Phylogeography of Mytilisepta virgata (Mytilidae: Bivalvia) in the northwestern Pacific: cryptic mitochondrial lineages and mito-nuclear discordance

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August 20, 2020

Abstract

The purplish bifurcate mussel Mytilisepta virgata is widely distributed and represents one of the major components of the intertidal community in the northwestern Pacific (NWP). Here, we characterized population genetic structure of NWP populations throughout their whole distribution range using both mitochondrial (mtDNA cox1) and nuclear (ITS1) markers. Population genetic analyses for mtDNA cox 1 sequences revealed two monophyletic lineages (i.e., southern and northern lineages) geographically distributed according to the two different surface water temperature zones in the NWP. The timing of the lineage split is estimated at the Pliocene- mid-Pleistocene (5.49-1.61 Mya), which is consistent with the timing of the historical isolation of the East Sea/Sea of Japan from the South and East China Seas caused by sea level decline during glacial cycles. Historical sea level fluctuation during the Pliocene-Pleistocene and subsequent adaptation of mussels to different surface water temperature zones may have contributed to shaping the contemporary genetic diversity and deep divergence of the two mitochondrial lineages. Unlike mtDNA sequences, a clear lineage splitting between the two mitochondrial lineages was not found in ITS1 sequences, showing a star-like structure that is composed of a mixture of southern and northern mitochondrial lineages. Possible scenarios are proposed to explain this type of mito-nuclear discordance: stochastic divergence in the coalescent processes of the two molecular markers, or balancing selection under different marine environments. Future work is required to address whether the thermal physiology of these mussels correlates with the deep divergence of their mitochondrial genes.

Introduction

The northwestern Pacific (NWP) is a vast marine realm characterized by unique tectonic and hydrologic features (Wang, 1999), providing a natural setting in which to investigate mechanisms and processes leading to marine diversification and speciation (Kong, Matsukuma, Hayashi, Takada, & Li, 2012; Ni, Li, Kong, & Yu, 2014; Ho, Kwan, Kim, & Won, 2015; Wang, Tsang, & Dong, 2015; Qiu, Li, Lin, Ding, & Miyamoto, 2016). Three continuous marginal seas are distributed from north to south in the NWP, including the East Sea (ES)/Sea of Japan (SJ), East China Sea (ECS) and South China Sea (SCS) (Tamaki & Honza, 1991). The size and connectivity of these seas probably dramatically changed throughout the Pleistocene (Wang et al., 1997). During past glacial epochs, sea levels dropped more than 120 m below the present level, resulting in the exposure of sills and continental shelves in the NWP (Wang & Sun, 1994; Voris, 2000) and effectively closing the sea passage (the Korean Strait, depth <130 m) between the ES/SJ and ECS, resulting in their geographic separation (Kitamura, Takano, Takata, & Omote, 2001). The ECS experienced pronounced

seawards migration (~1200 km), and was reduced to an elongated and closed trough (the Okinawa basin) (Xie, Jian, & Zhao, 1995), while the SCS became a semi-closed gulf with an area that was half its present size, connected to the Pacific through the Bashi Strait (Wang, 1999).

The dramatic paleohydrogeological changes in the NWP probably had a profound impact on marine species' distribution and evolution (Kong & Li, 2009; Liu, Li, Kong, & Zheng, 2011). An evolutionary paradigm emerging from marginal sea separation is that each sea basin served as an independent refugium for surviving populations during the glacial periods, and enhanced vicariant divergence among separated populations (Liu, Gao, Wu, & Zhang, 2007; Xu, Chan, Tsang, & Chu, 2009; Ni et al., 2014; Qiu et al., 2016). This pattern has been demonstrated in various marine faunas, including fishes (Shen, Jamandre, Hsu, Tzeng, & Durand, 2011; Song, Ma, Zhang, Gao, & Sun, 2014; Qiu et al., 2016), molluscs (Kong & Li, 2009; Liu et al., 2011; Ni, Li, Kong, & Zheng, 2012), barnacles (Chan, Tsang & Chu, 2007; Tsang et al. 2012) and mitten crab (Xu et al., 2009; Xu & Chu, 2012), all of which show substantial intraspecific evolutionary partitions among the seas. However, the paradigm is not applicable to all cases and is often complicated by biotic and/or abiotic factors, such as surface water temperate gradients, ocean currents, and diverse species-specific life-history traits (Dong, Han, Ganmanee, & Wang, 2015; He, Mukai, Chu, Ma, & Zhang, 2015; Ni, Li, Ni, Kong, & Yu, 2015; Li et al., 2017; Ni et al., 2020).

The surface water temperature gradient along the main coastline of the NWP is substantial. This coastline spans a broad latitudinal range roughly from 15 to 45° N and represents a transition zone connecting the tropical Indo-West Pacific and the cold North Pacific (Chen & Jiao, 1997; Briggs & Bowen, 2012). A biogeographic line, which begins at the Yangtze Estuary in China and extends northeastwards to Jeju Island (Korea) and southern Japan (Fig. 1), separates the North Pacific Temperate Biotic Region (characterized by cold-temperate fauna) and Indo-West Pacific Warm-water Biotic Region (characterized by tropical/subtropical fauna) (Liu, 2013; Ni, Kern, Dong, Li, & Park, 2017). Species with narrow thermal tolerances are restricted to one side of the boundary because of the steep temperature gradient across the Yangtze Estuary (Zhang, Qi, Zhang, & Ma, 1963; Liu, 2013): the annual sea surface temperature is >20°C on the south side of the estuary, and rapidly decreases to <15°C on the north side (Johnson & Boyer, 2015). The surface water temperature gradient in this region has been reported as an influential driver that accelerates genetic differentiation (e.g., seagrass *Zostera japonica* , Zhang, Zhou, Xue, & Liu, 2016) or cryptic speciation between neighboring populations (e.g., fish *Mugil cephalus* , Shen et al., 2011). However, whether this temperature gradient is a general driver of genetic differentiation in other marine species, especially coastal invertebrate species, is still largely unknown.

Ocean currents can also impact species' genetic patterns in this region (Dong et al., 2012; Han, Yanagimoto, Zhang, & Gao, 2012; He et al., 2015; Li et al., 2017). The main current systems in the NWP include the cyclonic circulation of the Kuroshio Current (KC) and coastal currents (Su & Yuan, 2005; Wei, Yu, Ran, & Zang, 2011). An extension of the North Equatorial Current, the KC is a major current that dramatically affects this region's environment and ecology (Kao, Wu, Hsin, & Dai, 2006; Fujikura, Lindsay, Kitazato, Nishida, & Shirayama, 2010). It originates east of the Philippines and flows north-eastwards through Taiwan and the Ryukyu Islands to the Pacific coast of southern mainland Japan (Su, 2004). The KC and its branch currents transport large volumes of water, salt, and nutrients from low latitude tropics to the northern reaches of the marginal seas (Chen, 1997; Heath, Zenitani, Watanabe, Kimura, & Ishida, 1998). This region is also characterized by diverse coastal currents (Su & Yuan, 2005; Wei et al., 2011), such as the Subei Coastal Current and the China Coastal Current along the coast of China, the Korean Coastal Current around the Korean Peninsula, and the Oyashio Current (a cold subarctic ocean current that originates in the Bering Sea flowing southwest off the Kuril Islands and meet the KS east of northern Japan). Oceanographic currents may potentially enhance population connectivity, often producing different outcomes from sea surface temperature gradient. Lack of genetic structuring among distant populations can be maintained by continuous gene flow of current-driven larval dispersal (Guo et al., 2015) and rafting events (Nikula, Fraser, Spencer, & Waters, 2010).

The purplish bifurcate mussel *Mytilisepta virgata* (Wiegmann, 1837), a member of the family Mytilidae,

is widely distributed in the NWP including Korea, Japan, mainland China and Taiwan and forms large mussel beds, constituting one of the major intertidal rocky shore communities (Iwasaki, 1994; Liu & Morton, 1994; Morton, 1995; Okutani, 2000; Min, Lee, Koh, & Je, 2004). These mussel beds contribute to marine biodiversity in the intertidal zone by providing habitat and shelter for diverse marine invertebrate species (August, 1985; Seed, 1996). This species spawns twice a year, in spring (from February to March) and autumn (from September to October) (Morton, 1995). The larval duration of this species' planktonic stage is 4 days (Sasaki, 1984), which can facilitate gene flow over a certain distance. These traits (distribution range and larval ecology) make this species ideal for testing the impacts and their relative strength of historical sea level fluctuations, surface water temperature, and ocean currents on marine phylogeography. In this study, based on comprehensive sampling throughout the mussel's entire distribution in the NWP, we assessed multiple lines of evidence (including mitochondrial and nuclear genes as well as morphological data) to (1) test whether there exist evolutionarily divergent linages in the NWP and (2) investigate driving factors that may have prompted cryptic divergence.

Materials and methods

Sampling and DNA sequencing

For mitochondrial *cox1* and nuclear ITS1 sequencing, a total of 680 *M. virgata* individuals were collected from the intertidal and/or subtidal zone at 34 localities on the seashores of China, Japan, Korea, and Taiwan, which covers almost the entire distribution range of*M. virgata* (Fig. 1; Table 1). MtDNA *cox1* and ITS1 from four individuals of *M. keenae* were also sequenced and used as outgroup sequences in the phylogenetic analyses. All samples were deposited as voucher specimens in the Marine Mollusk Resource Bank of Korea (MMRBK: http://mmrbk.org/). Total genomic DNA was extracted from the adductor muscle of each individual using a DNeasy tissue kit following the manufacturer's instructions. A partial fragment of the mtDNA *cox1* gene was PCR-amplified using the universal primers jgLCO1490 and jgHCO2198 (Geller, Meyer, Parker, & Hawk, 2013) in total reaction volumes of 50 μL, which included 2 μL of template DNA, 36.75 μL of D.W., 5 μL of 10x Taq buffer, 1 μL of each primer (10 pmol), 4 μL of 0.2mM dNTP mixture, and 0.25 μL of *Taq* polymerase (TaKaTa Ex*Taq*). The PCR amplification conditions were as follows: 1 cycle of denaturation at 95 for 3 min followed by 35 cycles of denaturation at 94 for 30 s, annealing at 45 for 30 s, elongation at 72 for 1 min, and a final extension at 72 for 10 min. To cross-reference the mtDNA*cox1* results, the nuclear ITS1 gene was PCR-amplified and sequenced for 101 individuals from 23 populations, which were subsampled so that mtDNA *cox1* haplotype diversity was well-represented in the ITS1 group (Table 1). The nuclear ITS1 gene fragment was PCR-amplified using ITS1 (5´-TCC GTA GGT GAA CCT GCG G-3´) (White, Bruns, Lee, & Taylor, 1990) and 5.8S-R (5´-TCG ATG AAG AAC GCA GC-3´) (Vilgalys & Hester, 1990). The PCR mixtures and conditions for the ITS1 gene fragment were the same as for mtDNA *cox1* , except that the annealing temperature was 55. PCR products were isolated from 1% agarose gels using a QIAquick gel extraction kit (QIAGEN Valencia, USA) following standard protocols and sequenced directly using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA).

Phylogenetic analyses and population structure

DNA sequences were aligned using Clustal W (Thompson, Higgins, & Gibson, 1994) with default options in Geneious Pro v8.1.9 (Biomatters, Auckland, New Zealand). Haplotypes were identified with a Bayesian coalescent-based program performed in DnaSP v5.10.1 (Librado & Rozas, 2009). All mtDNA *cox1* and ITS1 haplotypes were deposited in GenBank (*cox1* : MN728370-MN728538; ITS1: MN728333-MN728363). Phylogenetic relationships among haplotypes were inferred using maximum likelihood (ML) analysis and Bayesian inference (BI) implemented in RAxML v8.2.9 (Stamatakis, 2014) and MrBayes v3.2.6 (Huelsenbeck & Ronquist, 2001) using nucleotide sequences of *M. keenae* (GenBank Accession nos. for *cox1* : MN728366 – MN728369; GenBank Accession nos. for ITS1: MN728364 – MN728365) as an outgroup. The best fit models for the two genes were determined using the Akaike information criterion (AIC) in jModelTest 2.1.10 (number of substitution schemes = 11; Darriba, Taboada, Doallo, & Posada, 2012): GTR+I for *cox1* and TVM+G (0.47) for ITS1. The ML analysis was performed using 1,000 bootstrap replicates. The BI analysis was conducted with two independent runs, each with four simultaneous Monte Carlo Markov chain (MCMC)

runs for 1 x 10^6 generations. Trees were sampled in every 100 generations and posterior probability was estimated after removing an initial 25% of generations (reaching a stationarity of the chains) as burn-in. Haplotype networks were constructed using PopArt v1.7 (Leigh & Bryant, 2015) software via the TCS method (Clement, Posada, & Crandall, 2000).

Molecular diversity indices such as haplotype diversity (h), nucleotide diversity (π), and mean number of pairwise differences (k) of each population were estimated with ARLEQUIN v3.5 (Excoffier & Lischer, 2010). Pairwise genetic divergence between populations was evaluated using the fixation index (F_{st}) (Excoffier, Smouse, & Quattro, 1992). A hierarchical analysis of molecular variance (AMOVA) was conducted to estimate population structure among geographical groups, which were determined by a spatial analysis of molecular variance performed with SAMOVA v2 (Dupanloup, Schneider, & Excoffier, 2002). These analyses were carried out with 1,000 permutations after sequential Bonferroni adjustments using ARLEQUIN.

Morphological analysis

To confirm the molecular results (i.e., the presence of two distinct lineages detected from the molecular analysis), we examined shell morphometrics of *M. virgata* populations. A total of 15 shell characters (supplementary Fig. 1) were measured from 50 randomly selected individuals from each lineage (100 individuals in total). Measured shell characters were log-transformed, and principal components analysis (PCA) was performed in R-studio to assess morphological differences between the two lineages.

Divergence time and historical demography

The divergence time between the two *M. virgata cox1* lineages was estimated using a strict clock model and the HKY+I model in BEAST v2.4 (Drummond & Bouckaert, 2015). The mtDNA *cox1* molecular clock for this species have not been empirically assessed and we applied two different fossil-based divergence rates (a minimum rate of 0.7% and a maximum rate of 2.4%/Mya) for mtDNA *cox1* estimated from two different molluscan taxa (0.7%-1.2%/Mya [the ark clam; Marko, 2002]) and 2.4%/Mya; [teguline gastropod; Hellberg & Vacquier, 1999]). To estimate the timing of divergence between the two *M. virgata* lineages, two independent runs with an MCMC chain length of 2 x 10^8 were performed with sampling every 1,000 generations. Log and tree files were combined with LogCombiner v2.4.8 (Rambaut & Drummond, 2014a) with a burn-in of 25%. The effective sample size (ESS) values of all parameters above 1,000 were obtained in Tracer v1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). Trees were used to build a maximum clade credibility tree in TreeAnnotator v2.4.8 (Rambaut & Drummond, 2014b) and visualized in FigTree v1.4.3 (Rambaut, 2016). For testing historical demographic events, Tajima's D and Fu's F_S statistics were first calculated to test for neutrality using ARLEQUIN. We then used the pairwise mismatch distribution to test population expansion using DnaSP.

Results

Phylogenetic analyses and population structure

The mtDNA *cox1* gene fragment (661 bp in size) was sequenced for a total of 680 individuals collected from 34 locations across the northwestern Pacific (Fig. 1; Table 1). A total of 169 mtDNA *cox1* haplotypes were identified. Of these, 149 variable sites and 62 single-nucleotide polymorphic sites were detected, and 87 sites were parsimoniously informative. The *cox1* phylogenetic trees using ML and BI methods showed identical tree topology: they showed two monophyletic, divergent lineages (termed here the northern and southern mitochondrial lineages, according to their geographic distribution) with high support values (100% BP and 1.00 BPP) (Fig. 2A). The haplotype network displayed a dumbbell shape, with 48 steps (mutations) connecting two haplogroups with a "star genealogy" (Fig. 2B). Pairwise sequence divergence between the two lineages was unexpectedly very high, ranging from 7.4%-9.2%, but there was noticeably low divergence within the northern (0.5%) and southern lineages (0.6%), respectively. Distribution of the two lineages clearly corresponds to their geographic origins: the northern lineage includes all Korean populations and the northern Japan populations (JRK, JAM, JOG, JHS, JIB, JSM, JTZ), with a distribution range mainly in the cold surface water North Pacific region. In contrast, the southern lineage is composed of all Chinese

populations, the Taiwan population and the southern Japan populations (JBK, JAK, JTK, JHD, JKK), distributed northeastward along the tropical Indo-West Pacific region. In addition, six populations (JSH [8], JSS [9], JKS [11], JOI [16], JKG [17], JNG [18]) on the southeastern and western sides of the Japanese archipelago contained both lineages (Fig. 1).

In addition to the *cox1* data, 31 ITS1 genotypes were obtained from 101 individuals that contained 29 polymorphic sites. Their pairwise sequence divergence was very low (0.6%) compared to mtDNA *cox1* . In contrast to the *cox1* results, the ML and BI tree for the ITS1 data showed only a single clade where all individuals were mixed, with no segregation between individuals representing the northern and southern mitochondrial lineages (Fig. 3A). Network analysis of the ITS1 haplotype identified two major haplogroups that were only a single step (mutation) away from each other, each forming a star-like genealogy that comprised a mixture of northern and southern mitochondrial *cox1* members (Fig. 3B). Unlike the deep divergence detected between the two mtDNA *cox1* lineages, genotype distribution of ITS1 did not correspond to geographic location.

The NWP populations of *M. virgata* showed high haplotype diversity with overall h values of 0.874 for *cox1* and 0.824 for ITS1 (Table 1). Nucleotide diversity (π) and nucleotide differences (k) for *cox1* were high: 0.0417 and 27.539, respectively. In the six populations where the two divergent mtDNA lineages coexist, nucleotide diversity (π) and nucleotide difference (k) ranged from 0.0192 to 0.0418 and from 12.699 to 27.613, respectively, much higher than all other populations in this study (π =0.0008 to 0.0043, k =0.544 to 2.828). However, ITS1 sequences showed very low nucleotide diversity (π= 0.0026) and nucleotide differences (k= 0.909). In the SAMOVA test using the *cox1* gene,*M. virgata* populations were subdivided into the two regional groups consistent with the results from the phylogenetic analysis: one group comprises populations of the cold-temperate zone that includes the ES, Yellow Sea and northern Pacific coast of Japan (population nos. 1-9. 19-31), and the other group is composed of populations in the subtropical-tropical zone represented by the ECS and SCS and the southern Pacific coast of Japan (population nos. 10-18, 32-34). Genetic differentiation (F_{ST}) between populations belonging to different regions was substantial and statistically significant, ranging from 0.416 to 0.984 (Supplementary Table 1). However, F_{ST} values between populations within the same regional group were less than 0.223, and most of the comparisons were not statistically significant. The hierarchical AMOVA analyses with locality groups inferred from the result of the SAMOVA analysis indicated that 90.54% of the total genetic variation was contributed by 'among-groups' variation (Φ_{CT}=0.905,P <0.001), whereas genetic variation from 'among populations within groups' and 'within populations' was 1.07% (Φ_{SC}=0.113, P <0.001) and 8.40% (Φ_{ST}=0.916, P <0.001), respectively. The AMOVA analysis excluding the 6 'mixed' populations (contained individuals representing both lineages) shows that most of the genetic variation was observed from 'among groups' (97.33 %, Φ_{CT}=0.973, P <0.001; see Table 2 for more details).

Morphological analysis

Principle component analysis (PCA) of 15 morphological characters (Supplementary Fig. 1) extracted from 100 randomly-chosen individuals (n = 50 from the northern lineage, and n = 50 from the southern lineage) were examined (Fig. 4). The first two components (PC1 and PC2) accounted for 78.81% of the total variation. Ordination of PC1 and PC2 scores of 100 individuals delimited by *cox1* lineage indicated that the two lineages mostly overlap along both axes. Therefore, we conclude that these shell morphological characters do not differ between the two mitochondrial lineages, despite their substantial genetic divergence.

Divergence time and historical demography

The divergence time between the northern and southern lineages estimated using the two mtDNA *cox1* molecular clock settings (0.7% and 2.4%/Mya) was 5.49–1.61 Mya, corresponding to the Pliocene to early-to mid-Pleistocene, each coalescing at 0.94–0.30 Mya (the northern lineage) and 0.99–0.29 Mya (the southern lineage), respectively (Fig. 5). Neutrality test showed significant negative (-) values of Tajima'sD and Fu's F_S in both lineages (Table 3). The sequential mismatch distributions for the two lineages displayed a skewed unimodal peak close to the Y-axis (Fig. 6).

Discussion

Deep mitochondrial divergence

Recent molecular-based phylogeography studies in the NWP have uncovered some general patterns: marine species often harbor genetically divergent lineages in the SCS and ES (Cheang, Chu, & Ang Jr, 2010 [macroalga:*Sargassum hemiphyllum*]; Shen et al., 2011 [flathead grey mullet: *Mugil cephalus*]; Ni et al., 2012 [venus clam:*Cyclina sinensis*]; Cheng & Sha, 2017 [Japanese mantis shrimp: *Oratosquilla oratoria*]; Wang, Kong, Chen, Matsukuma, & Li, 2017 [spotted hard clam: *Meretrix petechialis*]). Here, using phylogenetic and network analyses of mtDNA *cox1* sequence data, we identified two distinct mitochondrial lineages within *M. virgata:* a northern lineage found in the cold surface-water temperature zone in the northern NWP (the Korean coast, the northern part of the ES/JS and the northern Pacific coast of Japan), and a southern lineage occupying the warm surface-water temperature zone in the NWP (ECS, SCS and the southern Pacific coast of Japan). The fixation index (F_{st}) and AMOVA analysis both support the conclusion that these lineages are genetically distinct and display a different biogeographic pattern except for some Japanese populations (JSH, JSS, JKS, JOI, JKG, JNG; Fig. 1 and Table 1).

Sequence divergence between the two lineages is extremely high (7.4-9.2%), while within-lineage distance is low (a maximum of 0.6%). DNA barcoding analyses of other molluscan species using mtDNA *cox1* have reported a mean intraspecific variation less than 2% (Meyer & Paulay, 2005; Zou, Li, & Kong, 2012). However, deeper intraspecific divergence (>2%) has also been detected in some Atlantic and Pacific molluscan species (gastropods and bivalve species); such deep divergence has been attributed to population subdivision by a biogeographic partition between geographic lineages (Layton, Martel, & Hebert, 2014). Very high levels of *cox1* divergence have also been reported in NWP bivalve species (Liu et al., 2011 [>4.3% in *Atrina pectinata*]; Wang et al., 2017 [5.85% in *Meretrix petechialis*]). Such divergence has been interpreted as molecular evidence of a physical barrier reducing gene flow. Historical isolation in marginal seas, caused by low sea level during past glaciations, might be the primary driver for deep intraspecific genetic splits in the NWP (Layton et al., 2014).

Discordance between mitochondrial and nuclear markers

The internal transcribed spacer (ITS1) of the ribosomal DNA has widely been used as a molecular marker for phylogenetic analysis at species level and for species identification of closely related molluscan taxa, including mussel species (Santaclara et al., 2006; Wood, Apte, MacAvoy, & Gardner, 2007). Nevertheless, unlike the deep divergence we uncovered in mitochondrial sequences, nuclear DNA sequences (ITS1) were not highly divergent (a maximum pairwise sequence divergence of 0.6%) and in the phylogenetic tree, all ITS1 sequences clustered together, irrespective of their geographic origin (Fig. 3A). The ITS1 network showed two genotype groups (a single step away from each other), each forming a star-like genealogy made of an admixture of northern and southern mitochondrial *cox1* members (Fig. 3B). This type of mito-nuclear discordance is often found in biogeography studies and can be explained by many possible hypotheses (reviewed in Toews and Brelsford 2012). Further investigation will be needed to define the precise cause of mito-nuclear discordance in this species, but we discussed two hypotheses in the case of *M. virgata* : incomplete lineage sorting and selection on mitochondrial genes.

The first hypothesis invokes the stochasticity of coalescent processes at the two independent loci, resulting in divergent patterns. Incomplete lineage sorting is often mentioned in studies that uncover mito-nuclear discordance because nuclear markers have a larger effective population size (i.e. slower coalescence) than mitochondrial DNA (Moore, 1995; Pesole, Gissi, De Chirico, & Saccone, 1999). However, in the present study the difference between the *cox1* and ITS1 results are so extreme (very deep divergence versus negligible divergence) that the incomplete lineage sorting hypothesis is inadequate given the inferred demographic history. Incomplete lineage sorting can only occur if two subpopulations maintain large population sizes before they merge into a common ancestral population; in contrast, our mtDNA *cox1* and ITS1 genealogies both support very rapid and recent population growth following a population bottleneck. This is indicated by the genealogies with very short external branches only (a "star genealogy;" see Figs. 2A, 2B and Fig. 3),

the significant negative values in Tajima's $D$and Fu's F_S test (Table 3), and the unimodal peak skewed over the Y-axis in the pairwise mismatch distribution analysis for the two mitochondrial lineages (Figs. 6A and 6B). Furthermore, the coalescence time between northern and southern *cox1* lineages is estimated to be more than five times older than those of within-lineage coalescences (Fig. 5). Given a small ancestral population(s) and/or a very old splits of an ancestral population, incomplete lineage sorting at ITS1 or a long waiting time prior to the coalescence of the mtDNA lineages are unlikely to be the reason for mito-nuclear discordance.

Another hypothesis worth considering is that natural selection may have produced long-term, balanced mtDNA polymorphism. Although it is a long-held assumption that most of the sequence variation in mtDNA is selectively neutral (Ballard & Whitlock 2004; Meiklejohn, Montooth, & Rand, 2007), it is also well documented that balancing selection can occur under spatial heterogeneity (Hedrick, 2006; Scott et al., 2011). We note that the spatial distribution of the northern and southern*cox1* lineages strongly correlates with surface seawater temperature (Fig. 1). Therefore, we suggest that these two divergent*cox1* lineages are a proxy for two mitochondrial genotypes adapted to different surface water temperature zones, possibly due to divergence in the energy-metabolic functions in mitochondria. To test for divergent natural selection on the mt genome, we compared the whole mitochondrial genome sequences of two individuals representing the northern and southern mitochondrial lineages. Calculating the ratio of nonsynonymous (dN) over synonymous (dS) substitutions for the 12 mitochondrial protein-coding genes of the complete mitochondrial genomes revealed that none of the 12 protein-coding genes (including*cox1* , the genetic marker used in this study) showed a significant excess of $\mathrm{dN/dS} > 1$ (Supplementary Table 2). These results do not provide supporting evidence for the selection on the mitochondrial genome; however, we cannot rule out the possibility that the two divergent lineages might still be maintained by balancing selection. The functional difference between two lineages might result from a small number of amino acid substitutions (not readily detectable in this case) or gene regulatory sequence differences. Additional mitochondrial genome sequencing that can cover the genetic diversity for the two mitochondrial lineages is further required to address whether deep divergence in mitochondrial genes is related to adaptation to different surface water temperature zones in the NWP.

Temperature-driven mitochondrial lineage splitting

Phylogeographic patterns of marine organisms in the NWP are shaped by the interplay of historical and/or contemporary oceanography, and present-day gene flow, which is often correlated with species-specific life-history features such as larval traits (Cheang et al., 2012). The timing of lineage splitting event between the two *M. virgata*lineages is estimated to be 5.49-1.61 Mya. Although there is substantial deviation between estimates using the lowest and highest calibration bounds of the molecular clock, the estimated divergence time coincides with the glacial periods from the Pliocene to Pleistocene epochs. Sea level fluctuation during this time affected population fragmentation and demographic changes for many coastal species, resulting in population structuring and/or lineage splitting in the NWP (Shen et al., 2011; Ludt & Rocha, 2014).Cyclic alternations of glacial-interglacial periods dramatically affected the seafloor topography and sea level dropped up to 120 m below its present level during the glacial periods (i.e., early Pleiostocene), resulting in historical isolation in the NW Pacific marginal seas: Pleiostocene isolation of the ES/JS, ECS, and SCS (Wang, 1999; Voris, 2000). The ES/JS was semi-enclosed and prevailed by cold surface water flows from the northern sea during the Northern Hemisphere glaciation and mid-Pleiostocene transition (Tada, 1994; Kitamura et al., 2001; Matsuzaki, Itaki, Tada, & Kamikuri, 2018). Meanwhile, the SCS was separated from the ECS (Ota, 1998; see Fig. 7 for details) by a large land bridge extending between eastern China and Taiwan Island (Kimura, 2000). Peng et al. (2019) found that there is a post-LGM expansion of*M. virgatus* in Zhejiang, China (belonging to the southern lineage in the present study), based on population genetic analysis of mtDNA*cox1* and 16S rDNA sequences.

In addition to historical geography in the NWP, some environmental factors (e.g., ocean current, surface water temperature and salinity) might act in combination with stochastic processes such as local selection and/or lineage sorting through time in shaping contemporary population structure (Palumbi, 1994; Miglietta, Faucci, & Santini, 2011). In our results, the geographic distribution of the two mitochondrial lineages agrees with two biogeographic regions with different surface water temperature zones: the northern lineage occupies the

North Pacific Temperate Biotic Region which is affected by the cold-water Liman Current flowing southward from the Sea of Okhotsk into in the East Sea (the Sea of Japan) and the Oyashio Current (also known as the Kurile Current) that flows south and meets the Kuroshio Current off the eastern shore of Japan. In contrast, the southern lineages are occupied by Chinese populations, Taiwan population and the southern Japan populations, all belonging to the Indo-West Pacific warm-water Biotic Region which is affected by the warm, northeasterly flowing Kuroshio Current. It is interesting to note that some Japanese populations where the two lineages co-occur (JSH, JSS, JKS, JOI, JKG, JNG; Table 1 and Fig. 1) agree well with the region where two currents with different surface water temperatures meet: JOI (16), JKG (17), and JNG (18) are found on the western side of Japanese archipelago where the cold-water carrying Liman Current (LC) and the warm-water carrying Tsushima Warm Current (TSWC) meet in the North Pacific Temperate Biotic Region, whereas JSH (8), JSS (9), and JKS (11) are found in southeastern side of Japan where the Kuroshio Current (KC) mixes with the southeasterly flowing, cold-water carrying Oyashio Current (OC) in the Indo-West Pacific warm-water Biotic Region.

Present-day oceanic currents are among the most influential factors that contribute to continuous gene flow over a wide geographic range in many marine invertebrates, including molluscan species (Dong et al., 2012; Guo et al., 2015; Li et al., 2017). *M. virgata* has a planktonic larval stage for 4 days, allowing them to disperse considerable distances via northeasterly flowing ocean currents (Sasaki, 1984). The present-day geographic distribution of the northern and southern lineages is assumed to be due to current-driven long-distance dispersal of planktonic larva from the northern and southern glacial refugia. Deep lineage splitting and geographic distribution of the two mitochondrial lineages uncovered in this study suggest that along with ocean currents, surface water temperature preference and/or differential selection between the respective ancestral populations of the southern and northern lineages have shaped contemporary phylogeographic patterns: The ancestral population of the northern lineage would have been under selection for adaptation to colder water, while the southern lineage might have adapted to a warmer environment. This thermal adaptation likely restricted gene flow between the two regional populations by reducing migrant fitness, resulting in lineage splitting along a temperature gradient (Teske, Von der Heyden, McQuaid, & Barker, 2011; Teske et al., 2019). Such a vicariant lineage splitting following long-term adaptation to different temperature zones has been reported in some other marine species including mantis shrimp (Cheng & Sha, 2017), mitten crab (Xu et al., 2009), barnacles (Tsang et al. 2012), and flathead mullet (Shen et al., 2011). We cautiously suggest that cryptic mitochondrial lineage splitting in *M. virgata* might be the result of an adaptive response (i.e., a balancing selection) to different environments, most likely different surface water temperature zones. An extensive genetic survey of nuclear genes, along with in-depth analysis of balancing selection from mitochondrial genome sequences, would be needed to clarify how these two mitochondrial lineages have adapted in response to thermal differences in the NWP.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (Nos. 2017R1A2B4011597, 2020R1A2C2005393).

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Data accesibility

Mitochondrial *cox1* and nuclear ITS1 sequences are available on GenBank (accession nos. MN728370-MN728538 and MN728333-MN728363).

Author contributions

Study conception and design: JKP. Data collection: YL, JS, TK, SC, BC, RG, YJW, YK, SCK, and TN. Data analysis and interpretation: YL, GN, YK, and JKP. Manuscript preparation: YL, GN, EK, YK, and JKP.

Figure legends

Figure 1. Sample locations of *M. virgata* and distribution of the two mtDNA *cox1* lineages. The map shows sea surface temperature in the northwestern Pacific in spring (2003 – 2011). CCC, China Coastal Current; TWC, Taiwan Warm Current; CRDW, Changjian River Diluted Water; SCC, Subei Coastal Current; YSWC, Yellow Sea Warm Current; EKWC, East Korea Warm Current; TSWC, Tsushima Warm Current; OC, Oyashio Current; LC, Liman Current. The dash/dotted line separates two biogeographic regions: the North Pacific Temperate Biotic Region; above the line) and Indo-West Pacific Warm-water Biotic Region; below the line). Populations are labelled with numbers (the southern and northern lineages are indicated in red and blue colored circles, respectively) that correspond with those shown in Table 1.

Figure 2. Phylogenetic tree and haplotype network constructed from mtDNA *cox1* . In the phylogenetic tree (A), numbers on branches are statistical support values for ML (bootstrap values)/BI (posterior probabilities). In the haplotype network (B), colors represent sampling locations of *M. virgata* . The size of each circle is proportional to the frequency of that haplotype. The crossbars on the lines indicate the number of nucleotide substitutions. Open circles represent hypothetical haplotypes.

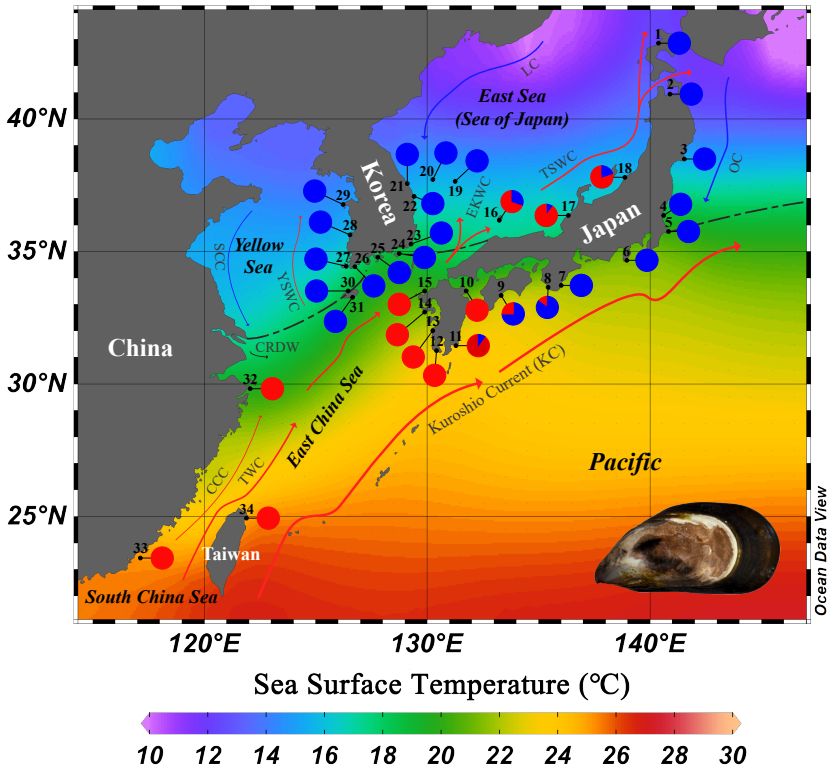
Figure 3. Phylogenetic tree and haplotype network constructed from nrDNA ITS1. In the phylogenetic tree (A), numbers at nodes are statistical support values for ML (bootstrap values)/BI (posterior probabilities). In the haplotype network (B), each circle represents one allele and color indicates lineage population. Circle size is proportional to the frequency of that haplotype. The crossbars on each branch indicate the number of nucleotide substitution.

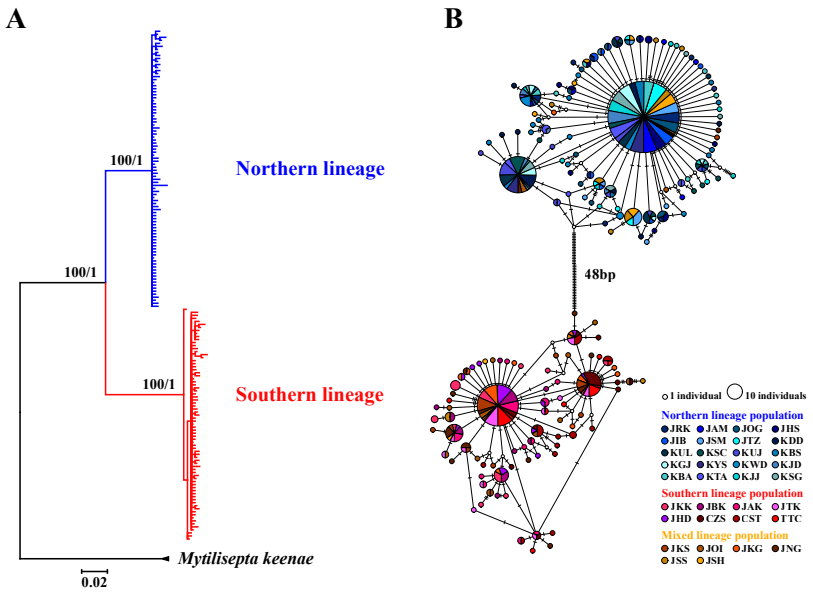
Figure 4. Plot of principal component analysis of northern and southern lineages using 15 shell morphological characters. PC1 explains 72.14% of the variation; PC2 6.67% of the variation.

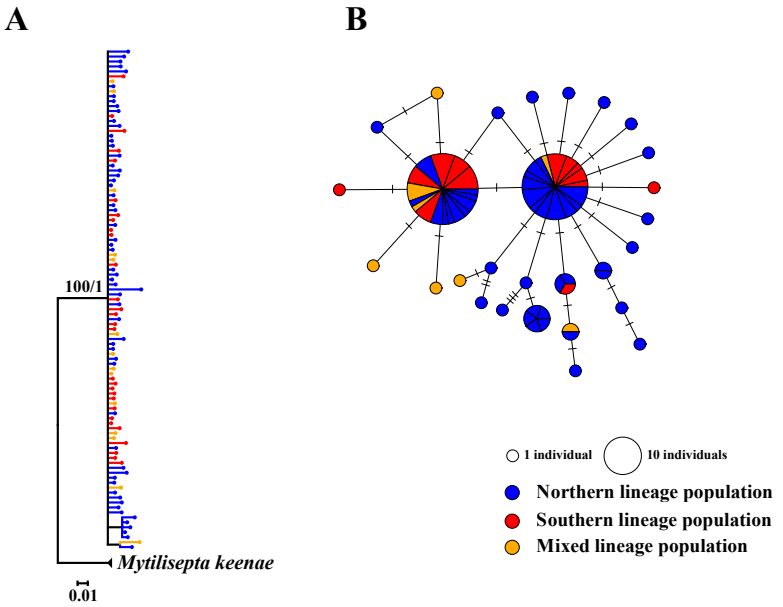
Figure 5. Estimates of divergence times using BEAST. A, 0.7%/Mya; B, 2.4%/Mya. Numbers above nodes are the mean divergence times. Horizontal bars represent 95% highest posterior densities of the divergence time estimates.

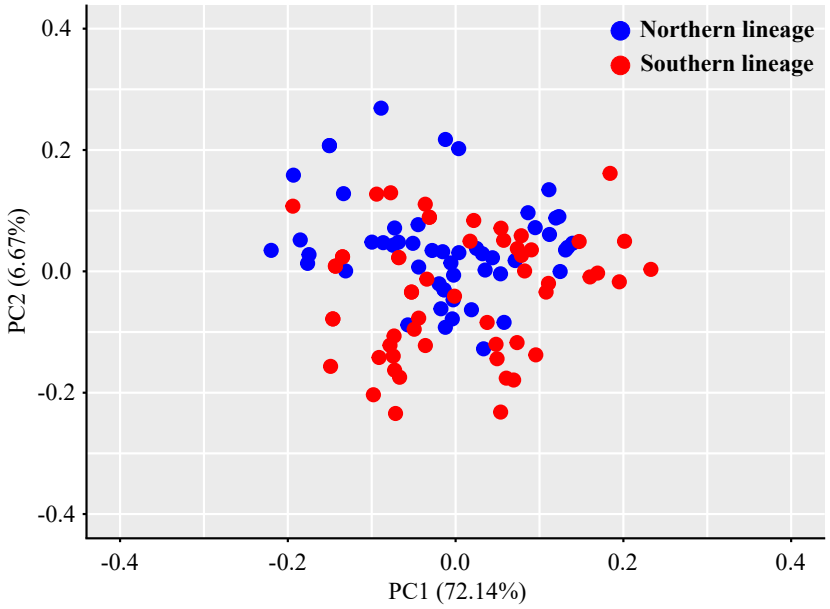
Figure 6. Mismatch distributions for the northern and southern lineages using mtDNA *cox1* sequence data. The red dotted lines represent the observed frequency of pairwise differences, and the blue solid lines indicate the expected values of the sudden population expansion model.

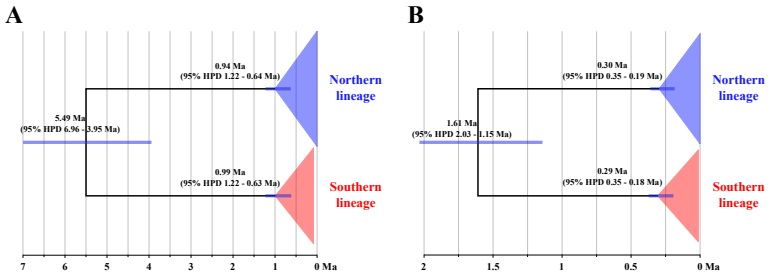
Figure 7. Hypothesized NW Pacific coastline (dotted line) during early Pleistocene showing that three NWP marginal seas (the East Sea [ES]/the Sea of Japan [SJ], East China Sea [ECS] and South China Sea [SCS]) were isolated due to sea level decline (modified from Ota, 1998). The current land mass topography is indicated in dark grey.

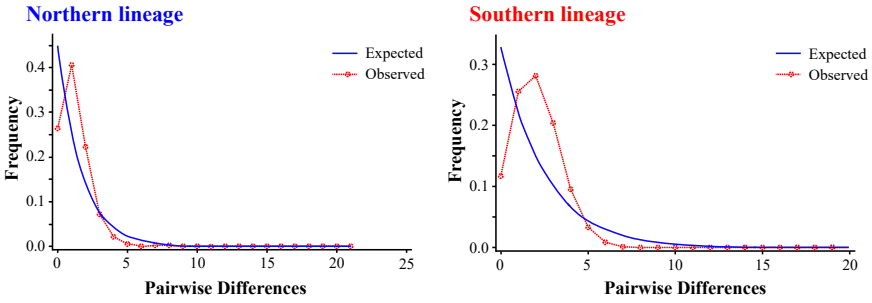


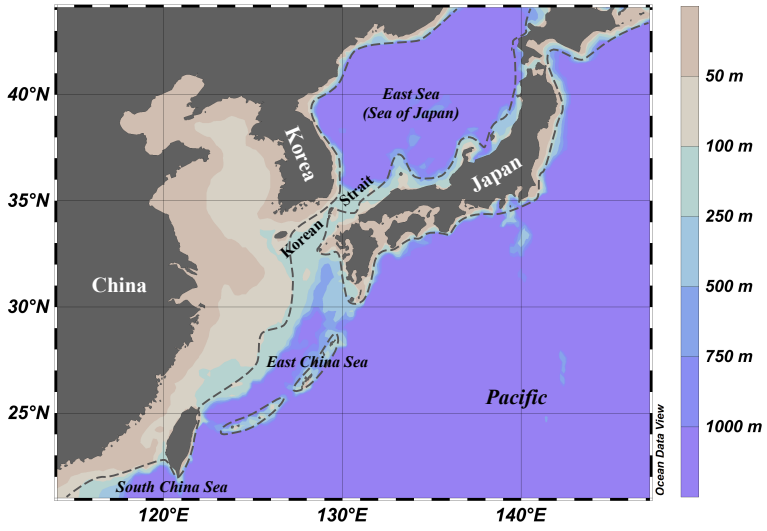












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