Journal of Experimental Marine Biology and Ecology 407 (2011) 171–181

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Molecular phylogeography of *Ruditapes philippinarum* in the Northwestern Pacific Ocean based on COI gene

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ARTICLE INFO

Article history:
Received 26 September 2010
Received in revised form 27 April 2011
Accepted 1 June 2011
Available online 19 July 2011

Keywords:
Cytochrome oxidase I
Isolation by distance
Neutrality test
Population expansion
Ruditapes philippinarum

ABSTRACT

To examine the pattern of phylogeography of *Ruditapes philippinarum*, a length of 644-bp gene fragment of the mtDNA COI was sequenced. A total of 170 individuals from 19 locations were analyzed yielding 74 haplotypes, most of which were unique. The levels of haplotype diversity and nucleotide diversity for the species ranged from 0.80 ± 0.16 (Notsuke Bay) to 1.00 ± 0.13 (Nanao Bay, Miyazu Bay, Dalian 1 and Ariake), and from 0.002 ± 0.001 (Notsuke Bay) to 0.011 ± 0.006 (Qingdao), respectively. Both the phylogenetic (NJ tree) and minimum spanning trees (MST) showed three significant genealogical clusters corresponding to sampling localities, a genetic differentiation speculated to be caused by the isolation of the marginal seas of the Northwestern Pacific during Pleistocene low sea-level stands. Both AMOVA and pairwise *Fst* analyses revealed significant genetic differentiation between the populations from Japan and China. The pattern of isolation by distance was also detected in this species ($r = 0.50$, $P < 0.001$). Both mismatch distribution analysis and the neutrality tests showed that *R. philippinarum* had undergone a recent population expansion. The estimate of population expansion time was about 425 kya–1580 kya.

1. Introduction

Due to periodic climatic oscillations over the Pleistocene, range contractions and expansions are thought to have greatly influenced the amount and distribution of intraspecific genetic variation in many species (Avise, 2000; Hewitt, 2000). The late Quaternary period (the past one million years) was characterized by a series of large glacial-interglacial changes (Imbrie et al., 1992). The major climatic oscillations occurred during the past ~800 kyr with a ~100 kyr dominant cycle. During glacial maxima, declines in sea levels of 120–140 m have been noted (Lambeck et al., 2002). Severe climatic shifts can produce great changes in species' geographical distribution and abundance, which can be expected to have genetic consequences (Dynesius and Jansson, 2000; Hewitt, 2000).

In the modern oceans, most of (75%) the marginal basins are concentrated in the western Pacific continental margin. During the Pleistocene glacial cycles, the sea level-induced environmental signal was amplified in the marginal seas of Western Pacific, giving rise to drastic changes in areas and configurations of these seas (Voris, 2000; Wang, 1999). As one of the most extensive continental shelves in the Western Pacific, the East China Sea Shelf was exposed during the Pleistocene ice ages (Xu and Oda, 1999). The Sea of Japan is a semi-enclosed marginal sea and was almost isolated from the Pacific Ocean during glaciation events (Kitamura et al., 2001). During the Pleistocene glacial period, the South China Sea was an enclosed inland sea connected to the Pacific through the Bashi Strait between Taiwan and Luzon (Wang, 1999). For these reasons the Northwestern Pacific appears to provide one of the best natural settings to study how colonization events, population bottlenecks, long term isolation and subsequent mixing have affected the lineage structure and geographical differentiation of marine species.

The Manila clam, *Ruditapes philippinarum* (Adams and Reeves, 1850), belongs to the family Veneridae (Rafinseque, 1815). *R. philippinarum* is a Pacific Asian subtropical low-boreal species distributed from the Philippines, the South China, Yellow, Japan, and Okhotsk Seas to the shoals near the South Kurils (Ponurovskii, 2008). The bivalve *R. philippinarum* is a highly preferred seafood, especially in China and Japan and has been cultured commercially in the West Pacific for decades (Fan et al., 2007; Watanabe et al., 2009).

R. philippinarum has spread successfully to many parts of the world, beyond its range of natural distribution, mostly through aquaculture (Melià et al., 2004; Melia and Gatto, 2005). However, in recent years the yield of *R. philippinarum* has decreased quickly, including many parts of Japan during the last two decades, because of overexploitation and coastal pollution (Liu et al., 1999; Watanabe et al., 2009). Given that *R. philippinarum* has previously supported significant commercial fisheries, attempts at recovery of affected populations are of great

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doi:10.1016/j.jembe.2011.06.002

importance. For example, as an effort to promote stock restoration, Japanese fishermen have imported seeds from China and released them to their coastal waters (Sekine et al., 2006). *R. philippinarum*, like other invertebrates, has pelagic larvae with a relatively long larval duration extending over 2–3 weeks (Yasuda et al., 2007). Being planktonic, the larvae are subjected to long distance dispersal and subsequent gene flow among populations. The early life-history characteristic indicates that potential larval dispersal of *R. philippinarum* is high. If *R. philippinarum* larvae could travel on the currents, the connectivity should be high among populations within this region (as evidenced by limited genetic structure). However, one previous study (Sekine et al., 2006) has found the Chinese and Japanese populations of *R. philippinarum* to be disparate.

To date, most researches on *R. philippinarum* have been focused mainly on its biology, ecology, and aquaculture (Drummond et al., 2006; Li et al., 2005; Park and Choi, 2004; Trinkler et al., 2010; Uchida et al., 2010; Xiao et al., 2006) with scarce work on its genetics. Isozyme studies on *R. philippinarum* have been done by Ge et al. (2008) and Ren et al. (2006) while Sekine et al. (2006) have reported on the genetic structure and diversity for the species. The results of isozyme analysis showed that there were genetic differences existing between the northern and southern populations (Ren et al., 2006). Meanwhile, molecular methods revealed genetic differentiations between Chinese and Japanese populations (Sekine et al., 2006).

With the advent of advanced molecular methods, genetic data now play an important role in guiding the management, conservation, and assessment of marine organisms (Avise, 1994). Recently, the increased use of mitochondrial DNA (mtDNA) has resulted in deeper insights into the molecular phylogenesis, population genetics, and conservation aspects of which detection of polymorphism at molecular level for natural populations is necessary. Mitochondrial cytochrome oxidase I gene (mtDNA COI) has been used widely in marine species, such as *Tegillarca granosa* (Zheng et al., 2009) and *Lasmigona subviridis* (King et al., 1999) due to adequate levels of variability and ease of amplification via universal primers (Folmer et al., 1994).

In the present study, we examined the genetic diversity and the pattern of phylogeography for *R. philippinarum* using sequence analysis from the cytochrome oxidase subunit I (COI) of mitochondrial DNA (mtDNA). This genetic survey is intended to serve as a baseline for future genetic monitoring of *R. philippinarum* populations.

2. Material and methods

2.1. Sampling and sequencing

Samples were collected between 2004 and 2009 at 8 sampling sites in China (Dalian2, Tianjin, Laizhou, Rushan, Qingdao, Ningbo, Putian and Guangzhou) and 2 in Japan (Akkeshi, Kagawa). Existing data of 6 Japanese populations (Nanao Bay, Miyazu Bay, Ariake Bay, Notsuke Bay, Mikawa Bay and Tokyo Bay) and 3 Chinese populations (Kiaochow Bay, Xiamen and Dalian1) were also analyzed in the present study. Sampling locations and related details are in Table 1 and Fig. 1. Pieces of muscle tissue were preserved in 95% ethanol before DNA extraction.

Genomic DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method (Sambrook et al., 1989). The universal invertebrate COI primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTT-CAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) were routinely used to amplify COI. If COI amplification was unsuccessful under these conditions, the HCO2198 primer was replaced with the alternative HCO2198-mod1 primer (5′-CCAAAAAATCAAAATAAATGAATAA-3′).

PCR were carried out with 1.25U *Taq* DNA polymerase (TaKaRa, Dalian) in 50 μL volumes. The final concentrations were 200 nM forward and reverse primers, 200 μM of each dNTP, 10 mM Tris, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$. All sets of PCR included one negative control reaction to check for contamination. The PCR amplification was carried out in a Biometra thermal cycler under the following conditions: initial 4 min denaturation at 94 °C and 35 alternating cycles of 40 s at 94 °C for denaturation, 30 s at 48 °C for annealing, and 1 min at 72 °C for extension, and a final extension at 72 °C for 7 min. A total of 2 μL of each PCR product was used for 1% agarose gel electrophoresis for verifying the amplified fragment length with a standard size marker (TaKaRa, Dalian). PCR products were purified with Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai). Both strands were sequenced using the BigDye Terminator Cycle Sequencing Kit (ver.2.0, PE Biosystems, Foster City, California) and run on an ABI Prism 3700 (Applied Biosystems) automatic sequencer according to the manufacturer's recommendations. The primers used for sequencing were the same as those for PCR amplification.

COI gene sequences were submitted to NCBI (http://www.ncbi.nlm.nih.gov/) under accession letter JN054502–JN054632, respectively (Table 1).

Table 1
Sample abbreviation (ID), sample sites, number of sequences (n), number of halpotypes, haplotype diversity (h), nucleotide diversity (π) based on mitochondrial cytochrome oxidase I sequence of *Ruditapes philippinarum* across the coastlines of China and Japan.

ID	Sample	Date of collection	Sample size (n)	No. of haplotypes	*h*	π	*k*	Reference
Kia	Kiaochow Bay	2004	4	3	0.83 ± 0.22	0.008 ± 0.006	5.46 ± 3.32	AB244404-AB244407
Xm	Xiamen	2004	5	4	0.90 ± 0.16	0.006 ± 0.004	3.65 ± 2.22	AB244408-AB244412
Nan	Nanao Bay	2004	5	5	1.00 ± 0.13	0.005 ± 0.004	3.26 ± 2.01	AB244384-AB244388
Miy	Miyazu Bay	2004	3	3	1.00 ± 0.27	0.007 ± 0.006	4.80 ± 3.21	AB244389-AB244391
Dl1	Dalian1	2004	2	2	1.00 ± 0.50	0.003 ± 0.004	2.03 ± 1.76	AB244402-AB244403
Ari	Ariake Bay	2004	5	5	1.00 ± 0.13	0.006 ± 0.004	3.69 ± 2.24	AB244392-AB244396
Not	Notsuke Bay	2004	5	3	0.80 ± 0.16	0.002 ± 0.001	1.01 ± 0.80	AB244397-AB244401
Mik	Mikawa Bay	2004	5	4	0.90 ± 0.16	0.004 ± 0.003	2.66 ± 1.69	AB244379-AB244383
Tok	Tokyo Bay	2004	5	4	0.90 ± 0.16	0.004 ± 0.003	2.46 ± 1.59	AB244374-AB244378
Nb	Ningbo	2004	13	7	0.85 ± 0.09	0.007 ± 0.004	4.82 ± 2.51	Present study
Gz	Guangzhou	2005	7	6	0.95 ± 0.10	0.007 ± 0.005	4.67 ± 2.60	Present study
Qd	Qingdao	2004	20	13	0.94 ± 0.03	0.011 ± 0.006	6.65 ± 3.27	Present study
Akk	Akkeshi	2005	12	9	0.91 ± 0.08	0.004 ± 0.003	2.67 ± 1.53	Present study
Dl2	Dalian2	2004	19	12	0.90 ± 0.06	0.007 ± 0.004	4.32 ± 2.24	Present study
Lz	Laizhou	2004	11	7	0.82 ± 0.12	0.005 ± 0.003	3.26 ± 1.82	Present study
Tj	Tianjin	2004	17	10	0.88 ± 0.07	0.006 ± 0.003	3.71 ± 1.97	Present study
Rs	Rushan	2009	9	7	0.92 ± 0.09	0.009 ± 0.005	5.48 ± 2.91	Present study
Pt	Putian	2004	16	6	0.84 ± 0.06	0.006 ± 0.003	3.59 ± 1.92	Present study
Kag	Kagawa	2005	7	4	0.81 ± 0.13	0.010 ± 0.006	6.21 ± 3.36	Present study
	Total	2004–2009	170	74	0.96 ± 0.01	0.010 ± 0.005	6.62 ± 3.14	

Dl1 population data was gained from the GenBank, Dl2 was investigated in present study.

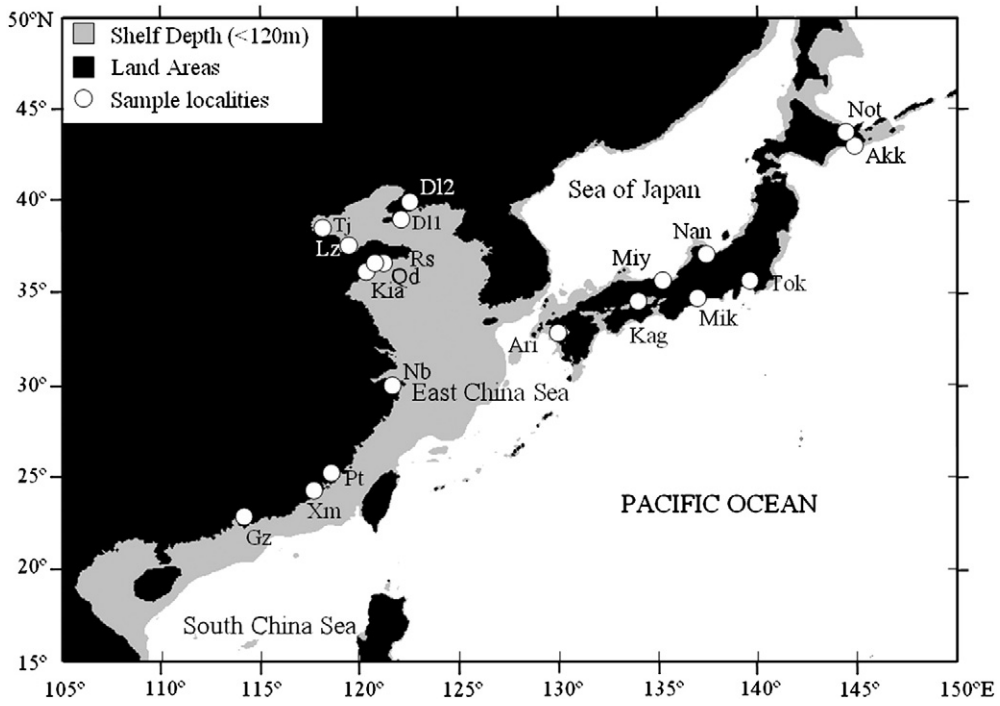

Fig. 1. Map showing sample locations of *Ruditapes philippinarum*, samples are marked by abbreviations that correspond to Table 1. Shaded sea areas are continental shelves that would have been exposed to the air during periods of low sea-level.

2.2. Sequences alignment and data analysis

Sequences were edited and aligned using Dnastar software (DNASTAR Inc., Madison, USA). Molecular diversity indices such as number of haplotypes, polymorphic sites, transitions, transversions, and indels were obtained using the program ARLEQUIN (ver. 2.000; Schneider et al., 2000). Haplotype diversity (h), nucleotide diversity (π) and the mean number of pairwise differences (k) and their corresponding variances were calculated following Nei (1987) as implemented in ARLEQUIN version 2.0 (Schneider et al., 2000). A gamma shape parameter of 0.101 and the Tamura-Nei substitution model was used, based on the result obtained with the programs PAUP* (version 4.0b10; Swofford, 1998) and Modeltest (version 3.7; Posada and Crandall, 1998).

Genetic relationships among haplotypes of the *R. philippinarum* for the COI gene sequence data were reconstructed using the neighbor-joining method implemented in PAUP* (version 4.0b10; Swofford, 1998) and the robustness of each internal branch of the phylogenetic trees was evaluated with 1000 bootstrap replicates (Saitou and Nei, 1987). Genealogical relationships were also examined by constructing haplotype networks using reduced median-network approach (Bandelt et al., 1995, 2000).

Significance of population structure was tested with analysis of molecular variance (AMOVA; Excoffier et al., 1992) and pairwise F_{ST} values. Both statistical calculations were carried out using the software ARLEQUIN (version 2.0, Schneider et al., 2000). The significance of the F_{ST} was tested by 10,000 permutations for each pairwise comparison in ARLEQUIN. When multiple comparisons were performed, P values were adjusted using the sequential Bonferroni procedure (Rice, 1989). Several groupings were tested in a hierarchical AMOVA, considering the geography of the region.

In order to detect the genetic similarities among samples, non-metric multidimensional scaling was used on the matrix of F_{ST} values (MDS; Kruskal and Wish, 1978). To test for isolation by distance (Slatkin and Hudson, 1991; Wright, 1943), pairwise values of $F_{ST}/(1-F_{ST})$ (Rousset, 1997) were plotted against geographical distance between sample sites. The strength and significance of the relationship between genetic distances and geographic distances was assessed using Reduced Major Axis (RMA) regression and Mantel tests using IBDWS (Fig. 7) (Jensen et al., 2005; http://ibdws.sdsu.edu/).

The null hypothesis of neutral evolution of the COI gene was tested using Fu's *Fs* test (Fu, 1997) and Tajima's *D* test (Tajima, 1989a) with the program ARLEQUIN (ver. 2.0; Schneider et al., 2000). Significant *D* values can be due to factors such as selection, population expansion and bottlenecks (Tajima, 1989b). Historical demographic expansions were investigated by examination of frequency distributions of pairwise differences between sequences (mismatch distribution; Excoffier, 2004; Ray et al., 2003; Rogers and Harpending, 1992). Mismatch distributions can be used to test the hypotheses about the population demographic history and selection (Rogers and Harpending, 1992). The distribution is usually multimodal in samples drawn from populations at demographic equilibrium, but it is usually unimodal in populations following a recent population demographic expansion and population range expansion (Excoffier, 2004; Ray et al., 2003; Rogers and Harpending, 1992; Slatkin and Hudson, 1991). The parameters of the demographic expansion τ, θ_0, and θ_1 are estimated by a generalized non-linear least-square approach and confidence intervals of the parameters are computed using a parametric bootstrap approach (Schneider and Excoffier, 1999). The estimator τ (tau, an estimate of the mode of the mismatch distribution) is an index of time since expansion is expressed in units of mutational time (Rogers and Harpending, 1992). The values of τ were transformed to estimates of real time since expansion with the equation $\tau = 2ut$, where u is the mutation rate for the sequence under study per generation and t is the time measured in generations since expansion. θ_0 and θ_1 correspond to the mutation parameter before and after population growth, which is defined as $2Nu$ for mitochondrial loci, where N is the effective female population size and u is the mutation rate per gene per generation. The mismatch analysis was performed in ARLEQUIN.

In the present study, 0.14%–0.52% divergence per nucleotide site per million years ($0.90*10^{-6}$—$3.35*10^{-6}$ mutations per sequence per year) was applied for the cytochrome oxidase I sequences of *R. philippinarum* (Luttikhuizen et al., 2003).

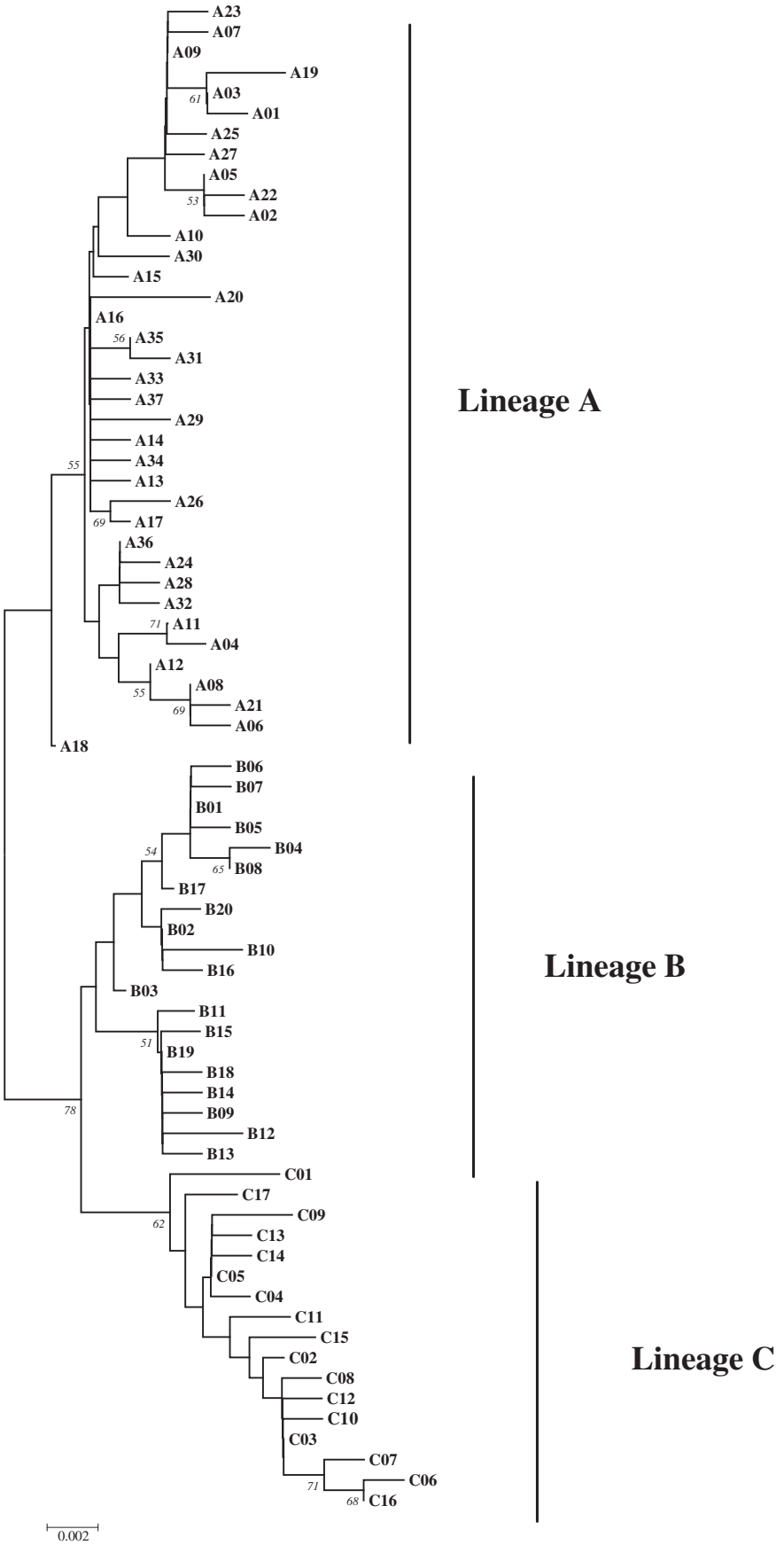

Fig. 2. Neighbor-joining tree of cytochrome oxidase I haplotypes constructed using Tamura and Nei distances for *Ruditapes philippinarum*. Bootstrap supports of >50% in 1000 replicates are shown above branches.

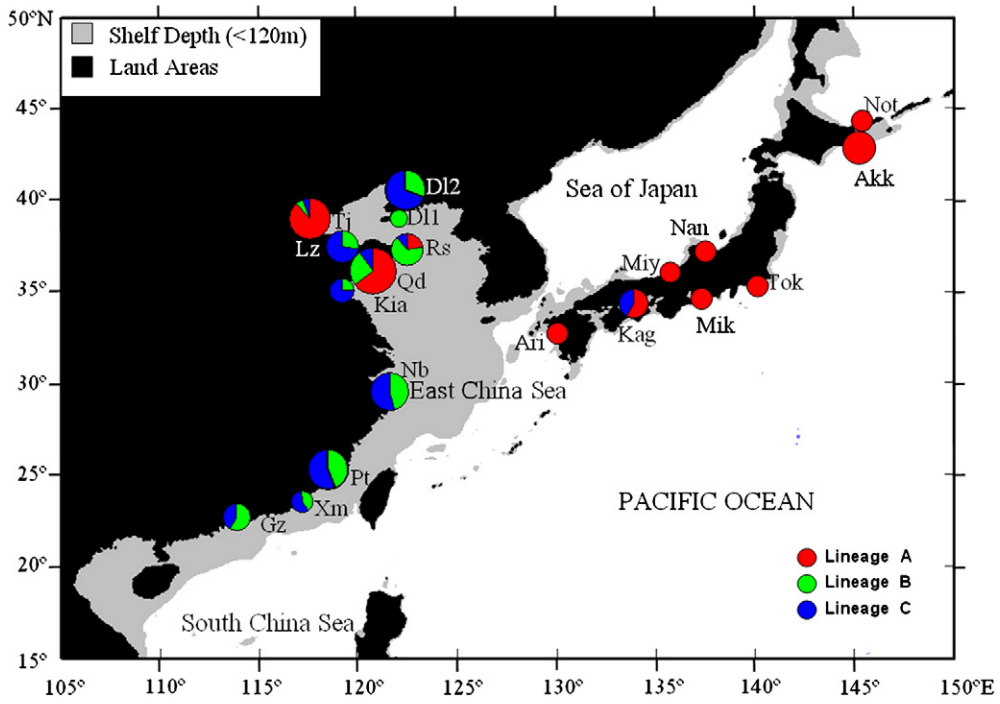

Fig. 3. Haplotype frequencies for *Ruditapes philippinarum* from Guangzhou to Notsuke Bay. The area of the circle is proportional to sample size.

3. Results

3.1. Genetic diversity

A sequence alignment of 644-bp of the COI fragment from 170 individuals of *R. philippinarum* collected from 19 localities across the coastlines of China and Japan (Table 1) was obtained. Sequence comparisons of the 644-bp segment of COI revealed 69 polymorphic sites (10.7%) with 73 substitutions, defining 74 haplotypes. No insertion/deletion was detected. The nucleotide composition of the fragment was A^T-rich (A, 23.58%; T, 41.56%), and variations consisted predominantly of transition substitutions (Ti:Tv = 6.3). The most abundant haplotype was shared by 20 specimens (6 from Tianjin, 4 from Akkeshi, 4 from Qingdao, 2 from Notsuke Bay, one from Ariake Bay, one from Mikawa Bay, one from Tokyo Bay and one from Kagawa). The second most abundant haplotype was shared by 18 individuals. The

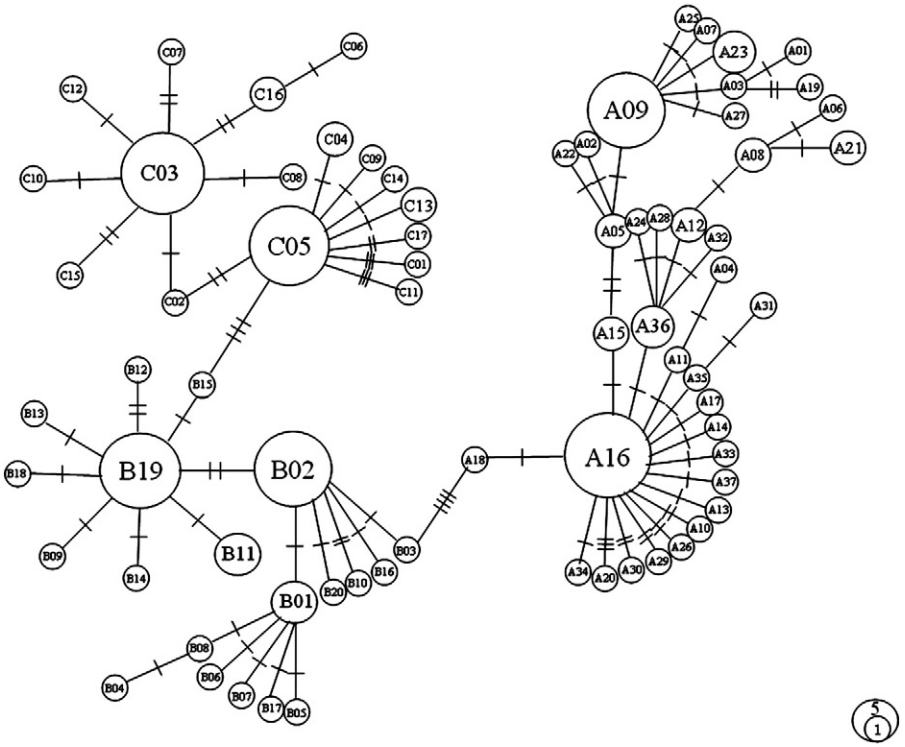

Fig. 4. Unrooted minimum spanning trees showing genetic relationship among COI region haplotypes for *Ruditapes philippinarum*. The sizes of circles are proportional to haplotype frequency. Haplotypes are marked by names that correspond to Table 2. Perpendicular tick marks on the lines joining haplotypes represent the number of nucleotide substitutions.

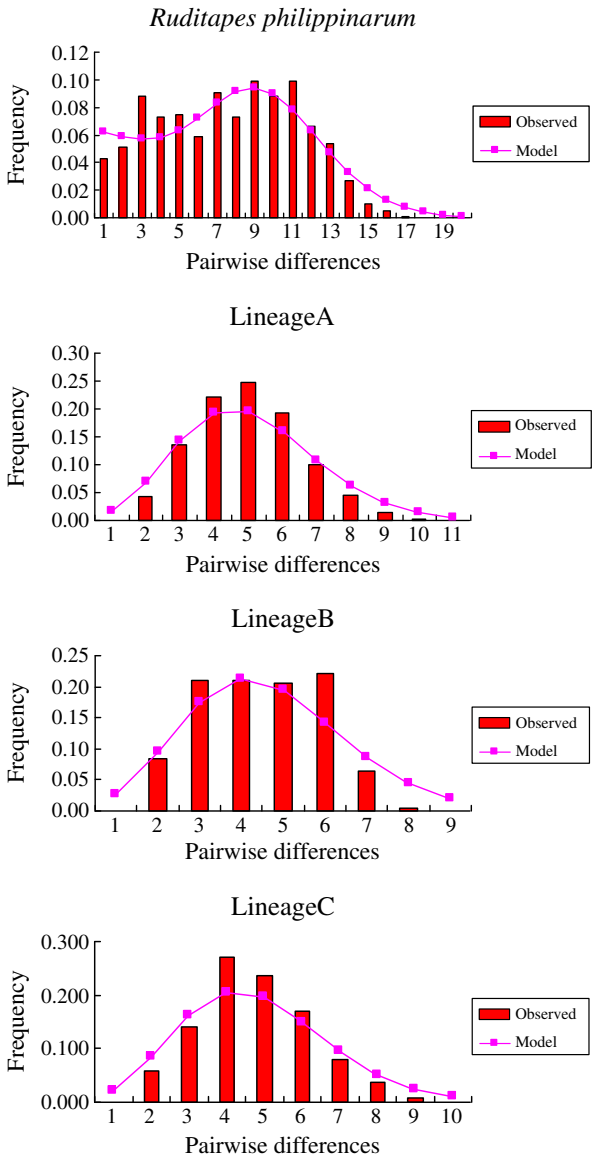

Fig. 5. The observed pairwise difference (bars), and the expected mismatch distributions under the sudden expansion model (solid line) of COI region haplotypes in *Ruditapes philippinarum*.

majority of haplotypes (75.7%) were singletons (haplotypes represented by a single sequence in the sample). Of the remaining 18 haplotypes, 13 were shared among populations and 5 were found in more than one individual, but only in one population (Table 2).

The sequences of these haplotypes are available at the NCBI sequence database under the accession letter JN054502–JN054632. High levels of haplotype diversity were detected within each population which showed high level of genetic diversity in this species. But, in contrast, low levels of nucleotide diversity were observed, except for the populations from Qingdao, Rushan and Kagawa. Haplotype diversity of the species ranged from 0.80 ± 0.16 (Notsuke Bay) to 1.00 ± 0.13 (Nanao Bay, Miyazu Bay, Dalian1 and Ariake Bay), while nucleotide diversity varied from 0.002 ± 0.001 (Notsuke Bay) to 0.011 ± 0.006 (Qingdao) (Table 1). Overall, the average values of haplotype diversity (h) and nucleotide diversity (π) were 0.96 ± 0.01 and 0.010 ± 0.005, respectively.

3.2. Phylogeographical patterns and genetic structure

The NJ tree constructed using the complete data set of 74 haplotypes identified three genealogical lineages (labeled A, B, C; Fig. 2). Net average genetic distances (Tamura and Nei with gamma correction) between lineages (± SE) were A/B: 0.9% (0.30%); A/C: 1.3% (0.4%); B/C: 0.6% (0.3%). Applying the COI sequence divergence rate (0.9–3.35%/MY) (Luttikhuizen et al., 2003), the divergence of lineage A from B occurred about 269,000–1,000,000 years before present (BP). The genetic distance between lineage C and A corresponds to 388,000–1,440,000 years BP. The divergence of lineage C from B dates back to 179,000–667,000 years BP. These results indicated middle to late Pleistocene divergence among these lineages. The average pairwise divergences between individuals within each of the lineages (±SE) were 0.60% (±0.1%), 0.50% (±0.1%) and 0.60% (±0.10%) for lineages A, B and C, respectively.

Significant geographical differences in the distribution of haplotype frequencies were detected in the three lineages. The Japanese populations were composed mainly of lineage A with the exception of Kagawa which also included some part of lineage C. Lineage A was also found in three Chinese populations (Tianjin 88%, Qingdao 65% and Rushan 22%). Lineage B and lineage C dominated the coastline of China. Lineage B mainly dominated the East China Sea (52%) and lineage C dominated the South China Sea (53%). One haplotype in lineage C was also found in Kagawa population (Fig. 3, Table 1).

Network of lineage A showed a star-like structure with a dominant haplotype (27%) shared by 6 Japanese populations and 2 Chinese populations (Fig. 4, Table 2). Furthermore, the most common haplotype was not found in Nanao, Miyazu and Rushan populations. In lineage B, the dominant (~23%) haplotype formed the center of a star-like network (Fig. 4). In lineage C, the second dominant (~30%) haplotype formed the center of a star-like network (Fig. 4). Most of the lineage C haplotypes found in Dalian 2 were confined to a subclade of the network, which also contained haplotypes from the East China Sea populations (Fig. 4, Table 2).

Significant genetic differentiations were checked among populations based on the MDS analysis, which showed significant population genetic structure maybe existed in the species (Fig. 6). A hierarchical AMOVA was carried out with different groupings based on the *F*st analysis and MDS analysis, which also revealed significant genetic subdivision existed among groups ($\Phi_{CT} = 0.478$; $P < 0.01$) and among populations within groups ($\Phi_{SC} = 0.019$; $P < 0.01$) (Fig. 6, Table 3, Table 5).

3.3. Historical demography

As expected from the star-like networks, the mismatch distributions for lineages A, B and C were unimodal (Fig. 5), closely fitted into the expected distributions under the sudden expansion model. For all populations, the mismatch distribution was clearly bimodal with one mode corresponding to the number of differences among three lineages, and the other to differences among individuals within lineages (Fig. 5). Fu's tests and Tajima's *D* tests of lineage A, B and C were negative and highly significant ($P = 0.00$) indicating a recent population demographic expansion of the species (Table 4).

The tau value (τ), which reflects the location of the mismatch distribution crest, provides a rough estimate of time when rapid population expansion started. The observed values of the age expansion parameter (τ) were 3.81 and 3.98 of mutational time for lineage B and C, respectively. The tau value of lineage A (4.06) was larger than those of lineage B and lineage C (Table 4). The estimate of expansion time based on the rates mentioned above for COI region were about 177,000–657,000 years ago (lineage B), 184,000–687,000 years ago (lineage C), and 188,000–700,000 years ago (lineage A).

4. Discussion

4.1. Genetic diversity

The mtDNA COI gene fragment used as molecular marker in this study showed a high level of polymorphism and was suitable for studying the population genetic diversity and the pattern of phylogeography of *R. philippinarum*. Levels of genetic diversity in

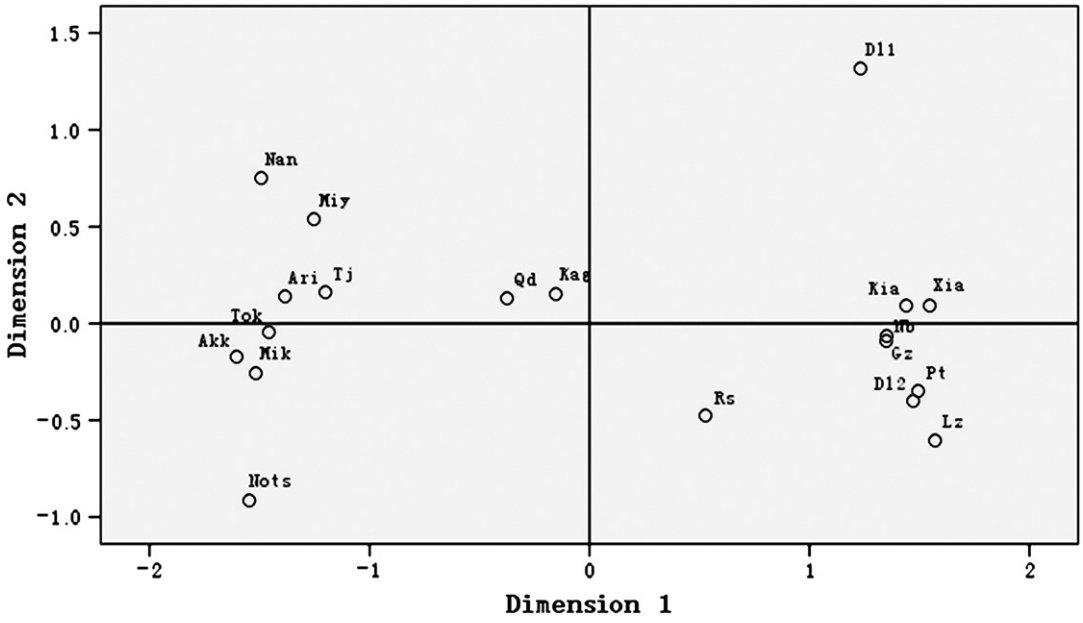

Fig. 6. Bidimensional representations of multidimensional scaling (MDS) based on F_{ST} values calculated between all pairs of samples at COI locus. See Fig. 1 for sample abbreviations.

the present study are comparable with other studies on marine bivalves (e.g. Luttikhuizen et al., 2003; Shefer et al., 2004). High levels of polymorphism and genetic diversity were also observed in *Tridacna crocea* from the Indo-Malay Archipelago (DeBoer et al., 2008; Kochzius and Nuryanto 2008) and other studies on marine invertebrates (e.g. Barber et al., 2002; Duran et al., 2004; Luttikhuizen et al., 2003; Shefer et al., 2004). A pattern of high level of haplotype diversity and moderate level of nucleotide diversity was observed for *R. philippinarum*. There are two possible reasons for such pattern. Theoretically, the level of genetic variability held in a population tends to be positively correlated with the effective population size (Fujii and Nishida, 1997). High level of haplotype diversity (h) suggests large, stable, effective population sizes over time in the species of the continental shelf (Stepien, 1999). Thus, one possible reason is that *R. philippinarum* might have continued having large population size and the high level of haplotype diversity (h) has been maintained in it.

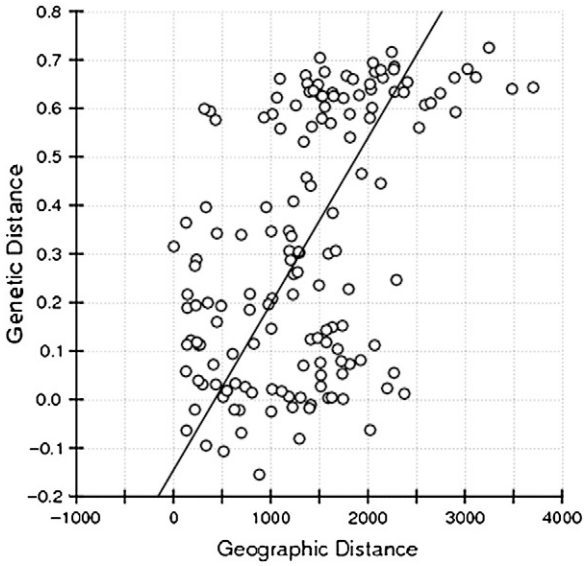

Fig. 7. Relationship between genetic vs. geographical distances in *Ruditapes philippinarum* using reduced major axis (RMA) regression without considering populations from Dalian 1 studied by Sekine et al. (2006).The regressions are $y = 3.422*10^{-4}*-0.1460$.

Indeed, the species is distributed widely along the coastlines of China and Japan. A period of rapid population growth (sudden population expansion) could also have contributed to the pattern of mtDNA COI genetic diversity in *R. philippinarum* (Rogers and Harpending, 1992). During sudden population expansion, the rate of stochastic loss of haplotypes slows down resulting in more haplotypes being retained than can be lost by genetic drift (Avise et al., 1984).

High levels of haplotype diversity (≥ 0.85) were found in all populations, except in Kiaochow Bay, Notsuke Bay, and Kagawa, which might be due to small effective population sizes. The level of nucleotide diversity in Qingdao was rather high compared to other sampling sites in the present study; Notsuke Bay and Dalian 1 populations showed low values. The result of genetic diversity in the present study was consistent with the previous study on isozyme (Ren et al., 2006). The values of average heterozygosity of the Qingdao population were higher than the rest, which indicated that the genetic diversity of the Qingdao population was more abundant comparatively, and the genetic background was more complex. The low genetic diversity in Notsuke Bay and Dalian 1 populations might be due to small number of individuals, overexploitation or both.

4.2. Phylogeographical patterns and genetic structure

Three distinct lineages were found in *R. philippinarum,* likely reflecting isolation of the marginal seas of the Northwestern Pacific during Pleistocene low sea-level stands. The frequency distribution of the lineage A indicated an origin in the Sea of Japan. Lineage B and C dominated the Chinese populations, indicating that these two lineages were isolated and diverged in the coastline of China. Two lineages (B and C) were found in all Chinese populations, indicating secondary contact following inundation of the barriers. Our results on *R. philippinarum* agreed with previous findings of genealogical structure in coastal regions of Japan and China based on mitochondrial COI sequence data (Sekine et al., 2006). According to the previous study, there were no shared haplotypes between Chinese and Japanese populations. However, shared haplotypes were found among Chinese (Qingdao, Tianjin) and Japanese populations in the present study. One reason is related to the connections generated by sea surface currents. The other possible reason may be the importation of *R. philippinarum* seeds from China to Japan. Imported *R. philippinarum* are generally released in areas already inhabited by

Table 2
Distribution of haplotypes among localities.

	Lineage A population												Lineage B population												Lineage C population											
Hap.*	Nan	Miy	Ari	Not	Mik	Tok	Qd	Akk	Tj	Rs	Kag	Tot.n*	Hap.*	Kia	Xia	Dl1	Nb	Gz	Qd	Dl2	Lz	Tj	Rs	Pt	Tot.n*	Hap.*	Kia	Xia	Nb	Gz	Qd	Dl2	Lz	Tj	Rs	Pt	Kag	Tot.n*
A01	1											1	B01	1		1							1		3	C01	1											1
A02		1										1	B02		2		2	2		2				2	10	C02		1									1
A03	1											1	B03			1									1	C03	2		5		1	6				4		18
A04		1										1	B04										1		1	C04		1								2		3
A05			1								1	2	B05							1					1	C05		1		1	1	2	5	1	1	1	3	16
A06			1									1	B06							1					1	C06			1									1
A07	1											1	B07					1							1	C07				1								1
A08					2							2	B08									1			1	C08						1						1
A09			1		1	2	1		1		2	8	B09						1						1	C09							1					1
A10	1											1	B10				1								1	C10						1						1
A11		1										1	B11						3						3	C11							1					1
A12					1	1						2	B12						1						1	C12						1						1
A13				1								1	B13								1				1	C13										2		2
A14			1									1	B14								1				1	C14				1								1
A15				2								2	B15										1		1	C15						1						1
A16			1	2	1	1	4	4	6		1	20	B16					1							1	C16			1			1						2
A17	1											1	B17				1								1	C17							1					1
A18						1						1	B18							1					1													
A19							1					1	B19				2				1		3	5	11													
A20								1				1	B20							1					1													
A21							2					2																									
A22								1				1																									
A23							2		2			4																									
A24									1			1																									
A25							1					1																									
A26								1				1																									
A27										1		1																									
A28									1			1																									
A29									1			1																									
A30								1				1																									
A31								1				1																									
A32							1					1																									
A33									1			1																									
A34								1				1																									
A35								1				1																									
A36							1		2	1		4																									
A37								1				1																									

*Hap., Haplotype; Tot. n, total number.

Table 3
Pairwise F_{ST} (below diagonal) and associated P values (above diagonal) among *Ruditapes philippinarum* populations.

	Kia	Xia	Nan	Miy	Dl1	Ari	Not	Mik	Tok	Nb	Gz	Qd	Akk	Dl2	Lz	Tj	Rs	Pt	Kag
Kia		0.4990^{NS}	0.0059*	0.0352*	0.4014^{NS}	0.0059*	0.0059*	0.0049*	0.0049*	0.6377^{NS}	0.4170^{NS}	0.0078*	0.0000**	0.8184^{NS}	0.1719^{NS}	0.0010*	0.0693^{NS}	0.3662^{NS}	0.1299^{NS}
Xia	−0.081		0.0137*	0.0195*	0.2080^{NS}	0.0078*	0.0098*	0.0068*	0.0127*	0.4150^{NS}	0.9697^{NS}	0.0059*	0.0000**	0.3418^{NS}	0.3154^{NS}	0.0010*	0.0967^{NS}	0.7363^{NS}	0.0840^{NS}
Nan	0.649	0.680		0.1113^{NS}	0.0430*	0.2266^{NS}	0.0186*	0.0440*	0.2305^{NS}	0.0000**	0.0010*	0.0527^{NS}	0.0059*	0.0000**	0.0000**	0.0410*	0.0020*	0.0000**	0.1191^{NS}
Miy	0.531	0.601	0.194		0.1065^{NS}	0.4033^{NS}	0.0986^{NS}	0.2383^{NS}	0.2998^{NS}	0.0039*	0.0078*	0.2100^{NS}	0.0352*	0.0000**	0.0029*	0.0713^{NS}	0.0039*	0.0000**	0.2900^{NS}
Dl1	0.258	0.222	0.665	0.589		0.0674^{NS}	0.0449*	0.0488*	0.0410*	0.1836^{NS}	0.4316^{NS}	0.1182^{NS}	0.0117*	0.0742^{NS}	0.0127*	0.0283*	0.7119^{NS}	0.0361*	0.1748^{NS}
Ari	0.588	0.625	0.026	0.005	0.601		0.5322^{NS}	0.4746^{NS}	0.9453^{NS}	0.0000**	0.0000**	0.3037^{NS}	0.2881^{NS}	0.0000**	0.0000**	0.4893^{NS}	0.0117*	0.0000**	0.2041^{NS}
Not	0.686	0.725	0.346	0.258	0.817	0.053		0.0830^{NS}	0.1914^{NS}	0.0010*	0.0020*	0.2363^{NS}	0.6484^{NS}	0.0000**	0.0010*	0.2744^{NS}	0.0088*	0.0010*	0.0791^{NS}
Mik	0.626	0.663	0.288	0.122	0.694	−0.021	0.306		0.2979^{NS}	0.0000**	0.0010*	0.1719^{NS}	0.0098*	0.0000**	0.0000**	0.1172^{NS}	0.0049*	0.0000**	0.0957^{NS}
Tok	0.621	0.654	0.031	0.072	0.671	−0.155	0.146	0.039		0.0000**	0.0000**	0.3740^{NS}	0.3359^{NS}	0.0000**	0.0000**	0.8467^{NS}	0.0205*	0.0000**	0.2178^{NS}
Nb	−0.069	−0.022	0.632	0.562	0.207	0.581	0.607	0.603	0.588		0.2959^{NS}	0.0000**	0.0000**	0.5625^{NS}	0.0420*	0.0000**	0.0176*	0.2461^{NS}	0.0078*
Gz	0.003	−0.107	0.631	0.560	−0.022	0.580	0.643	0.611	0.592	0.017		0.0029*	0.0000**	0.0996^{NS}	0.0635^{NS}	0.0010*	0.1143^{NS}	0.2246^{NS}	0.0752^{NS}
Qd	0.315	0.302	0.127	0.070	0.199	0.021	0.055	0.076	0.001	0.339	0.300		0.0166*	0.0000**	0.0000**	0.1387^{NS}	0.0420*	0.0000**	0.4844^{NS}
Akk	0.675	0.681	0.217	0.208	0.657	0.027	−0.021	0.196	0.014	0.633	0.640	0.112		0.0000**	0.0000**	0.1973^{NS}	0.0000**	0.0000**	0.0166*
Dl2	−0.095	0.004	0.668	0.606	0.314	0.622	0.639	0.636	0.625	−0.025	0.081	0.396	0.660		0.0225*	0.0000**	0.0010*	0.1025^{NS}	0.0000**
Lz	0.058	−0.011	0.704	0.651	0.419	0.661	0.716	0.675	0.667	0.115	0.104	0.364	0.694	0.118		0.0000**	0.0049*	0.2861^{NS}	0.0205*
Tj	0.576	0.569	0.152	0.149	0.500	−0.018	0.012	0.073	−0.063	0.558	0.540	0.031	0.023	0.594	0.599		0.0049*	0.0000**	0.0264*
Rs	0.189	0.124	0.457	0.408	−0.066	0.396	0.445	0.440	0.384	0.185	0.079	0.113	0.465	0.275	0.216	0.342		0.0166*	0.1104^{NS}
Pt	0.006	−0.064	0.679	0.627	0.306	0.634	0.664	0.650	0.634	0.018	0.033	0.347	0.663	0.050	0.004	0.579	0.160		0.0049*
Kag	0.216	0.227	0.193	0.112	0.238	0.113	0.235	0.199	0.094	0.287	0.246	−0.016	0.262	0.336	0.304	0.143	0.118	0.306	

*Significant at $P<0.05$ by the permutation test; **significant P values after Bonferroni correction; NS, not significant.

Table 4
Tajima's D and Fu's Fs , corresponding P-value, and mismatch distribution parameter estimates.

Groups	Tajima'D		Fu's F_S		Mismatch distribution		
	D	P	F_S	P	τ	θ_0	θ_1
All	−1.52	0.04	−24.79	0	9.17	0.00	15.02
Lineage A	−1.95	0.01	−25.86	0	4.06	0.00	9539.55
Lineage B	−1.80	0.02	−24.72	0	3.81	0.00	9517.91
Lineage C	−1.70	0.03	−20.70	0	3.98	0.00	9480.88

the same species, and they may interact with the native populations (Sekine et al., 2006). Differences in results of the two studies could be attributed to several reasons, including small population size and limited sampling locations.

Geological events during this interval evidently created vicariant barriers among populations in the Sea of Japan, the East China Sea and the South China Sea. The Sea of Japan was almost isolated from the Pacific Ocean and the East China Sea during Pleistocene glaciation events due to the shallow sills (<135 m) (Kitamura et al., 2001; Wang, 1999). A large land bridge extended from eastern China to Taiwan, the Ryukyus and probably to the main islands of Japan formed in the late Pleistocene, which is likely to have isolated the East China Sea from Pacific Ocean and the South China Sea (Kimura, 1996, 2000). We concluded that populations of *R. philippinarum* could thereby have become isolated in the Sea of Japan, the East China Sea and the South China Sea, resulting in the three lineages observed in the mitochondrial genome.

As in our study, strong genetic break between the Japanese populations and Chinese populations has also been reported for marine fishes, such as *Chelon haematocheilus* (Liu et al., 2007), *Sardinella zunasi* (Wang et al., 2008) and some marine invertebrates (Gao and Watanabe, 1998; Yokogawa, 1997). Fluctuating sea levels in Pleistocene have also been hypothesized to be the factor causing strong genetic divergence in this species. Similar genetic breaks have also been described between East China Sea and South China Sea populations of shellfish (Li et al., 2003; Pan et al., 2005). Genetic breaks have previously been described in other marine systems, strong differentiation has been observed in bivalve species in the North Sea–Baltic Sea transition zone. Two separate lineages were detected, one present in the NE Atlantic and the other in the Baltic Sea. These lineages are assumed to have come into secondary contact after extensive isolation, and it is suggested that repeated invasions of these species from the Pacific to the Atlantic Ocean gave rise to the two lineages (Johannesson and André, 2006). These genetic breaks cited above in Northwestern Pacific and North Sea–Baltic Sea transition zone reveal that historical geographical factors greatly influenced the evolutionary genetic structure of marine organisms in the three marginal seas of the Northwestern Pacific.

Table 5
Hierarchical analysis of molecular variance (AMOVA) of mtDNA COI sequences in *Ruditapes philippinarum* from the coastlines of China and Japan. For abbreviations of sample sites, see Table 1.

Region groupings	Φ_{CT}	Percentage variance among groups
(Nan,Miy,Ari,Akk,Tok,Mik,Not,Kag) (Kia,Lz,Dl2,Tj,Qd,Rs,Dl1,Xia,Gz,Nb,Pt)	0.32**	31.57
(Nan,Miy,Ari,Akk,Tok,Mik,Not,Kag) (Kia,Lz,Dl2,Tj,Qd,Rs,Dl1) (Xia,Gz,Nb,Pt)	0.26**	26.07
(Nan,Miy,Ari,Akk,Tok,Mik,Not,Tj) (Kag,Qd) (Rs) (Dl1,Xia,Gz,Kia,Nb,Pt,Lz,Dl2)	0.46***	45.85
(Nan,Miy,Ari,Akk,Tok,Mik,Not,Tj) (Kag,Qd) (Rs) (Dl1) (Xia,Gz,Kia,Nb,Pt,Lz,Dl2)	0.48***	46.09

*$0.05 \geq P \geq 0.01$; **$0.01 > P \geq 0.001$; ***$P < 0.001$; NS, not significant.

Strong gene frequency changes of the three lineages among the three marginal seas revealed highly limited genetic exchange between populations of *R. philippinarum* in the absence of contemporary dispersal barriers. The AMOVA showed high levels of genetic structuring among populations, which indicated low level of dispersal in this species. The results have important implications for fisheries management of the species. However, as individuals of each population are less than 30 which can't stand for the whole population, more samples should be used in the future work.

4.3. Historical demography

Population expansion and mutation rate heterogeneity have opposite effects on Tajima's *D* statistics. Population expansion leads to large negative values and considerably reduces its variance, however, mutation rate heterogeneity leads to less negative values and also increases its variance. Tajima's *D* test cannot reject the null hypothesis of neutrality and is overly conservative after a large population expansion combined with mutation rate heterogeneity (Aris-Brosou and Excoffier, 1996). The gamma distribution shape parameter was 0.101 for *R. philippinarum*, indicating high mutation rate heterogeneity among sites, which might be responsible for the insignificant Tajima's *D* test. This study points to the need for a more careful examination of test results, particularly when several statistical tests show different results.

Both the neutrality tests and mismatch distribution analysis indicated a recent population expansion in *R. philippinarum*. Both population range expansion and demographic expansion might have had an effect on the pattern of genetic diversity for *R. philippinarum*. Furthermore, the star-like median network of *R. philippinarum* is also consistent with a population expansion. The estimate of population expansion time suggested a population expansion in the middle to late Pleistocene (about 425 kya–1580 kya) for *R. philippinarum*. The late Pleistocene Period (the past one million years) was punctuated by a series of large glacial-interglacial changes (Imbrie et al., 1992). Late Pleistocene glaciations and associated temperature and salinity shifts, global shifts in ocean circulation patterns and altered productivity regimes, likely had great effect on the demographic history of *R. philippinarum*.

More than 75% of the marginal basins in the modern global sea are in the Western Pacific continental margin (Tamaki and Honza, 1991). During Quaternary glacial cycles, most of the East China Sea was exposed. Under the most severe environmental conditions in glacial periods, *R. philippinarum* might have become extinct and survived only in glacial refuges. Population expansions from glacial refugium are expected in *R. philippinarum* when more favorable conditions returned during interglacials and lower genetic diversities are expected in the postglacially colonized regions. Since the end of Last Glacial Maximum, the Sea of Japan, the East China Sea and the South China Sea must have been recolonized by *R. philippinarum*.

According to previous studies, the same signal of a sudden population expansion was found in invertebrates, such as *Tridacna crocea* from the Indo-Malay Archipelago (Kochzius and Nuryanto, 2008), the giant clam *T. maxima* in the Indo-West Pacific(Nuryanto and Kochzius, 2009)and a freshwater crab *Geothelphusa tenuimanus* from Okinawa (Naruse et al., 2004).

The results of the present study provide evidence for strong genetic divergence among *R. philippinarum* in the marginal seas of Northwestern Pacific, which coincides with expected patterns of vicariance due to sea level changes during the Pleistocene. This hypothesis should be further tested by comparative studies of other marine organisms in this region. In general, the marginal seas of the Northwestern Pacific could prove to be an interesting system for investigating the impact of major geological events on the distribution of genetic diversity in marine taxa. Finally, the surprisingly strong genetic differentiation among *R. philippinarum* populations illustrates that the genetic diversity among populations of marine invertebrates are often significantly underestimated, and that informed management and conservation of marine fishery resources must integrate results from molecular population genetic studies.

When managing a species as a resource, the ability to assess genetic diversity and population structure can be a vital tool for maintaining a productive fishery (Seeb et al., 1990). Severe population declines can be recognized by decreased genetic diversity (Glenn et al., 1999; Lavery et al., 1996). Our genetic assessment of *R. philippinarum* provides a baseline from which *R. philippinarum* populations can be appraised in the future. In order to have a comprehensive understanding of population genetic structure in *R. philippinarum*, and have a further insight into the genetic differentiation of the species, it is necessary to use more sensitive DNA markers (such as microsatellite DNA and AFLP).

Acknowledgments

We thank Ms. Tian-Yan Yang and Ms. Na Song for providing samples. We are grateful to Dr. Ming Liu, Dr. Na Song and Dr. Qiao-Li Yang for checking the contents of the paper. We thank Dr. Nwafili Sylvanus Anene and Ravindra Pawar for correcting the manuscript. This work was supported by the Special Research Foundation for Public Welfare Marine Programme (200905019). **[RH]**

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