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Detection of a highly divergent population structure and identification of a cryptic species in the East Asian dogwhelk *Nucella heyseana*

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ABSTRACT: The dogwhelk (genus *Nucella*) includes intertidal carnivorous snails that are widely distributed on the North Pacific and North Atlantic coasts. There is little knowledge on the taxonomy of *Nucella* species in East Asia, and classification, naming and description of East Asian *Nucella* species is far from being established. We performed population genetics analyses on Japanese *Nucella* spp., including samples from the continental coast of the Sea of Japan. We used microsatellite DNA markers and mtDNA-COI sequencing to determine the species composition, the species' classification status, and the population structure within the species. The specimens were genetically divided into 2 species, *Nucella heyseana* and the cryptic *N. freycineti*. In addition, *N. heyseana* was found to exhibit a highly divergent population structure among localities, which is attributable to 2 possible factors: direct development and ocean currents. These findings provide new insights into the taxonomy of East Asian *Nucella* species and management strategies for intertidal organisms of this region.

KEY WORDS: *Nucella* · Population structure · mtDNA-COI sequence · Microsatellite

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INTRODUCTION

The rich biodiversity of the Japanese coast, a biodiversity hotspot, is currently threatened (Myers et al. 2000). Conservation of this biodiversity is a serious concern that has prompted extensive DNA marker-based research on species diversity and the intraspecific diversity of coastal species. While much of this research has been devoted to commercial organisms, a relatively small amount of information has been collected for intertidal species of small size and limited commercial value, such as the gastropod *Batillaria cumingi* (Kojima et al. 2004) and the intertidal goby *Chaenogobius annularis* (Hirase et al. 2012).

The intertidal rocky shore exhibits some of the richest biodiversity in the maritime environment as a result of tidal influences, wave action, sun, and wind. Rocky shores are most abundant along the open coast of Japan; however, the intertidal rocky shore habitat is rapidly decreasing as artificial coastline increases (Nature Conservation Bureau Environment Agency 1994). In order to develop appropriate conservation and management strategies for this complex ecosystem, it is necessary to accumulate new information regarding the correct taxonomies of morphologically similar species, the manner in which the intraspecific population structure is affected by the environmental features of the intertidal coast, and the biological connections among rocky shore sites mediated by ocean currents.

Among intertidal organisms, the genus *Nucella* includes intertidal carnivorous snails that are widely distributed on the North Pacific and North Atlantic coasts. These snails include species that inhabit rocky

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intertidal habitats throughout their life cycle, which lacks a planktonic stage (Gallardo 1979, Crothers 1980, Tsuchiya 2000). As a result of this habitat dependence, the population structure of these snails is likely to be strongly influenced by the geological features of the region and the environmental features of the intertidal zone. Colson & Hughes (2007) investigated the population structure of *N. lapillus* along the Atlantic coast by using microsatellite markers. They showed that there is a close link between coastal hydrography and population structure. Marko (2004) performed comparative research on the population structures of *N. lamellose* and *N. ostrina* along the west coast of North America using mtDNA markers and found different biogeographical histories that reflected the habitat characteristics of the intertidal zone.

For East Asian *Nucella* species, the use of molecular markers has only been applied to a limited extent to date in East Asia, including allozyme variation (Park & Choe 1999, Zaslavskaya & Kolotuchina 2003) and phylogenetic analysis by mtDNA cytochrome b sequencing, which has been applied to a small number of specimens (Collins et al. 1996). In addition, there are few papers on the taxonomy of East Asian *Nucella* species, and the classification, naming and description of *Nucella* species in East Asia is far from being well established. For example, in Japan, *Nucella* individuals have been found in the northern regions, from Hokkaido to the coast of Sanriku and are generally described as a single species, *N. heyseana* (Japanese common name 'chidimibora'), which is distributed from the Aleutian Islands to the Korean Peninsula and from Hokkaido to the coast of Sanriku (Tsuchiya 2000). However, chidimibora exhibits various morphological variations in shell color and shape, and its taxonomy is still unclear. For example, Habe & Ito (1965) classified 4 species of *Nucella* in northern Japan on the basis of distribution area and shell morphology (roughness of shell surface; shell height to shell width proportion; screw head length to shell height proportion; thickness and width of aperture; and number, width, depth, and interval of spiral cords): *N. freycineti* (sic = *freycinetti*), *N. freycineti longata* (sic = *elongata*), *N. freycineti alabaster*, and *N. heyseana*, with *N. heyseana* referred to as chidimibora. They further classified *N. freycineti* as a geographical variation of *N. lima*; however, chidimibora was referred to as *N. lima*, not *N. heyseana*, in a study by Tsuchiya (2000). In Russia, 4 species, namely *N. lima*, *N. freycineti*, *N. heyseana*, and *N. elongata* have been reported (Egorov 1992, Zaslavskaya & Kolotuchina 2003). These studies did not report the distribution areas for these species, nor did they discuss any correlations with the *Nucella* species found in and around Japan.

In this study we performed population genetics analyses on Japanese *Nucella* spp., including samples from the continental coast of the Sea of Japan. We used microsatellite DNA markers and mtDNA-COI sequencing to determine the species composition in the coastal region surrounding Japan, the species' classification status, and the population structure within the species.

MATERIALS AND METHODS

Sampling

The *Nucella* samples used in this study were collected from Vostok (VSK) in Russia, Gangneung (GNG) in Korea, and 9 localities in Japan, namely Otaru (OTR), Wakkanai (WKN), Okoppe (OKP), Abashiri (ABS), Akkeshi (AKS), Shizunai (SZN), Erimo (ERM), Hachinohe (HCH), and Onagawa (ONG) (Table 1, Fig. 1). All individuals were randomly caught by hand from the intertidal rocky shore during low tide, regardless of their morphological features. The samples were then transported to our laboratory and DNA was extracted by the conventional phenol-chloroform method. The quality and concentration of the extracted DNA were checked by the GeneQuant II RNA/DNA calculator (GE Healthcare) and the DNA concentration was adjusted for all experiments.

Table 1. *Nucella heyseana* sampling data

Site name	Location	Sampling date	Sample size	
			Microsatellite	mtDNA-COI
VSK	Vostok, Russia	Jul 02	30	27
GNG	Gangneung, Korea	Mar 05	52	31
OTR	Otaru, Hokkaido, Japan	May 05	57	41
WKN	Wakkanai, Hokkaido, Japan	Apr 05	33	15
OKP	Okoppe, Hokkaido, Japan	Apr 05	56	40
ABS	Abashiri, Hokkaido, Japan	Apr 05	28	15
AKS	Akkeshi, Hokkaido, Japan	Apr 05	30	18
SZN	Shizunai, Hokkaido, Japan	Apr 05	51	26
HCH	Hachinohe, Aomori, Japan	Apr 05	35	19
ONG	Onagawa, Miyagi, Japan	May 05	36	20
ERM	Erimo, Hokkaido, Japan	Apr 05	–	19

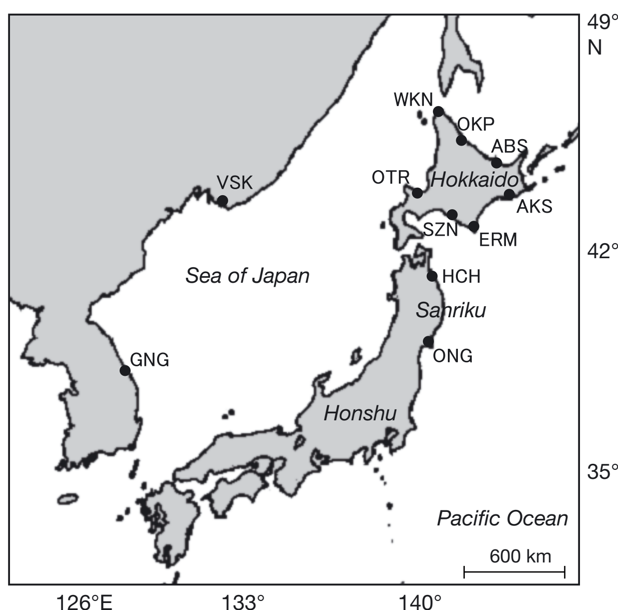


Fig. 1. *Nucella* spp. sampling sites (●); abbreviations in Table 1

Microsatellite DNA analyses

Microsatellite markers have been developed for *Nucella lapillus* by Kawai et al. (2001) and 14 loci were successfully cross-amplified for the *Nucella* specimens described as *N. heyseana* in this manuscript. Of these 14 loci, we achieved high resolution during the PCR-based amplification of 6 loci (*Nlw2*, *Nlw3*, *Nlw11*, *Nlw13*, *Nlw18* and *Nlw27*) in preliminary experiments and employed these in the current study. The forward primer for each marker locus was fluorescently labeled by 1 of 4 different dyes (6-FAM, VIC, NED or PET) and multiplex PCR (set 1: *Nlw2* + *Nlw3* + *Nlw13*, annealing temperature (Ta) = 57°C; set 2: *Nlw11* + *Nlw18* + *Nlw27*, Ta = 52°C) was performed. The PCR solution contained 1 ng of template DNA, 3 µL of QIAGEN MultiplexPCR kit Master Mix Solution (QIAGEN), and 2.5 µM forward and reverse primers, in a total volume of 6 µl. The PCR conditions involved an initial denaturing step of 15 min at 95°C, 35 cycles of 30 s at 94°C, 90 s at the annealing temperature and 90 s at 72°C, and a final extension step at 72°C for 10 min. The PCR products were resolved on an ABI 3130 Genetic Analyzer (Applied Biosystems) and alleles were scored with GeneMapper Software version 4.0 (Applied Biosystems). Each allele was named according to its size.

Allele frequencies and the observed (H_o) and expected (H_e) heterozygosities were calculated using GENEPOP v.3.4 (Raymond & Rousset 1995). Allelic richness (R_s) and the inbreeding coefficient for each locus-location combination, F_{IS} (Weir & Cockerham 1984), were calculated using FSTAT 2.9.3 (Goudet 2001). R_s is the number of alleles per locus standardized to the smallest sample size (N = 28) and F_{IS} values address deviations from the Hardy-Weinberg equilibrium (HWE). F_{IS} values were tested for significant departure from zero by using random permutation procedures (2200 randomizations).

PCR amplification errors, such as priming site polymorphisms (null alleles) leading to genotyping errors, and deviations from the HWE were estimated in accordance with previously described methods (Brookfield 1996), using MICRO-CHECKER software (van Oosterhout et al. 2004). Genotype frequencies not conforming to the HWE were adjusted using the first equation provided by Brookfield (1996) for comparison with nonadjusted data in the subsequent analysis.

Genetic divergence between pairs of sampling locations was quantified using conventional F_{ST} values (Weir & Cockerham 1984) and Nei's unbiased genetic distance, D_s (Nei 1978). The multilocus estimates of F_{ST} were calculated from null-corrected genotype data using SPAGeDi 1.1 software (Hardy & Vekemans 2002). D_s values were used to construct the unrooted neighbor-joining (NJ) tree with PHYLIP 3.66 software. The tree was constructed with Tree Explorer 2.12 software (http://www.ctu.edu.vn/~dvxe/Bioinformatic/Software/BIT%20Software/TE_man.html). The population structure of *Nucella heyseana* was further investigated using spatial analysis of molecular variance (SAMOVA) (Dupanloup et al. 2002). SAMOVA calculates the largest F_{CT} value corresponding to an arbitrary number of groups (K) (Dupanloup et al. 2002). We ran the SAMOVA program with 100 simulated annealing processes for various numbers of groups (K = 2 to 6) and compared simulated F_{CT} values to determine the best grouping of samples.

mtDNA analyses

A primer set for amplifying a 580-bp mtDNA partial COI region (NCOF: 5′ CTC CTG CTG GAT CAA AAA AAG 3′, NCOR: 5′ TCG AGC TGA ATT AGG CAA C 3′) was designed on the basis of the sequence of the amplicon, using mtDNA-COI universal primers (Folmer et al. 1994). PCR amplification was carried out in a 25 µl volume containing 0.5 U TaKaRa r*Taq* (TaKaRa), 2.5 ng template DNA, 0.5 µM forward and reverse primers, 0.2 mM dNTP mix, and 2.5 µl of 10× PCR buffer for TaKaRa r*Taq*. The ampli-

fication was performed under the following conditions: 3 min at 94°C for initial denaturation, 7 cycles of 30 s at 94°C for denaturation, 30 s at 53°C for annealing and 1 min at 72°C for extension, 25 cycles of 30 s at 90°C for denaturation, 30 s at 53°C for annealing and 1 min at 72°C for extension, and a final extension at 72°C for 5 min. PCR products were purified by USB ExoSAP-IT (Affymetrix). Cycle sequencing reactions were performed using the Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare) and a DNA sequencer (DSQ-2000, Shimadzu), in accordance with the manufacturer's protocol. A 471-bp mtDNA-COI fragment that could be clearly sequenced in all individuals was used for population genetic analyses.

Nucleotide sequences were aligned using CLUSTAL W v.1.83 (Thompson et al. 1994). Molecular diversity indices, such as number of haplotypes, haplotype frequency, polymorphic sites, transitions, transversions, and indels were obtained using the program ARLEQUIN 3.11 (Schneider et al. 2006). Haplotype diversity (h) and nucleotide diversity (π) were calculated following the method of Nei (1987) as implemented in ARLEQUIN 3.11. A statistical parsimony haplotype network of unique COI sequences was constructed using the program TCS 1.21 (Clement et al. 2000).

Genetic population structure was evaluated with F_{ST} statistics and the net mean nucleotide distance (d_A) using Kimura's 2-parameter method (Kimura 1980) in ARLEQUIN 3.01. The significance of F_{ST} was tested by 10 000 permutations. The unrooted NJ tree inferred from d_A was constructed using PHYLIP 3.66. The population structure was further tested using SAMOVA, in the same manner as used for the microsatellites.

RESULTS

Microsatellite DNA analyses

The sample from ERM showed poorly reproducible PCR amplification for *Nlw13*, *Nlw18,* and *Nlw27* and ERM was therefore excluded from microsatellite analyses. Allele frequencies and parameters of genetic variability of each locus and sampling location are detailed in Table S1 in the Supplement at www.int-res.com/articles/suppl/m484p131_supp.pdf and Table 2, respectively. The global F_{ST} values for each locus were all significantly greater than zero, with the exception of *Nlw2,* which was monomorphic in many of the sampling locations (Table 2). These values represent the overall genetic divergence across all sampling locations. The levels of genetic variability differed among sampling locations and were higher in samples from the 2 localities of Honshu Island (HCH and ONG) (R_s = 7.6, H_e = 0.581 on average), but lower in the samples from the 6 localities of Hokkaido (R_s = 5.0, H_e = 0.481 on average), as well as the 2 localities from the Eurasian coast (R_s = 4.6, H_e = 0.495 on average). Significant deviations from the HWE were detected in *Nlw11*, *Nlw13*, *Nlw18,* and *Nlw27* with heterozygote deficiency, suggesting the existence of null alleles. The existence of null alleles was also estimated using the method of Brookfield (1996) in *Nlw11*, *Nlw13*, *Nlw18*, and *Nlw27*, with a 95% confidence interval, but the frequencies of null alleles were all under 30%, with the highest value of 23.9% observed in GNG (data not shown). We then calculated all population genetic parameters by using both null-adjusted and nonadjusted data. Both sets of results showed consistent patterns and tree topologies; therefore, we only present the nonadjusted data in this study.

F_{ST} values were calculated to estimate the genetic divergence among populations. Global F_{ST} values across 10 sampling locations were relatively high (F_{ST} = 0.176) and pairwise F_{ST} values were also significantly different from zero in every pair of sampling locations (Table 3). Pairwise F_{ST} values were the lowest between OKP and ABS (0.013) and the highest between VSK and SZN (0.319). Pairwise F_{ST} values tended to be lower among sampling locations within the same water body and higher among sampling locations belonging to different water bodies.

D_s values were calculated between 10 sampling locations and were adopted to construct NJ trees. The NJ tree (Fig. 2a) was composed of 3 major clusters: the first consisted of 2 localities from the Eurasian coast (VSK and GNG), 4 localities from the northern coast of Hokkaido (OTR, WKN, OKP, and ABS), and HCH; the second consisted of 2 localities from the Pacific coast (AKS and SZN); and the third consisted of ONG. The first cluster was divided into 4 subclusters, namely VSK and GNG from the Eurasian coast, OTR and WKN from the Sea of Japan, OKP and ABA from the Okhotsk Sea, and HCH from Honshu Island. VSK and GNG from the Eurasian coast subcluster were relatively divergent from each other (D_s = 0.1613, F_{ST} = 0.1397). In addition, the hierarchical population structure estimated by SAMOVA demonstrated the highest F_{CT} value at K = 4 (F_{CT} = 0.271: VSK vs. GNG, OTR, WKN, OKP, ABS vs. AKS, SZN, HCH vs. ONG) and K = 5 (F_{CT} = 0.270: VSK vs. GNG vs. OTR, WKN, OKP, ABS vs. AKS, SZN, HCH vs. ONG), which was to a large extent consistent with the tree topology.

Table 2. *Nucella heyseana*. Genetic variability of 6 microsatellite loci at 10 sampling sites. Abbreviations are as follows: N: no. of individuals; *Na*: no. of alleles observed per locus; *Rs*: no. of alleles observed per locus standardized to the smallest sample size (N = 28); R: allelic range in bp; H_e: expected heterozygosity; H_o: observed heterozygosity; F_{IS}: inbreeding coefficient; (–) division by zero; F_{ST} and R_{ST}: single locus estimates of F_{ST} and R_{ST} over all samples. *Significant departure from zero (p < 0.05)

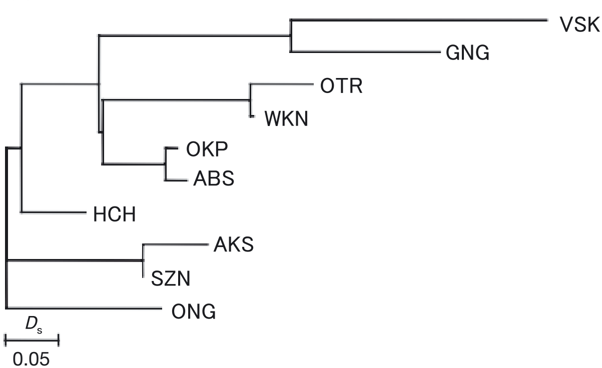
Site		Locus						Mean
		Nlw2	*Nlw3*	*Nlw11*	*Nlw13*	*Nlw18*	*Nlw27*	all loci
VSK	N	30	30	30	30	30	30	30.0
	Na	1	10	5	2	2	5	4.2
	Rs	1.0	9.8	4.9	2.0	2.0	4.9	4.1
	R	121	197-219	191-201	172-184	179-203	152-172	
	He	0	0.782	0.619	0.499	0.210	0.586	0.449
	Ho	0	0.767	0.633	0.533	0.233	0.667	0.472
	F_{IS}	–	0.021	–0.024	–0.069	–0.115	–0.141	–0.052
GNG	N	52	52	52	52	52	52	52.0
	Na	1	12	8	6	2	6	5.8
	Rs	1.0	9.8	6.7	5.1	2.0	5.8	5.1
	R	121	171-225	152-203	168-188	179-203	152-172	
	He	0	0.812	0.726	0.712	0.369	0.629	0.541
	Ho	0	0.846	0.308	0.731	0.365	0.692	0.490
	F_{IS}	–	–0.022	0.579*	–0.006	0.015	–0.113	0.103*
OTR	N	57	57	57	57	57	57	57.0
	Na	1	16	5	5	4	8	6.5
	Rs	1.0	11.3	4.0	5.0	2.9	6.2	5.0
	R	121	166-225	191-201	164-180	177-204	142-172	
	He	0	0.723	0.542	0.730	0.086	0.678	0.460
	Ho	0	0.842	0.526	0.719	0.088	0.526	0.450
	F_{IS}	–	–0.167*	0.029	0.014	–0.024	0.225*	0.021
WKN	N	33	33	33	33	33	33	33.0
	Na	1	7	7	5	2	5	4.5
	Rs	1.0	6.7	6.4	5.0	1.8	4.8	4.3
	R	121	175-215	162-201	164-180	179-203	142-170	
	He	0	0.620	0.704	0.779	0.030	0.651	0.464
	Ho	0	0.515	0.515	0.697	0.030	0.606	0.394
	F_{IS}	–	0.171	0.271*	0.106	0.000	0.070	0.153*
OKP	N	56	56	56	56	56	56	56.0
	Na	1	12	7	6	2	7	5.8
	Rs	1.0	9.2	5.9	5.5	1.9	5.3	4.8
	R	121	175-205	162-203	164-184	184-203	142-172	
	He	0	0.774	0.697	0.637	0.069	0.434	0.435
	Ho	0	0.750	0.446	0.518	0	0.464	0.363
	F_{IS}	–	0.031	0.361*	0.188*	–	–0.071	0.167*
ABS	N	28	28	28	28	28	28	28.0
	Na	1	6	5	5	2	7	4.3
	Rs	1.0	5.9	5.0	5.0	2.0	6.9	4.3
	R	121	175-203	191-201	164-180	177-203	142-172	
	He	0	0.645	0.721	0.605	0.070	0.457	0.416
	Ho	0	0.536	0.429	0.607	0	0.393	0.327
	F_{IS}	–	0.142	0.410*	–0.013	–	0.121	0.202*

Site		Locus						Mean
		Nlw2	*Nlw3*	*Nlw11*	*Nlw13*	*Nlw18*	*Nlw27*	all loci
AKS	N	30	30	30	30	30	30	30.0
	Na	1	16	4	3	5	6	5.8
	Rs	1.0	15.7	4.0	3.0	5.0	5.9	5.8
	R	121	167-221	191-199	172-188	177-203	132-162	
	He	0	0.913	0.704	0.515	0.641	0.656	0.572
	Ho	0	0.900	0.567	0.400	0.300	0.667	0.472
	F_{IS}	–	0.014	0.198*	0.226	0.536*	–0.016	0.176*
SZN	N	51	51	51	51	51	51	51.0
	Na	2	15	7	3	4	5	6.0
	Rs	1.8	13.5	6.1	2.9	4.0	5.0	5.5
	R	113-121	168-227	162-199	172-188	176-203	132-162	
	He	0.039	0.899	0.779	0.519	0.603	0.405	0.541
	Ho	0.039	0.902	0.745	0.588	0.392	0.392	0.510
	F_{IS}	–0.010	0.017	0.059	–0.120	0.352*	0.096	0.078*
HCH	N	35	35	35	35	35	35	35.0
	Na	1	24	9	8	7	5	9.0
	Rs	1.0	21.8	8.6	7.6	6.8	4.6	8.4
	R	121	167-229	189-211	160-188	175-205	126-162	
	He	0	0.943	0.837	0.697	0.702	0.417	0.600
	Ho	0	0.914	0.629	0.800	0.543	0.400	0.548
	F_{IS}	–	0.031	0.252*	–0.150	0.230*	0.042	0.088*
ONG	N	36	36	36	36	36	36	36.0
	Na	1	20	7	7	4	4	7.2
	Rs	1.0	18.1	6.8	6.9	3.8	3.7	6.7
	R	121	163-223	187-199	162-188	177-204	132-162	
	He	0	0.911	0.836	0.810	0.544	0.273	0.562
	Ho	0	0.889	0.833	0.833	0.361	0.250	0.528
	F_{IS}	–	0.024	0.003	–0.029	0.339*	0.084	0.062
Mean all lots	N	40.8	40.8	40.8	40.8	40.8	40.8	40.8
	Na	1.1	13.8	6.4	5.0	3.4	5.8	5.9
	Rs	1.1	12.2	5.8	4.8	3.2	5.3	5.4
	R	113-121	163-229	162-211	160-188	175-205	132-172	
	He	0.004	0.802	0.716	0.650	0.332	0.519	0.504
	Ho	0.004	0.786	0.563	0.643	0.231	0.506	0.455
	F_{ST}	0.007	0.121*	0.131*	0.153*	0.365*	0.191*	0.176*
	R_{ST}	0.007	0.119	0.075	0.228	0.472	0.142	0.238*

Table 3. *Nucella heyseana*. Pairwise estimates of F_{ST} between localities based on 6 microsatellite loci (lower matrix) and mtDNA-COI sequencing (upper matrix)

	VSK	GNG	OTR	WKN	OKP	ABS	AKS	SZN	HCH	ONG
VSK		0.116**	0.620**	0.862**	0.886**	0.741**	0.773**	0.779**	0.644**	0.640**
GNG	0.140**		0.609**	0.806**	0.842**	0.684**	0.726**	0.738**	0.604**	0.612**
OTR	0.215**	0.149**		0.759**	0.812**	0.701**	0.704**	0.725**	0.663**	0.663**
WKN	0.229**	0.163**	0.027**		−0.002	0.053	0.892**	0.872**	0.450**	0.634**
OKP	0.221**	0.155**	0.092**	0.064**		0.106*	0.879**	0.869**	0.551**	0.715**
ABS	0.227**	0.174**	0.130**	0.098**	0.013*		0.580**	0.607**	0.194*	0.397**
AKS	0.287**	0.220**	0.237**	0.195**	0.238**	0.226**		−0.003	0.146*	−0.004
SZN	0.319**	0.235**	0.249**	0.200**	0.219**	0.211**	0.025**		0.169**	0.008*
HCH	0.253**	0.183**	0.198**	0.160**	0.149**	0.139**	0.075**	0.044**		0.063*
ONG	0.302**	0.203**	0.228**	0.211**	0.212**	0.197**	0.088**	0.074**	0.052**	

*$p < 0.05$, **$p < 0.01$



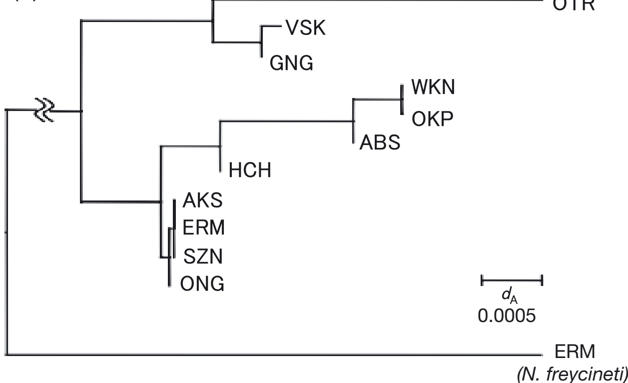


Fig. 2. *Nucella heyseana*. Neighbor-joining tree of genetic relationships among sampling locations (a) based on 6 microsatellite loci, using null-corrected data, (b) based on mtDNA-COI sequencing. D_S: Nei's unbiased genetic distance; d_A: net nucleotide distance

mtDNA analyses

A 471-bp mtDNA-COI fragment revealed 51 polymorphic sites defining 41 putative haplotypes (DDBJ accession number submitted) in 271 individuals across 11 sampling locations (Table S2 in the Supplement). All variable sites were substitutions, and there were no indels. Among the 51 variable sites, 60.7% were classified as transitions, 33.3% were classified as transversions, and 5.8% included both types. The percentages of synonymous and nonsynonymous substitutions were 61.2 and 32.2%, respectively.

The parsimony network of 41 haplotypes and their frequencies are summarized in Fig. 3 and Table S2. Among the 41 haplotypes, 6 haplotypes (Hn36 to Hn41), observed in 14 individuals from ERM, clearly diverged from the other haplotypes with at least 23 substitutions. These 14 individuals exhibited shorter screw head length compared to the other specimens, corresponding to *Nucella freycineti* morphology, as identified by Zaslavskaya & Kolotuchina (2003). Therefore, these 14 individuals were classified as a different data set and were not included in the further calculations of haplotype diversity or F_{ST} values among populations. The other 35 haplotypes exhibited a simple network with 4 major haplotypes (Hn01, Hn08, Hn16, and Hn26) connected by 1 to 2 substitutions and several minor satellite haplotypes (Fig. 3). Based on the geographic location and shell morphological features (3 to 14 deep, clear spiral cords), we designated the individuals using Hn01 to Hn35 as *N. heyseana*, as identified by Zaslavskaya & Kolotuchina (2003). Among *N. heyseana*, the major haplotypes differed between sampling locations: Hn08 in VSK (71.4%) and GNG (71.0%); Hn26 in OTR (73.1%); Hn16 in WKN (93.3%), OKP (92.5%), and ABS (73.3%); and Hc01 in AKS (94.4%), ERM (92.3%), except for *N. freycineti*, SZN (100%), HCH (57.9%), and ONG (70%).

The molecular diversity of each sampling location is summarized in Table 4. Among 10 sampling locations, *h* was on average 0.351 and, except for ERM, was lowest in AKS (0.111). The highest value was found for HCH (0.620). Average π was 0.0011 and,

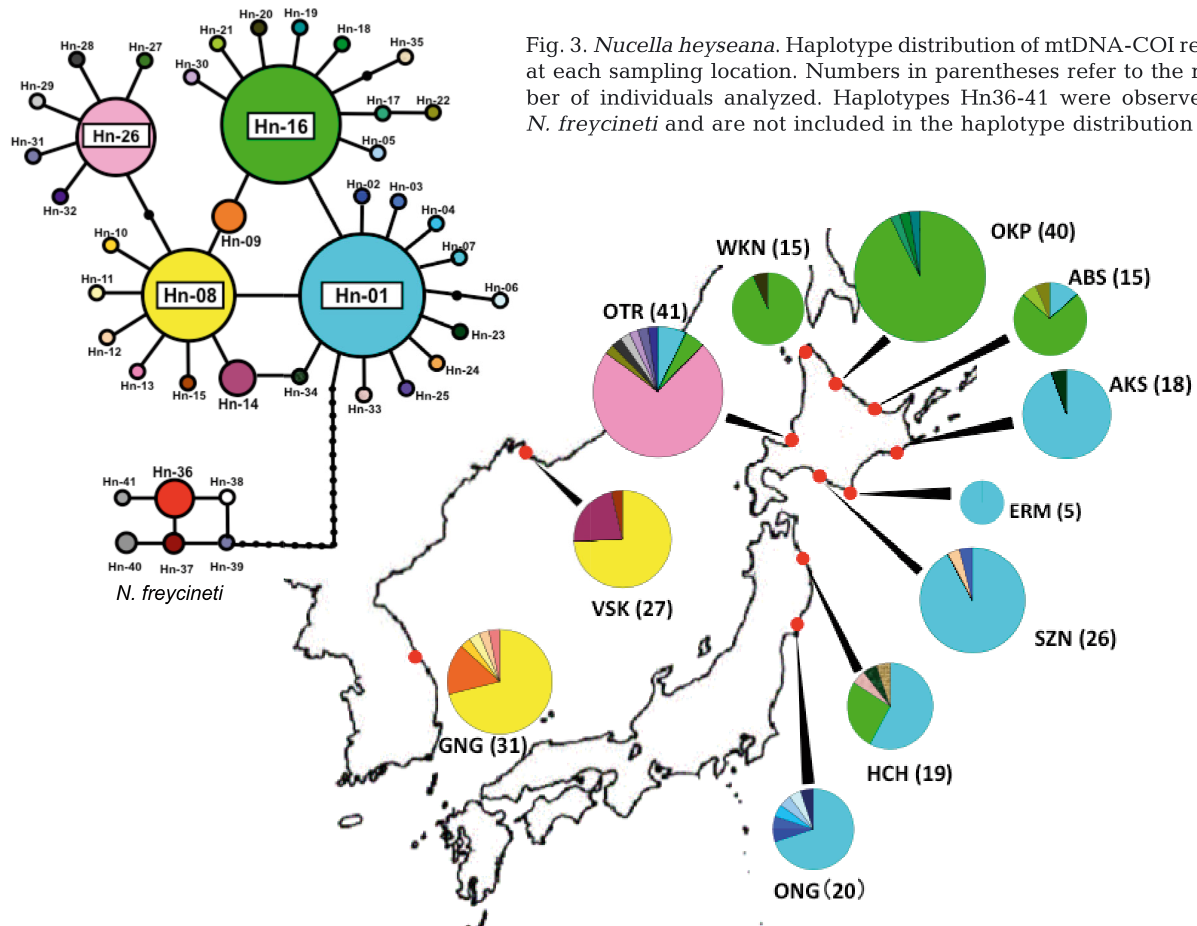


Fig. 3. *Nucella heyseana*. Haplotype distribution of mtDNA-COI region at each sampling location. Numbers in parentheses refer to the number of individuals analyzed. Haplotypes Hn36-41 were observed in *N. freycineti* and are not included in the haplotype distribution map

except for ERM, the lowest value was found for AKS (0.0002). The highest value was found for OTA (0.0026). Genetic variability was much higher in HCH and ONG from the Pacific Ocean side than other sampling locations, which was identical to the results obtained from microsatellites.

Table 4. *Nucella heyseana* and *N. freycineti*. Haplotype diversity (*h*) and nucleotide diversity (π) of mtDNA-COI fragments

Site	Sample size	Haplotype Numbers	Haplotype h	Haplotype π
VSK	27	3	0.416	0.0009
GNG	31	6	0.482	0.0011
OTR	41	9	0.465	0.0026
WKN	15	2	0.133	0.0003
OKP	40	4	0.146	0.0003
ABS	15	4	0.467	0.0019
AKS	18	2	0.111	0.0002
SZN	26	3	0.151	0.0003
HCH	19	5	0.620	0.0019
ONG	20	7	0.521	0.0017
ERM (*N. freycineti*)	14	6	0.747	0.0024
Mean of 10 lots[a]			0.351	0.0011

[a]Except for *N. freycineti* from ERM

The global F_{ST} value across 10 sampling locations was extremely high (0.701), suggesting high genetic divergence among local populations in *Nucella heyseana*. Pairwise F_{ST} values ranged from 0.005 to 0.8920 and all pairs except WKN–OKP, WKN–ABS, AKS–SZN, and AKS–ONG exhibited significant pairwise F_{ST} values ($p < 0.05$) (Table 3, upper matrix). F_{ST} values tended to increase between sampling locations belonging to different water bodies.

The NJ tree based on d_A demonstrated that *Nucella freycineti* from ERM diverged significantly from other *N. heyseana* samples (Fig. 2b). Four major clusters were classified among *N. heyseana*: the Eurasian coast (VSK and GNG); the Sea of Japan side of Hokkaido (OTR); the Okhotsk Sea side of Hokkaido (WKN, OKP, and ABA); and the Pacific coast (AKS, SZN, ERM, HCH, and ONG) (Fig. 2b). OTR was highly independent from the other 3 clusters, but was most similar to the Eurasian coast cluster. Among the other 3 clusters, the Okhotsk and Pacific Ocean clusters were most closely related. The level of genetic divergence was relatively low within the cluster, except between HCH and 3 other localities. The hierarchical

population structure estimated by SAMOVA demonstrated the highest F_{CT} value at K = 4 (F_{CT} = 0.726: VSK, GNG vs. OTR vs. WKN, OKP, ABS vs. AKS, SZN, HCH, ONG), consistent with the tree topology.

DISCUSSION

Species composition of *Nucella* species along the Japanese coast

In our samples there were 2 genetically isolated groups, one that comprised 14 individuals from ERM and one that contained all of the other specimens. The morphological features of these 14 specimens were slightly different from those of the others, namely the length of the spire was shorter and there was a lack of constriction between the spire and the body whorl. We compared the registrations, drawings, and photos of Habe (1958), Egorov (1992), Tsuchiya (2000), and Zaslavskaya & Kolotuchina (2003) carefully and concluded that the 14 ERM specimens met the definition of *Nucella freycineti*. *N. freycineti* was initially identified in the Kamchatka Peninsula and was more recently found at Etorofu Island (Zaslavskaya & Kolotuchina 2003) and Akkeshi in Hokkaido (Yamazaki 2011). Specimens from this species were thought to be more cryophilic than the specimens from the other species found in this study (presumed species *N. heyseana*). The Erimo region therefore likely represents the edge of the distribution area of *N. freycineti*, because this region is located at the southern tip of the cold Oyashio Current and is therefore unaffected by the warmer Tsushima Current. It is very interesting that the genetic variability of the mtDNA of *N. freycineti* was higher (h = 0.747) than that observed in *N. heyseana* populations (h = 0.111 to 0.620), despite its low incidence and it is highly possible that a cryptic viable population exists in this region.

The populations of *Nucella heyseana* examined in this study were highly divergent from one another; however, haplotype networks showed that the 4 major haplotypes (Hn01, Hn08, Hn16, and Hn26) observed in *N. heyseana* were connected to one another with only 1 to 2 substitutions. This suggests that all the samples collected in this study should be classified within the same species. We could not find any other *Nucella* species along the coast of Japan; this suggests that the majority of intertidal *Nucella* individuals in Japan are either *N. heyseana* or, in some northern regions, *N. freycineti*.

Genetic variability of *Nucella heyseana*

The genetic variability of *Nucella heyseana* differed among sampling locations. Although genetic variability is generally lower at the edge of a species' range, because of the small population size (Marko 2004, Bell & Okamura 2005), we found the opposite in our study: higher variability in both microsatellites and mtDNA in the populations at HCH and OGN. In contrast, research has demonstrated that genetic variability is higher when the breeding population is large (Duran et al. 2004). We estimated large population sizes based on observations of high densities of adult individuals from the coast of Onagawa, as described by Kijima et al. (2004). However, an accurate estimation of the actual population size is required to assess the effective population size of each site.

Genetic population structure of *Nucella heyseana*

A highly subdivided population structure was observed in *Nucella heyseana*, with relatively high F_{ST} values across all sampling locations in both microsatellites (0.177) and mtDNA (0.701). It seems likely that the highly divergent population structure of this species is closely related to its breeding ecology. *Nucella* species are direct developmental snails and do not spawn directly in seawater (Gallardo 1979, Crothers 1980, Tsuchiya 2000). A male *Nucella* and a female *Nucella* mate, and the female deposits egg capsules on the intertidal rocks. The larvae develop in the egg capsule until the latter stage of veliger, and then, following a short pelagic duration, they settle on suitable intertidal rocks as adults. This short pelagic duration restricts gene flow among localities, which leads to a pronounced population structure.

A negative correlation between the duration of the pelagic larval stage and the level of genetic divergence among local populations has been demonstrated in a number of different studies (Helmuth et al. 1994, Hoskin 1997, Todd et al. 1998). However, the precise larval duration of *Nucella heyseana* has not been determined, and the relationship between the larval period and the population structure in this species should be evaluated by further ecological research, including mating experiments, to reveal the process of development.

In addition, the limitation of mobility in adult individuals of the *Nucella* species is likely to prevent migration among local populations. Hughes (1972) reported that adult individuals of *N. lapillus* moved only

2 to 4 m over a period of 1 yr. Similarly, our investigation of the mobility of adult individuals of *N. heyseana* in Onagawa demonstrated that 52% of the marked individuals remained on the same rocky shore for the entire 2 mo of the study (data not shown).

The highly divergent population structure observed in *Nucella heyseana* is not an uncommon feature for marine animals lacking a pelagic larval stage. However, a previous study of *N. lapillus*, which is also a direct developer, showed relatively low levels of genetic structure (Wares & Cunningham 2001, Colson & Hughes 2004, 2007). The authors of these studies further suggested that rafting could be a major agent of dispersal for marine species lacking planktonic life stages. They pointed out the inadequacy of systematically estimating potential levels of gene flow in marine organisms from developmental mode alone.

In the present study, 3 related groups (Eurasian coast, northern Hokkaido, and Pacific coast), were identified based on the analysis of microsatellites and 4 related groups (Eurasian coast, Okhotsk coast, Sea of Japan side of Hokkaido, and Pacific coast), were identified based on the analysis of mtDNA. These results suggest that the population structure of *Nucella heyseana* is also influenced by geographical distance and ocean current, in addition to breeding ecology. For example, 2 neighboring populations of *N. heyseana*, belonging to different bodies of water, had clearly divergent distinctive characteristics, suggesting that the gene flow of *N. heyseana* is strongly controlled by the ocean current (Fig. 4). For HCH, an intermediate genetic composition between the Pacific Ocean side and Sea of Japan side was observed, in accordance with the mixture of the Tsushima and Oyashio Currents. Similar results have also been reported for other snails along the coast of Japan (*Batillaria cumingi* by mtDNA-COI sequencing by Kojima et al. (2004), *Littorina sitkana* by protein electrophoresis by Nohara (1999)).

Additionally, the historical background of the population plays an important role in the current population structure. Marko (2004) reported markedly different genetic structures between 2 species of *Nucella* (*N. ostrina* and *N. lamellosa*) and attributed this to differences in biogeographical response to climate change. In our study region, ocean currents have been drastically changed by glacial and interglacial cycles. During glacial maxima, the Sea of Japan is believed to have been isolated from the Pacific Ocean by the connecting Eurasian continent, Sakhalin, and the Hokkaido and Honshu Islands, due to the lowering sea level (Nishimura 1974). This historical hydrographic change has had a considerable impact on the phylogeographic structure of many marine species and the deep divergence of the lineage between the populations of the Pacific coast and the Sea of Japan coast was also observed in the intertidal gastropod *Batillaria cumingi* (Kojima et al. 2004) and the intertidal goby *Chaenogobius annularis* (Hirase et al. 2012). However, the haplotypes for *N. heyseana* showed low divergence from one another, indicating that the present populations of *N. heyseana* represent a single lineage and that the high levels of population structure observed in this study were recently formed, presumably after postglacial expansion and colonization events following the Last Glacial Maximum. Similar biogeographical histories were also suggested for *N. lapillus* (Wares & Cunningham 2001) and *N. ostrina* (Marko 2004); this suggests that the *Nucella* species inhabiting the exposed intertidal rocky shore are highly sensitive to climate change.

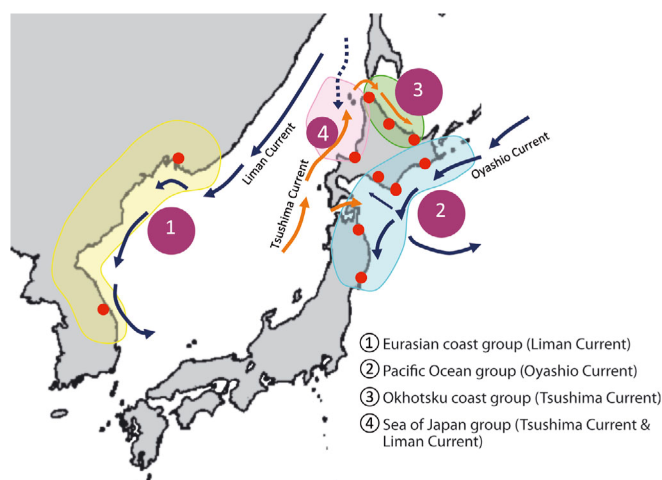


Fig. 4. *Nucella heyseana*. Relationship between ocean current and population structure, based on mtDNA analyses

Discrepancies between microsatellite and mtDNA analyses in population clustering were also observed in the present study. The chief difference between the 2 markers was the position of OTR, which was in a cluster with neighboring populations for microsatellites, while being isolated from any other populations for mtDNA, as shown both in the NJ tree and SAMOVA. This may be explained by 2 possible hypotheses. The first hypothesis suggests that in this study, the mtDNA-COI sequence was more sensitive than the microsatellite loci. This is well established and is a consequence of the maternal haploid inheritance of mtDNA (Karl et al. 2012). The effective population size (Ne) of mtDNA is only one-fourth that of nuclear genes; therefore, mtDNA is expected to

diverge 4 times faster than nuclear DNA when gene flow is restricted (Birky et al. 1989). This hypothesis is supported by our results demonstrating higher F_{ST} values for the mtDNA marker (global F_{ST} = 0.701) than for the microsatellites (global F_{ST} = 0.177). The ability of DNA markers to detect population structure usually depends on their genetic variability. However, except for *Nlw2*, the genetic variability of the microsatellite loci used in this study was sufficiently high to detect differentiation among local populations (H_e = 0.332 to 0.802). It is therefore possible that the microsatellites with high variability could not detect genetic differentiation due to the allele homoplasy prevalent in microsatellite DNA (Estoup et al. 2002, Mank & Avise 2003). This problem can be resolved by using multiple marker loci to minimize the effects of homoplasy at the particular locus.

The second hypothesis suggests a difference in the diffusion speed (i.e. the level of gene flow) between the nuclear genome and mtDNA. mtDNA analyses suggested that the OTR population could previously have diverged from the population on the Eurasian coast and subsequently gradually intermixed with other populations in Hokkaido. If the diffusion speed is much faster in microsatellite DNA, the clustering by microsatellites will reflect a more recent population structure of *Nucella heyseana*, while the mtDNA will still carry the distinct appearance of past populations. However, it should be noted that the difference in diffusion speed between the nuclear genome and mtDNA is usually caused by the difference in mobility between males and females (Prugnolle & de Meeus 2002). In the present study, we did not collect any data related to the mobility of adult individuals according to sex; however, the gene flow of *N. heyseana* may correlate more strongly with the movement of small juveniles by such processes as rafting. Therefore, this hypothesis is difficult to support.

CONCLUSIONS

Most of the snails in our samples were identified as *Nucella heyseana*. In addition, some individuals from ERM were identified as *N. freycineti*. We therefore identified 2 species in our samples that were clearly genetically different from each other. These results should contribute to improving our understanding of the taxonomy of *Nucella* species around East Asia, which has long been the subject of debate. The samples from our study have been stored in the National Museum of Nature and Science, Tokyo, as voucher specimens.

In addition, the *Nucella heyseana* samples examined in this study formed highly divergent local populations, suggesting that direct development influences population structure and demonstrating the dominant effect of the main ocean current specific to each body of water. These data should be useful not only for conservation of this species, but also for developing conservation strategies for intertidal communities in this region.

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