



Title	Phylogeography of Neptune whelk (<i>Neptunea arthritica</i>) suggests sex-biased impact of tributyltin pollution and overfishing around northern Japan
Author(s)	Azuma, Noriko; Miranda, Richard M.; Goshima, Seiji; Abe, Syuiti
Citation	Journal Of Molluscan Studies, 81, 131-138 https://doi.org/10.1093/mollus/eyu068
Issue Date	2015-02
Doc URL	http://hdl.handle.net/2115/59770
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Journal of Molluscan Studies following peer review. The definitive publisher-authenticated version J. Mollus. Stud. (2015) 81 (1): 131-138 is available online at: http://mollus.oxfordjournals.org/content/81/1/131 .
Type	article (author version)
File Information	JMS2013115txt(1).pdf



[Instructions for use](#)

1 PHYLOGEOGRAPHY OF NEPTUNE WHELK (*Neptunea arthritica*) SUGGESTS SEX-BIASED
2 IMPACT OF TRIBUTYLTIN POLLUTION AND OVERFISHING AROUND NORTHERN
3 JAPAN.

4

5 NORIKO AZUMA^{1*}, RICHARD M. MIRANDA², SEIJI GOSHIMA¹ AND SYUITI ABE³

6

7 ¹*Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1, Minato-cho, Hakodate*
8 *041-8611, Japan*

9 ²*Universidad Austral de Chile, Sede Puerto Montt, Región de Los Lagos, Chile.*

10 ³*Sanriku Fisheries Research Center, Department of Revitalization for Sanriku-region, Iwate*
11 *University, 3-75-1 Heita, Kamaishi 026-0001, Japan*

12

13

14

15 Running head: PHYLOGEOGRAPHY OF NEPTUNEA WHELK

16

17

18

19

20

21

*Correspondence: Noriko Azuma, E-mail: nazu@fish.hokudai.ac.jp

22

ABSTRACT

23 The Neptune whelk, *Neptunea arthritica*, is a sublittoral sea snail from Pacific waters that has been
24 a food resource and is commercially important for the coastal fisheries in northern Japan. This
25 species showed a severe decline during the 1970s and 1980s, possibly because of overfishing,
26 imposex caused by tributyltin (TBT) pollution and parasite infection. In the present study, we
27 investigated genetic variation among the populations of *N. arthritica* from eight localities in
28 northern Japan, including Hokkaido and Aomori, using a mitochondrial DNA (mtDNA) marker,
29 partial sequence of the cytochrome *c* oxidase subunit I (COI) gene, to compare the obtained results
30 with those from previous microsatellite analyses. We also addressed the evolutionary history of *N.*
31 *arthritica* and human impact on the population genetic profiles of this species. The parsimony
32 network showed 14 COI haplotypes separated into 2 groups (Groups A and B), with an intermediate
33 haplotype connecting both of the groups. Among eight populations, six were fixed for only one or
34 two haplotypes, and any geographic–genetic correlation was not found; they were probably
35 affected by random drift of the mtDNA lineage. Thus, the results from mtDNA contrasted with
36 those from previous microsatellite analysis, indicating that geographic structure was affected by the
37 restricted gene flow between populations. Our results suggested that *N. arthritica* diversified into
38 Groups A and B during the Pliocene; however, recent TBT pollution and size-selective fishing
39 pressure have reduced genetic diversity and concealed the natural population structure. The present

40 study also suggested that human impact may cause long and possibly irreversible modification of
41 ecosystems, particularly for species forming discrete and relatively small local populations, such as
42 *N. arthritica*. Thus, the combined use of mtDNA and microsatellite genetic data provides a
43 powerful tool to investigate the health of biodiversity in molluscs and shows that the output results
44 of such analyses are of great interest for the conservation and management of molluscan species.
45

INTRODUCTION

47 *Neptunea arthritica* (Bernardi, 1857) is a dioecious gastropod with internal fertilisation and
48 direct development in the sublittoral zone to a depth of a few tens of metres. The egg masses are
49 deposited on hard substrata such as rocks and boulders, and it takes 3 years or more for maturation
50 (Fujinaga, 2003). The typical *N. arthritica* (*N. arthritica arthritica*) is distributed in the Pacific
51 Ocean, the Sea of Japan and the Sea of Okhotsk along the coasts of northern Japan and Sakhalin in
52 southern Russia, whereas a subspecies *N. arthritica cumingii* (hereafter *N. a. cumingii*) is found
53 from the western part of the Sea of Japan to the East China and Yellow Sea (Okutani, 2000), with
54 the range partly overlapping with typical *N. arthritica*. Sea snails, including *N. arthritica*, have
55 been a food resource and commercially important in the coastal fisheries in northern Japan
56 (Mizushima & Torisawa, 2003); thus, several biological studies of *N. arthritica* have been mainly
57 conducted for resource management (Kawai *et al.*, 1994; Fujinaga & Nakao, 1996; Suzuki *et al.*,
58 2002; Fujinaga, 2003; Fujinaga *et al.*, 2006; Miranda *et al.*, 2007 & 2009; Miranda, Fujinaga &
59 Nakao, 2008; Lombardo & Goshima, 2010). However, none of the population genetic studies had
60 appeared before our recent microsatellite DNA analysis in *N. arthritica* around Hokkaido (Azuma
61 *et al.*, 2011). Using five loci of microsatellite DNA markers in seven populations of *N. arthritica*
62 around Hokkaido, we suggested the restricted gene flow among populations with increasing genetic
63 differentiation among populations separated by increasing geographic distances, *i.e.* following the

64 isolation-by-distance model (Azuma *et al.*, 2011). The observed restricted gene flow between local
65 populations was most probably influenced by the balance of the transport force of sea water and the
66 low level of dispersal potential of this species (Azuma *et al.*, 2011). Therefore, the suggested
67 genetic structure was considered to be a result of natural distribution without strong anthropogenic
68 disturbance.

69 However, the microsatellite data could not provide much knowledge regarding phylogeny and
70 evolutionary history of this species in a palaeontological time scale. To reconstruct the evolutionary
71 history of *N. arthritica*, we chose nucleotide sequence variation in the 5' portion of the cytochrome
72 *c* oxidase subunit I (COI) gene in the mitochondrial genome as a genetic marker. This marker was
73 used in the present study for the following reasons: (1) Genetic markers from mtDNA have an
74 advantage in genealogy analyses because they lack recombination and uniparental (maternal)
75 inheritance (which results in the absence of heterozygotes); this makes it feasible to clarify lineages
76 in comparison with markers from nuclear DNA (Harrison, 1989; Avise, 2000; Freeland, 2005). (2)
77 The COI region examined in the present study showed sufficient variation within species and
78 included a barcoding portion where sequence data were accumulated in many taxa; thus, it was
79 used to compare sequences with those from other species. (3) mtDNA sequence data are available
80 for molecular clock estimation, from which the divergence time of lineages can be estimated
81 (Kumar, 2005).

82 Besides utility for the reconstruction of the evolutionary process, the mtDNA marker shows a
83 higher ability to disclose the past bottleneck effect because of the small effective population size,
84 which is a quarter of that of nuclear DNA (Moore, 1995). This indicates that mtDNA has
85 sufficient sensitivity for detecting past population declines. Previous microsatellite DNA
86 analyses for *N. arthritica* did not reveal any evidence of a recent decline in each population
87 (Azuma *et al.*, 2011). However, around Hokkaido, *N. arthritica* showed a severe decline during the
88 1970s and 1980s, possibly because of overfishing, imposex caused by tributyltin (TBT) pollution
89 and parasite infection (Kawai *et al.*, 1994; Fujinaga *et al.*, 2006; Miranda *et al.*, 2007 & 2009).
90 Using the genetic profile of mtDNA, we can expect to detect such a recent decline better compared
91 with when using microsatellites. In particular, if the main factors for population declines are severe
92 in females, we can expect a drastic reduction in genetic diversity in mtDNA, which represents
93 variability in the matriline. The skewed sex ratio caused by size-selective fishing has been reported
94 in many fishery resource species (Rowe & Hutchings, 2003; Fenberg & Roy, 2008; Kendal & Quin,
95 2013). Similarly, size-selective harvesting, in which larger snails are caught, may cause more
96 serious fishing pressure on females than on males in *N. arthritica* because the maturing size is
97 larger in females than in males (Fujinaga, 2003; Miranda *et al.*, 2008). Imposex induced by TBT
98 was observed in many species of gastropods modifying genitals and sterilizing females (Gibbs,
99 1996; Blackmore, 2000; Pavoni *et al.*, 2007; Bigatti *et al.*, 2009). Thus, human impact, overfishing,

100 and TBT pollution are likely to have affected populations in a sex-biased manner (more severe in
101 females than in males), while parasite infection seemed to damage reproduction in both sexes
102 (Miranda *et al.*, 2009).

103 The present study aimed to genetically characterise *N. arthritica* populations around Hokkaido
104 using an mtDNA marker and to compare the obtained results with those from previous
105 microsatellite analyses to address the following two topics: (1) evolutionary history of *N. arthritica*
106 and (2) human impact on population genetic profiles of this species. For (1), we tried to clarify the
107 evolutionary scenario in the paleontological time scale using mtDNA data, which included genetic
108 diversity, haplotype genealogy and spatial distribution of haplotypes. For (2), we analysed mtDNA
109 data in comparison with results of microsatellite analyses. If the sign of genetic drift such as low
110 level of genetic diversity within population appeared in mtDNA, definitely conflicting with the
111 results from microsatellite DNA, we assumed sex-biased damage by human impact, which was
112 more severe in females than in males in the examined populations.

113

114 MATERIALS AND METHODS

115 *Specimens*

116 We used 238 individuals of *N. arthritica* from seven locations in Hokkaido, namely Wakkanai
117 (WA), Rumoi (RU), Kumaishi (KU) and Shiriuchi (SH) on the Sea of Japan coast; Toyoura (TO)

Table 1.

Fig. 1.

118 and Nemuro (NE) on the Pacific Ocean coast and Saroma (SA) on the Sea of Okhotsk coast, as
119 well as from Aomori (AO) in northernmost Honshu (Table 1, Fig. 1). Hereafter, the term ‘sample’
120 is used for a group of individuals collected from each of the abovementioned localities, as
121 representative of the local population. The samples were identical to those used for our previous
122 microsatellite DNA analysis (Azuma *et al.*, 2011), except for RU, which was recruited in the
123 present study. Genomic DNA of the RU sample was extracted using the Pure Gene Kit (Qiagen)
124 according to the manufacturer’s protocol, as described previously (Azuma *et al.*, 2011), and used
125 for analysis.

126

127 *Nucleotide sequencing*

128 The 5' region of mtDNA COI was amplified by polymerase chain reaction (PCR) in a 30 µl
129 reaction mixture containing template DNA (approximately 500 pg), dNTPs, a pair of primers
130 [LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT
131 TCA GGG TGA CCA AAA AAT CA-3'; Folmer *et al.*, 1994)] and *Taq* DNA polymerase (Sigma),
132 according to the manufacturer’s instructions. The thermal cycling profile included precycling
133 denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at
134 45°C for 45 s and extension at 72°C for 45 s. After electrophoretic examination on a 2% agarose
135 gel, the PCR products were purified with magnetic beads (AMPure, Agencourt), cycle-sequenced

136 using the abovementioned forward and reverse primers and the BigDye[®] Terminator v3.1 Cycle
137 Sequencing Kit (Applied Biosystems) and loaded onto an automated sequencer, ABI PRISMTM
138 3130 (Applied Biosystems). The obtained sequences of both directions were aligned and edited to
139 428 bp using DNASIS-Mac v.3.5 (Hitachi) and ClustalX 1.81 software (Thompson *et al.*, 1997) for
140 defining haplotypes and deposited in the DDBJ/Genbank database with accession Nos.
141 AB432872–AB432884 and AB811355.

142

143 *Molecular phylogeny*
144 A phylogenetic tree of the obtained COI haplotypes was reconstructed using Bayesian algorithm in
145 MrBayes 3.12 (Ronquist & Huelsenbeck, 2003). The sequences with high similarity (>92%) to the
146 obtained data were searched by Basic Local Alignment Search Tool (BLAST) in the
147 DDBJ/Genbank database. HQ834061 (in *N. a. cumingii*), FJ710085 (in *N. arthritica*) and FJ710084
148 (in *N. a. cumingii*) were found and included in the present phylogenetic tree and haplotype network
149 analyses. A COI sequence from *N. eulimata*, accession No. EU883634, was used as an outgroup for
150 phylogenetic analysis. We applied the substitution model GTR + G + I, which was recommended
151 as the best fitting substitution model for our data set by jModelTest 0.1.1 (Posada, 2008; Guindon
152 & Gascuel, 2003). In Bayesian analysis, the posterior probability distribution of trees was
153 approximated by drawing a sample every 100 steps over 1,000,000 Markov chain Monte Carlo

154 (MCMC) cycles, in which the average standard deviation dropped to less than 0.00001, after
155 discarding a burn-in of 250,000 cycles. The length of burn-in was determined by the number of
156 cycles reaching the stability of log likelihood values. The haplotype genealogy within species was
157 resolved with a parsimony network using the TCS Network Program (Clement, Posada & Crandall,
158 2000) under 95% connection limit, with gaps as the 5th state.

159 The divergence time within species was estimated following the calibration detailed in
160 Nakano *et al.* (2010), which assumed that the subgenus *Barbitonia*, including *N. arthritica*,
161 diverged from other *Neptunea* species approximately 11 million years ago (MYA) on the basis of
162 reliable fossil records of the oldest *Barbitonia*.

163

164 *Population genetic analyses*

165 We used a program package of Arlequin version 3.1 (Excoffier, Laval & Shneider, 2005) to
166 estimate haplotype (h) and nucleotide diversity (π) in each sample and to detect genetic
167 differentiation among samples by the calculation of pairwise F_{ST} (Weir & Cockerham, 1984).
168 Genetic differentiation between the samples was visualised on a two-dimensional surface by
169 non-metric multidimensional scaling (nMDS) plotting on the basis of pairwise F_{ST} using the
170 statistical software R version 2.9.0 (R Development Core Team). To test the significance of the
171 hierarchical population structure, analysis of molecular variance (AMOVA; Excoffier, Smouse &

172 Quattro, 1992) was conducted with Arlequin version 3.1 (Excoffier *et al.*, 2005) assuming the three
173 categories that were suggested by haplotype distribution and geography: 1. [WA, SA, RU] and [KU,
174 SH, AO, TO, NE], 2. [WA, SA, RU, NE] and [KU, SH, AO, TO] and 3. [WA, SA, RU], [KU, SH,
175 AO, TO] and [NE].

176 Evaluation of the isolation-by-distance (IBD) model (Wright, 1943) to assess the level of
177 gene flow was performed using the abovementioned Arlequin program. For the IBD test, the
178 geographic distance between sample locations was determined from the putative migration routes
179 of whelks (Fig. 1). The distance matrix determined in this manner was compared with the F_{ST}
180 matrix, and the significance of correlations was evaluated by the Mantel test.

181

182 **RESULTS**

183

184 *COI sequence variation and haplotype genealogy*

185 PCR amplification of approximately 650-bp fragments was not always successful in the examined
186 whelk specimens, probably because of the low quality of extracted DNA. To eliminate unreliable
187 sequences, a confirmed part of the 428-bp sequence was used for haplotype identification. Thus, 19
188 polymorphic nucleotide sites were found in the aligned sequences of COI from 238 analysed
189 individuals, which defined a total of 14 haplotypes, *NACO1HI–H13* and *NACO1A1* (Fig. 2). The

Fig. 2.

190 results of BLAST search revealed that the most frequent haplotype in our analysis, *NACOIH1*, was
191 identical to five 428-bp sequences in the DDBJ/Genbank database (accession Nos. JN053005,
192 JN053006 and EU883627 from *N. a. cumingii*; EU883629 from *Neptunea* sp.1 and FJ710078 from
193 *N. arthritica*). In the BLAST search, we also found that the database sequence of FJ710085 for *N.*
194 *arthritica* was identical to the sequences of *N. arthritica* (AB498776, AB498777 and AB498778)
195 and *N. a. cumingii* (FJ710083 and FJ710079). Thus, typical *N. arthritica* and *N. a. cumingii* shared
196 at least two haplotypes, *NACOIH1* and FJ710085 (Fig. 2). In the Bayesian tree (Fig. 2), all the
197 haplotype sequences observed herein and those from databank, except for EU883634, were
198 separated in three clusters, Group A (*NACOIH1–5*, *NACOIH7*, *NACOIA1* and *HQ83061* from
199 databank), Group B (*NACOIH6* and *NACOIH8–13*) and the third group consisting of two other
200 databank sequences, FJ710085 and FJ710084, whereas *NACOIH10* was intermediate to these
201 groups. Although the posterior probabilities for Group A and the third group (0.61 and 0.80,
202 respectively) were not high enough to support the monophyly, the three groups were discriminated
203 in a parsimony haplotype network (see below).

Fig. 3.

204 The haplotype network (Fig. 3) was three forked, also showing two groups of haplotypes as
205 seen in the Bayesian tree, Groups A and B, with core haplotypes (*NACOIH1* and *NACOIH6*) and
206 derived haplotypes around core ones. *NACOIH10* was present in the centre of the network,
207 connecting Groups A and B and the third one containing databank sequences FJ710084 and

208 FJ710085. Several missing haplotypes appeared between groups, indicating lineage sorting within
209 each group, and a star-like shape with core and derived haplotypes in each group suggested recent
210 radiation.

211 Based on the GTR + G + I model, the genetic distance was estimated to be 6.7% between
212 EU883634 (*Neptunea eulimata*) and *NACOIH1*. Given the 6.7% divergence for 11 MYA in the
213 separation of *Barbitonia* from other *Neptunea*, the divergence rate per million years was estimated
214 to be 0.609%. Considering the genetic distance of 1.6%–2.3% between Groups A and B, the
215 divergence time between the two groups was estimated to be 4.67–2.65 MYA during the Pliocene.
216 The divergence time of haplotypes within each group (0.2%–0.4% difference from each other) was
217 estimated to be approximately 0.3–0.65 MYA during the Pleistocene.

218

219 *Genetic population structure*

220 The haplotype distribution within samples is shown in Figure 4 and Supplementary Data. The
221 haplotype *NACOIH1* was common among the examined samples, except for SA. The SA sample
222 contained only *NACOIH5*, which was probably derived from *NACOIH1* with two substitutions
223 (Fig. 3). Haplotypes from Group A occurred in every sample, whereas haplotypes from Group B
224 were found in only two samples, KU and TO. Haplotype *NACOIH10*, connecting both the groups,
225 occurred only in NE.

Fig. 4.

226 As shown in Table 1, the haplotype diversity (h) was moderate, and the nucleotide diversity
227 (π) was low as a whole. Both h and π were the highest in KU, which had two Group A and six
228 haplotypes from Group B (Supplementary Data), followed by WA, which had five haplotypes from
229 Group A. The WA sample showed low π because of a lack of haplotype from Group B. The third
230 highest h and the second highest π were observed in TO. Both h and π were zero in three
231 monomorphic samples, SA (only *NACOIH5*), RU (only *NACOIH1*) and SH (only *NACOIH1*).
232 This diversity profile was contrasting with the results of previous microsatellite marker analysis
233 (Azuma *et al.*, 2011; Table 1), in which the expected heterozygosity in each sample (0.577–0.729)
234 was comparable with the total estimate (0.673).

235 F_{ST} analysis (Table 2) revealed that 20 out of 28 pairs of samples were genetically different (Table 2.
236 bold letter); however, the difference/similarity pattern strikingly differed from the results of
237 previous microsatellite analysis (Azuma *et al.*, 2011). In nMDS plotting, the F_{ST} estimates using
238 microsatellite markers showed a correlation between the geographic and genetic structure (Fig. Fig. 5.
239 5-A), whereas the genetic distance of mtDNA haplotypes between samples did not show a
240 correlation with their spatial distribution (Fig. 5-B). The SH sample was distinctly separated from
241 neighbouring KU and TO but completely overlapped with RU. On the other hand, KU and TO were
242 in close proximity to each other. The SA sample was clearly distant from other populations,
243 probably reflecting the exclusive occurrence of *NACOIH5* but lack of *NACOIH1*, a major common

244 component in the other populations.

245 AMOVA failed to support any of the hierarchical structures in the category 1, 2 and 3

246 suggested by haplotype distribution and geography ($P = 0.15, 0.12$ and 0.51 , respectively).

247 The Mantel test did not show a significant correlation between genetic (F_{ST}) and geographic

248 distance ($P = 0.22$), indicating that the examined *N. arthritica* populations did not follow the IBD

249 model with the current mtDNA data.

250

251 DISCUSSION

252 *mtDNA phylogeny and phylogeography of N. arthritica*

253 Haplotype distribution was heterogeneous among the localities, and the localisation of

254 lineages was probably due to the historical dispersal pattern. Considering the limited distribution of

255 Group B, only in KU and TO in southern Hokkaido, and the results of F_{ST} analysis using

256 microsatellite DNA (Fig. 5), the genetic differentiation between southern and northern population is

257 plausible. The haplotype *NACOIH10*, present in the centre of the haplotype network and thus

258 potentially ancestral to all other haplotypes observed herein, was found only in NE, and it may

259 indicate that the species possibly originated from the east, the Kuril Islands. However, we could not

260 delineate a certain structure or evolutionary process because F_{ST} analyses, AMOVA and IBD test

261 failed to capture a reasonable geographic–genetic structure in mtDNA. The loss of genetic diversity

262 in some populations may hide the structure in these analyses. It is likely that SH may have
263 possessed haplotypes from Group B in the past, similar to neighbouring KU and TO. If haplotypes
264 from Group B had remained in SH populations, the geographic structure would have been easily
265 described as north–south differentiation. The possible cause of the genetic loss in SH, genetic drift,
266 is discussed later, with comparison of results from the present mtDNA and previous microsatellite
267 DNA analyses.

268 Sharing of some Group A sequences and those retrieved from databank (FJ710084 and
269 FJ710085) in both *N. arthritica arthritica* and *N. a. cumingii* indicates that the latter is genetically
270 indistinguishable from the former. Sometimes, the name of *N. cumingii* appeared as a full species
271 (WoRMS Editorial Board, 2014); however, Hou *et al.* (2013) suggested that *N. cumingii* and *N. a.*
272 *cumingii* are the same species in molecular phylogenetic analysis using mtDNA and nuclear DNA.
273 Combined results of Hou *et al.* (2013) and the present study suggest that *N. a. cumingii* and
274 so-called *N. cumingii* are not full species but subspecies or a geographic form of *N. arthritica*.

275
276 *Discordance of mtDNA and microsatellite DNA phylogeography of N. arthritica*
277 The population genetics inferred from mtDNA analysis was not consistent with that inferred
278 from previous microsatellite DNA analysis, and recent genetic drift is the most plausible reason for
279 the discordance. The appropriate sample collection in the present study was proved by various

280 alleles and HWE in each sample in microsatellite analyses using same individuals; thus, the
281 discordance of the results from two markers was not due to the artefact in the field or laboratory
282 works but reflected the actual property of *N. arthritica* around Hokkaido.

283 In our previous microsatellite DNA analyses, each of the examined sample (local population)
284 of *N. arthritica* showed genetic diversity ($H_E = 0.577$ to 0.729) that was comparable with the total
285 diversity estimation ($H_E = 0.673$), and the population structure was correlated to geography
286 (Supplementary File of the present study; figs. 3 and 4 in Azuma *et al.*, 2011). In contrast, the
287 present mtDNA analyses provided different population genetic profiles: three of eight samples (SA,
288 RU and SH) were monomorphic, showing extremely lower diversity ($h = 0$ and $\pi = 0$) than the total
289 estimation ($h = 0.57$ and $\pi = 0.0061$) (Table 1). Such situation can be generally considered to be a
290 result of the bottleneck by a founder effect or genetic drift in a small size of the local population. In
291 some species of a low dispersal ability and small local population size, the local population is likely
292 fixed for one or a few haplotypes, as seen in the Japanese crayfish (Koizumi *et al.*, 2012). Such
293 species usually showed apparent genetic–geographic correlation, and it seems reasonable that the
294 low dispersal ability caused both low genetic diversity within the population and geographic
295 structure among populations, probably by stepwise migration in their evolutionary history.
296 However, in *N. arthritica*, the departure from IBD (Mantel test), negative AMOVAs and
297 unreasonable nMDS plotting pattern based on F_{ST} revealed no genetic–geographic correlation. In

summary, it is conceivable that the observed mtDNA phylogeographical pattern in *N. arthritica* was influenced by very recent genetic drift. Genetic drift stochastically left a small number of genotypes (Harrison, 1989), and the natural genetic structure related to geography may be hidden after the drift. 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. However, if the bottleneck occurred a long time ago, genetic diversity should be more or less recovered even in mtDNA by gene flow, as suggested by our microsatellites analysis (Azuma et al., 2011). Thus the genetic drift was thought to be recent. Possible causes of the genetic drift in *N. arthritica* include natural biotic and abiotic factors, e.g. predation, parasitism, disease, change in climate and topology and human impact such as exploiting, environmental modification and pollution. Among these, the human impact, overfishing and imposex caused by TBT pollution, was 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 specific causes of the extreme population decline in the 1970s and 1980s (Fujinaga et al., 2006; Miranda et al., 2007 &2009), and they were reported to be surely related to the skewed sex ratio in the reproductive stage. Recently, Toews & Brelsford (2012) reviewed 126 studies exhibiting discordant biogeography of mtDNA and nuclear DNA (mito–nuclear discordance) in animal species. They concluded that the most frequent reason for mito–nuclear discordance was

316 sex-biased asymmetries, including sex-biased offspring production, and that very rare cases were
317 able to be solely explained by genetic drift in both sexes and the small effective population size in
318 mtDNA. The sex-biased asymmetry could be the reason for the striking mito–nuclear discordance
319 in *N. arthritica*. The sex ratio (male/female) in prosobranch gastropods has been generally reported
320 to be 1:1 (Hughes, 1986; Power & Keegan, 2001; Ilano, Fujinaga & Nakao, 2003); the ratio in *N.*
321 *arthritica* was reported to be 0.82 in 2003–2004 in Lake Saroma (Miranda *et al.*, 2009). This
322 suggests that the female number is surely equal to or a little greater than the male number in stable
323 *N. arthritica* populations. Miranda *et al.* (2009) also observed that almost all normal adult females
324 (i.e. without imposex or parasites) had abundant sperm in their capsule gland in April–June 2003
325 and June 2004 in Lake Saroma, suggesting that all mature females join the annual reproduction.
326 Thus, it is not likely that a lesser number of females than males produce offspring under normal
327 condition. However, if imposex occurs in females, it causes sex-biased asymmetry in reproduction,
328 a decrease in the number of females involved in reproduction. Thus we conclude that the recent
329 imposex caused by TBT pollution and severe matrilineal decline was considered to be the most
330 plausible cause of mito–nuclear discordance in *N. arthritica*. Fujinaga *et al.* (2006) reported virtual
331 recovery from imposex in *N. arthritica* populations around Hokkaido after banning TBT use. In
332 addition, previous microsatellite DNA analyses in *N. arthritica* (Azuma *et al.*, 2011) and *Nucalla*
333 *lapillus* (Colson & Hughes, 2004) revealed a substantial level of genetic diversity in each

334 population of the two species, suggesting a rapid recovery of genetic diversity in the nuclear
335 genome from the genetic disturbance of TBT-induced imposex. Nevertheless, the present mtDNA
336 analysis suggested that a bottleneck effect caused by TBT pollution is still responsible for the lack
337 of diversity in matrilines of *N. arthritica* around Hokkaido.

338 Another cause of deficiency of mtDNA genetic diversity is overfishing. Fujinaga *et al.*
339 (2006) described an exceptionally high sex ratio (male/female > 1.4) in the four localities around
340 Hokkaido in 2002, attributing it to fishing pressure. If the fishing pressure is higher on females than
341 on males, it reduces the effective population size of females to a greater extent than that of males.
342 For example, in Hiyama district in southern Hokkaido, Fisherman's Cooperative Association
343 prohibits the catch of sea snails, mainly *N. arthritica*, of a small size, i.e. less than 6 cm of shell
344 height. The mature size of shell height was reported to be 50 mm in males and 60 mm in females at
345 Usu Cove (Fujinaga, 2003) and 60 mm in males and 75 mm in females in Lake Saroma (Miranda *et*
346 *al.*, 2009). This may suggest that the fishery restriction as that seen in Hiyama area caused
347 sex-selective fishery, in which more reproductive females would be caught than males. Other
348 factors, anthropogenic transplantation and/or population decline by parasite infection, are not likely
349 to be responsible for the striking mito-nuclear discordance in *N. arthritica* because these factors
350 should affect microsatellite DNA variation as well as mtDNA. The level of genetic impact of TBT
351 pollution and/or overfishing probably differs between localities. Some of the examined populations,

352 WA, KU and TO, have maintained a high level of genetic diversity with regard to the haplotype

353 diversity index h , and it may indicate that the negative impact was low in these populations.

354 The SA population in Lake Saroma showed a low level of genetic diversity both in mtDNA

355 and microsatellite analyses as the monomorphic haplotype component in mtDNA and the lowest

356 genetic diversity in microsatellites. Lack of genetic diversity in both markers is attributable to

357 specific reasons in this population, namely a founder effect in recent population establishment and

358 parasite infection, in addition to TBT pollution and overfishing. In SA, haplotype *NACOHI*, which

359 was ubiquitous and the most abundant in total samples, was not found, and all individuals had

360 haplotype *NACOIH5*, which was found in only two individuals in WA, the closest to SA among the

361 populations examined in the present study (Fig. 1). The founder effect by recent establishment of

362 this population probably caused this particular phenomenon. Lake Saroma is connected with the

363 Sea of Okhotsk by a channel, which was first opened in 1929. The diatom assemblages and data of

364 sedimentary ages from bore hole samples revealed that the salinity of Lake Saroma had increased

365 in 1929 (Kashima, 1996), thereby indicating that the time of migration or introduction of *N.*

366 *arthritica*, the species unable to survive in the low salinity, was after 1929. It is likely that many or

367 all of the founders derived from the source population had *NACOIH5* at that time. Because two

368 individuals in WA also had this haplotype, it is not likely that *NACOIH5* originally evolved in SA.

369 Of course, imposex by TBT pollution, overfishing and parasite infection threatened this population

370 as well as other populations, and it might enhance the founder effect, reducing genetic diversity.

371 Severe parasite infection was observed in SA (Miranda, 2009), and it may be more severe in SA

372 than in other habitat of *N. arthritica* around Hokkaido because of the enclosed water, a feature

373 different from other coastal habitats.

374

375 *Evolutionary history of N. arthritica*

376 As mentioned above, the loss of genetic diversity in many populations makes it difficult to

377 reconstruct the evolutionary history of *N. arthritica* around Hokkaido. Thus, the following is a very

378 rough sketch of the evolution of this species. The main diversification of species into Groups A and

379 B was estimated during the Pliocene, 4.67–2.65 MYA, at the onset of global cooling, and this

380 dating does not contradict the fossil record of *N. arthritica* in a deposit of the Pliocene (Amano,

381 1997). By the late Pliocene, endemic speciation of many molluscan species, which characterise the

382 Ommma–Manganji fauna (Otuka, 1939; Amano, 2007), occurred in the Sea of Japan. This event

383 was influenced by the environmental change in the Sea of Japan, which was semi-closed by a land

384 bridge connecting the Korea Peninsula and Kyushu and lifting backbone range of mountains on the

385 Japanese Archipelago (Chinzei, 1978). The diversification of *N. arthritica* may be enhanced by such

386 environmental change. The eurythermal capacity of *N. arthritica* may have allowed it to survive

387 through the drastic climate and topological changes in the Pleistocene, as hypothesised by Amano

388 (1997), while many sympatric *Neptunea* species went extinct. The several missing haplotypes in
389 each branch of Groups A and B in the haplotype network (Fig. 3) may suggest that *N. arthritica* had
390 suffered climate oscillation as well as other species and lost many lineages. The present haplotype
391 distribution, which showed that Group B was found only in southern Hokkaido, may suggest that
392 Groups A and B were allopatrically established and contacted later. The diversification within each
393 group started at the middle of Pleistocene, as inferred by genetic diversity between core haplotypes
394 and derived ones (0.2%); however, the star-like shape of each group in the haplotype network may
395 indicate more recent radiation. In the present study, there appeared to be no reproductive isolation
396 between Groups A and B within each population because no deviation from the Hardy–Weinberg
397 equilibrium was found at any of the microsatellite DNA loci examined in KU or TO (Appendix in
398 Azuma *et al.*, 2011), both of which included Groups A and B (Fig. 4 and Supplementary Data). The
399 restricted gene flow found in the microsatellite DNA analyses suggested that the settled local
400 populations were somewhat isolated from each other and a small number of migrants may be
401 responsible for gene flow. Each of local population has evolved on the balance of such isolation
402 and migration; in other words, local decline was rescued by recruitment from neighbouring
403 populations. Some local populations have shrunk since the 1970s (Kawai *et al.*, 1994; Fujinaga *et*
404 *al.*, 2006) because of TBT pollution and/or overfishing. This reduction in the population size is
405 reflected by poor genetic diversity in mtDNA and it has erased important genetic evidence to detect

406 precise evolutionary history in this species. At present, the population size appears to be recovering
407 in each locality; however, the genetic diversity that once decreased in the matriline is likely
408 unrecovered. The matrilineal diversity in each population may partly recover with gene flow in the
409 future; however, genetic recovery definitely needs much longer time than the recovery of the
410 population size.

411

412 Conclusion

413 In the present study, comparison of mtDNA data with microsatellite DNA indicated that sex-biased
414 asymmetry in population genetics was probably affected by anthropogenic pollution and fishing
415 pressure in *N. arthritica*. The legislation prohibiting TBT usage as an antifouling agent for coastal
416 boats and aquaculture constructions was implemented in 1990 in Japan, and many countries,
417 including Japan, ratified a total TBT ban proposed by International Marine Organization. However,
418 the effect may persist for a considerable period of time. Both TBT pollution and overfishing were
419 stopped around Hokkaido more than 15 years before sample collection for the present study;
420 however, loss of genetic diversity was not recovered in the matriline of *N. arthritica*. It is important
421 to know that human impact may cause long and possibly irreversible modification in ecosystems,
422 particularly in species forming discrete and relatively small local populations, such as *N. arthritica*.

423

424

425

ACKNOWLEDGEMENTS

426 This work was partly supported by MEXT Revitalization Project for the Creation of Fisheries

427 Research and Education Center in Sanriku. The authors would like to thank Enago (www.enago.jp)

428 for the English language review.

429

430

431

REFERENCES

- 432 AMANO, K. 1997. Biogeography of genus *Neptunea* (Gastropoda: Buccinidae) from the Pliocene
433 and lower Pleistocene of the Japan Sea borderland. *Paleontological Research*, **1**: 274–284.
- 434 AMANO, K. 2007. The Omma-Manganji fauna and its temporal change. *Fossils*, **82**: 6–12. (In
435 Japanese with English abstract)
- 436 AVISE, J.C. 2000. *Phylogeography: the history and formation of species*. Harvard University Press,
437 Cambridge.
- 438 AZUMA, N., MIRANDA, R.M., GOSHIMA, S. & ABE, S. 2011. Genetic population structure of
439 Neptune whelk in northern Japan inferred from microsatellite DNA variation. *Plankton and*
440 *Benthos Research*, **6**: 42–50.
- 441 BIGATTI, G., PRIMOST, M.A., CLEDON, M., AVERBUJ, A., THEOBALD, N., GERWINSKI,
442 W., ARNTZ, W., MORRICONI, E. & PENCHASZADEH, P.E. 2009. Biomonitoring of TBT
443 contamination and imposex incidence along 4700 km of Argentinean shoreline (SW Atlantic:
444 from 38S to 54S). *Marine Pollution Bulletin*, **58**: 695–701.
- 445 BLACKMORE, G. 2000. Imposex in *Thais clavigera* (Neogastropoda) as an indicator of TBT
446 (Tributyltin) bioavailability in coastal waters of Hong Kong. *Journal of Molluscan Studies*, **66**:
447 1–8.
- 448 CHINZEI, K. 1978. Neogene molluscan faunas in the Japanese Islands: An ecologic and
449 Zoogeographic synthesis. *Veliger*, **21**: 155–170.
- 450 CLEMENT, M., POSADA, D. & CRANDALL, K.A. 2000. TCS: a computer program to estimate
451 gene genealogies. *Molecular Ecology*, **9**: 1657–1659.
- 452 COLSON, L. & HUGHES, N. 2004. Rapid recovery of genetic diversity of dogwhelk (*Nucella*
453 *lapillus* L.) populations after local extinction and recolonization contradicts predictions from
454 life-history characteristics. *Molecular Ecology*, **13**: 2223–2233.
- 455 EXCOFFIER, L., LAVAL, G. & SCHNEIDER, S. 2005. Arlequin v. 3.0: an integrated software
456 package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**: 47–50.

- 457 EXCOFFIER, L., SMOUSE, P.E. & QUATTRO, J.M. 1992. Analysis of molecular variance
458 inferred from metric distances among DNA haplotypes: application to human mitochondrial
459 DNA restriction data. *Genetics*, **131**: 479–491.
- 460 FENBERG, P.B. & ROY, K. 2008. Ecological and evolutionary consequences of size-selective
461 harvesting: how much do we know? *Molecular Ecology*, **17**: 209–220.
- 462 FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA primers for
463 amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan
464 invertebrates. *Molecular Marine Biology and Biotechnology*, **3**: 294–297.
- 465 FREELAND, J.R. 2005. *Molecular Ecology*. John Wiley and Sons, Chichester.
- 466 FUJINAGA, K. 2003. Ecological studies on the life history of the Neptune Whelk *Neptunea*
467 *arthritica*. *Memoirs of the Graduate School of Fisheries Sciences, Hokkaido University*, **50**: 1–61.
- 468 FUJINAGA, K. and NAKAO, S. 1996. Migration pattern of *Neptunea arthritica* Bernardi, with
469 special reference to relations with reproductive and feeding activities. *Japanese Journal of
470 Ecology*, **44**: 331–338. (In Japanese with English abstract)
- 471 FUJINAGA, K., ILANO A.S., NOMURA, H., MIRANDA, R.T. & NAKAO, S. 2006. Present state
472 of imposex in neptune whelk *Neptunea arthritica* inhabiting shallow waters around Hokkaido,
473 Japan. *Fisheries Science*, **72**: 995–1003.
- 474 GIBBS, P.E. 1996. Oviduct malformation as a sterilising effect of Tributyltin (tbt)-induced
475 imposex in *Ocenebra erinacea* (gastropoda: muricidae). *Journal of Molluscan Studies*, **62**:
476 403–413.
- 477 GUINDON, S. & GASCUEL, O. 2003. A simple, fast, and accurate algorithm to estimate large
478 phylogenies by maximum likelihood. *Systematic Biology*, **52**: 696–704.
- 479 HARRISON, R.G. 1989. Animal mitochondrial DNA as a genetic marker in population and
480 evolutionary biology. *Trends in Ecology & Evolution*, **4**: 6–11.
- 481 HUGHES, R.N. 1986. *A functional biology of Marine Gastropods*. John Hopkins University Press,
482 Baltimore.

- 483 HOU L., DAHMS, H.-U., DONG, C., CHEN, Y., HOU, H., YANG, W. & ZOU, X. 2013.
- 484 Phylogenetic positions of some genera and species of the family Buccinidae (Gastropoda:
- 485 Mollusca) from China based on ribosomal RNA and COI sequences. *Chinese Science Bulletin*,
- 486 **58**: 2315–2322.
- 487 ILANO, A.S., FUJINAGA, K. & NAKAO, S. 2003. Reproductive cycle and size at sexual maturity
- 488 of the commercial whelk *Buccinum isaotakii* in Funka Bay, Hokkaido, Japan. *Journal of the*
- 489 *Marine Biological Association of United Kingdom*, **83**: 1287–1294.
- 490 KAWAI, K., YAMAGUCHI, S., IDE, N., GOSHIMA, S. & NAKAO, S. 1994. Reproductive cycle
- 491 and parasite infection in the neptune whelk *Neptunea arthritica* in Lagoon Saroma. *Venus*, **53**:
- 492 105–112.
- 493 KASHIMA, K. 1996. Diatom assemblages from lake sediments of Lake Abashiri and Lake Saroma,
- 494 Hokkaido, northern part of Japan. *Laguna*, **3**: 33–39. (In Japanese with English abstract)
- 495 KENDAL, N.W. & QUIN, T.P. 2013. Size-selective fishing affects sex ratios and the opportunity
- 496 for sexual selection in Alaskan sockeye salmon *Oncorhynchus nerka*. *Oikos*, **122**: 411–420.
- 497 KOIZUMI, I., USIO, N., KAWAI, T., AZUMA, N. & MASUDA, R. 2012. Loss of genetic
- 498 diversity means loss of geological information: the endangered Japanese crayfish exhibits
- 499 remarkable historical footprint. *PLoS ONE*, **7**: e33986. doi:10.1371/journal.pone.0033986.
- 500 KUMAR, S. 2005. Molecular clocks: Four decades of evolution. *Nature Reviews Genetics*, **6**:
- 501 654–662.
- 502 LOMBARDO, R.C. & GOSHIMA, S. 2010. Female copulatory status and male mate choice in
- 503 *Neptunea arthritica* (Gastropoda: Buccinidae). *Journal of Molluscan Study*, **76**: 317–322.
- 504 MIRANDA, R.M., FUJINAGA, K., ILANO, A.S. & NAKAO, S. 2007. Incidence of imposex and
- 505 parasite infection in *Neptunea arthritica* at Saroma Lagoon and their relationship. *Aquaculture*
- 506 *Science*, **55**: 9–15.
- 507 MIRANDA, R.M., FUJINAGA, K. & NAKAO, S. 2008. Age and growth of *Neptunea arthritica*
- 508 estimated from growth marks in the operculum. *Marine Biology Research*, **4**: 224–235.

- 509 MIRANDA, R.M., FUJINAGA, K., ILANO, A.S & NAKAO, S. 2009. Effects of imposex and
510 parasite infection on the reproductive features of the Neptune whelk *Neptunea arthritica*.
511 *Marine Biology Research*, **5**: 268–277.
- 512 MIZUSHIMA, T. & TORISAWA, M. 2003. *Fisheries and aquatic life in Hokkaido*. Hokkaido
513 Shinbun Press, Sapporo, Japan. (In Japanese)
- 514 MOORE, W.S. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees
515 versus nuclear-gene trees. *Evolution*, **49**:718–726.
- 516 NAKANO, T., KURIHARA, Y., MIYOSHI, H. & HIGUCHI, S. 2010. Molecular phylogeny of
517 Neptunea (Gastropoda: Buccinidae) inferred from mitochondrial DNA sequences, with
518 description of a new species. *Venus*, **68**: 121–137.
- 519 OKUTANI, T. 2000. *Marine Mollusks in Japan*. Tokai University Press, Kanagawa.
- 520 OTUKA, Y. 1939. Mollusca from Cainozoic Systems of eastern Aomori Prefecture. *Journal of*
521 *Geological Society of Japan*, **44**: 23–31.
- 522 PAVONI, B., CENTANNI, E., VALCANOVER, S., FASOLATO, M., CECCATO, S. &
523 TAGLIAPIETRA, D. 2007. Imposex levels and concentrations of organotin compounds (TBT
524 and its metabolites) in *Nassarius nitidus* from the Lagoon of Venice. *Marine Pollution Bulletin*,
525 **55**: 505–511.
- 526 POSADA, D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*,
527 **25**: 1253–1256.
- 528 POWER, A.J. & KEEGAN, B.F. 2001. Seasonal patterns in the reproductive activity of the red
529 whelk, *Neptunea antiqua* (Mollusca: Prosobranchia) in the Irish Sea. *Journal of the Marine*
530 *Biological Association of the United Kingdom*, **2**: 243–250.
- 531 RONQUIST, F. & HUELSENBECK, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference
532 under mixed models. *Bioinformatics*, **19**: 1572–1574.
- 533 ROWE, R. & HUTCHINGS, J.A. 2003. Mating systems and the conservation of commercially
534 exploited marine fish. *Trends in Ecology & Evolution*, **18**: 567–571.

- 535 SUZUKI, K., HIRASHI, T., YAMAMOTO, K. & NASHIMOTO, K. 2002. Estimation of natural
536 mortality and exploitation rates of whelk *Neptunea arthritica* by multiple tagging experiment.
537 *Fisheries Science*, **68**: 87–94.
- 538 THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997.
539 The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by
540 quality analysis tools. *Nucleic Acids Research*, **24**: 4876–4882.
- 541 TOEWS, D.P. & BRELSFORD, A. 2012. The biogeography of mitochondrial and nuclear
542 discordance in animals. *Molecular Ecology*, **21**: 3907–3930.
- 543 WEIR, B.S. & COCKERHAM, C.C. 1984. Estimating F-statistics for the analysis of population
544 structure. *Evolution*, **38**: 1358–1370.
- 545 WoRMS EDITORIAL BOARD. 2014. World Register of Marine Species. available from
546 <http://www.marinespecies.org> at VLIZ. Accessed 2014-01-14
- 547 WRIGHT, S. 1943. Isolation by distance. *Genetics*, **28**: 114–138.
- 548

549

FIGURE CAPTIONS

550 **Figure 1.** Map of sampling locations of *Neptunea arthritica* in northern Japan, Wakkai (WA),
551 Rumoi (RU), Kumaishi (KU), Shiriuchi (SH), Toyoura (TO), Aomori (AO), Nemuro (NE) and
552 Saroma (SA). Dashes indicate putative migration pathways.

553

554 **Figure 2.** Fifty-percent majority-rule Bayesian tree inferred from partial mtDNA CO1 sequences of
555 *Neptunea arthritica* using the GTR + G + I model. Bold and italic OTU indicates the haplotype
556 found in the present study, and the others are the accession numbers of sequences retrieved from
557 DDBJ/GenBank. The tree was rooted using EU883634 from *N. eulimata* as an outgroup. Nodal
558 numbers represent Bayesian posterior probability values.

559

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

565

566 **Figure 4.** Distribution of the mtDNA COI haplotypes in each sampling locality of *Neptunea*

567 *arthritica*. Note that six of eight samples have only one or two haplotypes, and distant RU and SH
568 share only *NACOIH1*.

569

570 **Figure 5.** The non-metric multidimensional scaling (nMDS) plotting of *Neptunea arthritica*
571 samples with pairwise F_{ST} values. **A:** based on five loci of microsatellite DNA markers (Azuma *et*
572 *al.*, 2011), **B:** based on a 428-bp sequence of partial mtDNA COI. In **A**, the horizontal long
573 scattering plot, which is consistent with geographic relationships between samples, suggests a
574 population structure with a one-dimensional genetic cline, from eastern and northeastern Hokkaido
575 to southern Hokkaido and northernmost Honshu (Azuma *et al.*, 2011).

576

Table 1. Informations of Neptune whelk samples analyzed in the present study.

Sample name, Collection date, Sample size (number of individuals), and diversity indices, haplotype diversity (h) and nucleotide diversity (π) estimated in partial COI sequence, and mean expected heterozygosity (H_E) estimated in five loci of microsatellite (Azuma et al., 2011). Sample of RU were not analyzed with microsatellites because of poor amplification in PCR.

Sample name	Collection date (year, month)	Sample size	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

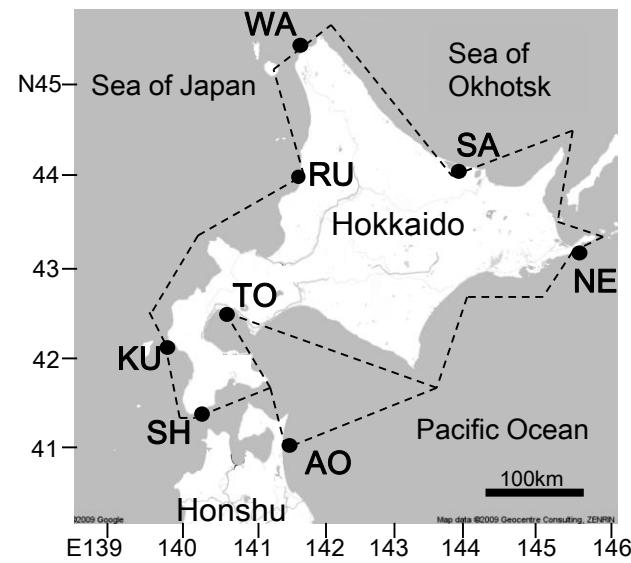
Table 2. Pairwise F_{ST} between *Neptunea arthritica* samples based on partial COI sequence. Bold letter indicates significant deviation from 0 at $p < 0.01$ after Bonferroni correction.

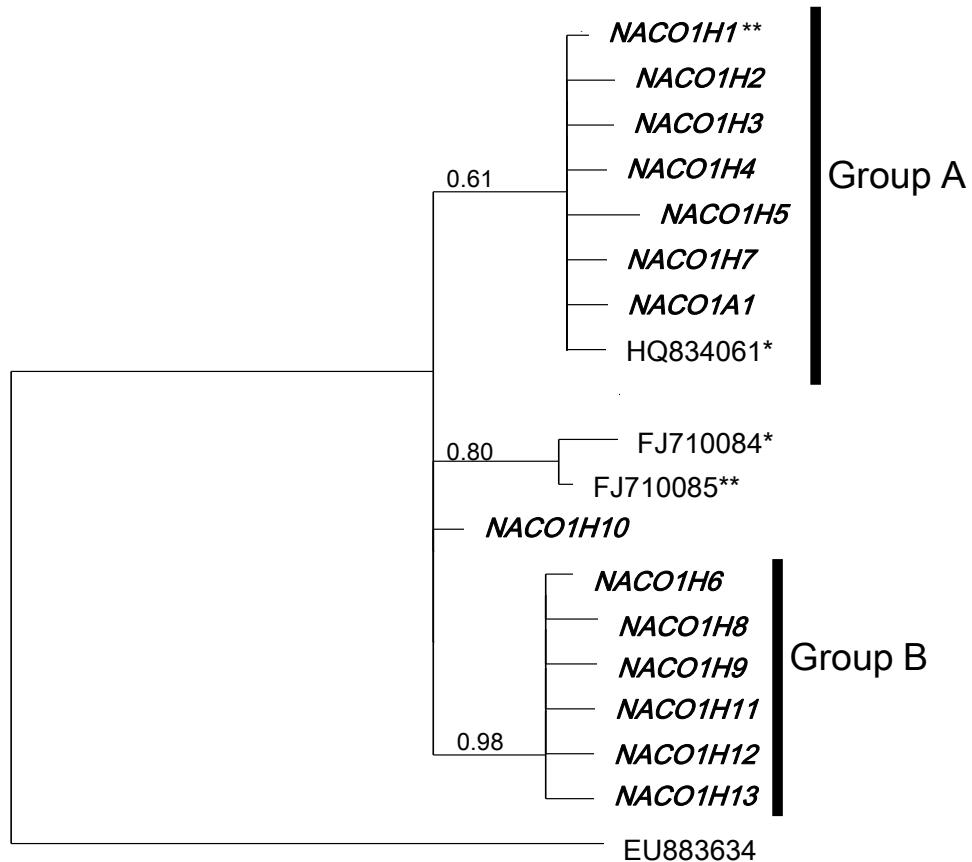
	NE	SA	WA	RU	KU	SH	TO
NE							
SA	0.763						
WA	0.153	0.813					
RU	0.172	1.000	0.118				
KU	0.422	0.714	0.549	0.590			
SH	0.172	1.000	0.118	0.000	0.590		
TO	0.445	0.739	0.574	0.621	-0.0315	0.621	
AO	0.156	0.968	0.103	0.041	0.574	0.041	0.604

Supplementary File

Haplotype frequency of partial COI (number of individuals which showed the haplotype) in each sample. Bold letter indicates the highest frequency in each sample.

	<i>NACO1</i>	<i>NACO1H</i>	<i>NACO1H</i>	<i>NACO1H</i>	<i>NACO1H</i>	<i>NACO1</i>									
	<i>H1</i>	<i>H2</i>	<i>H3</i>	<i>H4</i>	<i>H5</i>	<i>H6</i>	<i>H7</i>	<i>H8</i>	<i>H9</i>	<i>10</i>	<i>11</i>	<i>12</i>	<i>13</i>	<i>A1</i>	Total
NE	24	0	0	0	0	0	0	0	0	6	0	0	0	0	30
SA	0	0	0	0	30	0	0	0	0	0	0	0	0	0	30
WA	18	2	7	1	2	0	0	0	0	0	0	0	0	0	30
RU	30	0	0	0	0	0	0	0	0	0	0	0	0	0	30
KU	10	0	0	0	0	13	1	1	2	0	1	1	1	0	30
SH	30	0	0	0	0	0	0	0	0	0	0	0	0	0	30
TO	11	0	0	0	0	19	0	0	0	0	0	0	0	0	30
AO	26	0	0	0	0	0	0	0	0	0	0	0	0	2	28
Total	149	2	7	1	32	32	1	1	2	6	1	1	1	2	238





* found in only *N. a. cumingii*

**shared by *N. arthritica* and *N. a. cumingii*

