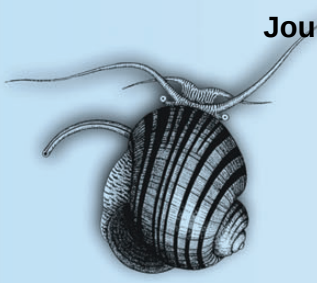
Journal of Molluscan Studies Advance Access published 1 September 2014



Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2014) 1–8. doi:10.1093/mollus/eyu068

Phylogeography of Neptune whelk (*Neptunea arthritica*) suggests sex-biased impact of tributyltin pollution and overfishing around northern Japan

Noriko Azuma[1], Richard M. Miranda[2], Seiji Goshima[1] and Syuiti Abe[3]

[1]*Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1, Minato-cho, Hakodate 041-8611, Japan;*
[2]*Universidad Austral de Chile, Sede Puerto Montt, Región de Los Lagos, Chile; and*
[3]*Sanriku Fisheries Research Center, Department of Revitalization for Sanriku-region, Iwate University, 3-75-1 Heita, Kamaishi 026-0001, Japan*

Correspondence: N. Azuma; e-mail: nazu@fish.hokudai.ac.jp

(Received 30 September 2013; accepted 16 July 2014)

ABSTRACT

The Neptune whelk, *Neptunea arthritica*, is a sublittoral snail from Pacific waters that is a food resource and supports a commercially important fishery in northern Japan. This species showed a severe decline during the 1970s and 1980s, possibly because of overfishing, imposex caused by tributyltin (TBT) pollution and parasite infection. In the present study, we investigated genetic variation among the populations of *N. arthritica* from eight localities in northern Japan, including Hokkaido and Aomori, using a mitochondrial DNA (mtDNA) marker, a partial sequence of the cytochrome *c* oxidase subunit I (COI) gene. We also addressed the evolutionary history of *N. arthritica* and human impact on the population genetic profiles of this species. The parsimony network showed 14 COI haplotypes separated into two groups (Groups A and B), with an intermediate haplotype connecting both groups. Among eight populations, six were fixed for only one or two haplotypes, and no geographic-genetic correlation was found; they were probably affected by random drift. These results contrasted with those from previous microsatellite analysis, which indicated that geographic structure was the result of restricted gene flow between populations. Our results suggested that *N. arthritica* diverged into Groups A and B during the Pliocene; however, recent TBT pollution and size-selective fishing pressure have reduced genetic diversity and concealed the natural population structure. The present study also suggested that human impact may cause longstanding and possibly irreversible modification of ecosystems, particularly for species forming discrete and relatively small local populations, such as *N. arthritica*. Thus, the combined use of mtDNA and microsatellite genetic data provides a powerful tool to investigate the health of biodiversity in molluscs.

INTRODUCTION

Neptunea arthritica (Bernardi, 1857) is a dioecious gastropod with internal fertilization and direct development, found in the sublittoral zone to a depth of a few tens of metres. The egg masses are deposited on hard substrata such as rocks and boulders, and maturation takes 3 years or more (Fujinaga, 2003). The typical *N. arthritica* (*N. arthritica arthritica*) is distributed in the northern Pacific Ocean, the Sea of Japan and the Sea of Okhotsk, along the coasts of northern Japan and Sakhalin in southern Russia, whereas a subspecies *N. arthritica cumingii* (hereafter *N. a. cumingii*) is found from the western part of the Sea of Japan to the East China and Yellow Seas (Okutani, 2000), with the range partly overlapping with typical *N. arthritica*. Sea snails, including *N. arthritica*, have long been a food resource and commercially important in coastal fisheries in northern Japan (Mizushima & Torisawa, 2003); thus, several studies of *N. arthritica* have been mainly conducted for the purposes of resource management (Kawai *et al.*, 1994; Fujinaga & Nakao, 1996; Suzuki *et al.*, 2002; Fujinaga, 2003; Fujinaga *et al.*, 2006; Miranda *et al.*, 2007, 2009; Miranda, Fujinaga & Nakao, 2008; Lombardo & Goshima, 2010). However, no population genetic studies had appeared until our recent microsatellite DNA analysis of *N. arthritica* (Azuma *et al.*, 2011). Using five loci of microsatellite DNA markers in seven populations of *N. arthritica* around Hokkaido, we suggested that restricted gene flow among populations resulted in increased genetic differentiation with increasing geographic separation, i.e. following the isolation-by-distance (IBD) model (Azuma *et al.*, 2011). The observed restricted gene flow probably reflected the balance between dispersal by marine currents and the low vagility of this species (Azuma *et al.*, 2011). Therefore, the genetic structure was considered to be natural and without strong anthropogenic disturbance.

However, the microsatellite data could not provide information on phylogeny and evolutionary history. To reconstruct the evolutionary history of *N. arthritica*, we chose sequence variation in the 5′ portion of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene. This marker was used for the following

reasons. (1) Markers from mitochondrial DNA (mtDNA) lack recombination and have uniparental (maternal) inheritance (which results in the absence of heterozygotes), facilitating lineage analysis (Harrison, 1989; Avise, 2000; Freeland, 2005). (2) The COI region examined showed sufficient intraspecific variation and included a barcoding portion; thus, it could be used for comparison with other species. (3) Molecular clock estimates are available for mtDNA sequence data, from which divergence times of lineages can be estimated (Kumar, 2005).

Mitochondrial DNA markers reveal past bottleneck effects because of the small effective population size, which is a quarter of that of nuclear DNA (Moore, 1995), so they are sensitive for detecting past population declines. Previous microsatellite analyses of *N. arthritica* did not reveal any evidence of a recent decline in the populations (Azuma *et al.*, 2011). However, around Hokkaido, *N. arthritica* did show a severe decline during the 1970s and 1980s, possibly because of overfishing, imposex caused by tributyltin (TBT) pollution and parasite infection (Kawai *et al.*, 1994; Fujinaga *et al.*, 2006; Miranda *et al.*, 2007, 2009). Using the genetic profile of mtDNA, we can expect to detect such a recent decline better than with microsatellites. In particular, if the main factors causing population declines are severe in females, we can expect a drastic reduction in genetic diversity in mtDNA, which represents variability in the matriline. A skewed sex ratio caused by size-selective fishing has often been reported (Rowe & Hutchings, 2003; Fenberg & Roy, 2008; Kendal & Quin, 2013). Selective harvesting of larger snails should increase fishing pressure on females of *N. arthritica*, because females mature at larger size than males (Fujinaga, 2003; Miranda *et al.*, 2008). Imposex induced by TBT has been observed in many species of gastropods, modifying genitalia and sterilizing females (Gibbs, 1996; Blackmore, 2000; Pavoni *et al.*, 2007; Bigatti *et al.*, 2009). Thus, human impact by overfishing and TBT pollution has likely affected populations in a sex-biased manner, being more severe in females. Parasite infection, on the other hand, damages reproduction in both sexes (Miranda *et al.*, 2009).

The present study aimed to genetically characterize *N. arthritica* populations around Hokkaido using an mtDNA marker and to compare the results with those from previous microsatellite analyses to address (1) the evolutionary history of *N. arthritica* and (2) human impact on population genetic profiles of this species. For (1), we analysed genetic diversity, haplotype genealogy and spatial distribution of haplotypes. For (2), we compared the mtDNA data with previous results of microsatellite analysis. We suggest that if the signature of genetic drift and low genetic diversity within populations are revealed in mtDNA, contradicting the results from microsatellite DNA, this will be evidence of sex-biased damage by human impact, more severe in females than in males.

MATERIAL AND METHODS

Specimens

We used 238 individuals of *Neptunea arthritica* from seven locations in Hokkaido, namely Wakkanai (WA), Rumoi (RU), Kumaishi (KU) and Shiriuchi (SH) on the Sea of Japan coast, Toyoura (TO) and Nemuro (NE) on the Pacific Ocean coast, and Saroma (SA) on the Sea of Okhotsk coast, as well as from Aomori (AO) in northernmost Honshu (Table 1, Fig. 1). Hereafter, the term 'sample' is used for a group of individuals collected from each of these localities, as representative of the local population. The samples were identical to those used for our previous microsatellite DNA analysis (Azuma *et al.*, 2011), except for RU, which was analysed only in the present study. Genomic DNA of the RU sample was extracted using the Pure Gene Kit (Qiagen) according to the manufacturer's protocol, as described previously (Azuma *et al.*, 2011).

Nucleotide sequencing

The 5′ region of COI was amplified by PCR in a 30-μl reaction mixture containing template DNA (*c.* 500 pg), dNTPs, a pair of primers [LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′; Folmer *et al.*, 1994)] and *Taq* DNA polymerase (Sigma), according to the manufacturer's instructions. The thermal cycling profile included precycling denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 45 s and extension at 72 °C for 45 s. After electrophoretic examination on a 2% agarose gel, the PCR products were purified with magnetic beads (AMPure, Agencourt), cycle-sequenced using the same forward and reverse primers and the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and loaded onto an automated sequencer, ABI PRISM™ 3130 (Applied Biosystems). The obtained sequences of both directions were aligned and edited to 428 bp using DNASIS-Mac v. 3.5 (Hitachi) and ClustalX v. 1.81 software (Thompson *et al.*, 1997) for defining haplotypes and deposited in the DDBJ/GenBank database (accession nos AB432872–AB432884 and AB811355).

Molecular phylogeny

A phylogenetic tree of the COI haplotypes was reconstructed using the Bayesian algorithm in MrBayes v. 3.12 (Ronquist & Huelsenbeck, 2003). Sequences with high similarity (>92%) to the obtained data were found with the BLAST tool in the DDBJ/GenBank database and added to the phylogenetic and haplotype network analyses (HQ834061 and FJ710084 for *N. a. cumingii*, FJ710085 for *N. arthritica*). A COI sequence from *N. eulimata* (EU883634) was used as outgroup. We applied the substitution model GTR+G+I, recommended as the best fitting substitution model by jModelTest v. 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). In the Bayesian analysis, the posterior probability distribution of trees was approximated by drawing a sample every 100 steps over 1,000,000 Markov chain Monte Carlo cycles, in which the average standard deviation dropped to <0.00001, after discarding a burn-in of 250,000 cycles. The haplotype genealogy was resolved with a parsimony network using the TCS Network Program (Clement, Posada & Crandall, 2000) under a 95% connection limit, with gaps coded as a 5th state.

Divergence time was estimated following the calibration by Nakano *et al.* (2010), which assumed that the subgenus *Barbitonia*, including *N. arthritica*, diverged from other *Neptunea* species *c.* 11 Mya on the basis of the oldest fossil *Barbitonia*.

Population genetic analyses

We used Arlequin v. 3.1 (Excoffier, Laval & Schneider, 2005) to estimate haplotype (h) and nucleotide diversity (π) in each sample and to detect genetic differentiation among samples by pairwise F_{ST} (Weir & Cockerham, 1984). Genetic differentiation between samples was visualized by a nonmetric multidimensional scaling (nMDS) plot of pairwise F_{ST} using the statistical software R v. 2.9.0 (R Development Core Team). To test the significance of the hierarchical population structure, analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was conducted with Arlequin, assuming the three categories that were suggested by haplotype distribution and geography: (1) [WA, SA, RU] and [KU, SH, AO, TO, NE], (2) [WA, SA, RU, NE] and [KU, SH, AO, TO] and (3) [WA, SA, RU], [KU, SH, AO, TO] and [NE].

Evaluation of the IBD model (Wright, 1943) to assess the level of gene flow was performed using Arlequin. For the IBD test, the geographic distance between sample locations was determined from putative migration routes (Fig. 1). The

Table 1. Samples of *Neptunea arthritica*, with haplotype diversity (h) and nucleotide diversity (π) estimated in the present study from partial COI sequences, and mean expected heterozygosity (H_E) estimated from five microsatellite loci by Azuma *et al.* (2011).

Sample abbreviation	Collection date (year, month)	Sample size n	haplotype diversity (h)	Nucleotide diversity (π)	Expected heterozygosity (H_E)
NE	2007, 03	30	0.33 ± 0.08	0.0030 ± 0.0013	0.643
SA	2006, 09	30	0	0	0.577
WA	2007, 09	30	0.59 ± 0.08	0.0019 ± 0.0015	0.726
RU	2007, 10	30	0	0	–
KU	2006, 11	30	0.71 ± 0.05	0.0088 ± 0.0028	0.717
SH	2006, 09	30	0	0	0.720
TO	2007, 03	30	0.48 ± 0.05	0.0078 ± 0.0019	0.729
AO	2007, 11	28	0.13 ± 0.08	0.0032 ± 0.0006	0.606
Total		238	0.57 ± 0.03	0.0061 ± 0.0036	0.673

Sample RU was not analysed with microsatellites because of poor amplification in PCR. For sample localities, see Figure 1.

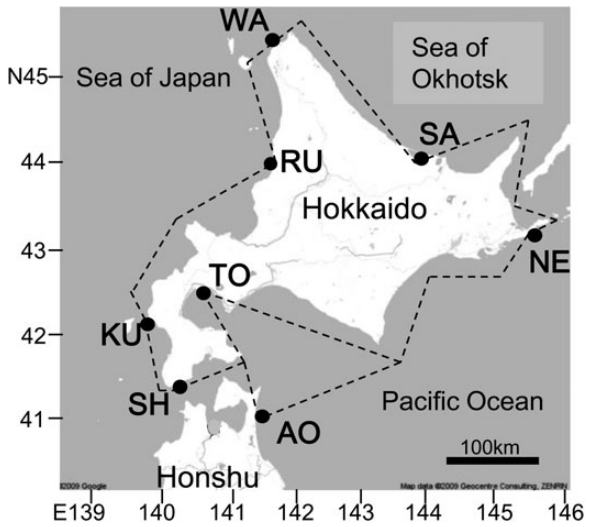


Figure 1. Map of sampling locations of *Neptunea arthritica* in northern Japan. Abbreviations: WA, Wakkanai; RU, Rumoi; KU, Kumaishi; SH, Shiriuchi; TO, Toyoura; AO, Aomori; NE, Nemuro; SA, Saroma. Dashes indicate putative migration pathways.

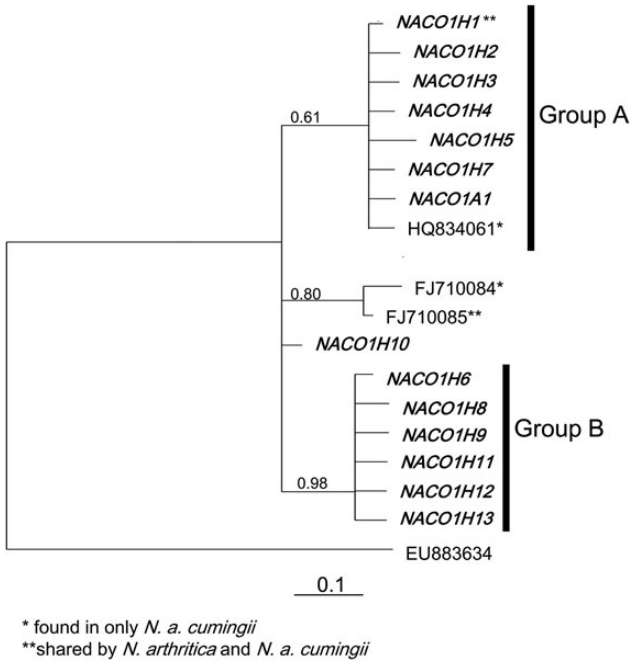


Figure 2. Fifty percent majority-rule Bayesian tree inferred from partial mtDNA COI sequences of *Neptunea arthritica*. Bold and italic OTUs indicate haplotypes found in the present study; others are accession numbers of sequences retrieved from DDBJ/GenBank. The tree was rooted using EU883634 from *N. eulimata* as outgroup. Nodal numbers represent Bayesian posterior probability values.

resulting distance matrix was compared with the F_{ST} matrix, and the significance of correlations evaluated by the Mantel test.

RESULTS

COI sequence variation and haplotype genealogy

PCR amplification of *c.* 650 bp fragments was not always successful, probably because of low DNA quality. To eliminate unreliable sequences, a confirmed part of the 428-bp sequence was used for haplotype identification. Thus, 19 polymorphic nucleotide sites were found in the aligned sequences of COI from 238 individuals, which defined 14 haplotypes, *NACO1H1–H13* and *NACO1A1* (Fig. 2). BLAST search revealed that the most frequent haplotype, *NACO1H1*, was identical to five 428-bp sequences in the DDBJ/GenBank database (accession nos JN053005, JN053006 and EU883627 from *N. a. cumingii*, EU883629 from *Neptunea* sp.1 and FJ710078 from *N. arthritica*). In the BLAST search, we also found that the database sequence of FJ710085 for *N. arthritica* was identical to those of *N. arthritica* (AB498776, AB498777 and AB498778) and *N. a. cumingii* (FJ710083 and FJ710079). Thus, *N. arthritica* and *N. a. cumingii* shared at least two haplotypes, *NACO1H1* and FJ710085 (Fig. 2). In the Bayesian tree (Fig. 2), all the new haplotype sequences and the ingroup sequences from the databank were grouped into three clusters: Group A (*NACO1H1–5*, *NACO1H7*, *NACO1A1* and HQ83061 from databank), Group B (*NACO1H6* and *NACO1H8–13*) and a third group consisting of two databank sequences (FJ710085 and FJ710084), whereas *NACO1H10* was intermediate. Although the posterior probabilities for Group A and the third group (0.61 and 0.80, respectively) were not high enough to support monophyly, the three groups were also discriminated in the parsimony haplotype network (see below).

The haplotype network (Fig. 3) was three-forked, showing the same two groups seen in the Bayesian tree, Groups A and B, with core haplotypes *NACO1H1* and *NACO1H6*. *NACO1H10* was present in the centre of the network, connecting Groups A and B and a third group containing databank sequences FJ710084 and FJ710085. Several missing haplotypes appeared between

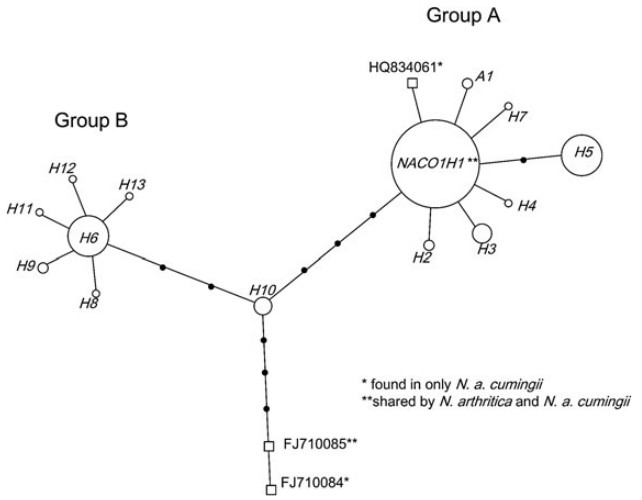


Figure 3. Parsimony network of the mtDNA COI haplotypes of *Neptunea arthritica*. Open circles indicate a haplotype observed in the present study and circle size reflects haplotype abundance (number of individuals that had the haplotype). Squares and closed circles indicate a sequence retrieved from the DDBJ/GenBank database and a missing haplotype, respectively. A solid line between circle/square indicates a single nucleotide substitution.

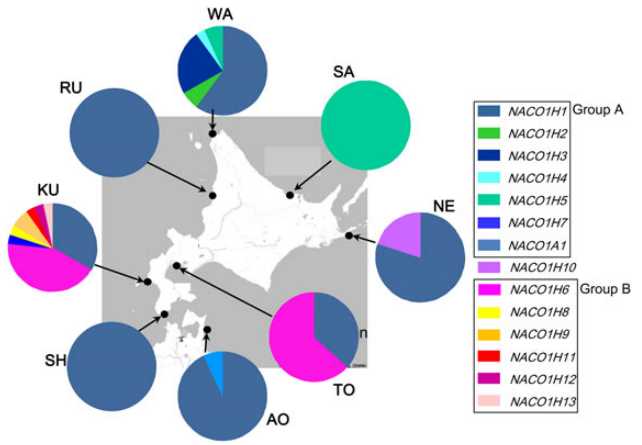


Figure 4. Distribution of the mtDNA COI haplotypes in each sampling locality of *Neptunea arthritica*. Note that six of eight samples have only one or two haplotypes, and distant localities RU and SH share only *NACO1H1*.

groups, indicating lineage sorting within each group, and the star-like shapes (with core and derived haplotypes) in each group suggest recent radiation.

Based on the GTR+G+I model, the genetic distance was estimated to be 6.7% between EU883634 (the outgroup *N. eulimata*) and *NACO1H1*. Given the 11 Mya separation of subgenus *Barbitonia* from other *Neptunea*, the divergence rate per million years was estimated to be 0.609%. The genetic distance between Groups A and B was 1.6–2.3% and their divergence time was therefore estimated to be 4.67–2.65 Mya, during the Pliocene. The divergence times of haplotypes within each group (0.2–0.4% difference) were estimated to be 0.3–0.65 Mya, during the Pleistocene.

Genetic population structure

The haplotype distribution within samples is shown in Figure 4 and in the Supplementary material. The haplotype *NACO1H1* was common among the examined samples, except for SA. The

Table 2. Pairwise F_{ST} between *Neptunea arthritica* samples based on partial COI sequences.

	NE	SA	WA	RU	KU	SH	TO
NE							
SA	**0.763**						
WA	**0.153**	**0.813**					
RU	0.172	**1.000**	0.118				
KU	**0.422**	**0.714**	**0.549**	**0.590**			
SH	0.172	**1.000**	**0.118**	0.000	**0.590**		
TO	**0.445**	**0.739**	**0.574**	**0.621**	−0.0315	**0.621**	
AO	0.156	**0.968**	**0.103**	0.041	**0.574**	0.041	**0.604**

Bold letter indicates significant deviation from 0 at $P < 0.01$ after Bonferroni correction. For sample localities, see Figure 1.

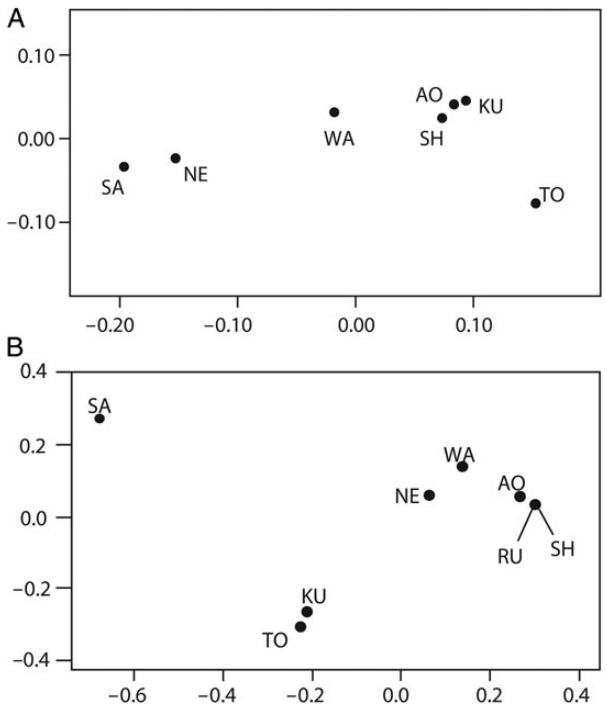


Figure 5. Nonmetric multidimensional scaling plots of *Neptunea arthritica* samples with pairwise F_{ST} values. **A.** Based on five microsatellite loci (Azuma *et al.*, 2011). **B.** Based on a 428-bp sequence of partial mtDNA COI. In **A** the plot suggests a population structure with a one-dimensional genetic cline, from eastern and northeastern Hokkaido to southern Hokkaido and northernmost Honshu (Azuma *et al.*, 2011).

SA sample contained only *NACO1H5*, derived from *NACO1H1* by two substitutions (Fig. 3). Haplotypes from Group A occurred in every sample, whereas haplotypes from Group B were found in only two samples, KU and TO. Haplotype *NACO1H10*, connecting the two groups, occurred only in NE.

As shown in Table 1, h was moderate and π was low. Both h and π were highest in KU, which had two Group A and six Group B haplotypes (Supplementary material), followed by WA, which had five Group A haplotypes. The WA sample showed low π because of lack of haplotypes from Group B. The third highest h and second highest π were observed in TO. Three samples were monomorphic (h and $\pi = 0$), SA (only *NACO1H5*), RU (only *NACO1H1*) and SH (only *NACO1H1*). This diversity profile contrasts with the results of previous microsatellite analysis (Azuma *et al.*, 2011; Table 1), in which the expected heterozygosity in each sample (0.577–0.729) was

comparable with the total estimate (0.673). F_{ST} analysis (Table 2) revealed that 20 out of 28 pairs of samples were genetically different, differing strikingly from the results of microsatellite analysis (Azuma *et al*., 2011). In nMDS plots, the F_{ST} estimates using microsatellite markers showed a correlation between the geographic and genetic structure (Fig. 5A), whereas the genetic distance of mtDNA haplotypes between samples showed no geographical correlation (Fig. 5B). In the latter, the SH sample was separated from neighbouring KU and TO, but overlapped with RU, whereas KU and TO were in close proximity. The SA sample was distant from other populations, probably reflecting the exclusive occurrence of *NACO1H5* but lack of *NACO1H1*, a major common component in the other populations.

AMOVA failed to support any hierarchical structure in the categories 1, 2 and 3 suggested by haplotype distribution and geography ($P = 0.15$, 0.12 and 0.51, respectively).

The Mantel test did not show a significant correlation between genetic (F_{ST}) and geographic distances ($P = 0.22$), indicating that according to mtDNA data the populations did not follow the IBD model.

DISCUSSION

mtDNA phylogeny and phylogeography of Neptunea arthritica

Haplotype distribution was heterogeneous among the localities, and the localization of lineages was probably due to the historical dispersal pattern. Considering the limited distribution of Group B, only in KU and TO in southern Hokkaido, and the results of F_{ST} analysis using microsatellite DNA (Fig. 5), genetic differentiation between southern and northern population is plausible. Haplotype *NACO1H10*, present in the centre of the haplotype network and thus potentially ancestral to all other haplotypes, was found only in NE, possibly indicating that the species originated in the Kuril Islands to the east. However, neither F_{ST} analysis, AMOVA or the IBD test revealed significant geographic-genetic structure in mtDNA. The loss of genetic diversity in some populations may explain the lack of structure in these analyses. It is likely that SH may have possessed haplotypes from Group B in the past, similar to neighbouring KU and TO. If haplotypes from Group B had remained in the SH population, the geographic structure would have been one of clear north-south differentiation. The possible cause of the genetic loss in SH, genetic drift, is discussed.

Sharing of some Group A sequences and two retrieved from the databank in both *N. arthritica arthritica* and *N. a. cumingii* indicates that the two are genetically close. *Neptunea cumingii* has sometimes been considered to be a full species (WoRMS Editorial Board, 2014); however, Hou *et al*. (2013) suggested that *N. cumingii* and *N. a. cumingii* are the same species based on phylogenetic analysis of mtDNA and nuclear DNA. Combined the results of Hou *et al*. (2013) and the present study suggest that *N. a. cumingii* is most appropriately regarded as a subspecies or geographic form of *N. arthritica*.

Discordance of mtDNA and microsatellite DNA phylogeography of N. arthritica

The population genetics inferred from mtDNA analysis was not consistent with that inferred from our previous microsatellite analysis (Azuma *et al*., 2011). The samples used in the two analyses were largely the same, and results from microsatellites demonstrated Hardy-Weinberg equilibrium in the samples using several alleles. Thus, the discordance of the results from two markers was not due to artefacts arising from sampling or laboratory work. Recent genetic drift is the most plausible reason for the discordance.

In our previous microsatellite analyses, each sample of *N. arthritica* showed genetic diversity ($H_E = 0.577$–0.729) that was comparable with the estimated total diversity ($H_E = 0.673$) and the genetic structure was correlated with geography (Supplementary material; Azuma *et al*., 2011: figs 3, 4). In contrast, the present mtDNA analyses gave different population genetic profiles: three of eight samples (SA, RU and SH) were monomorphic ($h = 0$ and $\pi = 0$), therefore showing far lower diversity than the total estimation ($h = 0.57$ and $\pi = 0.0061$) (Table 1). Such a situation can be considered to be a result of the bottleneck caused by a founder effect or genetic drift in a small local population. In some species with low dispersal ability and small local population size, local populations are often fixed for one or a few haplotypes, as seen in the Japanese crayfish (Koizumi *et al*., 2012). Such species usually show apparent genetic-geographic correlation, and it seems reasonable that low dispersal ability is the cause of both low genetic diversity within populations and geographic structure among them, probably by stepwise migration in their evolutionary history. However, in *N. arthritica*, the departure from the IBD model (Mantel test), negative AMOVAs and nMDS plots based on F_{ST} revealed no genetic-geographic correlation. It is conceivable that the observed mtDNA phylogeographic pattern in *N. arthritica* was influenced by very recent genetic drift. Genetic drift acting stochastically left a small number of genotypes (Harrison, 1989) and the natural genetic structure related to geography was therefore hidden. Thus, genetic drift could be a reason for the genetic-geographic inconsistency as well as for the lack of genetic diversity in some *N. arthritica* populations. If the bottleneck occurred a long time ago, genetic diversity should have recovered even in mtDNA as a result of gene flow, as suggested by our microsatellites analysis (Azuma *et al*., 2011). Therefore the genetic drift is thought to be recent. Possible causes of the bottleneck in *N. arthritica* include natural biotic and abiotic factors, e.g. predation, parasitism, disease, change in climate and topology and human impact such as exploitation, environmental modification and pollution. Among these, the human impact of overfishing and imposex (caused by TBT pollution) is considered to be the most plausible cause of the contrasting results obtained from mtDNA and microsatellite analyses. In the *N. arthritica* population around Hokkaido, overfishing and TBT pollution were reported to be the causes of the extreme population decline in the 1970s and 1980s (Fujinaga *et al*., 2006; Miranda *et al*., 2007, 2009) and are related to the skewed sex ratio of reproductive adults. Recently, Toews & Brelsford (2012) reviewed 126 studies exhibiting discordant phylogeography of mtDNA and nuclear DNA ('mito–nuclear discordance') in animal species. They concluded that the most frequent reason for mito–nuclear discordance was sex-biased asymmetry, including sex-biased offspring production, and that only very rare cases could be explained solely by genetic drift in both sexes and the small effective population size of mtDNA. Such sex-biased asymmetry could be the reason for the striking mito–nuclear discordance in *N. arthritica*. The sex ratio (male/female) in caenogastropods has been generally reported to be 1 : 1 (e.g. Hughes, 1986; Power & Keegan, 2001; Ilano, Fujinaga & Nakao, 2003) and that in *N. arthritica* was reported to be 0.82 in 2003–2004 in Lake Saroma (Miranda *et al*., 2009). This suggests that female numbers are equal to or greater than male numbers in stable *N. arthritica* populations. Miranda *et al*. (2009) also observed that almost all normal adult females (i.e. without imposex or parasites) had abundant sperm in their capsule glands in April–June 2003 and June 2004 in Lake Saroma, suggesting that all mature females participate in annual reproduction. Thus, it is not likely that fewer females than males produce offspring under normal conditions. However, if imposex occurs in females, it causes sex-biased asymmetry in reproduction—a decrease in the number of females involved in reproduction.

Thus we conclude that the recent imposex caused by TBT pollution and resulting matrilineal decline are the most plausible causes of mito–nuclear discordance in *N. arthritica*. Fujinaga *et al.* (2006) reported recovery from imposex in *N. arthritica* populations around Hokkaido after the use of TBT was banned. In addition, the microsatellite analyses of *N. arthritica* revealed a substantial level of genetic diversity (Azuma *et al.*, 2011), suggesting a rapid recovery of genetic diversity in the nuclear genome following the genetic disturbance of TBT-induced imposex. A similar result has been shown in *Nucella lapillus* (Colson & Hughes, 2004). Nevertheless, the present mtDNA analysis suggests that a bottleneck effect caused by TBT pollution is still responsible for the low diversity in matrilines of *N. arthritica* around Hokkaido.

Another cause of disruption of the natural sex ratio is overfishing. Fujinaga *et al.* (2006) described an exceptionally high sex ratio (male/female >1.4) at four localities around Hokkaido in 2002, attributing it to greater fishing pressure on females than on males. As an example, in Hiyama district in southern Hokkaido, the Fisherman's Cooperative Association prohibits the catch of sea snails, mainly *N. arthritica*, <6 cm in shell height. The size of mature shells was reported to be 50 mm in male *N. arthritica* and 60 mm in females at Usu Cove (Fujinaga, 2003) and 60 and 75 mm, respectively, in Lake Saroma (Miranda *et al.*, 2009). In this way, size-selective fisheries may also be sex-selective, removing more reproductive females than males. Other factors, such as anthropogenic transplantation and/or population reduction by parasite infection, are not likely to be responsible for the mito–nuclear discordance in *N. arthritica*, because these factors should affect microsatellite DNA variation as well as mtDNA. The level of genetic impact of TBT pollution and/or overfishing probably differs between localities. Some of the examined populations (WA, KU and TO) have maintained a high level of genetic diversity (h), suggesting that the negative impact was low in these populations.

The population in Lake Saroma (SA) showed only a single mtDNA haplotype and the lowest microsatellite diversity among the samples. Low genetic diversity in both markers is attributable to specific reasons in this population, namely a recent founder effect and parasite infection, in addition to the general causes of TBT pollution and overfishing. In SA, haplotype *NACOH1*, which was otherwise ubiquitous and the most abundant in the total of samples, was not found. All individuals had haplotype *NACO1H5*, which was found elsewhere in only two individuals in WA, the closest sampled population to SA (Fig. 1). The founder effect caused by recent establishment of this population probably explains this particular case. Lake Saroma is connected with the Sea of Okhotsk by a channel, which was first opened in 1929. The diatom assemblages and sedimentary ages from borehole samples have revealed that the salinity of Lake Saroma increased in 1929 (Kashima, 1996), thereby indicating that the time of migration or introduction of *N. arthritica*, was after 1929, since the species is unable to survive in low salinity. It is likely that many or all of the founders derived from a source population carried haplotype *NACO1H5*. Because two individuals in WA also had this haplotype, it is not likely that *NACO1H5* originally evolved in SA. Of course, imposex, overfishing and parasite infection threatened this population as well as others and might have enhanced the founder effect, reducing genetic diversity. Severe parasite infection has been observed in SA (Miranda *et al.*, 2009) and may be more severe than elsewhere around Hokkaido because of the enclosed nature of the locality.

Evolutionary history of N. arthritica

The loss of genetic diversity in many populations makes it difficult to reconstruct the evolutionary history of *N. arthritica* around Hokkaido in detail. The differentiation into Groups A and B was estimated as 4.67–2.65 Mya, during the Pliocene, at the onset of global cooling. This dating does not contradict the earliest fossil record of *N. arthritica* in a Pliocene deposit (Amano, 1997). By the late Pliocene, many endemic molluscan species characterized the Omma–Manganji fauna in the Sea of Japan (Otuka, 1939; Amano, 2007). Their appearance was influenced by isolation of the Sea of Japan, which was semi-closed by a land bridge connecting the Korean Peninsula with Kyushu at the same time as the uplifting of the backbone range of mountains on the Japanese Archipelago (Chinzei, 1978) and these events may also have enhanced diversification in *N. arthritica*. As hypothesized by Amano (1997), the eurythermal capacity of *N. arthritica* may have allowed it to survive through the drastic climatic and topological changes in the Pleistocene, when many sympatric *Neptunea* species went extinct. The several missing haplotypes in the branches leading to Groups A and B in the haplotype network (Fig. 3) may suggest that *N. arthritica* lost many lineages at this time. At present, Group B haplotypes are found only in southern Hokkaido, suggesting that Groups A and B were established allopatrically and subsequently came back into contact. Diversification within each group started in the middle Pleistocene; however, the star-like shape of each group in the haplotype network may indicate more recent radiation. In the present study, there appeared to be no reproductive isolation between Groups A and B, because no deviation from Hardy-Weinberg equilibrium was found at any of the microsatellite DNA loci in KU or TO (Azuma *et al.*, 2011: appendix), both of which included Groups A and B (Fig. 4; Supplementary material).

The restricted gene flow found in the microsatellite analyses suggests that local populations are somewhat isolated from each other, with a small number of migrants responsible for gene flow. Each local population reflects the balance of such isolation and migration, so that local decline can potentially be reversed by recruitment from neighbouring populations. Some local populations have shrunk since the 1970s (Kawai *et al.*, 1994; Fujinaga *et al.*, 2006) because of TBT pollution and/or overfishing and this, as argued above, is reflected by low genetic diversity of mtDNA; this has erased important genetic evidence for reconstructing the precise evolutionary history of this species. At present, the population size appears to be recovering in each locality, but matrilineal genetic diversity does not appear to have recovered. Such recovery may occur with gene flow in the future, but will take much longer than recovery of the population size.

Conclusion

In the present study, comparison of mtDNA data with that from microsatellite DNA has indicated that sex-biased asymmetry in population genetics of *N. arthritica* was probably affected by anthropogenic pollution and fishing pressure. The legislation prohibiting use of TBT as an antifouling agent for coastal boats and aquaculture facilities was implemented in 1990 in Japan, and many countries, including Japan, ratified a total TBT ban. However, the effect may persist for a considerable period of time. Both TBT pollution and overfishing were stopped around Hokkaido >15 years before sample collection for the present study; however, matrilineal genetic diversity of *N. arthritica* has not recovered. It is important to recognize that human impact may cause long and possibly irreversible modifications in ecosystems, particularly in species forming discrete and relatively small local populations, such as *N. arthritica*.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

ACKNOWLEDGEMENTS

This work was partly supported by MEXT Revitalization Project for the Creation of Fisheries Research and Education Center in Sanriku. The authors thank Enago (www.enago.jp) and editor David Reid for the English language review.

REFERENCES

AMANO, K. 1997. Biogeography of genus *Neptunea* (Gastropoda: Buccinidae) from the Pliocene and lower Pleistocene of the Japan Sea borderland. *Paleontological Research*, **1**: 274–284.

AMANO, K. 2007. The Omma-Manganji fauna and its temporal change. *Fossils*, **82**: 6–12. (In Japanese with English abstract)

AVISE, J.C. 2000. *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge.

AZUMA, N., MIRANDA, R.M., GOSHIMA, S. & ABE, S. 2011. Genetic population structure of Neptune whelk in northern Japan inferred from microsatellite DNA variation. *Plankton and Benthos Research*, **6**: 42–50.

BIGATTI, G., PRIMOST, M.A., CLEDON, M., AVERBUJ, A., THEOBALD, N., GERWINSKI, W., ARNTZ, W., MORRICONI, E. & PENCHASZADEH, P.E. 2009. Biomonitoring of TBT contamination and imposex incidence along 4700 km of Argentinean shoreline (SW Atlantic: from 38S to 54S). *Marine Pollution Bulletin*, **58**: 695–701.

BLACKMORE, G. 2000. Imposex in *Thais clavigera* (Neogastropoda) as an indicator of TBT (tributyltin) bioavailability in coastal waters of Hong Kong. *Journal of Molluscan Studies*, **66**: 1–8.

CHINZEI, K. 1978. Neogene molluscan faunas in the Japanese Islands: an ecologic and zoogeographic synthesis. *Veliger*, **21**: 155–170.

CLEMENT, M., POSADA, D. & CRANDALL, K.A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**: 1657–1659.

COLSON, L. & HUGHES, N. 2004. Rapid recovery of genetic diversity of dogwhelk (*Nucella lapillus* L.) populations after local extinction and recolonization contradicts predictions from life-history characteristics. *Molecular Ecology*, **13**: 2223–2233.

EXCOFFIER, L., LAVAL, G. & SCHNEIDER, S. 2005. Arlequin v. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**: 47–50.

EXCOFFIER, L., SMOUSE, P.E. & QUATTRO, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**: 479–491.

FENBERG, P.B. & ROY, K. 2008. Ecological and evolutionary consequences of size-selective harvesting: how much do we know? *Molecular Ecology*, **17**: 209–220.

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**: 294–297.

FREELAND, J.R. 2005. *Molecular ecology*. John Wiley, Chichester.

FUJINAGA, K. 2003. Ecological studies on the life history of the Neptune Whelk *Neptunea arthritica*. *Memoirs of the Graduate School of Fisheries Sciences, Hokkaido University*, **50**: 1–61.

FUJINAGA, K., ILANO, A.S., NOMURA, H., MIRANDA, R.T. & NAKAO, S. 2006. Present state of imposex in neptune whelk *Neptunea arthritica* inhabiting shallow waters around Hokkaido, Japan. *Fisheries Science*, **72**: 995–1003.

FUJINAGA, K. & NAKAO, S. 1996. Migration pattern of *Neptunea arthritica* Bernardi, with special reference to relations with reproductive and feeding activities. *Japanese Journal of Ecology*, **44**: 331–338. (In Japanese with English abstract)

GIBBS, P.E. 1996. Oviduct malformation as a sterilising effect of tributyltin (tbt)-induced imposex in *Ocenebra erinacea* (Gastropoda: Muricidae). *Journal of Molluscan Studies*, **62**: 403–413.

GUINDON, S. & GASCUEL, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52**: 696–704.

HARRISON, R.G. 1989. Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Trends in Ecology & Evolution*, **4**: 6–11.

HOU, L., DAHMS, H.-U., DONG, C., CHEN, Y., HOU, H., YANG, W. & ZOU, X. 2013. Phylogenetic positions of some genera and species of the family Buccinidae (Gastropoda: Mollusca) from China based on ribosomal RNA and COI sequences. *Chinese Science Bulletin*, **58**: 2315–2322.

HUGHES, R.N. 1986. *A functional biology of marine gastropods*. John Hopkins University Press, Baltimore.

ILANO, A.S., FUJINAGA, K. & NAKAO, S. 2003. Reproductive cycle and size at sexual maturity of the commercial whelk *Buccinum isaotakii* in Funka Bay, Hokkaido, Japan. *Journal of the Marine Biological Association of United Kingdom*, **83**: 1287–1294.

KASHIMA, K. 1996. Diatom assemblages from lake sediments of Lake Abashiri and Lake Saroma, Hokkaido, northern part of Japan. *Laguna*, **3**: 33–39. (In Japanese with English abstract)

KAWAI, K., YAMAGUCHI, S., IDE, N., GOSHIMA, S. & NAKAO, S. 1994. Reproductive cycle and parasite infection in the Neptune whelk *Neptunea arthritica* in Lagoon Saroma. *Venus*, **53**: 105–112.

KENDAL, N.W. & QUIN, T.P. 2013. Size-selective fishing affects sex ratios and the opportunity for sexual selection in Alaskan sockeye salmon *Oncorhynchus nerka*. *Oikos*, **122**: 411–420.

KOIZUMI, I., USIO, N., KAWAI, T., AZUMA, N. & MASUDA, R. 2012. Loss of genetic diversity means loss of geological information: the endangered Japanese crayfish exhibits remarkable historical footprint. *PLoS ONE*, **7**: e33986.

KUMAR, S. 2005. Molecular clocks: four decades of evolution. *Nature Reviews Genetics*, **6**: 654–662.

LOMBARDO, R.C. & GOSHIMA, S. 2010. Female copulatory status and male mate choice in *Neptunea arthritica* (Gastropoda: Buccinidae). *Journal of Molluscan Studies*, **76**: 317–322.

MIRANDA, R.M., FUJINAGA, K., ILANO, A.S. & NAKAO, S. 2007. Incidence of imposex and parasite infection in *Neptunea arthritica* at Saroma Lagoon and their relationship. *Aquaculture Science*, **55**: 9–15.

MIRANDA, R.M., FUJINAGA, K., ILANO, A.S. & NAKAO, S. 2009. Effects of imposex and parasite infection on the reproductive features of the Neptune whelk *Neptunea arthritica*. *Marine Biology Research*, **5**: 268–277.

MIRANDA, R.M., FUJINAGA, K. & NAKAO, S. 2008. Age and growth of *Neptunea arthritica* estimated from growth marks in the operculum. *Marine Biology Research*, **4**: 224–235.

MIZUSHIMA, T. & TORISAWA, M. 2003. *Fisheries and aquatic life in Hokkaido*. Hokkaido Shimbun Press, Sapporo, Japan. (In Japanese)

MOORE, W.S. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution*, **49**: 718–726.

NAKANO, T., KURIHARA, Y., MIYOSHI, H. & HIGUCHI, S. 2010. Molecular phylogeny of *Neptunea* (Gastropoda: Buccinidae) inferred from mitochondrial DNA sequences, with description of a new species. *Venus*, **68**: 121–137.

OKUTANI, T. 2000. *Marine molluscs in Japan*. Tokai University Press, Kanagawa.

OTUKA, Y. 1939. Mollusca from Cainozoic systems of eastern Aomori Prefecture. *Journal of the Geological Society of Japan*, **44**: 23–31.

PAVONI, B., CENTANNI, E., VALCANOVER, S., FASOLATO, M., CECCATO, S. & TAGLIAPIETRA, D. 2007. Imposex levels and concentrations of organotin compounds (TBT and its metabolites) in *Nassarius nitidus* from the Lagoon of Venice. *Marine Pollution Bulletin*, **55**: 505–511.

POSADA, D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**: 1253–1256.

POWER, A.J. & KEEGAN, B.F. 2001. Seasonal patterns in the reproductive activity of the red whelk, *Neptunea antiqua* (Mollusca: Prosobranchia) in the Irish Sea. *Journal of the Marine Biological Association of the United Kingdom*, **2**: 243–250.

RONQUIST, F. & HUELSENBECK, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**: 1572–1574.

ROWE, R. & HUTCHINGS, J.A. 2003. Mating systems and the conservation of commercially exploited marine fish. *Trends in Ecology & Evolution*, **18**: 567–571.

SUZUKI, K., HIRAISHI, T., YAMAMOTO, K. & NASHIMOTO, K. 2002. Estimation of natural mortality and exploitation rates of whelk *Neptunea arthritica* by multiple tagging experiment. *Fisheries Science*, **68**: 87–94.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**: 4876–4882.

TOEWS, D.P. & BRELSFORD, A. 2012. The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology*, **21**: 3907–3930.

WEIR, B.S. & COCKERHAM, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution*, **38**: 1358–1370.

WoRMS EDITORIAL BOARD. 2014. World Register of Marine Species. Available from http://www.marinespecies.org at VLIZ (accessed 14 January 2014).

WRIGHT, S. 1943. Isolation by distance. *Genetics*, **28**: 114–138.