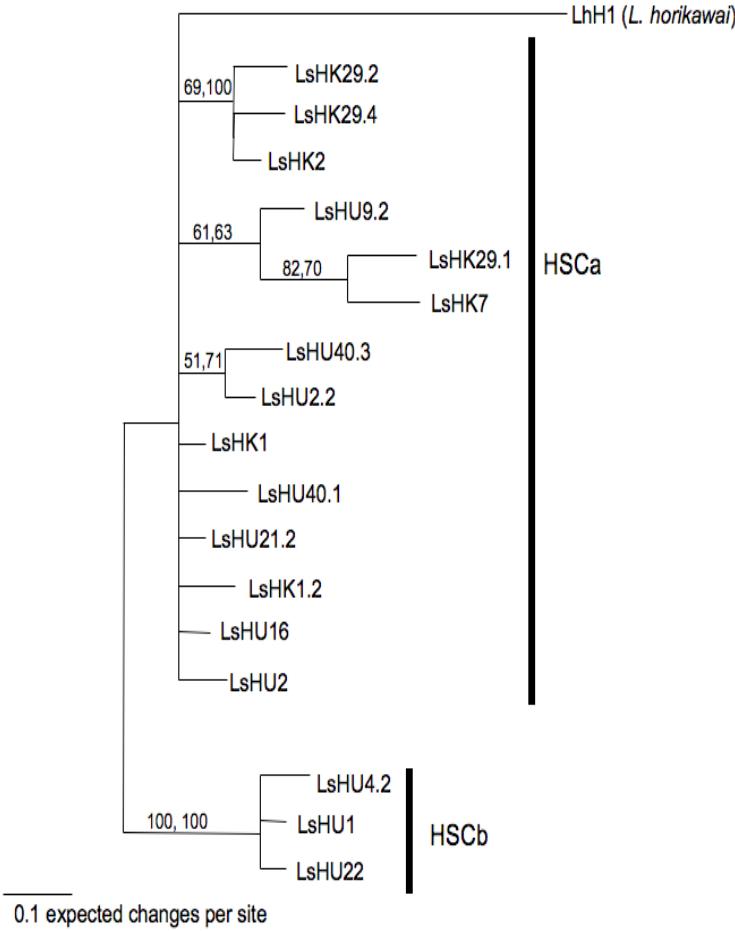


Fig. 5



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Table 1. Collection locality, date, species, and sample size (no. of individuals) of the *Littorina* periwinkles examined in the present study. *Includes cryptic species revealed in the present study.

Locality	(lat., long.)	Date (year, month)	Species	Sample size
Hokkaido	Utoro (44°04'48"N, 145°00'25"E)	2010, Oct.	<i>L. sitkana</i> *	39
		2010, Oct.	<i>L. kasatka</i>	3
	Kushiro (42°96'80"N, 144°22'46"E)	2010, Oct.	<i>L. sitkana</i>	42
Kyushu	Hirado (33°18'36"N, 129°27'28"E)	2011, Oct.	<i>L. horikawai</i>	8

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Table 2. Variation in observed partial HSC70 intron sequences in nominal *Littorina sitkana*. Haplotypes were separated into 2 of the diverged clade, HSCa (Clade A, typical *L. sitkana*) and HSCb (Clade B, *L. sp.*). N indicates a deletion and dots indicate similar nucleotides/deletions to the first listed haplotype (LsHU2). Nucleotides at sites 42, 57, 93, 107, 108, 183, and 290 (in bold letters) are different between the 2 clades, and were used as discriminative keys for the 2 clades.

Clade	Haplotype	Site No. 17	42	57	82	93	107	108	102	110	141	142	146	176	181	183	227	228	233	255	257	262	279	290	316
HSCa	LsHU2		T	G	A	A	G	N	N	A	G	A	A	G	A	A	C	T	C	T	T	T	A	T	T
	LsHU9-2		•	•	G	•	•	•	•	A	•	•	•	A	•	•	N	N	•	•	•	•	G	•	•
	LsHU16		•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	LsHU17		•	•	•	•	•	•	•	A	•	•	G	C	C	•	•	•	•	•	•	•	•	•	•
	LsHU21.2		•	•	•	•	•	•	•	N	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•
	LsHU40		•	•	•	•	•	•	•	A	•	•	G	•	•	•	•	•	•	•	•	•	•	•	•
	LsHU40-1		•	•	•	•	•	•	•	A	•	•	G	•	•	•	•	•	•	•	•	•	G	•	•
	LsHU40-3		•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	C	•	•	•	•	•
	LsHU40-4		•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•
	LsHK1		•	•	•	•	•	•	•	A	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•
	LsHK1.2		•	•	•	•	•	•	•	A	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•
	LsHK2		C	•	•	•	•	•	•	A	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•
	LsHK7		•	•	G	G	•	•	•	A	•	•	•	A	•	•	•	G	A	•	•	•	G	•	•
	LsHK29-1		C	•	•	•	•	•	•	A	•	•	•	A	•	•	•	G	A	•	•	•	G	•	•
	LsHK29-2		C	•	•	•	•	•	•	A	•	•	•	C	•	•	•	•	•	•	•	C	•	•	•
	LsHK29-4		C	•	•	•	•	•	G	A	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•
HSCb	LsHU1		•	C	C	A	C	G	A	•	A	•	•	A	•	G	•	•	•	•	•	•	•	G	•
	LsHU4.2		•	C	C	G	C	G	A	•	A	•	•	A	•	G	•	•	•	•	•	•	•	G	•
	LsHU10.2		•	C	C	•	C	G	A	•	A	•	•	A	•	G	•	•	•	•	C	•	•	G	•
	LsHU11.2		•	C	C	•	C	G	A	•	A	•	•	A	•	G	•	•	•	•	•	•	•	G	A
	LsHU14		•	C	C	•	C	G	A	•	A	•	•	A	•	G	•	•	•	•	•	•	•	G	•
	LsHU22		•	C	C	•	C	G	A	•	A	N	N	•	A	•	G	•	•	•	•	•	•	G	•

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Table 3. Numbers of individuals (N) for each combination of DNA types of mtDNA Cytb (CBa or CBb), 16SrRNA (16Sa or 16Sb), and nuclear DNA HSC70 (HSCa or HSCb).

	N	DNA type
Utoro	11	CBa + 16Sa + HSCa
	3	CBb + 16Sb + HSCa
	25	CBb + 16Sb + HSCb
Kushiro	6	CBa + 16Sa + HSCa