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

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15 Abstract

16

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

35

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

37

INTRODUCTION

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

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

62 In Sitka Periwinkles, *Littrina sitkana* Philippi, the main focus of the present study,
63 extreme variability in shell form has been observed (reviewed by Reid 1996). The
64 phenotypic characters of the shell surface, such as smooth or riblet surface phenotypes,
65 are thought to be plastic as Boulding et al. (1993) observed and related to environment
66 gradients, especially wave action (Yamazaki and Goshima 2012). *L. sitkana* develops
67 without a pelagic larval stage, and is common in intertidal zones on rocky and boulder
68 shores throughout the northern Pacific, from Oregon to the Bering Sea, the Okhotsk Sea,
69 and the northern Sea of Japan (Reid 1996). The species was first described by Philippi
70 (1846) based on a ribbed shell from Sitka, Alaska (lectotype figured by Reid, 1996: fig.
71 56A).
72 The smooth-shelled type, which is abundant on the eastern Hokkaido coast, has
73 been considered as one of the intraspecific variants (e.g., Reid 1996; Zaslavskaya 2006).
74 Revealing the taxonomy of *L. sitkana* has been complicated due to the variability in
75 shell characters and the wide distribution. Many phenotypes have been reported, but
76 these have been classified as intraspecific variants as reviewed by Reid (1996). Some
77 authors have considered smooth-shelled forms (named as *L. kurila*, *L. subtenebrosa*, *L.*
78 *atkana* and others) to represent distinct species, and their synonymy with L. sitkana (see
79 Reid 1996) needs to be tested with molecular data.
80 Genetic variation within *L. sitkana* has been investigated for Russian (Zaslavskaya
81 1995 and 2006; Zaslavskaya and Pudovkin 2005), North American (Boulding et al.
82 1993; Kyle and Boulding 2000, Lee and Boulding 2009, Marko et al. 2010), and
83 Japanese (Nohara 1999) populations. Zaslavskaya (1995 and 2006) reported 3 unknown
84 species, including *L.* sp1, sp2, and sp3, based on allozyme studies. The collected
85 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 cytchrome *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, Bervery, 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.

156 A phylogenetic tree was reconstructed using Bayesian methods in MrBayes 3.12
157 (Ronquist and Huelsenbeck 2003). We applied the substitution model GTR + G + I,
158 which was recommended as the best fitting substitution model for our data set by
159 jModelTest 0.1.1 (Posada 2008; Guindon and Gascuel 2003). In the Bayesian analysis,
160 the posterior probability distribution of trees was approximated by drawing a sample
161 every 100 steps over 1,000,000 MCMC cycles, after discarding a burn-in of 250,000
162 cycles.
163 We used an additional mtDNA marker, 16S, and a nuclear DNA marker, HSC70,
164 to resolve the following issues. Usage of multiple markers was expected to confirm and
165 compliment the results of the Cytb analysis.
166 1. In the Cytb analysis, we could not compare our samples to *Littrina natica*, which
167 is distributed in the neighboring area, the Kurile Islands, and sometimes has a
168 similar shell shape to *L. sitkana*, because no Cytb sequence for this species was
169 available in the database. Therefore, the possibility that samples of the newly
170 found lineage were members of *L. natica* could not be rejected.
171 2. The observed haplotypes might have included pseudogene sequences. As our data
172 did not include the entire full-length Cytb gene, the possibility that our
173 sequences represented a pseudogene remained, even after we found no stop
174 codon in the observed sequences.
175 3. The observed genetic divergence should be regarded as intraspecific variation if
176 there was no reproductive barrier between the 2 lineages.
177 *L. natica* 16S sequences were available from the database, and examining linkage
178 between 16S and Cytb types could be used as an approach to distinguish pseudogene
179 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.

204 **Nuclear DNA analysis of HSC70 intron variation**

205 The most important aim of nuclear DNA analysis was to test for reproductive isolation
206 between 2 clades that were identified in the mtDNA analysis. Because samples from
207 Kushiro included only 1 clade of Cytb, CBa (see Results), we used 6 representative
208 individuals with different Cytb haplotypes. However, as samples from Utoro included
209 members of both clades, CBa and CBb (see Results), all samples from this area were
210 examined, with the exception of 3 individuals that were assumed to be *L. kasatka*
211 following the mtDNA analysis. A partial fragment (*ca*. 650 bp) of HSC70 was amplified
212 by PCR using the primers F: GGCACCTTTGACGTGTCAGTCC and R:
213 TCCACAGGCTCCAGTGTGCCAC (Lee and Boulding 2009). Amplification by PCR,
214 purification of the products, and direct sequencing were carried out in the same manner
215 as for Cytb. Some samples showed heterozygosity; therefore, the corresponding PCR
216 products were cloned with a TOPO TA Cloning® Kit (invitrogen) and were sequenced
217 using universal M13 primers. Sequence variation was observed only in the intron region,
218 so we trimmed both ends of each sequence to remove the exons, and edited the
219 sequences to 287 bp. Unique sequences were deposited in DDBJ/GenBank with
220 accession nos. AB665223–665239. Expected and observed heterozygosities for 32
221 individuals from Utoro, for which haplotypes were successfully identified, were
222 calculated using Genepop (Raymond and Rouset 1995; Rouset 2008). Hardy-Weinberg
223 equilibrium (HWE) was also tested using Genpop. Phylogenetic trees for the haplotypes
224 were reconstructed in 2 ways, using both Bayesian and MP methods, because, similar to
225 the 16S region, indels were observed within the HSC70 introns. For the Bayesian
226 method, we selected the GTR + G + I model by using jModelTest 0.1.1. Indels were
227 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

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

261 In the analysis of partial 16S sequences, 4 haplotypes were found among the
262 Hokkaido samples (Fig.4). Haplotype Lk16SU36 occurred in individuals that were
263 regarded as *L. kasatka* following the Cytb analysis, and again formed a monophyletic
264 cluster with a previously reported *L. kasatka* sequence. The 8 *L. horikawai*
265 individuals had a single haplotype that was similar to the reported sequence for this
266 species (GenBank accession no. U46796). Both the Bayesian and MP trees for the
267 16S region indicated an apparent separation of the 16Sa and 16Sb clades, and these
268 corresponded to CBa and CBb, respectively (Fig. 4). The posterior probability and
269 bootstrap support for clade 16Sb in the tree was in fact below significance level;
270 however, non-monophyly of *L. sitkana* was confirmed, because *L. horikawai* was
271 inlcuded. At the individual level, all of the individuals with CBa haplotypes showed
272 16Sa haplotypes, and all of the CBb individuals had 16Sb haplotypes. The 16Sb clade
273 appeared to be independent from *L. natica*. These results indicated that members of
274 the CBb clade were not *L. natica*, and that the Cytb region that we used in this study
275 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.

300 **Linkage of mtDNA and HSC70 haplotypes**

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

308

309 **DISCUSSION**

310 Molecular evidence revealed separation of 2 clades of nominal *Littorina sitkana* in the
311 east coast of Hokkaido. The sequence from GenBank with the accession number
312 GQ902751 was reported in Marko et al. (2010) as the most abundant haplotype along
313 the northeastern Pacific coast, including Sitka in Alaska, the type locality from whre
314 Philippi's specimens were collected. The sequence appeared in the CBa group in the
315 phylogenetic tree, as shown in Fig. 3, thus we believe that CladeA with the CBa and
316 HSCa haplotypes contains typical *L. sitkana* specimens. The other, CladeB with the
317 CBb and HSCb haplotype, may be a sister taxon of *L. sitkana*. We are aware that the
318 phylogenetic trees based on 2 mtDNA markers, Cytb and 16S, were inconsistent with
319 each other in some points, and that they failed to reconstruct precise phylogenies among
320 the species. Other markers, such as mtDNA 12S rRNA or nuclear DNA 18S or
321 28SrRNA, may supply more accurate results. However, for the present study, we
322 required molecular markers that exhibit variations within the species of interest. We
323 detected high levels of genetic differentiation and reproductive isolation between *L.*

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

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

Among 3 of the new species suggested by Zaslavskaya (2006), *Littorina* sp1 is most likely identical to CladeB. *Littorina* sp1 was genetically close to *L. sitkana*, but was independent from it, like as CladeB. *Littorina* sp1 was collected from Kasatka Bay, 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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497 **Figure legends**

498

499 Fig. 1

500 Map of Japan displaying the 2 sampling sites in Hokkaido (inset A, *Littorina sitkana*)

501 and 1 in Kyushu (inset B, *L. horikawai*).

502

503 Fig. 2

504 Shells of the examined *Littorina* samples. A: *L. sitkana* from Utoro, Hokkaido. B: *L.* sp.

505 from Utoro, Hokkaido. C: *L. sitkana* from Kushiro, Hokkaido. D: *L. horikawai* from

506 Hirado. Kyushu. Sample B was pottentially new species, CladeB, having CBb + 16Sb +

507 HSCb genotype.

508 Fig.3

509 Fifty-percent majority-rule Bayesian tree inferred from partial mitochondrial DNA Cytb

510 sequences (428bp) using the GTR + G + I model. The tree was rooted using *L. kasatka*

511 as the outgroup. Numbers above the branches represent Bayesian posterior probability

512 values (%). Species names in italics correspond to sequences of related *Littorina* species

513 previously reported in GenBank, and other names indicate haplotypes that were

514 identified in the present study.

515

516 Fig. 4

517 Fifty-percent majority-rule Bayesian tree inferred from partial mitochondrial DNA 16S

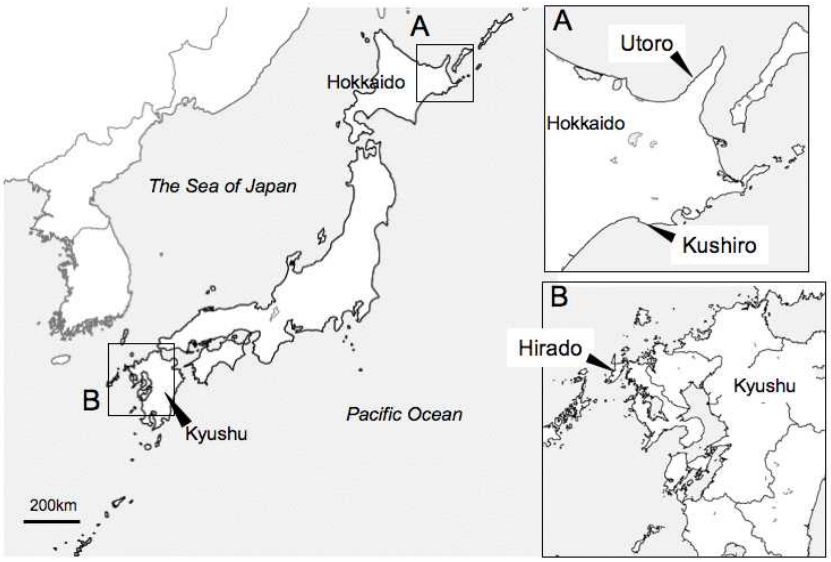
518 sequences (434 bp) using the GTR + G + I model. The tree was rooted using *L. kasatka*

519 as the outgroup. Numbers above the branches represent Bayesian posterior probability

520 values (%) and bootstrap values (%) from 1000 replications. The latter were calculated

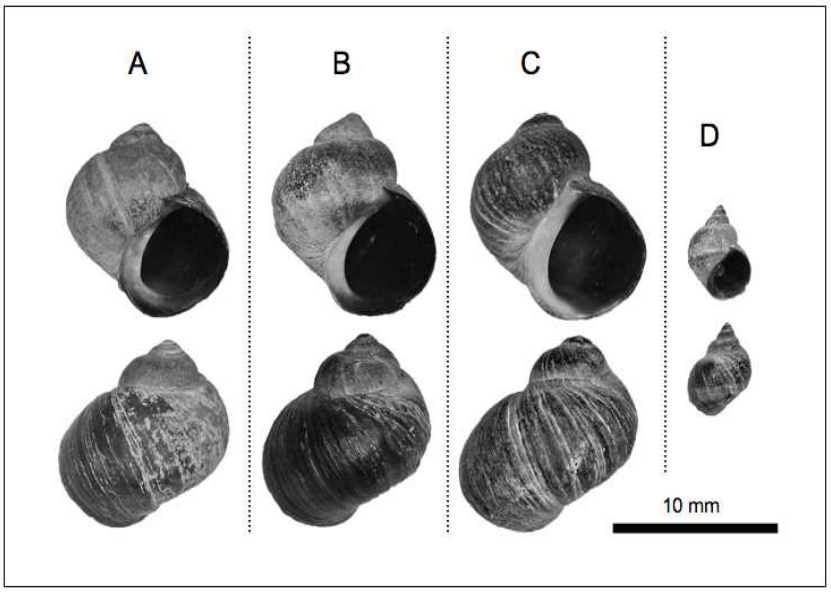
521 after reconstruction of a maximum parsimony consensus tree, treating gaps as a fifth
522 element. Species names in italics correspond to sequences of related *Littorina* species
523 previously reported in GenBank, and other names indicate haplotypes that were
524 identified in the present study.
525
526 Fig. 5
527 Fifty-percent majority-rule Bayesian tree (unrooted) inferred from partial nuclear DNA
528 HSC70 intron sequences (287bp) using the GTR + G + I model. Numbers above the
529 branches represent Bayesian posterior probability values (%) and bootstrap values (%)
530 from 1000 replications, which were calculated after reconstruction of a maximum
531 parsimony consensus tree treating gaps as a fifth element. Haplotypes were separated
532 into 2 of the diverged clade, HSCa (CladeA, typical *L. sitkana*) and HSCb (CladeB, *L.*
533 *sp.*).

Fig. 1


A
Hokkaido
The Sea of Japan
B
Kyushu
Pacific Ocean
200km
A
Utoro
Hokkaido
Kushiro
B
Hirado
Kyushu

534

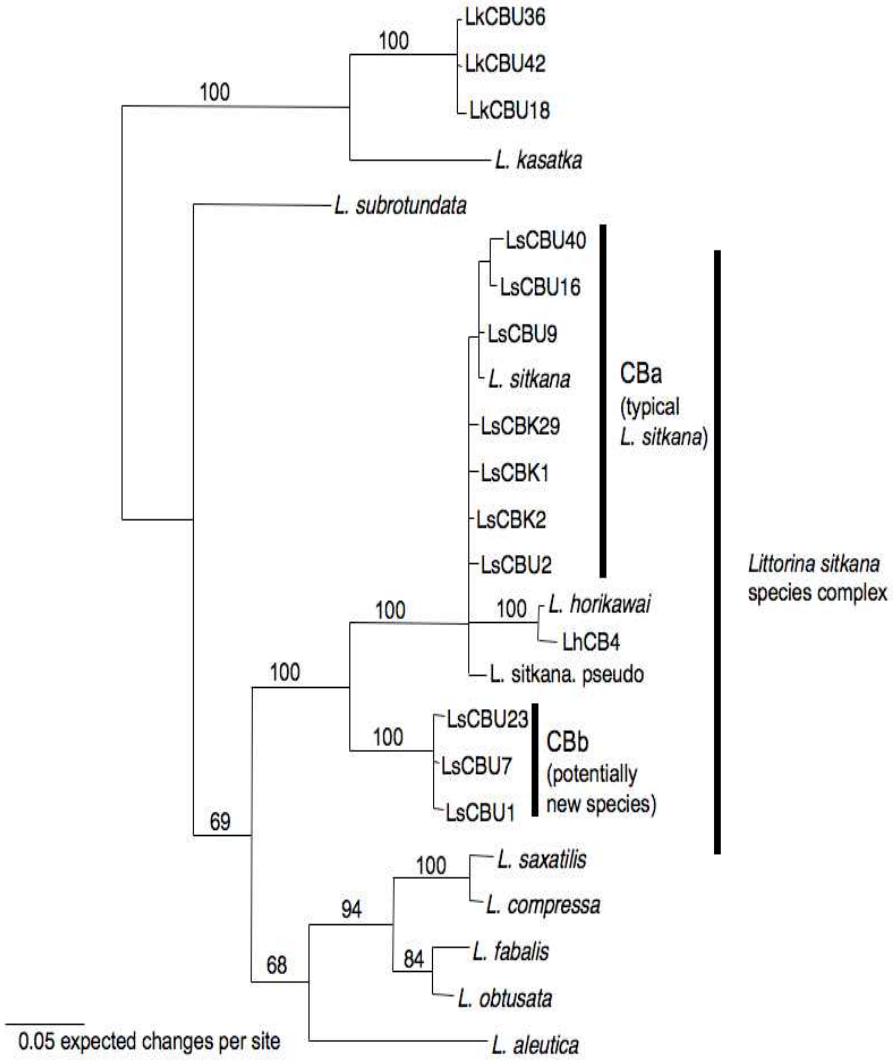
Fig. 2



535

536

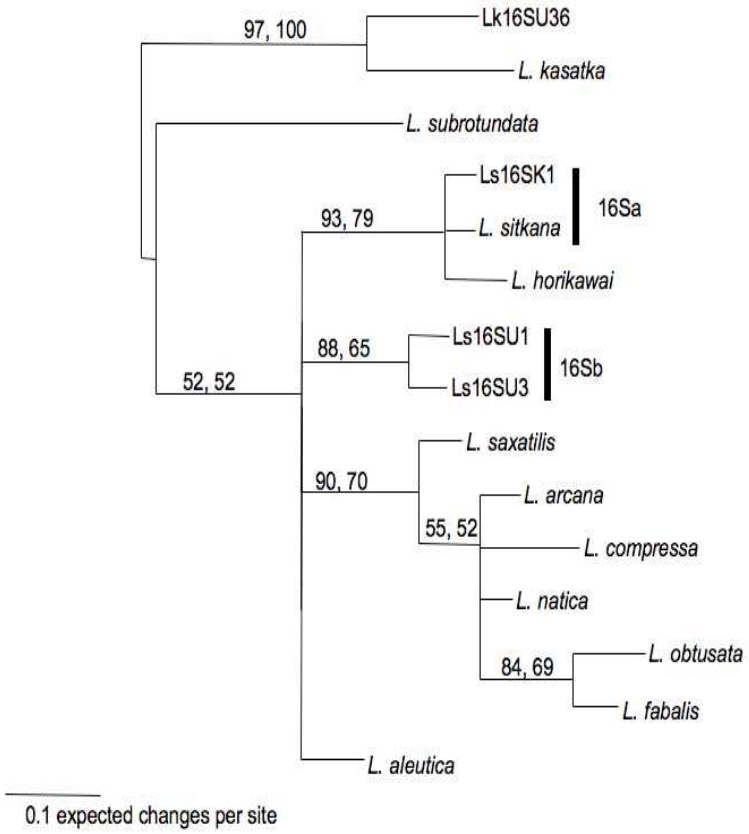
Fig. 3


LkCBU36
LkCBU42
LkCBU18
L. kasatka
L. subrotundata
LsCBU40
LsCBU16
LsCBU9
L. sitkana
LsCBK29
LsCBK1
LsCBK2
LsCBU2
CBa
(typical
L. sitkana)
Littorina sitkana
species complex
L. horikawai
LhCB4
L. sitkana. pseudo
LsCBU23
LsCBU7
LsCBU1
CBb
(potentially
new species)
L. saxatilis
L. compressa
L. fabalis
L. obtusata
L. aleutica
100
100
100
100
100
100
94
84
69
68
0.05 expected changes per site

537

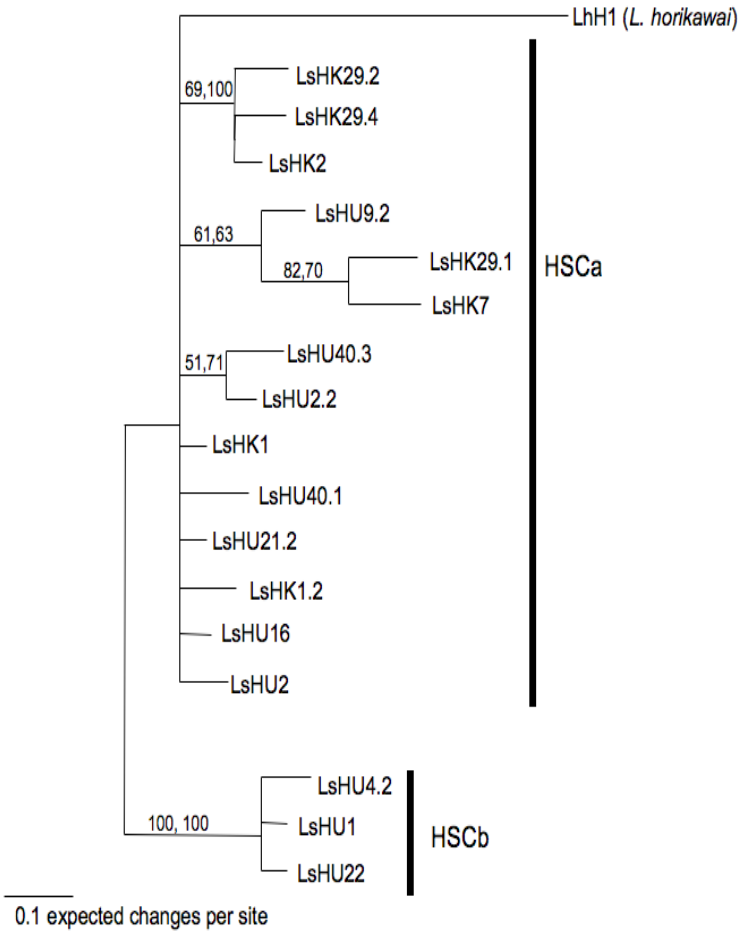
538

Fig. 4


97, 100
Lk16SU36
L. kasatka
L. subrotundata
Ls16SK1
L. sitkana
16Sa
93, 79
L. horikawai
88, 65
Ls16SU1
Ls16SU3
16Sb
52, 52
L. saxatilis
90, 70
L. arcana
55, 52
L. compressa
L. natica
84, 69
L. obtusata
L. fabalis
L. aleutica
0.1 expected changes per site

539

Fig. 5



540

541

542

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.	*L. sitkana**	39
		2010, Oct.	*L. kasatka*	3
	Kushiro (42°96′80″N, 144°22′46″E)	2010, Oct.	*L. sitkana*	42
Kyushu	Hirado (33°18′36″N, 129°27′28″E)	2011, Oct.	*L. horikawai*	8

543

Table 2. Variation in observed partial HSC70 intron sequences in nominal*Littrina 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	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**	·

544

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

545