Genetic population structure of Neptune whelk in northern Japan inferred from microsatellite DNA variation

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Abstract: Genetic population structure of the Neptune whelk (Neptunea arthritica) in northern Japan was estimated from six samples collected from Hokkaido and one sample from Aomori, in northernmost Honshu, using five polymorphic microsatellite DNA loci. Pairwise $F_{ST}$ estimates indicated a genetic cline from eastern and northeastern Hokkaido to southern Hokkaido and northernmost Honshu. The individual-based assignment method and analyses of molecular variance suggested three geographic groups within this cline. The observed population structure was most likely influenced by isolation-by-distance with restricted gene flow, as suggested by the significant correlation between genetic and geographic distance for the entire region examined. The inferred restriction of gene flow is likely due to the poor dispersal ability of this species, which has a benthic, sedentary life history and passive dispersal along the coasts. The observed genetic structure of N. arthritica will be useful for conservation and fisheries management of this species.

Key words: $F_{ST}$, gastropod, genetic cline, isolation-by-distance, microsatellite, Neptunea arthritica, population genetics

Introduction

Marine species generally tend to show little genetic differentiation among local populations because of the lack of apparent geographic barriers leading to increase in the gene flow. Dispersal ability also is thought to influence the level of gene flow in marine species, as is often correlated negatively with the level of genetic differentiation among local populations (Bohnak 1999 for review). However, the correlation between dispersal ability estimated by the length of planktonic larval period and population structure is still controversial. For example, levels of geographic differentiation are elevated in species without planktonic larval stage in marine gastropods (Collin 2001, Lee & Bouldings 2009).

Hair crab, Erimacrus isenbeckii (Brandt) (Azuma et al. 2007) and Japanese scallop, Patinopecten yessoensis (Jay) (Nagashima et al. 2003), both having inshore planktonic stages for a month or more, show indistinct genetic differentiation. On the other hand, Japanese sand fish, Arctosco pus japonicus (Steindachner), having no or little planktonic larval stage and inert swimming ability in adult form, exhibits distinct genetic structure probably influenced by the gene flow through the sea current-dependent dispersal (Yanagimoto 2004). Also, poor correlation between pelagic larval duration and genetic structure has been suggested by an extensive literature survey of marine species (Weersing & Toonen 2009). These discrepant observations likely indicate that the length of planktonic larval stage may not be a sole determinant for the current and recent gene flow in marine organisms in general, as population structure may also be influenced by other factors, such as the whole life history and evolutionary history in a species concerned (Kyle & Bouldings 2000, Collin 2001, Lee & Bouldings 2009).

Neptune whelk, Neptunea arthritica (Bernardi, 1857) (Gastropoda: Buccinidae), is distributed in the intertidal zone to 100-m depth in the Pacific Ocean, the Sea of Japan, and the Sea of Okhotsk along the coasts of northern Japan (Okutani 2000) and is commercially important in coastal fisheries. However, a severe decline in whelk resources occurred during the 1970s and 1980s, probably because of overfishing and imposex (Fujinaga et al. 2002, Miranda et al. 2007). N. arthritica has internal fertilization, spawning egg masses with less than 100 egg capsules on hard sub-
strates of rocks or shells, and showing direct larval development around two months before hatching within egg masses without planktonic stage (Fujinaga 1985). Adults have a sedentary lifestyle, which will bring about a low level of gene flow and genetic differentiation between local populations. Also, this lifestyle likely causes a low level of recruitment from neighboring areas, which makes it difficult to recover the size of local populations after a dramatic decline. This species, however, has recently become regarded as an invasive exotic species along the coasts of the United States (O’Connor et al. 2008) and the Black Sea (Shadrin et al. 2002), most likely by incidental transportation through human activity. Thus, *N. arthritica* must be controlled both as a fisheries resource and a nuisance by conservation and management on the basis of population genetic findings. However, no population genetic study of this species has been conducted so far with molecular genetic markers.

The present study aims to estimate the genetic diversity and population structure of *N. arthritica* around Hokkaido and to obtain basic genetic information using microsatellite (ms) DNA markers recently developed for this species (Azuma et al. 2009). Microsatellites, also known as short tandem repeats, are currently the most powerful set of loci for population genetics, because of the high variability at individual level with biparental inheritance, co-dominance, and the high rate of molecular evolution. msDNA markers have proven useful to estimate the genetic population structure in several gastropods, including a freshwater snail (Meunier et al. 2004) and some marine species (Weetman et al. 2006). Considering the results of genetic analysis, we discuss the effect of current and recent gene flow and historical factors for population structure of *N. arthritica*. The genetic findings to be obtained will be essential to understand the life history, migration pattern, and invasion of *N. arthritica*, besides estimation of the conservation unit and the resource abundance that must be useful for fishery management of this species.

### Materials and Methods

Two hundreds and forty-seven individuals of *Neptunea arthritica* were collected from northern Japan, including from Hokkaido and from Aomori in northernmost Honshu, at the following seven locations: Wakkanaï (WA), Kumaishi (KU), and Shiriuichi (SH), on the Sea of Japan coast; Toyoura (TO), Higashidori, Aomori (AO), and Nemuro (NE), on the Pacific Ocean coast; and Saroma (SA), on the Sea of Okhotsk coast (Table 1, Fig. 1). Hereafter, the term of “a sample” is used for each group of individuals collected from each locale.

A small piece of foot muscle was cut from each of individuals (28 to 32 per sampling location) and stored in 99% ethanol at room temperature until use. Genomic DNA was extracted from about 20 mg of muscle tissue with a Pure Gene Kit (Qiagen) in accordance with the manufacturer’s protocol. Extracted DNA was dissolved in 200 μL Tris-EDTA (pH 8.0).

Seven msDNA loci (*TMS4*, *TMS9*, *TMS11*, *TMS19*, *TMS23*, *TMS26*, *TMS33*) were amplified by PCR. Amplification was carried out in 15-μL reaction mixtures containing template DNA, dNTPs, a set of fluorescent forward and reverse primers, and Taq DNA polymerase (Sigma) in accordance with the manufacturer’s instruction. The thermal cycling profile included precycling denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 s and annealing at a suitable temperature (58°C for *TMS4* and *TMS23*, 50°C for *TMS9* and *TMS11*, 54°C for *TMS19*, and 53°C for *TMS26* and *TMS33*) for 30 s; and extension at 72°C for 20 s. The PCR products were electrophoresed on an ABI 3130xl Genetic Analyzer (Applied Biosystems) with a LIZ-500 size standard (Applied Biosystems), and fragment sizes were estimated using GeneMapper Software (Applied Biosystems). Both *TMS4* and *TMS23* showed nonspecific amplification in several individuals, and so it was difficult to identify some alleles at these loci. Therefore, these two loci were excluded from the present analysis.

### Table 1. Sample information of *Neptunea arthritica* analyzed in the present study.

| Sample name (region*) | Collection date (year, month) | No. of individuals |
|-----------------------|-----------------------------|-------------------|
| NE (PO)               | 2007, 03                    | 30                |
| SA (SO)               | 2006, 09                    | 32                |
| WA (SJ)               | 2007, 09                    | 32                |
| KU (SJ)               | 2006, 11                    | 32                |
| SH (SJ)               | 2006, 09                    | 31                |
| AO (PO)               | 2007, 11                    | 28                |
| TO (PO)               | 2007, 03                    | 32                |

* PO, Pacific Ocean. SO, Sea of Okhotsk. SJ, Sea of Japan.
To evaluate the basic utility of markers in population analysis, the occurrence of null alleles was tested with MICRO-CHECKER ver. 2.2.3 (Oosterhout et al. 2004). The observed ($H_o$) and expected heterozygosities ($H_e$), deviation of genotype frequencies from Hardy–Weinberg expectations (HWE), allelic richness ($Ar$), and linkage disequilibria were tested with GENEPop ver. 3.4 (Raymond & Rousset 1995, Rousset 2008) at a significance level 0.05 after Bonferroni correction.

Genetic differentiation among samples was examined with pairwise $F_{ST}$ (Weir & Cockerham 1984) using Arlequin ver. 2.000 (Schneider et al. 2000). The genetic relationship among samples was visualized two-dimensionally by nonmetric multidimensional scaling (nMDS) plotting based on the pairwise $F_{ST}$ using the statistical software R ver. 2.9.0 (R Development Core Team).

To estimate population structure in another way, the individual-based assignment method also was employed to detect the number of distinct original populations of all individuals using STRUCTURE ver. 2.1 (Prichard et al. 2000, Falush et al. 2003). This method tested a certain number of assumed source populations ($K$) that are differentiated by allele frequencies. The likelihood of candidate populations ($K=1–7$) was determined by comparing the estimated probability to identify the most likely number of populations. STRUCTURE ver. 2.1 also was used to assign each individual to a plausible population. We used an admixture model in which the parameter $Q$ (estimated membership coefficient for each individual in each assumed population) was calculated as an indicator of the degree of accurate membership in each assumed population. Genotypes were analyzed with correlated allele frequencies assumed. Runs were performed with a burn-in period of 100,000 simulations followed by 1 million Markov chain Monte Carlo simulations and 10 iterations for each $K$. The average $Q$ for each sample represented the probability that the sample belonged to the assumed population.

To test the significance of hierarchical population structure suggested by $F_{ST}$ analysis and assignment methods, Arlequin ver. 2.0 was again employed for analysis of molecular variance (AMOVA). The variance components were estimated on the basis of the grouping suggested by $F_{ST}$ based nMDS plotting and $Q$ from the assignment method.

The correlation between geography and genetic structure and the effect of restricted gene flow were evaluated for Isolation-by-Distance (IBD) model (Wright 1943) using $F_{ST}$ estimates and geographic distance. Many software programs automatically calculate the Euclidean distance between localities using their coordinates irrespective of geography, which often crosses land areas where no marine species can move. To avoid this problem, the distance between sample locations was determined from the putative migration routes of whelks, shown in Fig. 1. The distance matrix determined in this manner was compared with the $F_{ST}$ matrix, and the significance of correlations was evaluated with the Mantel test in Arlequin ver. 2.0.

**Results**

Linkage disequilibrium was not significant in any of the examined loci, with $p$ values ranging from 0.073 (between TMS9 and TMS11) to 0.998 (between TMS9 and TMS19). No evidence of scoring error due to stuttering, large allele dropout, or null alleles was detected on the basis of 95% confidence intervals. In all samples, the number of alleles ranged from 11 to 35 and $H_e$ ranged from 0.580 (TMS26) to 0.811 (TMS11) for each locus, indicating the utility of these markers for population analysis. The number of alleles and the $H_e$ and $H_o$ values of each locus in each location are shown in the Appendix. When all individuals were treated as one population, significant deviations from HWE were observed for all loci, suggesting the existence of multiple subpopulations in the examined area. Each locus in each location followed HWE, except TMS19 at SA ($p=0.0004$), suggesting that each sample represent a single subpopulation. Among the seven samples, SA had the lowest average $H_e$ for all loci, the lowest $A_r$ for each locus, and an extremely small number of alleles at each locus (Fig. 2), indicating that this subpopulation had the lowest genetic diversity.

Pairwise $F_{ST}$ estimates are shown in Table 2. $F_{ST}$ values ranged from 0.0426 (KU and SH) to 0.3909 (SA and TO). All pairs showed significant differentiation, but the $F_{ST}$ values were relatively low (<0.1) in the pairs of KU–SH, WA–KU, and SA–WA.

![Fig. 2. Allelic richness ($A_r$) in each sample corrected with the smallest sample size. All loci showed the lowest value for the SA sample.](image)

**Table 2.** Pairwise $F_{ST}$ estimates between samples. Asterisk indicates significant deviation from 0 at $p<0.01$ after Bonferroni correction.

|        | NE  | SA  | WA  | KU  | SH  | TO  | AO  |
|--------|-----|-----|-----|-----|-----|-----|-----|
| SA     | 0.0920* |     |     |     |     |     |     |
| WA     | 0.1196*  0.2062* |     |     |     |     |     |     |
| KU     | 0.2546*  0.2986*  0.0997* |     |     |     |     |     |     |
| SH     | 0.2143*  0.2694*  0.0549*  0.0426* |     |     |     |     |     |     |
| AO     | 0.2455*  0.2926*  0.1046*  0.0900*  0.0617* |     |     |     |     |     |     |
| TO     | 0.3435*  0.3909*  0.2416*  0.2006*  0.1684*  0.1974* |     |     |     |     |     |     |
SH–WA and AO–SH, suggesting relatively high level of gene flow along the coast of the Sea of Japan.

The nMDS plot based on $F_{ST}$ estimates is shown in Fig. 3. After 100 iterations, the stress value converged on 0.0083, indicating the high reliability of this plot. The samples from southern region (TO, AO, KU, SH) and from northeastern (NE, SA) were apparently distant, and the sample of WA settled between them. As a whole, the horizontal long scattering plot, which is consistent with geographic relationships between samples, suggests a population structure with a one-dimensional genetic cline, gradually differentiating from eastern and northeastern Hokkaido to southern Hokkaido and northernmost Honshu through the Sea of Okhotsk coast, the Soya Strait, the Sea of Japan, and the Tsugaru Strait. In the cline, three types of clustering were suggested: two groups of [NE, SA] and [WA, KU, SH, AO], and [TO]; three groups of [NE, SA], [WA, KU, SH, AO], and [TO]; and four groups of [NE, SA], [WA], [KU, SH, AO], and [TO].

The plausible population number was estimated from the probability test of $K$ populations using the STRUCTURE program. The distribution of mean ln $p (K=X)$ in 10 iterations for $K=1–7$ was $-4721.8, -4155.0, -3816.3, -3757.7, -3660.4, -3740.3, -3853.8$. The probability was highest when $K=5$. However, because of the occasional overestimate of $K$ with the STRUCTURE program (see documentation for STRUCTURE ver. 2.3, available at http://pritch.bsd.uchicago.edu/structure.html), clustering into more than five populations was excluded in the assignment of individuals to each population. As shown in Table 3, the three types of clustering (i.e., samples divided into three, four, or five populations) were used to estimate the average $Q$ for each sample. The value of ln $p (K=X)$ were lower in $K=1, 2$ than in $K=3, 4$ or 5, thereby suggesting elimination of clustering in that samples divided into one or two. When three populations were assumed, the average $Q$ values from NE and SA were highest for population 3 (0.965 and 0.987, respectively), from WA, KU, SH, and AO for population 2 (0.563, 0.912, 0.804 and 0.931, respectively), and from TO for population 1 (0.966), suggesting the groups to be [NE, SA], [WA, KU, SH, AO], and [TO]. The results were consistent with the geographic grouping of sampling locations suggested from nMDS plotting based on $F_{ST}$ estimates. WA showed remarkable $Q$ values both for population 2 (0.563) and for population 3 (0.374), suggesting the intermediate status of this sample as shown in the nMDS plotting. When four populations were assumed, the clustering appeared to be [NE, WA, SA], [KU, SH, AO], and [TO], with $Q$ values of 0.755, 0.973, 0.973, 0.891, 0.580, 0.915 and 0.964, respectively, and the assumption of five populations resulted in [NE, WA, SA], [KU, SH, AO], and [TO], with $Q$ values of 0.758, 0.759, 0.908 and 0.941, respectively.

For AMOVA, samples were divided into the following groups (Table 4): (1) two groups of [NE, SA] and [WA, KU, SH, AO], and [TO]; (2) three groups of [NE, SA], [WA, KU, SH, AO], and [TO]; (3) four groups of [NE, SA], [WA, KU, SH, AO], and [TO]; (4) four groups of [NE, WA, SA], [KU, SH, AO], and [TO]; and (5) five groups of [NE], [WA], [SA], [KU, SH, AO], and [TO]. Groupings (1) and (3) refer to the results of the nMDS plotting, and groupings

### Table 3. Proportion of assigned membership to each pre-defined population in the 3 to 5 clusters.

|       | 3 populations | 4 populations | 5 populations |
|-------|---------------|---------------|---------------|
|       | Pop 1 | 2 | 3 | 4 | 5 |
| NE    | 0.011 | 0.024 | 0.965 |       |       |       |
| SA    | 0.006 | 0.007 | 0.987 |       |       |       |
| WA    | 0.063 | 0.563 | 0.374 |       |       |       |
| KU    | 0.074 | 0.912 | 0.014 |       |       |       |
| SH    | 0.131 | 0.804 | 0.064 |       |       |       |
| AO    | 0.041 | 0.931 | 0.029 |       |       |       |
| TO    | 0.966 | 0.024 | 0.010 |       |       |       |

#### Sampling locations suggested from nMDS plotting based on $F_{ST}$ estimates.

![nMDS plotting of samples with pairwise $F_{ST}$ value, showing three approximate clusters: [SA, NA], [WA, KU, SH, AO], [TO].](image)
(4) and (5) refer to the individual-assignment test. Grouping (2) was based on both methods. According to AMOVAs, groupings (2) and (3) indicated significant differentiation at the highest hierarchical level among groups, and the fixation index was higher in (2) than in (3) (Table 4).

The Mantel test showed a significant correlation between genetic ($F_{ST}$) and geographic distance for all samples ($p = 0.006$), suggesting that the gene flow in *N. arthritica* fits the IBD model (Fig. 4).

| No of group | % of variation | Fixation indices | $F_{ST}$ | p value |
|-------------|----------------|-----------------|----------|---------|
| Among groups | 16.21 | 0.162 | 0.0390 |
| Among samples within groups | 10.53 | 0.125 | 0.0000 |
| Within samples | 73.26 | 0.267 | 0.0000 |
| Among groups | 17.81 | 0.178 | 0.0059* |
| Among samples within groups | 6.82 | 0.083 | 0.0000 |
| Within samples | 75.37 | 0.246 | 0.0000 |
| Among groups | 16.02 | 0.160 | 0.0078* |
| Among samples within groups | 6.30 | 0.074 | 0.0000 |
| Within samples | 77.69 | 0.223 | 0.0000 |
| Among groups | 14.49 | 0.145 | 0.0195 |
| Among samples within groups | 7.60 | 0.089 | 0.0000 |
| Within samples | 77.91 | 0.221 | 0.0000 |
| Among groups | 13.60 | 0.136 | 0.0362 |
| Among samples within groups | 7.41 | 0.086 | 0.0000 |
| Within samples | 78.99 | 0.210 | 0.0000 |

**Table 4.** Results of AMOVA when samples were divided into two groups of [NE, SA] and [WA, KU, SH, AO, TO], three groups of [NE, SA], [WA, KU, SH, AO] and [TO], four groups (A) of [NE, SA], [WA], [KU, SH, AO] and [TO], four groups (B) of [SA], [NE, WA], [KU, SH, AO] and [TO], and five groups, [SA], [NE, WA], [KU, SH] [AO] and [TO]. * indicate significant at the level of $p<0.01$.

**Discussion**

Iguchi et al. (2007) investigated the genetic population structures of the deep-sea whelks *Buccinum tsubai* (Kuroda) and *Neptunea constricta* (Dall) in the Sea of Japan by using a partial sequence of the mtDNA COI gene and found clear genetic differentiation among distant populations, however, could not show genetic differentiation among populations around Hokkaido, probably because there were insufficient numbers of samples and individuals collected in the area. Very recently, Shirai et al. (2010) revealed population structure of *B. tsubai* with three groups in the Sea of Japan off the mainland Honshu of Japan, although their main interest seemed to be intraspecific phylogeny. They well documented the population structure probably affected by the sedentary life style of direct-developmental gastropod. However, they did not sample around Hokkaido, and did not use msDNA markers, which is generally expected to clarify the gene flow better than mitochondrial DNA. Therefore, to our knowledge, the present study is the first to report the population genetic structure of sea whelks around Hokkaido and the first to apply msDNA markers for the population genetics of sea whelks around Japan.

In the present study, the genetic differentiation of *N. arthritica* was estimated mainly using $F_{ST}$ not $R_{ST}$. Generally, if the molecular evolution of msDNA follows stepwise
mutation model, and if the species is so sedentary that migration is less important for population structure than mutation, \( R_{ST} \) is expected to give more accurate differentiation estimates than \( F_{ST} \) (Slatkin 1995, Balloux & Lugon-Moulin 2002). However, performance of \( F_{ST} \) would be better than \( R_{ST} \) in \( Nm \) estimates if sample size and the number of loci are small (\( n_i \leq 10 \) and \( n_i < 20 \), respectively) (Gaggiotti et al. 1999). In addition, unreliability of \( R_{ST} \) has been suggested with msDNA loci, as deviation from a single-step mutation model was observed (Balloux et al. 2000). This situation may also hold true for the present study with the medium size of specimens (ca. 35 per sample) but small number of loci examined (7), and hence \( R_{ST} \) analysis was not included.

The observed variation of the five msDNA loci employed was sufficient to estimate the genetic diversity in the \( N. \ arthritica \) samples examined (Appendix). The lowest genetic diversity at SA among the examined samples, as estimated by \( H_e \) and \( A \), (Fig. 2, Appendix), may be ascribed to the small population size and isolation in Lake Saroma, a lagoon connected to the Sea of Okhotsk. The \( H_e \) and \( A \) values in the other samples were comparable, even between AO in northernmost Honshu and the samples in southern Hokkaido.

All results indicate that the examined population structure of \( N. \ arthritica \) was basically more influenced by mild gene flow than genetic drift. Gene flow is likely restricted by their sedentary life style, but occasional migration could occur accidentally. The nMDS plot based on \( F_{ST} \) estimates suggested a genetic cline along Hokkaido and northernmost Honshu (Fig. 3). This structure is different from the previous observations in marine species around Hokkaido, such as Japanese scallop (Nagashima et al. 2003, Sato et al. 2005), Japanese sandfish (Yanagimoto 2004), and hair crab (Azuma et al. 2007). Clear discrimination of samples from eastern Hokkaido and those from the Sea of Japan was suggested in the study of hair crab (Azuma et al. 2007) and the present study, but no other genetic relationships between samples were common in the previous and the present studies. In hair crab, genetic similarity was observed in the samples from the Sea of Okhotsk and the Funka Bay, but, in \( N. \ arthritica \), SA from the Sea of Okhotsk was the most distant from TO in the Funka Bay (Table 2). Therefore, the genetic structure of \( N. \ arthritica \) inferred herein may be unique and species-specific around Hokkaido.

As shown in Fig. 3, both ends of the linear nMDS plotting based on \( F_{ST} \) are TO and SA. However, SA may be thought to be out of the cline, because the lowest level of diversity within SA (Fig. 2) suggested biased estimation of genetic differentiation between SA and other samples (Table 3). Excluding SA, a gradual genetic differentiation was seen along the Sea of Okhotsk and Sea of Japan coasts, but a deep genetic break likely lay somewhere in the Pacific Ocean between TO/AO and NE, as suggested by the large interregional genetic distance (Fig. 3). Analysis with more samples from the Pacific Ocean coast is needed to ascertain the occurrence of such a break and its border.

The genetic cline also was basically favored by IBD, as suggested by the Mantel test of all samples, indicating restricted gene flow among locations, probably because of the poor dispersal ability of \( N. \ arthritica \) (Fig. 4). Therefore, occasional migration between populations likely follows the stepping-stone model, in which migrants can move to neighboring populations but not to distant populations.

Within the observed genetic cline, the individual-assignment test (highest \( Q \) value) and AMOVA (highest fixation index) suggested the [NE, SA], [WA, KU, SH, AO], and [TO] geographic grouping as the most plausible, although four or five groups also were possible according to the individual-assignment test (Tables 3 and 4). These groupings commonly cluster KU, SH, and AO together but distinguish TO from the other samples, mostly reflecting the genetic distance among the samples (Table 2). The sample of WA appeared in the group of the Sea of Japan with KU, SH probably due to relatively low to moderate genetic differentiation (Table 2), despite the substantial geographic distances among these sampling locations (Fig. 1). Genetic similarity between KU, SH and AO may suggest a metapopulation structure in this area.

Clustering of KU, SH, and AO suggests possible gene flow across the Tsugaru Strait, with at least 140 m depth on the way from Hokkaido to Aomori. Such gene flow, if any occurs, might be caused by passive dispersal during a chance event such as a storm, as these intertidal whelks would not be able to crawl across the deep sea floor of the strait. However, the substantial divergence between these three samples and the relatively nearby sample of TO (Fig. 1) is puzzling, even though the TO sample is distinctly divergent from any other samples examined here (Fig. 3, Table 3). TO is located within Funka Bay, which has rather unique bathymetric and hydrographic conditions (Ohtani 1971). The Tsushima Warm Current flows northward along Honshu, and the current’s major outflow from the Sea of Japan through the Tsugaru Strait to the Pacific Ocean. This water, named the Tsugaru warm-water mass, intrudes into Funka Bay in late summer and/or autumn, when the whelks are hatching and beginning their sedentary life stage, and the water mass occupies the whole bay through winter. From January to February, the Oyashio coastal cold-water mass enters the bay and replaces the Tsugaru warm-water mass. Therefore, the unique hydrographic environment in the Funka Bay may partly restrict gene flow between TO and the neighboring locations examined.

In addition to the present gene flow, historical demography also might have influenced the observed structure as suggested by Kyle & Boulding (2000), which compared the population genetic structure of gastropods with and without pelagic larval dispersal. Iguchi et al. (2007) also suggested influence of evolutionary history on different genetic population structure of two species of gastropods, probably both of which have similar level of dispersal ability. Unfortunately, microsatellite data cannot be applied to molecular clock calibration for the correlation of demo-
graphic changes and the glacial effect that caused changes in sea level and topography around the Sea of Japan with the closing or narrowing of the Soya, Tsushima, and Tsugaru Straits. However, sedentary lifestyle of *N. arthritica* and significant genetic distances in every pair of samples (Table 3) suggest that the genetic structure of this species may still be informative to discuss the paleontology of this species. Amano (1997) reported that fossilized remains of *N. arthritica* were found in a deposit of the Pliocene and hypothesized that the species’ eurythermal capacity might have allowed them to survive in the Sea of Japan through the dramatic climate changes during the glacial and interglacial periods in the Pliocene, when many *Neptunea* species went extinct in the Sea of Japan. Amano (2002) also suggested another evolutionary scenario in which some neogastropod species, including *N. arthritica*, suffered a decline due to low salinity of surface waters and/or anoxia of deep waters during the Pliocene in the Sea of Japan, and afterward Pacific populations reinvaded the Sea of Japan. The present observation that average $H_g$ values were higher in the samples from the Sea of Japan (WA, KU, and SH) than in those from the Pacific Ocean (NE, TO, and AO; see Appendix) may favor a long history without extreme population declines in the Sea of Japan. The Tsugaru Strait was formed around 0.10–0.15 million years ago, whereas the Soya Strait was completed much more recently, around 12,000 years ago (Oshshima 1991). Considering the suggested topographical change, migration of *N. arthritica* from the Sea of Japan to the Pacific Ocean might have occurred in the late Pliocene, near the Last Glacial Maximum. This and the present findings seem to favor the former hypothesis of Amano (1997), not the latter one (Amano 2002), although more precise estimation of the historical demography of *N. arthritica* should be attempted using more samples and other informative markers, such as mtDNA, which often allows analysis of the evolutionary and demography history of a species.

In conclusion, the present study revealed the characteristic population structure of *Neptunea arthritica* in its natal distribution, in which gene flow was likely restricted between locales because of the whelks’ sedentary lifestyle. Although invasion of *N. arthritica* into nonnatal areas along the coasts of the United States (O’Connor et al. 2008) and the Black Sea (Shadrin et al. 2002) suggests that it is highly adaptable to new environments, conservation and management plans for *N. arthritica* should be devised carefully on a population-by-population basis, in consideration of the genetic differentiation among locales that was achieved via natural dispersal over the species’ long evolutionary history.

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**Appendix.** The number of alleles (N.A.) and expected and observed heterozygosity ($H_E$ and $H_O$) at each locus in each sample. * and ** indicate significant deviation from HWE at $p<0.05$ and $p<0.01$ levels after Bonferroni correction.

|     | $TMS9$ | $TMS11$ | $TMS19$ | $TMS26$ | $TMS33$ | All loci |
|-----|--------|---------|---------|---------|---------|----------|
| NE  | N.A.   | 7       | 14      | 9       | 4       | 3        | 7.4      |
|     | $H_E$  | 0.719   | 0.876   | 0.667   | 0.438   | 0.514    | 0.643    |
|     | $H_O$  | 0.700   | 0.900   | 0.667   | 0.433   | 0.500    | 0.640    |
| SA  | N.A.   | 3       | 5       | 5       | 2       | 2        | 3.4      |
|     | $H_E$  | 0.662   | 0.684   | 0.775   | 0.268   | 0.495    | 0.577    |
|     | $H_O$  | 0.719   | 0.781   | 0.625*  | 0.250   | 0.469    | 0.568    |
| WA  | N.A.   | 12      | 17      | 14      | 2       | 7        | 10.4     |
|     | $H_E$  | 0.799   | 0.930   | 0.748   | 0.396   | 0.755    | 0.726    |
|     | $H_O$  | 0.813   | 0.906   | 0.781   | 0.344   | 0.750    | 0.719    |
| KU  | N.A.   | 10      | 19      | 14      | 5       | 6        | 10.8     |
|     | $H_E$  | 0.796   | 0.888   | 0.777   | 0.534   | 0.563    | 0.717    |
|     | $H_O$  | 0.719   | 0.938   | 0.813   | 0.594   | 0.594    | 0.732    |
| SH  | N.A.   | 11      | 16      | 12      | 5       | 5        | 9.8      |
|     | $H_E$  | 0.795   | 0.892   | 0.861   | 0.508   | 0.424    | 0.720    |
|     | $H_O$  | 0.710   | 0.871   | 0.774   | 0.581   | 0.419    | 0.671    |
| AO  | N.A.   | 6       | 15      | 8       | 5       | 5        | 7.8      |
|     | $H_E$  | 0.758   | 0.842   | 0.829   | 0.549   | 0.660    | 0.729    |
|     | $H_O$  | 0.750   | 0.750   | 0.750   | 0.464   | 0.571    | 0.570    |
| TO  | N.A.   | 10      | 12      | 13      | 3       | 4        | 8.4      |
|     | $H_E$  | 0.498   | 0.783   | 0.859   | 0.381   | 0.503    | 0.606    |
|     | $H_O$  | 0.469   | 0.719   | 0.875   | 0.312   | 0.469    | 0.572    |
| Total| N.A.   | 23      | 35      | 34      | 11      | 11       | 22.8     |
|     | $H_E$  | 0.750   | 0.811   | 0.791   | 0.580   | 0.638    | 0.673    |
|     | $H_O$  | 0.611** | 0.737*  | 0.664** | 0.372** | 0.474**  | 0.572    |