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

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ABSTRACT

The Neptune whelk, *Neptunea arthritica*, is a sublittoral sea snail from Pacific waters that has been a food resource and is commercially important for the coastal fisheries 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, partial sequence of the cytochrome *c* oxidase subunit I (COI) gene, to compare the obtained results with those from previous microsatellite analyses. 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 2 groups (Groups A and B), with an intermediate haplotype connecting both of the groups. Among eight populations, six were fixed for only one or two haplotypes, and any geographic–genetic correlation was not found; they were probably affected by random drift of the mtDNA lineage. Thus, the results from mtDNA contrasted with those from previous microsatellite analysis, indicating that geographic structure was affected by the restricted gene flow between populations. Our results suggested that *N. arthritica* diversified 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

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

Neptunea arthritica (Bernardi, 1857) is a dioecious gastropod with internal fertilisation and direct development 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 it takes 3 years or more for maturation (Fujinaga, 2003). The typical *N. arthritica* (*N. arthritica arthritica*) is distributed in the 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 Sea (Okutani, 2000), with the range partly overlapping with typical *N. arthritica*. Sea snails, including *N. artheritica*, have been a food resource and commercially important in the coastal fisheries in northern Japan (Mizushima & Torisawa, 2003); thus, several biological studies of *N. arthritica* have been mainly conducted for 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, none of the population genetic studies had appeared before our recent microsatellite DNA analysis in *N. arthritica* around Hokkaido (Azuma *et al*., 2011). Using five loci of microsatellite DNA markers in seven populations of *N. arthritica* around Hokkaido, we suggested the restricted gene flow among populations with increasing genetic 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.

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 the abovementioned 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 recruited 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), and used for analysis.

Nucleotide sequencing

The 5′ region of mtDNA COI was amplified by polymerase chain reaction (PCR) in a 30 μl reaction mixture containing template DNA (approximately 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

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 PRISM™
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, *NACO1H1–H13* and *NACO1A1* (Fig. 2). The

Fig. 2.

results of BLAST search revealed that the most frequent haplotype in our analysis, *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 the sequences of *N. arthritica* (AB498776, AB498777 and AB498778) and *N. a. cumingii* (FJ710083 and FJ710079). Thus, typical *N. arthritica* and *N. a. cumingii* shared at least two haplotypes, *NACO1H1*and FJ710085 (Fig. 2). In the Bayesian tree (Fig. 2), all the haplotype sequences observed herein and those from databank, except for EU883634, were separated in three clusters, Group A (*NACO1H1–5*, *NACO1H7*, *NACO1A1* and *HQ83061* from databank), Group B (*NACO1H6* and *NACO1H8–13*) and the third group consisting of two other databank sequences, FJ710085 and FJ710084, whereas *NACO1H10* was intermediate to these groups. Although the posterior probabilities for Group A and the third group (0.61 and 0.80, respectively) were not high enough to support the monophyly, the three groups were discriminated in a parsimony haplotype network (see below).

Fig. 3.

The haplotype network (Fig. 3) was three forked, also showing two groups of haplotypes as seen in the Bayesian tree, Groups A and B, with core haplotypes (*NACO1H1* and *NACO1H6*) and derived haplotypes around core ones. *NACO1H10* was present in the centre of the network, 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 *NACO1H1*. 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 Fig. 4.
221 haplotype *NACO1H1* was common among the examined samples, except for SA. The SA sample
222 contained only *NACO1H5*, which was probably derived from *NACO1H1* 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 *NACO1H10*, connecting both the groups,
225 occurred only in NE.

As shown in Table 1, the haplotype diversity (h) was moderate, and the nucleotide diversity (π) was low as a whole. Both h and π were the highest in KU, which had two Group A and six haplotypes from Group B (Supplementary Data), followed by WA, which had five haplotypes from Group A. The WA sample showed low π because of a lack of haplotype from Group B. The third highest h and the second highest π were observed in TO. Both h and π were zero in three monomorphic samples, SA (only *NACO1H5*), RU (only *NACO1H1*) and SH (only *NACO1H1*). This diversity profile was contrasting with the results of previous microsatellite marker 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 (i Table 2. bold letter); however, the difference/similarity pattern strikingly differed from the results of previous microsatellite analysis (Azuma *et al*., 2011). In nMDS plotting, the F_{ST} estimates using microsatellite markers showed a correlation between the geographic and genetic structure (Fig. Fig. 5. 5-A), whereas the genetic distance of mtDNA haplotypes between samples did not show a correlation with their spatial distribution (Fig. 5-B). The SH sample was distinctly separated from neighbouring KU and TO but completely overlapped with RU. On the other hand, KU and TO were in close proximity to each other. The SA sample was clearly distant from other populations, probably reflecting the exclusive occurrence of *NACO1H5* but lack of *NACO1H1*, 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 *NACO1H10*, 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. cumingi* 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 π = 0) than the total
289 estimation (h = 0.57 and π = 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

298 summary, it is conceivable that the observed mtDNA phylogeographical pattern in *N. arthritica*
299 was influenced by very recent genetic drift. Genetic drift stochastically left a small number of
300 genotypes (Harrison, 1989), and the natural genetic structure related to geography may be hidden
301 after the drift. Thus, genetic drift could be a reason for the genetic–geographic inconsistency as
302 well as for the lack of genetic diversity in some *N. arthritica* populations. However, if the
303 bottleneck occurred a long time ago, genetic diversity s3ld be more or less recovered even in
304 mtDNA by gene flow, as suggested by our microsatellites analysis (Azuma et al., 2011). Thus the
305 genetic drift was thought to be recent. Possible causes of the genetic drift in *N. arthritica* include
306 natural biotic and abiotic factors, e.g. predation, parasitism, disease, change in climate and
307 topology and human impact such as exploiting, environmental modification and pollution. Among
308 these, the human impact, overfishing and imposex caused by TBT pollution, was considered to be
309 the most plausible cause of the contrasting results obtained from mtDNA and microsatellite
310 analyses. In the *N. arthritica* population around Hokkaido, overfishing and TBT pollution were
311 reported to be specific causes of the extreme population decline in the 1970s and 1980s (Fujinaga
312 *et al*., 2006; Miranda *et al*., 2007 &2009), and they were reported to be surely related to the skewed
313 sex ratio in the reproductive stage. Recently, Toews & Brelsford (2012) reviewed 126 studies
314 exhibiting discordant biogeography of mtDNA and nuclear DNA (mito–nuclear discordance) in
315 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 *NACOH1*, which
359 was ubiquitous and the most abundant in total samples, was not found, and all individuals had
360 haplotype *NACO1H5*, 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 *NACO1H5* at that time. Because two
368 individuals in WA also had this haplotype, it is not likely that *NACO1H5* 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. arthiritica* 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

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548

549 FIGURE CAPTIONS

550 **Figure 1.** Map of sampling locations of *Neptunea arthritica* in northern Japan, Wakkanai (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 *NACO1H1*.

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

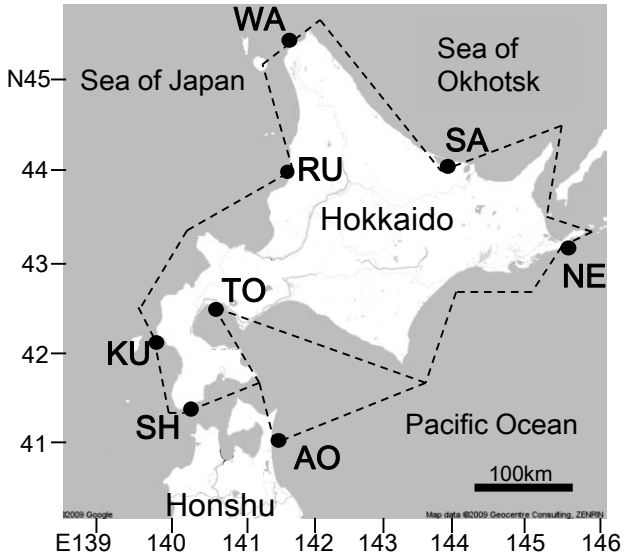
Table 2. Pairwise F_{ST} between *Neptunea arthritica* samples based on partial COI sequence. Bold letter indicates significant deviation from 0 at $p<0.01$ after Bonferroni correction.

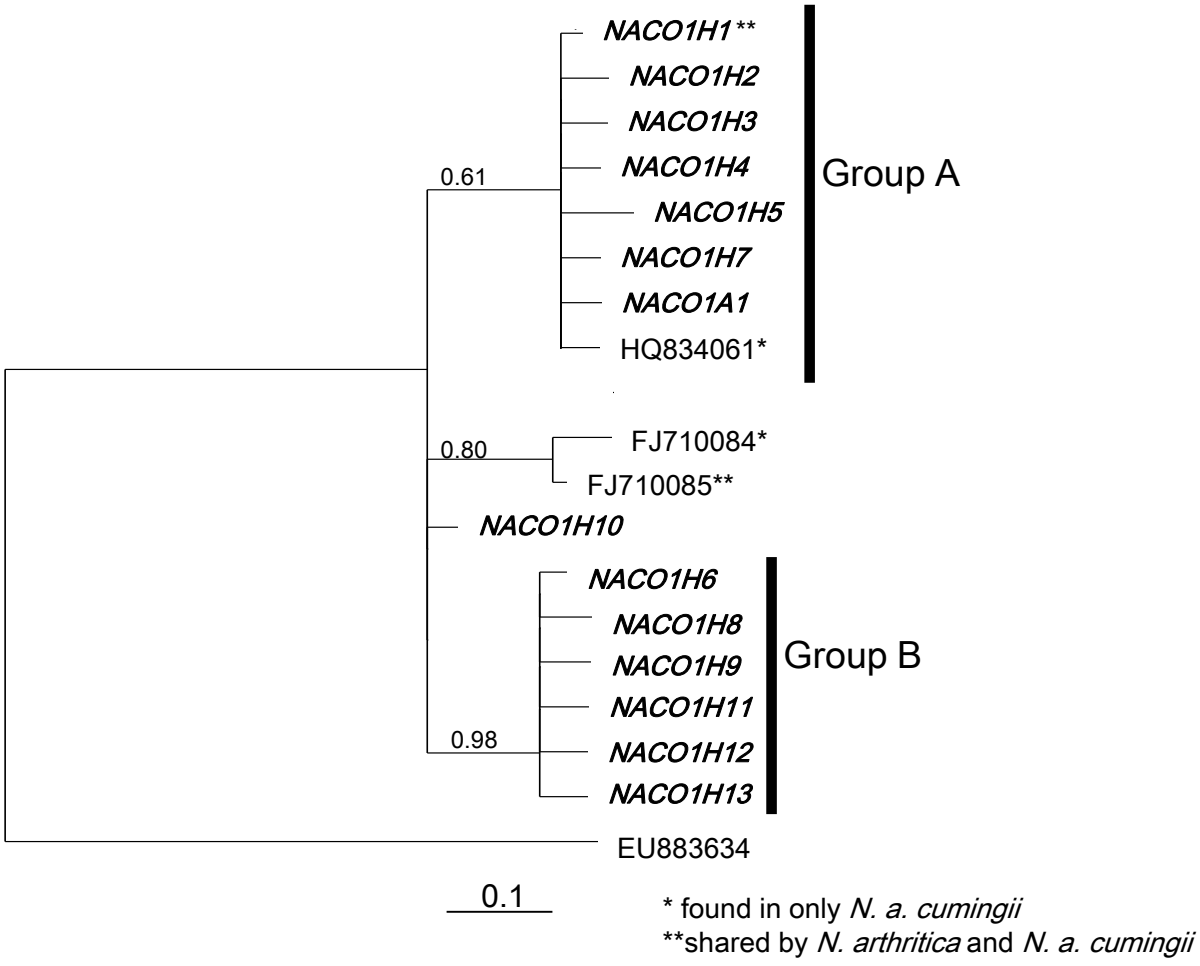
	NE	SA	WA	RU	KU	SH	TO
NE							
SA	**0.763**						
WA	**0.153**	**0.813**					
RU	0.172	**1.000**	0.118				
KU	**0.422**	**0.714**	**0.549**	**0.590**			
SH	0.172	**1.000**	**0.118**	0.000	**0.590**		
TO	**0.445**	**0.739**	**0.574**	**0.621**	-0.0315	**0.621**	
AO	0.156	**0.968**	**0.103**	0.041	**0.574**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

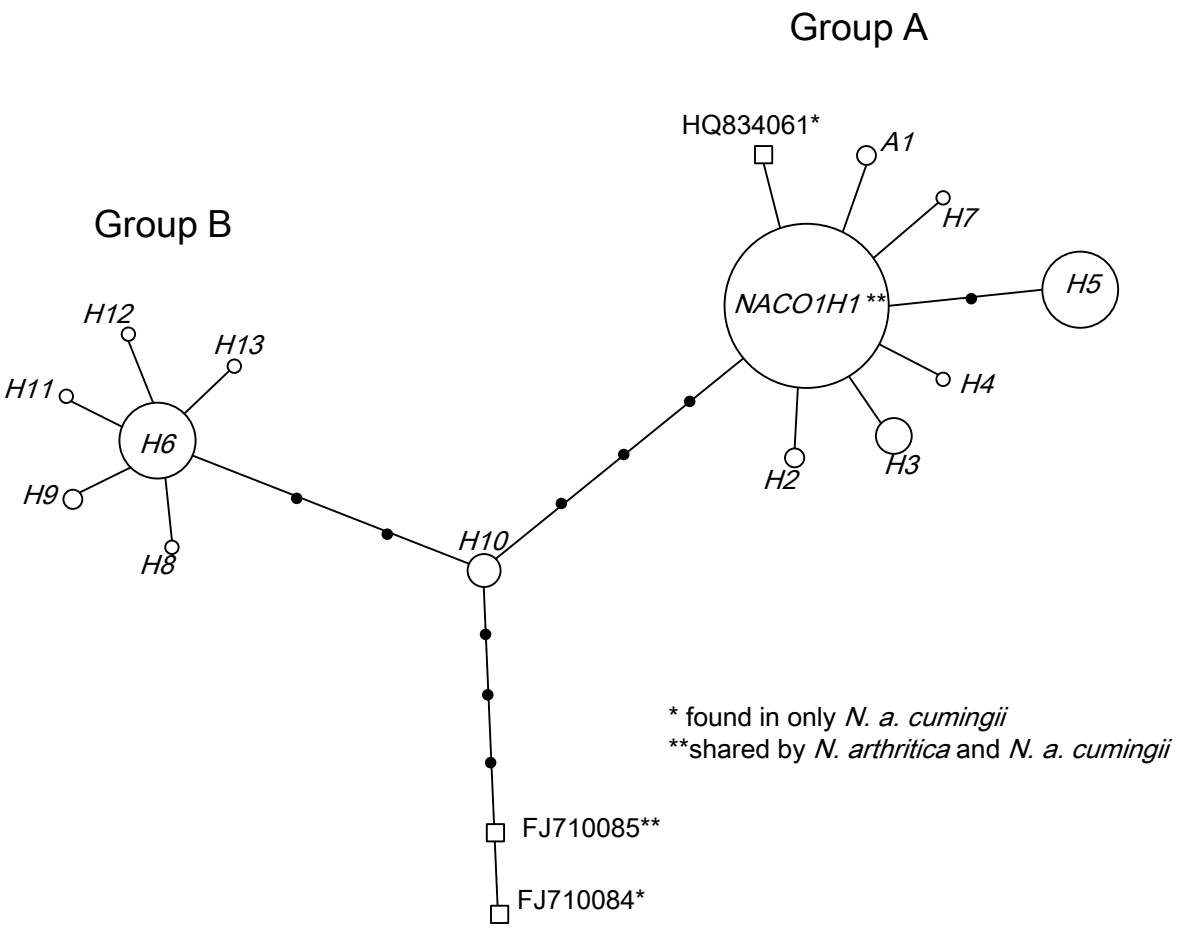
Supplementary File

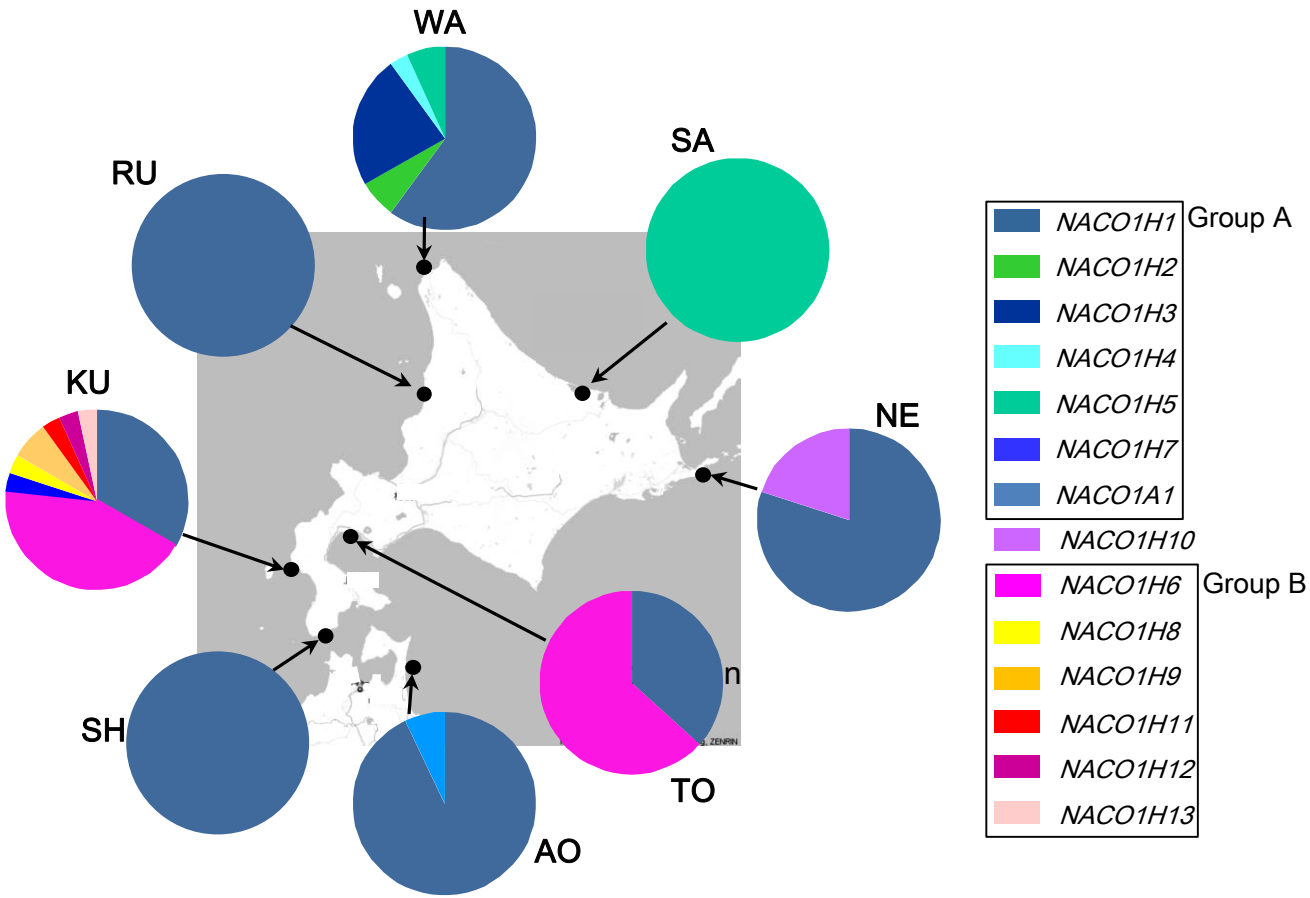
Haplotype frequency of partial COI (number of individuals which showed the haplotype) in each sample. Bold letter indicates the higest frequency in each sample.

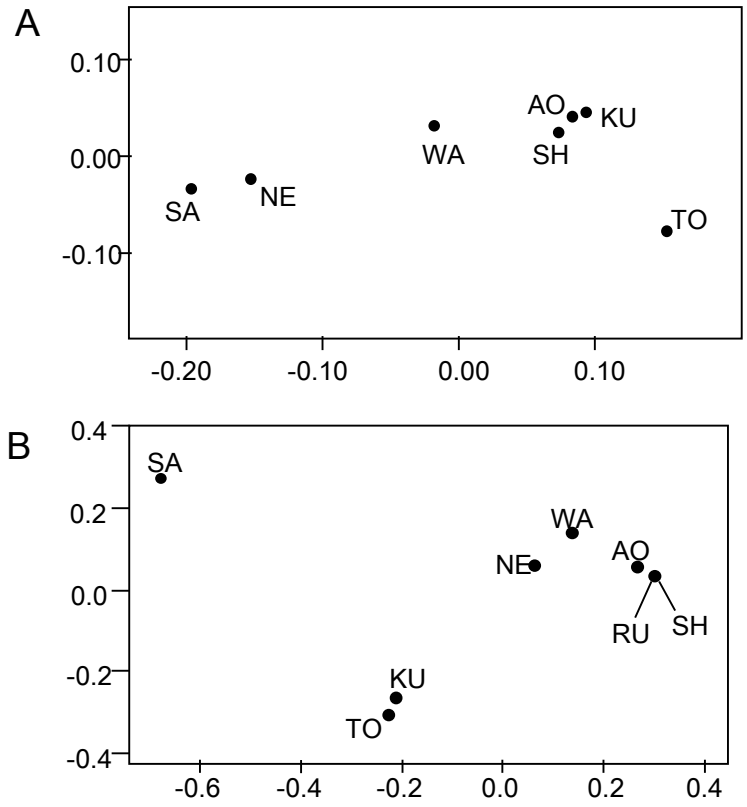
	NACO1 H1	***NACO1 H2***	***NACO1 H3***	***NACO1 H4***	***NACO1 H5***	***NACO1 H6***	***NACO1 H7***	***NACO1 H8***	***NACO1 H9***	***NACO1H 10***	***NACO1H 11***	***NACO1H 12***	***NACO1H 13***	***NACO1 A1***	**Total**
NE	**24**	0	0	0	0	0	0	0	0	6	0	0	0	0	30
SA	0	0	0	0	**30**	0	0	0	0	0	0	0	0	0	30
WA	**18**	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


WA
Sea of
Okhotsk
N45
Sea of Japan
SA
RU
44
Hokkaido
43
NE
TO
KU
42
SH
Pacific Ocean
41
AO
100km
Honshu
E139
140
141
142
143
144
145
146


NACO1H1**
NACO1H2
NACO1H3
NACO1H4
NACO1H5
NACO1H7
NACO1A1
HQ834061*
Group A
0.61
FJ710084*
FJ710085**
0.80
NACO1H10
NACO1H6
NACO1H8
NACO1H9
NACO1H11
NACO1H12
NACO1H13
Group B
0.98
EU883634
0.1
* found in only N. a. cumingii
**shared by N. arthritica and N. a. cumingii


Group A
Group B
HQ834061*
A1
H7
H5
NACO1H1**
H4
H3
H2
H12
H13
H11
H6
H9
H8
H10
FJ710085**
FJ710084*
* found in only N. a. cumingii
**shared by N. arthritica and N. a. cumingii


WA
SA
RU
KU
NE
SH
AO
TO
Group A
NACO1H1
NACO1H2
NACO1H3
NACO1H4
NACO1H5
NACO1H7
NACO1A1
NACO1H10
Group B
NACO1H6
NACO1H8
NACO1H9
NACO1H11
NACO1H12
NACO1H13


A
0.10
0.00
-0.10
AO
KU
WA
SH
SA
NE
TO
-0.20
-0.10
0.00
0.10
B
0.4
0.2
0.0
-0.2
-0.4
SA
WA
NE
AO
RU
SH
KU
TO
-0.6
-0.4
-0.2
0.0
0.2
0.4