Three predominant species groups of deep-sea whelks (Gastropoda: Buccinidae) in the Sea of Japan: their molecular taxonomy and geographic distribution

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Abstract: Based on 3,484 specimens collected from a series of surveys, we revealed that three species groups of deep-sea whelks (Buccinidae) are distributed predominantly in the Sea of Japan (off Honshu Island, 200–1,900 m in depth). The first species group is composed of species of the genus Neptunea. Although N. intersculpta and N. constricta have been considered as representatives of the Sea of Japan, our mitochondrial (mt) 16SrDNA data showed no clear reason to recognize more than one taxonomic unit for them. The second is composed of Buccinum tsubai; three haplogroups off Honshu Island were reconfirmed using 16SrDNA sequences. The present study demonstrated two of these haplogroups distributed in the same region near the Noto Peninsula. The last group, made up of B. striatissimum and related taxa, is still problematic despite our present morphological and molecular analyses. In our tentative conclusion, two different species are widely distributed off Honshu Island, one is B. striatissimum inhabiting in shallower regions (< ca. 500 m in deep) and another is B. tenuissimum in deeper regions. To overcome the remaining uncertainty related to the genetic differentiation of these two species, further clarification of their taxonomic relationship is needed. Geographical distributions of these three species groups are detailed based on the present study.

Key words: buccinid whelks, geographic distribution, molecular taxonomy, predominant species, Sea of Japan

Introduction

An abundant fauna of the buccinid whelks is known around the Japanese Archipelago and adjacent seas (Okutani et al. 1988, Higo et al. 1999, Okutani 2000, Kantor 2006). In the deep-sea area of the Sea of Japan, the water mass named “Japan Sea Proper Water” (below 200–300 m in depth) characterized by low temperature and high dissolved oxygen, several species of the genera Buccinum and Neptunea are broadly distributed (e.g., Kato 1979, Doi 1997a, b, c, Michine et al. 2002, Maeda & Doi 2006). As major species in the genus Buccinum, B. striatissimum Sowerby (locally named “Ecchu-bai”), B. tenuissimum Kuroda in Teramachi (“Oo-ecchu-bai”), and B. tsubai Kuroda in Teramachi (“Tsubai”) are well-known, and all of their original localities are off Honshu Island, the Sea of Japan. Buccinum bayani Jousseaume (“Kaga-bai”), based on specimens from eastern Korea (Habe 1978, Kantor 2006), is another species name for organisms in the Sea of Japan. In the genus Neptunea, two species, N. intersculpta (Sowerby) (“Ezobora-modoki”) and N. constricta (Dall) (“Chijimi-ezobora”), are known off Honshu Island in the Sea of Japan. These species have been taxonomically confused, as already commented on by Kato (1979) and Tsuchida & Hayashi (1994). In both genera of Buccinum and Neptunea, previous workers described several more species and subspecies, but these taxa are mostly ambiguous due to the large degree of phenotypic variability in these genera. This lack of taxonomic clarity of the deep-sea whelks in the Sea of Japan limits advances in studies on their ecology as well as on the fishery for them.

Such difficulty in species identification may be due to various factors for each taxa. However, in molluscs with shells, it appears to be mainly due to the fact that they have been described primarily on the basis of the shell morphology (Schander & Sundberg 2001, Wagner 2001). Most features of shell morphologies are simple but show considerable variation, and are also characterized by morphological
plasticity. If only a few characteristics of shells are used for identification, taxonomic and other biological analyses may be extraordinarily difficult. For example, in the genera of *Buccinum* and *Neptunea*, thickness of shells, form of columella, and development of ribs, growth lines and/or sutures are frequently used, but these features are not necessarily useful for their identification. Furthermore, morphological changes with growth and other reasons (e.g., individuality and environmental conditions) have hardly been examined, and rather minor variations seem to have been used for establishing new taxa (species, subspecies, variations, etc.). Thus, this situation often results in difficulty in species identification, lack of confidence in systematic conclusions, and/or poorly resolved phylogeny.

Recently, it has become easier to acquire DNA data as characteristics of living organisms, and this data can be used to resolve taxonomic, phylogenetic and/or ecological uncertainties (e.g., Oliverio & Mariottini 2001, Collin 2003a, b, Kameda et al. 2007). In the Sea of Japan, Iguchi et al. (2004, 2005, 2007a, b) have examined the systematics, population biology, and biogeography of buccinid molluscs. In these studies, for example, *B. tsubai* in the Sea of Japan including the area of western Hokkaido, has been found to be composed of four genetically separate populations based on mitochondrial DNA (mtDNA) polymorphisms, and each population has been found to have unique morphological features (Iguchi et al. 2005).

In this paper, we analyze the species composition of deep-sea whelks in the Sea of Japan off Honshu Island mainly using DNA data, and discuss some problems associated with their taxonomy and the geographic distribution of each taxa.

**Materials and Methods**

**Research and samples**

In the following sections, “the Sea of Japan” refers to only a district off Honshu Island unless otherwise noted. For explanation, the Sea of Japan was divided into 11 sea areas as shown in Fig. 1. These sea areas, used to make statistical data for the bottom trawl fishery in the Sea of Japan, are called here, for example, *Area 2* and *Areas 6–8* with italicized characters. Most of the whelk samples (2,577 individuals) were collected through the following two primary surveys (Table 1, Fig. 1): (a) a bottom trawl survey of resources of the snow crab [*Chionoecetes opilio* (Fabricius)] in 2005 (105 sites performed at *Areas 4–10* at depths of 200–455 m), and (b) a series of the deep-sea dredge survey for biological studies of the beni-zuwai crab (*C. japonicus* Rathbun) in 2005–2007 (six localities named *b1–b6*, 115 sites at *Areas 1, 2*, and *9–11*: depths of 200–2,289 m). All of these surveys were performed by R/V *Tanshu-Maru*, 499 tons. The bottom trawl with otter boards (type “NT-4”; mesh size of cod end=15 mm; average mouth width=17.4 m throughout the survey) was operated for 30 minutes at a towing speed of 3.0 knots; accordingly,
the average dragged area was about 46,000 m². The deep-
sea dredge (type “BZ-1”: mesh size of cod end/H11005
20 mm; mouth width/H11005 6.8 m) was under operation for 30 minutes
and 2.0-knot speed (average dragged area: 6,060 m²). See
Hirose et al. (2006) for details of both fishing gears. An ad-
ditional 907 samples were collected during five other sur-
veys (c–g) to complement the two primary ones detailed
above.

All the samples (3,484 individuals) were brought back to
the laboratory, and then primary (morphological) identifica-
tions and measurements [shell length (SL), and wet weight
of whole body and of soft parts; data not shown here] were
made. Of these, 571 individuals were used for the molecu-
lar assay: a small volume of foot muscle was dissected and
stored individually at /H11002 20°C in 2 ml tubes containing
99.5% ethanol.

### Table 1. Collection data and number of sampling sites.

Survey a) Trawl for *Chionoecetes opilio* (2005).

| Area* | Date                | Number of sampling sites (depth range: m) |
|-------|---------------------|-------------------------------------------|
|       |                     | 200–250| 250–300| 300–350| 350–400| >400  | Total |
| 4     | Jun. 13 to 15, 2005 | 4      | 1      | 1      | 2      | 8     |
| 5     | Jun. 9 to 13, 2005  | 3      | 2      | 1      | 1      | 7     |
| 6     | Jun. 4 to 10, 2005  | 4      | 7      | 3      | 3      | 3     | 20    |
| 7     | May 31 to Jun. 7, 2005 | 2  | 3      | 3      | 2      | 10    |
| 8     | May 17 to Jun. 2, 2005 | 14 | 2      | 2      | 2      | 20    |
| 9     | May 23 to 28, 2005  | 5      | 4      | 9      | 2      | 2     | 22    |
| 10    | May 10 to 21, 2005  | 10     | 3      | 3      | 1      | 1     | 18    |
| Total |                     | 42     | 22     | 22     | 8      | 11    | 105   |

Survey b) Deep-sea dredge for *Chionoecetes japonicus* (2005–2007).

| Survey* | Area* | Date                | Number of sampling sites (depth range: m) | Remarks             |
|----------|-------|---------------------|-------------------------------------------|---------------------|
| b1       | 1     | Aug. 30 to Sep. 10, 2007 | 1 | 1 | 1 | 1 | 1 | 16 | Nishi-tsugaru Trough |
| b2       | 2     | Aug. 27 to 31, 2005   | 2 | 5 | 2 | 3 | 2 | 2 | 3 | 1 | 1 | 24 | Mogami Bank |
| b3       | 9     | Aug. 23, 2006         | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 1 | 18 | Shin'oki Bank |
| b4       | 9     | Aug. 21 to 23, 2005   | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 2 | 2 | 1 | 18 | |
| b5       | 10    | Jul. 5 to 8, 2005     | 1 | 2 | 2 | 2 | 3 | 1 | 2 | 1 | 3 | 17 | |
| b6       | 11    | Aug. 24 to 31, 2006   | 8 | 4 | 4 | 1 | 3 | 1 | 4 | 2 | 3 | 5 | 35 | Yamato Bank |
| Total    |       |                     | 13 | 17 | 12 | 14 | 12 | 10 | 11 | 9 | 10 | 7 | 115 | |

Other surveys for complemental DNA analysis

| Survey* | Area* | Date                | Number of sampling sites | Depth range (m) | Remarks |
|----------|-------|---------------------|--------------------------|-----------------|---------|
| c        | 2     | Jul. 6 to 8, 2006   | 3                        | 256–351         | Trap for *Chionoecetes opilio* |
| d        | 3     | Jul. 4 to 12, 2006  | 7                        | 246–527         | Trap for *C. opilio* |
| e        | 3     | Sep. 22 to 23, 2006 | 3                        | 266–321         | Bottom trawl for *Hippoglossoides dubius* |
| f        | 4     | Oct. 31, Dec. 6, 2006, Mar. 16, 2007 | 3 | 840–1100 | Trap for *C. japonicus* |
| g        | 4     | Jun. 6, 2007        | 1                        | ca. 400–500     | Bottom trawl (Toyama Bay: fish market) |

*See Fig. 1 for area codes (1–11) and surveys (a–g).
DNA extractions, PCR and sequencing

All DNA was extracted using DNeasy Tissue Kit (Qia-gen) following the manufacturer's instructions. Partial 16SrDNA of mtDNA was analyzed for these extracted DNA. Polymerase chain reaction (PCR) was performed with Z-taq (TaKaRa) on an ABI 9700 Cycler under the following conditions: 30–32 cycles with denaturation at 98°C for 5s, annealing at 50°C for 8s, and extension at 72°C for 20s (total reaction volume is 12 μL). The 16SrDNA was amplified using 16S universal primers (forward: 5'-CGG CCT GTT TAT CAA AAA CAT-3', reverse: 5'-GGA GCT CCG GTT TGA ACT CAG ATC-3'; Pulumbi et al. 1991); this region partially overlaps with the sequence data of Iguchi et al. (2004, 2005, 2007a, b, 2008). PCR products were purified using ExoSAP-IT (Amersham Biosciences), directly sequenced in both directions with the BigDye termination reaction kit (ver. 1.1) and the same primers noted above, and analyzed with an ABI 310 sequencer.

Sequence data were aligned by Clustal X (Thompson et al. 1997; parameters of “Gap Opening” and “Gap Extension” were 10.0 each) and eye, referring to the universal numbering system of the RNA secondary structure (De Rijk et al. 1999). Aligned data were imported into MacClade 3.07 (Sinauer Associates) to determine the number of unique haplotypes. For construction of the network, we used the computer program TCS 1.21 (Clement et al. 2000), which implements the statistical parsimony algorithm described by Templeton et al. (1992). Levels of genetic diversity within and among populations were tested (if possible) by a hierarchical analysis of molecular variance (AMOVA: Excoffier et al. 1992) using the phi statistics, which include information on mitochondrial haplotype frequency (Weir & Cockerham 1984) and genetic distance (pairwise difference). The name of a population is assembled from its haplogroup and locality (code of Area or survey). An exact test of population differentiation (Raymond & Rousset 1995, Goudet et al. 1996) was performed by multiple permutation (number of steps in Markov Chain 10000; number of dememorisation steps 20,000) of the original data set; p-values were adjusted with sequential Bonferroni correction (Rice 1989). All AMOVA tests were implemented in Arlequin 2.0 (Schneider et al. 2000).

Results

A total of 2,577 individuals of deep-sea whelks, caught during the two primary surveys, were identified morphologically and the weight and size of each individual measured in the laboratory. We found three morphological groups, each of which is composed of two or more related species or populations as shown in the following subsections. One of these came under the genus Neptunea characterized by a rotund and fusiform shell, an elongated anterior canal and an oval operculum with an inferior nucleus. Here it is called the “intersculpta species group.” The second was corresponding to Buccinum tsubai in having an ovoid and medium-sized shell with a narrow flat shelf below the strongly constricted suture. Here we treat it as the “tsubai species group.” The last group was the “striatissimum species group,” characterized by a conico-ovoid and large-sized shell with a tall spire and a more or less constricted suture.

These three species groups accounted for the overwhelming majority of the whole catches of molluscs excluding cephalopods. The averages of their densities (kg/km²) by depth range are shown for surveys a (bottom trawl: Fig. 2) and b (deep-sea dredge: Fig. 3). Gastropod molluscs were caught at depths of 200–1,902 m, and no catch was recorded at the sites to 2,289 m. These three groups accounted for over 90% of catches at all depth ranges in each area (trawl) and locality (dredge) except for the following: most catches were Fusitriton oregonensis (Redfield) in Ranellidae in the range of 200–250 m of Area 4 (Fig. 2A), and were Lussilulotusius furukawai (Oyama) in Buc- cinidae in the 300–350 m of Area 5 (Fig. 2B). Other gastropods less frequently sampled are: L. emphaticus (Dall) and Plicifusus aurantius (Dall) in Buccinidae; Cryptonica spp. in Naticidae; and Affria diomedea Bartsch in Turridae.

Each species group was examined based on molecular analysis below. Some notes on its morphology were also added.

Intersculpta species group

Our present specimens showed considerable variations of shell morphology including the general contour of shells, condition of the spiral ribs, and form of the aperture as shown in Fig. 4A–F. Of these, the specimen in Fig. 4A, characterized by strong spiral ribs with an indistinct secondary rib between them, is comparable to the original description of Neptunea intersculpta (Sowerby 1899); such a condition was observed in about a half of specimens in Areas 6–10, but not found in other areas. In the other specimens, the spiral ribs were rather weak, or almost absent as in Fig. 4D.

Conversely, the present mtDNA analysis showed very close genetic relations among specimens of this species group. Muscle tissues of 91 individuals having various conditions of the shell sculpture were obtained for this study. The aligned data of partial 16SrDNA sequences were 533bp in size including an insertion or deletion (indel) in some specimens (actual size: 532–533bp). Fourteen unique haplotypes were detected by 13 polymorphic sites including 12 substitutions (all were transitions); these are deposited in DDBJ/EMBL/GenBank (accession numbers AB439733–AB439746). These haplotypes differ from each other by only 1–5 mutations, and their network was drawn as shown in Fig. 4G. The numbers of specimens from each Area are too few to perform a comparison among Areas (AMOVA analysis) (Table 2). As shown in this table, two geographic groups of haplotypes are suggested, i.e., Areas 1–6 and 6–11 (Area 6 seems to be characterized by both groups of haplotypes).
Fig. 2. Average of density (kg km$^{-2}$) by depth range in the bottom trawl survey (a). A, Area 4; B, 5; C, 6; D, 7; E, 8; F, 9; G, 10. “X” shows the absence of sampling site. See Table 1 for number of sampling sites of each depth range.

Fig. 3. Average of density (kg km$^{-2}$) by depth range in the deep-sea dredge survey (b). A, Area I (survey b1); B, 2 (b2); C, 2 (b3); D, 9 (b4); E, 9 (b5); F, II (b6). For details see Fig. 2.
Fig 4. Morphological and molecular variations of the 
intersculpta species group. Samples from the following sea areas: (A) 
Area 8 (102.2 mm SL, 210 m in depth: survey a); (B) 4 (133.2 mm SL, 260 m: a); (C) 5 (146.9 mm SL, 300 m: a); (D) 10
(151.2 mm SL, 340 m: a); (E) 1 (116.2 mm SL, 819 m: b1); (F) 9 (140.7 mm SL, 701 m: b4). Diagram (G) shows the haplotype network of 16SrDNA generated with TCS 1.21 (Clement et al., 2000; 95% connection limit); each circle represents a single haplotype, and its size is proportional to the number of individuals with that haplotype; a small closed circle indicates a missing intermediate.

Fig 5. Morphological and molecular variations of the tsubai species group. Samples from (A) Area 2 (65.1 mm SL, 351 m in depth: c); (B) 1 (45.3 mm SL, 1197 m: b1); (C) 9 (82.6 mm SL, 312 m in depth: a); (D) 10 (65.2 mm SL, 1,196 m: b5); (E) 11 (53.2 mm SL, 706 m: b6); (F) 11 (47.4 mm SL, 1,474 m: b6). Diagram (G) shows the haplotype network of 16SrDNA, and short bars mean the same as closed circles; for details see Fig. 4.
Tsubai species group

We examined 1,572 individuals from all sea areas. As can be seen in several examples (Fig. 5A–F), considerable variations in shell morphology (e.g., general contour of a shell, ratio of shell height and shell width, coloration, and size and form of an aperture) were observed. Some comments on the morphology are given in the Discussion section. Mitochondrial 16SrDNA sequences (532–535 bp in size) were obtained from 152 specimens, which comprised 18 different haplotypes defined by 23 polymorphic sites including 21 substitutions (18 transitions and 3 transversions) and three observed indels. The haplotype network is given in Fig. 5G. Eighteen haplotypes (deposited DDBJ/EMBL/GenBank: accession numbers AB439747–AB439764) were subdivided into three distinct haplogroups, TB-I, TB-II, and TB-III, each of which are composed of haplotypes 01–08, 09–15, and 16–18, geographically distributed as in Fig. 6. Individuals of Area 4 (survey a: Fig. 6) were composed of two different haplogroups, having three haplotypes (01, 03, and 08) from TB-I and two haplotypes (09 and 11) from TB-II; abundant haplotypes 01 and 09 were obtained together at two sampling sites (near the headland of the Noto Peninsula) of 260 m and 420 m in depths.

Striatiissimum species group

Our 1,299 specimens of this species group could be substantially divided into two morphological groups. One is characterized by a somewhat thick and rigid shell ornamented by crowded and slender spiral threads, and also by a thick and reflected outer lip in a typical form (Figs. 7A–D, 8). These conditions are incorporated into the status of Buc- cinnium striatiissimum; this form is called “s-type” below. Another one has an extremely thin and fragile shell ornamented by a lattice-like sculpture made of slightly thicker spiral threads and growth lines (Fig. 7E–F); because of the fragility, the shell morphology could not be seen as a complete form in most cases. This group corresponds to B. tenuissimum, here called the “t-type” form. Considerable variations were found in their overall profile of shell, e.g., conditions of an outer lip and shell surface, size of an aperture, swell of the body whorl, height of the spire, and coloration, in both forms. In small specimens below ca. 50 mm in SL, these two forms are hardly distinguished because of their thin shells and similar pale shell color (Fig. 7G–H).

Table 2. Fourteen haplotypes and number of individuals for each haplotype by sampling areas (intersculpta species group).

| Haplotype | Area | total |
|-----------|------|-------|
| #01       | 3 3 3 | 17 2 28 |
| #02       | 1    | 1     |
| #03       | 3 6 2 | 2 13   |
| #04       | 1    | 1     |
| #05       | 1    | 1     |
| #06       | 1    | 1     |
| #07       | 1    | 1     |
| #08       | 1 3 4 | 1 6   |
| #09       | 1    | 1     |
| #10       | 5 3 8 5 | 9 30   |
| #11       | 1    | 1     |
| #12       | 1    | 1     |
| #13       | 1 3 1 | 1 6   |
| #14       | 2    | 2     |
| total     | 6 9 9 5 1 17 5 10 2 25 2 | 91     |

Fig. 6. Geographic distributions of three haplogroups of Buccinum tsubai within the Sea of Japan. Three kinds of symbols show the haplogroup in each survey (in the survey a, the haplogroup is indicated for each area). Open circle, solid circle, and open square represent the haplogroup TB-I (“Yamagata-Toyama” of Iguchi et al. (2004)), TB-II (“San’in”), and TB-III (“Yamato Bank”), respectively.
Although both forms are widespread in the Sea of Japan, their vertical distribution differs from each other, 200–503 m deep in s-type and 441–1,902 m deep in t-type, respectively.

Three hundred and thirty-four individuals were collected for DNA analysis from the whole of the Sea of Japan, and these were divided into 168 s-type and 166 t-type forms. The partial 16SrDNA sequences were 532–534 bp in size (aligned data was 537 bp). Thirty-one unique haplotypes were detected by 31 polymorphic sites including 26 substitutions (22 transitions and 4 transversions) and five observed indels; these are deposited in DDBJ/EMBL/GenBank (accession numbers AB439765–AB439795). As seen in the haplotype network (Fig. 7I), we found that two distinct haplogroups, named ST-I and ST-II here, were separated from each other by six nucleotides at least. Haplogroup ST-I was confined in Areas 4 and 6–10 (west of the Noto Peninsula), found at depths of 205–503 m, and ST-II was distributed in all 11 sea areas examined, and appeared at depths of 205–1,902 m (Table 3).

An AMOVA test was performed to examine the genetic structure of each haplogroup. Each population for the analysis was composed of 20 specimens or more as a rule, and s-typed specimens from Areas 2 and 3 were treated as different populations (Table 4; populations “II(c)” and “II(d–e),” respectively). Within-population genetic variation was rather low (number of haplotypes 2–8; nucleotide diversity 0.0001–0.0033). As in Table 5, the haplogroup ST-I showed no difference of haplotype composition among three sea areas, Areas 6, 8 and 10. By contrast, our analysis revealed
the haplogroup ST-II has distinct genetic differences. As seen in the exact test (of population differentiation) (Table 6), the population II(b6) (Area 11) was significantly different from the others excluding II(b3) (Area 9) (p<0.05 or p<0.01). Two populations comprising s-typed specimens [II(e) and II(d-e)] did not differ from the others significantly. When two geographical populations are hypothesized (divided into Areas 1–10 and 11), the ΔCT value was maximized (0.113, p<0.0001) and the genetic variability between them was 11.3% (Table 5), showing distinct genetic structure among the specimens with haplogroup ST-II.

### Discussion

Although Kato (1979) and Tsuchida and Hayashi (1994) surveyed the distribution of deep-sea gastropods in the Sea of Japan (off Honshu Island) in detail, here we found three major species groups which accounted for the fauna of macrobenthos in very high density. In each species group, some problems were found in recognition of species and/or populations. In each subsection below, we also give some notes on its geographic distribution that emerged through the present study.
Table 4. Mitochondrial 16SrDNA sequence diversity in the striatissimum species group.

| Haplogroup | Area | Population | Survey | Number of samples | Number of Haplotype | Number of Nucleotide diversity (×100) |
|------------|------|------------|--------|-------------------|---------------------|--------------------------------------|
| ST-I       | 6    | I (6)      | a      | 30                | 6                   | 0.2152                               |
|            | 8    | I (8)      | a      | 23                | 6                   | 0.3337                               |
|            | 10   | I (10)     | a      | 37                | 8                   | 0.2310                               |
| ST-II      | 2    | II (c)*    | c      | 22                | 7                   | 0.1519                               |
|            | 2    | II (b2)    | b2     | 28                | 2                   | 0.0134                               |
|            | 3    | II (d-e)*  | d, e   | 35                | 2                   | 0.0902                               |
|            | 4    | II (f)     | f      | 20                | 6                   | 0.1432                               |
|            | 9    | II (b3)    | b3     | 21                | 4                   | 0.0697                               |
|            | 9    | II (b4)    | b4     | 26                | 4                   | 0.1195                               |
|            | 10   | II (b5)    | b5     | 23                | 6                   | 0.1928                               |
|            | 11   | II (b6)    | b6     | 31                | 5                   | 0.2138                               |

Name of a population is assembled from its haplogroup and locality (code of Area or survey). Populations with an asterisk(*) were composed of s-typed specimens.

Table 5. Hierarchical analysis of mt16SrDNA in samples of the striatissimum species group.

| Analysis Source of variation | df | F statistics | Percentage of variation |
|-----------------------------|----|--------------|------------------------|
| ST-I (3 sampling sites)     |    |              |                        |
| One gene pool              |    |              |                        |
| Among sites                | 2  | 0.00553 (p=0.2950) | 0.55                  |
| Within sites               | 87 | 99.45        |                        |
| ST-II (8 sampling sites)   |    |              |                        |
| One gene pool              |    |              |                        |
| Among sites                | 7  | 0.08300 (p<0.0001) | 8.3                   |
| Within sites               | 195| 91.7         |                        |
| Two gene pools [(Areas 1–10, Area 11)] |    |              |                        |
| Among areas                | 1  | 0.11274 (p<0.0001) | 11.27                 |
| Within areas               | 6  | 0.04831 (p=0.0004) | 4.29                  |
| Within sites               | 195| 0.15561 (p<0.0001) | 84.44                 |

Table 6. Pairwise non-differentiation exact p-values between populations (striatissimum species group).

| ST-I     | I(6) | I(8) | I(10) |
|----------|------|------|-------|
| I (6)    | –    | –    | –     |
| I (8)    | 0.3724 | –    | –     |
| I (10)   | 0.2711 | 0.0766 | –     |

| ST-II    | II (c) | II (b2) | II (d–e) | II (f) | II (b3) | II (b4) | II (b5) | II (b6) |
|----------|---------|---------|-----------|--------|---------|---------|---------|---------|
| II (c)   | –       | –       | –         | –      | –       | –       | –       | –       |
| II (b2)  | 0.0257  | –       | –         | –      | –       | –       | –       | –       |
| II (d–e) | 0.0007* | 0.0008* | –         | –      | –       | –       | –       | –       |
| II (f)   | 0.5516  | 0.0163  | 0.0173    | –      | –       | –       | –       | –       |
| II (b3)  | 0.3941  | 0.0818  | 0.0020    | 0.2009 | –       | –       | –       | –       |
| II (b4)  | 0.0634  | 0.0252  | 0.0262    | 0.1978 | 0.2491  | –       | –       | –       |
| II (b5)  | 0.0179  | 0.0002  | 0.0038    | 0.1400 | 0.0622  | 0.5148  | –       | –       |
| II (b6)  | 0.0001** | 0.0000** | 0.0000** | 0.0008* | 0.0140 | 0.0000** | 0.0000** | –       |

Significant at p<0.05 (*) or p<0.01 (**) after Bonferroni correction. See Table 4 for the names of populations.
Intersculpta species group

In the present study, 429 specimens of the genus Neptunea were examined from the Sea of Japan. No specimen of N. cumingi Crosse was found, and all should be classified into N. intersculpta or N. constricta. Although several other species from Japanese waters, e.g., N. bulbacea (Valenciennes), N. elegantula Ito & Habe, N. frater (Pilsbry), N. heros (Gray), N. pribiloffensis (Dall), and N. rugosa Golikov, should be compared with our specimens, their status is too ambiguous to discuss in the present study. Although N. intersculpta and N. constricta have been distinguished from each other based on the development of the spiral ribs as a primary feature, this character cannot be used alternatively to distinguish them (Fig. 4) as demonstrated by Kato (1979). The apex of the spire, form of the operculum and/or color of the shell surface are other specific features (e.g., Golikov 1963, Okutani et al. 1988), but each of them cannot be divided into two forms among our specimens because of serious variations.

Iguchi et al. (2007a) conducted a population analysis of “Neptunea constricta” using the partial sequence of the COI region of mtDNA (about 700 bp) and a total of 40 specimens from the Sea of Japan, Hokkaido, and the Pacific coast of Honshu (off Fukushima Prefecture). They did not refer to morphological features of their specimens, which may fall into our intersculpta species group. They reported that there were significant differences among haplotype compositions of three populations, “Yamagata-Toyama” (Areas 2–4), “San’in” (Areas 6–10) and “Yamato Bank” (Area 11). Unfortunately their sampling was not sufficient for this kind of analysis as they collected only 1–4 individuals from each sampling site, and haplotype data from some sites were integrated into a haplotype composition of their “sea area,” which had been recognized a priori as being the same as the populations of Buccinum tsubai (Iguchi et al. 2004). Moreover, the number of specimens of each “sea area” was not sufficient for a study of population genetics; apart from “San’in” area using 19 specimens, only nine and five were for “Yamagata-Toyama” and “Yamato Bank” populations, respectively.

The results of the present 16SrDNA analysis (and morphological variations) indicate that there is no clear reason for recognizing more than one taxonomic unit among the intersculpta species group in the Sea of Japan. Since geographical distributions of Neptunea intersculpta and N. constricta are also known from the neighboring sea areas, taxonomic conclusions can only be reached by comparison with additional specimens from there.

Kato (1979) reported that this species group occurs from 250–1,250 m in depth (off the middle part of Honshu Island), and Tsuchida & Hayashi (1994) concluded it mainly inhabits 200–800 m on both coasts of Honshu Islands. According to our results (Figs. 2–3), this group occurs over an extensive geographic distribution (Fig. 3F) and shows a strong eurybathy (200–1,509 m: deepest record is at the survey b3). In the trawl survey, the volume of catches seems to be unrelated to the depth. Results of the deep-sea dredge show that the density is generally high at depths of 400–1,000 m. The intersculpta species group is also known from the following regions, but seems to be above ca. 500 m in depth: around Hokkaido, the Pacific coast of Honshu (north of Kanto district), the Tatar Straight, eastern-south Sakhalin, and southern Kurile Islands (Golikov 1963, Okutani 2000, Kantor 2006).

Tsubai species group

Iguchi et al. (2004, 2007a) revealed that Buccinum tsubai is composed of four local populations in the Sea of Japan (including a region off western Hokkaido) using partial sequences of 16SrDNA (44 samples) and COI (64). No common haplotype was found among these populations, and the authors considered that they had differentiated into a level of subspecies (Iguchi et al. 2007b). Three haplogroups presented here, TB-1, TB-II, and TB-III (Fig. 5G), should correspond to three local populations of Iguchi et al.’s (2004) “Yamagata-Toyama,” “San’in,” and “Yamato Bank,” respectively. Although our present surveys were performed at more numerous and deeper sampling sites than those of Iguchi et al. (2004, 2007a), we discovered no other population and strongly supported their conclusion.

In morphology, Iguchi et al. (2005) referred to the morphological differences among these populations. According to their result, the “Yamagata-Toyama” population is characterized as having a rather broader shell with a more massive body whorl (and accordingly a lower spire) than the other three populations; and the “Yamato Bank” population has a relatively high spire. Specimens of Fig. 5B and 5E may be typical examples of their statements, respectively; however, those of Fig. 5A and 5F were not rare examples in these two populations, respectively. Further detailed studies including not only morphological changes with growth and depth but individual and local variations are needed to understand morphological features of each population.

Buccinum tsubai was also known from the Sea of Okhotsk (Tiba & Kosuge 1984) or was treated as a junior synonym of B. rossicum Dall (Golikov 1980, Kantor 2006). Such a species complex needs to be reexamined using samples from such adjacent seas to the Sea of Japan.

The tsubai species group inhabits the whole of the Sea of Japan (Figs. 2–3). Previous records have shown it lives at depths of 250–1,250 m (Kato 1979; Tsuchida & Hayashi 1994). Our collection data revealed the shallowest record is 250 m (Area 6) and the deepest is about 1,500 m (Areas 2 and 11). Distribution density became higher at depths of ca. 300 m or more: in the deep-sea dredge, sampling stations at 400 to 1,000 m or more showed higher densities.

Striatissimum species group

This species group has been divided into two morphotypes by thickness and profile of shell and condition of its surface. Specimens with a thicker shell (s-type) are ordinarily treated as Buccinum striatissimum, and those with a thin
and fragile shell (t-type) are *B. tenuissimum*. The former category may include several other species, for example, *B. bayani, B. elatior* (Middendorff), *B. senshumaruae* Kosuge & Ishiyama, and *B. terebriforme* Habe & Ito. The status of these nominal species remains ambiguous, leading to inconsistent treatment among studies (e.g., Habe 1978, Okutani et al. 1988, Higo et al. 1999, Kantor 2006).

Iguchi et al. (2007b) analyzed the phylogeny among several species of the genus *Buccinum* in Japan using partial 16SrDNA sequences. They confirmed the monophyly of a clade composed of *B. striatissimum, B. tenuissimum, B. bayani,* and *B. aniwamum* Dall (all collected from the Sea of Japan), and a close relationship between *B. tenuissimum* and *B. bayani*. *Buccinum bayani* is generally known to be another species having a thicker shell, occurring almost throughout the whole of the Sea of Japan (e.g., Doi 1997b, Seto & Doi 1999, Okutani 2000, Borulya & Bregman 2002) and southern Sakhalin and Kurile Islands (Golikov 1980). Iguchi et al. (2007b) also referred to the taxonomy of these species, and suggested that *B. striatissimum* differs from *B. tenuissimum* at the level of subspecies, and that the species status of *B. bayani* was problematic because it had the same haplotype as that of *B. tenuissimum*. Their analysis provided a certain result as a phylogenetic study among these species, but failed as a taxonomic one because of inadequate information of intraspecific variations; the number of their specimens was only three to four for each species.

Iguchi et al. (2008) further reported the sympatric distribution of *Buccinum striatissimum* and *B. tenuissimum* in the San’in area based on 16SrDNA sequences. Although they noted that such samples could be distinguished by this short mtDNA data, we consider that our morphological and mtDNA data explains a more complex situation. We found 72 s-typed specimens have haplogroup of ST-II in areas west of Noto Peninsula (Areas 4–10); as shown in Fig. 8, two s-typed specimens, which are very similar in form and were collected together from 227 m in depth of Area 7, have different types of haplogroups. These data suggest that a kind of genetic confusion has occurred in two species, i.e., hybridization.

On the other hand, at shallower (< ca. 500 m) regions north of the Noto Peninsula (Areas 1–4), all of our specimens have the s-type form and ST-II nucleotides. Now we cannot explain such a combination of morphological and molecular features, but it is possible that this can be explained by an introgressive event, an advanced condition of the genetic confusion seen west of Noto Peninsula. Although Iguchi et al. (2007b) treated them as a third species called “*Buccinum bayani,*” we cannot agree with them here because of the serious ambiguity of its species status. Although Kira (1959) and Habe and Ito (1965) noted that *B. bayani* differs from its close relative, *B. striatissimum*, by coloration and thickness of shell, we could not divide our s-type specimens into two different forms by these features.

As noted above, the *striatissimum* species group should be composed of two or more taxonomic unit, and further studies including nuclear DNA are needed to differentiate this group. Here we tentatively refer to s- and t-typed forms as *Buccinum striatissimum* and *B. tenuissimum*, respectively.

This group inhabits the whole of the Sea of Japan, and shows the strongest eurybathy among the three species groups treated here. The deepest record was 1,902 m for *B. tenuissimum* (Area 9). *Buccinum striatissimum* are distributed along Honshu Island at depths shallower than about 500 m. In the areas west of the Noto Peninsula, *B. striatissimum* broadly inhabits depths of 200–500 m; no distinct tendency was found in the distribution with depth. In the areas north of the Noto Peninsula, it was collected below 300 m in depth, but is known at 200 m within Toyama Bay (Seto & Doi 1999). Tsuchida & Hayashi (1994) noted that *B. tenuissimum* was known from ca. 350–1,500 m (mainly seen in 750–1,200 m). In the present surveys, we found it appeared at depths of 300 m and distributed in deeper waters, and its inhabiting density was relatively high to 1,500 m.

**Concluding remarks**

On the bathyal gastropods in the Sea of Japan, here we examined what species are primarily distributed based on morphological and molecular analyses using as many samples as possible. As a result, three predominant species groups, all of which are important for fisheries, were found. Especially we referred to one of them composed of *Buccinum striatissimum* and related species in detail, and deliberated on their species composition including the possibility of hybridization between two major species (*B. striatissimum* and *B. tenuissimum*). Although the gastropod fauna of the Sea of Japan has been considered to differ from that of neighboring sea areas essentially (the Sea of Okhotsk and the Pacific coast of Honshu Island), the evolutionary timescale seems to be not so long (also see Nishimura 1978), and its taxonomy has to be reviewed based on comparison with the latter seas. In this reviewing work, an approach with molecular methods should bring better results.

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