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ITS_1 DNA sequences reveal population genetic differentiation and structure in the Chinese clam *Cyclina sinensis* (Veneridae: Bivalvia)

PAN BAOPING [1*], ZHANG LIYAN[1], SONG XIN[1], GAO WEIWEI [1] & BU WENJUN [2]
[1] School of Life Sciences, Tianjin Key Laboratory of Cyto-Genetical and Molecular Regulation, Tianjin Normal University, Tianjin, P R China, 300387
[2] School of Life Sciences, Nankai University, Tianjin, P R China, 300071
*Corresponding author—Email: panbaoping@yahoo.com.cn

Abstract

The genetic diversity and structure of 10 populations of *Cyclina sinensis* distributed along coastal regions in China were investigated by sequencing ribosomal DNA internal transcribed $spacer_1$ (ITS_1). The lengths of the ITS_1 sequences of *C. sinensis* ranged from 564 to 595 nucleotides. Forty-two allelic sequences [nucleotide diversity; π=0.033; θ (per site) based on the total number of mutations=0.048] have been identified from a total of 80 individuals. Phylogenetic analysis of these sequences, using a sample from Japan as outgroup, recovered a topology containing two major clades. One clade comprised the samples from the China Bohai Sea, the Yellow Sea and the Dong Sea (northern and middle parts of the China Sea), the other clade represented the those from the South China Sea. F_{ST} values indicated significant differences in each pairwise combination of populations representing each of the two clades, while the AMOVA analysis showed that the majority of genetic variation (67.7%) was attributable to variation between the two main clades, with 25.7% attributable to within-population variation and 6.6% to between populations within groups. These results suggest strong genetic structure among the Chinese populations of *C. sinensis*. Evolutionary rate analysis implies that the two main clades have experienced population isolation since the late Pleistocene (approximately 0.35and1.91MY ago), due to coastal freshwater intrusions and/or cold current upwelling.

Key words: Genetic differentiation; genetic structure; AMOVA; biogeographic barrier

Introduction

The venerid clam *Cyclina sinensis* (Gmelin, 1791) is a commercially important marine bivalve (Liu and Xu 2003) that is abundant and widely distributed around Asia. *C. sinensis* is commonly found in intertidal zones of muddy sand beaches along the north and south coasts of China, in Japan and in Korea. Its range extends to the Far East of Russia and Southeast Asia. It can tolerate wide temperature and salinity ranges. Recently, a number of studies have been carried out on its geographic distribution (Xu 1997; Zhuang 2001), anatomy (Yu and Zheng 2001; Zhao. *et al.* 2009), ecology and reproduction (Yu *et al.*1995; Xu 2000; Liu *et al*. 2002),genetic markers (Wang *et al*. 2001; Chen *et al*. 2004; Zhao *et al.* 2007; Feng *et al*. 2010),and population diversity and differentiation(Pan *et al.* 2005).Population genetic structure is dependent on the interaction of the biology of a species and the environment in which it resides. Marine organisms generally show low levels of genetic differentiation over large geographic distances (Avise 2000; Palumbi and Baker 1994), owing to the absence of obvious barriers to migration and to passive dispersal by pelagic larval stages. However, there are a number of exceptions due to biological mechanisms, water dynamics, or historical events (Shulman and Bermingham 1995; Shulman 1998; Palumbi *et al*.1997; Barber *et al*. 2002; Nelson *et al*. 2000). Recent phylogeographical investigations have revealed surprising levels of previously hidden marine biodiversity, casting doubt on the long-held paradigm that marine systems are largely open to movement among populations (Mathews 2006). Thus, a clearer understanding of the connectivity among marine populations may result in more effective designs for marine-protected areas and reserves.

In bivalve molluscs, a variety of methods, such as PCR amplification alone, or PCR amplification followed by restriction analysis or sequencing, have been used to differentiate related species (Ding *et al.* 2004) and to explore the phylogeographic and phylogenetic relationships (He *et al*. 2005; Vidigal *et al*. 2004; Reece *et al*. 2008; Shilts *et al.* 2007; Källersjö *et al*. 2005; Lee and Ó Foighil 2005). Eizadora *et al.*, (2000) demonstrated that ITS-1 sequence variations, identified at very high polymorphic sites in *Tridacna crocea* (Lamarck, 1819), could be appropriate markers for molecular systematic studies at the species and population levels. Ribosomal DNA internal transcribed spacer (ITS) sequence variation has generally proven to be a powerful tool for studying phylogenetics and for species identification (Mizukami and Kito1999), and has been used with a wide range of invertebrates (Chen and Miller 1996; Chu *et al.* 2001; Vogler and Desalle 1994; Vane *et al.* 1999) including molluscs (Stothard *et al.* 1996; Caporale *et al.* 1997; King *et al.*1999; Wilber *et al.* 2000; Kenchington *et al.* 2002; Ding *et al.* 2004; Vierna *et al.* 2010). Consequently for this study, the nucleotide sequence of ribosomal DNA internal transcribed spacers was examined to assess the genetic diversity and phylogeographic structure of *C. sinensis* from Chinese coastal populations.

Materials and Methods

Samples were collected from ten populations of *C. sinensis* from the maritime coasts of China (Fig.1) which encompass a wide range of geographic regions and habitats. Between 25 and 30 individuals representing each population were collected from Chinese Sanya (18.20°N) to Zhuanghe

(39.78°N).The adductor muscles of each specimen were dissected out and fixed in 70% ethanol. The details of the localities of the sampled populations are given in Table 1.

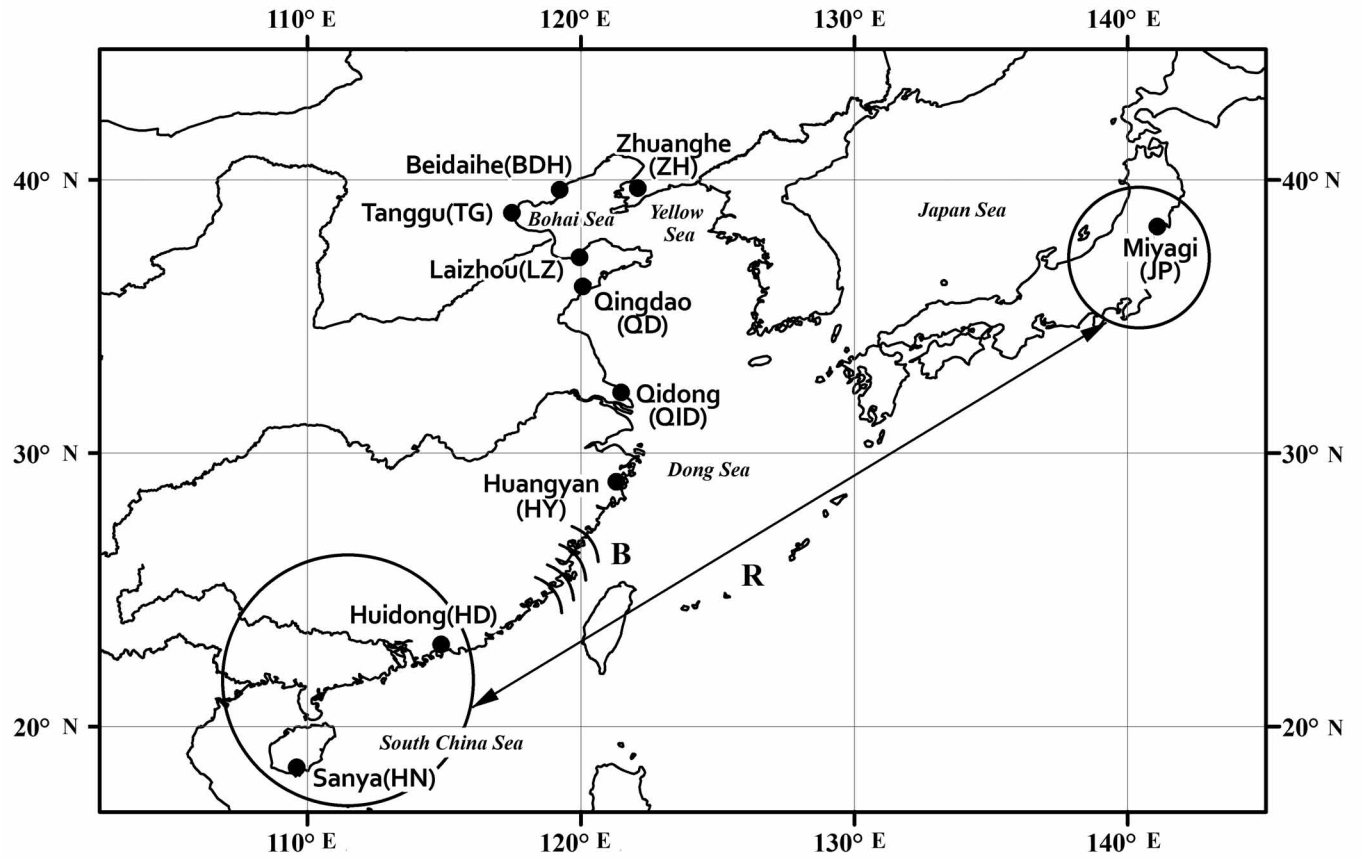

FIGURE 1. The sampled locations of *C. sinensis* populations. The line (R) indicates a molecular data similarity between the two areas and B indicates an apparent barrier (see text).

TABLE 1. The locations and geographic coordinates for the ten sampled populations of *C. sinensis.*

Analyzed Samples	Area	Abbre-viations	Geographic coordinates
Ingroup Samples	Sanya	HN	109.50°E, 18.20°N
	Huidong	HD	114.70°E, 22.97°N
	Huangyan	HY	121.27°E, 28.64°N
	Qidong	QID	121.67°E, 31.80°N
	Qingdao	QD	120.33°E, 36.07°N
	Laizhou	LZ	119.90°E, 37.10°N
	Beidaihe	BDH	119.57°E, 39.28°N
	Tanggu	TG	117.39°E, 39.00°N
	Zhuanghe	ZH	122.06°E, 39.78°N
Outgroup Sample	Miyagi (Japan)	JP	141.00°E, 38.23°N

Eight individuals were randomly selected from each population. Genomic DNA was extracted following the method given in Grewe *et al.*(l993). A 0.1 g sample of tissue was pulverized and incubated in 700 µl buffer (25 mmol/L Tris-HCl pH8.0, 0.3M NaCl, 5 mmol/L ETDA, 0.5% CTAB, 0.1% 2-mercaptoethanol, 100µg/ml proteinase K) at 60℃ for 2.5 h, and DNA was purified twice by chloroform/ isoamylalcohol extraction followed by ethanol precipitation. PCR was performed in a 25 µl volume containing 25 ng genomic DNA, 1×PCR buffer, 100 µM dNTP mix, l.5 mM $MgCl_2$, 0.2 µM of each primer and 1 Unit of Taq polymerase (*TaKaRa*). The primers described by Gaffney *et al.* (1998) for ITS1-a 5`-GGTTCTGTAGGTGAACCTGC-3' and ITS1-b 5`-CTGCGTTCTTCATCGACCC-3` were used. Amplification started at 94°C for 3 min for pre-denaturation, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 60 s and elongation at 72°C for 60 s, with 7 min at 72°C for final elongation. The amplified fragments were separated by agarose gel (1.2%) electrophoresis in 1×TBE (89 mmol/L Tris, 89 mmol/L boric acid, 5 mmol/L EDTA, pH 8.3), stained with ethidium bromide and observed under ultraviolet light. After purification using the UNIQ-10 Kit (Sangon, Shanghai), PCR products were ligated into pMD18-T Vector (*Takara*) and used to transform a competent cell of *Escherichia coli* Top10.Recombinant colonies were identified by IPTG/X-Gal blue-white screening. The positive clones were sequenced in both directions using a DNA sequencer (ABI PRISM 3730, Applied Biosystems).

The sequences were aligned using ClustalX 1.83 (Thompson *et al.* 1997). For the DNA sequence, full multiple alignment was executed using the default parameters. Allelic sequence diversity was analyzed by DNAsp 4.10 (Rozas *et al.*2003), and the nucleotide sequence data were submitted to GenBank (Accession numbers (DQ900882-DQ900895, EU979388- EU979417). The pairwise distance matrix of the allelic sequences was generated using the method of Hasegawa *et al.* (1985) to evaluate the ratio of transition to transversions. The Maximum-likelihood (ML) tree of the allelic sequences was produced using PAUP4.10 beta (Swofford 1998). For the ML analysis, the best-fitting nucleotide substitution model (GTR + I + G) were selected by Modeltest 3.7 (Posada and Crandall, 1998) using the Akaike Information Criterion (AIC). The ML trees were generated using a random stepwise heuristic search (only one tree was retained) based on 1000 replicates with random additions of sequence. Bootstrap analysis (1000 replication) was performed using a heuristic search procedure. The same likelihood parameters were used to test the values of pairwise distance among allelic sequence and a molecular evolution clock was calculated for the ML trees. Neighbor-joining (NJ) trees based on F_{ST} distances between the 10 populations were produced using Mega3.1 (Kumar *et al.*, 2005). Mega was also used to calculate the genetic distance between populations based on the Kimura 2-Parameter method.

The program AMOVA (Arlequin 3.1, Excoffier *et al.* 2006) was used to investigate the genetic spatial structure. This maximizes the proportion of the total genetic variation between groups of populations, without pre-defining the populations. The program package was used to analyze F_{ST}P values and its statistical significance from ITS1 sequence of *C. sinensis* across populations.

Results

The ITS1 sequences of *C. sinensis* obtained ranged from 564 (HD1, Hap36) to 595 (QD4, Hap20) nucleotides in length. The alignments were 621 nucleotides long (including sites with gaps/missing data). The sequence alignments contained 96 polymorphic sites (Table.2). In total, 42 Allelic sequences were identified among the ITS1 sequences. There was an overall nucleotide diversity of π=0.033, and the θ estimate based on the total number of mutations was 0.048. The distribution of the allelic sequences across populations and their GenBank Accession Numbers are also shown in Table 2.

The ML (Maximum-likelihood) phylogenetic tree based on the 42 identified allelic sequences (Fig. 2) has two major clades. One basic clade comprised the populations (ZH, QD, LZ, QID, BDH, TG and HY) from the China Bohai Sea, the Yellow Sea, and the Dong Sea. Allelic sequence 22 was found in all seven populations. Allelic sequence 10 was found in four populations (ZH, BDH, TG and LZ) Allelic sequence 13 was found in three (QD, QID and HY) and allelic sequence 27 in two (BDH and LZ). Other allelic sequences were population specific. The second clade contains the Allelic sequence of the populations of HD and HN from the South China Sea. Allelic sequence 31 and 42 were found in both populations. Notably, the haplotypes in the Miyagi JP population (outgroup) were associated with populations from the South China Sea (Fig. 1), this "association" is due to the sharing of allelic sequence 42 (Table 2). The minimum pairwise distance between allelic sequences was 0.134. Using an estimated divergence rates for ITS-1 of between 0.07 and 0.38 per MY (Page and Linse 2002), estimates of the time since population segregation distribution of *C. sinensis* in the study areas was (0.35–1.91MY before present) suggesting consistency with a sea level change since the late Pleistocene.

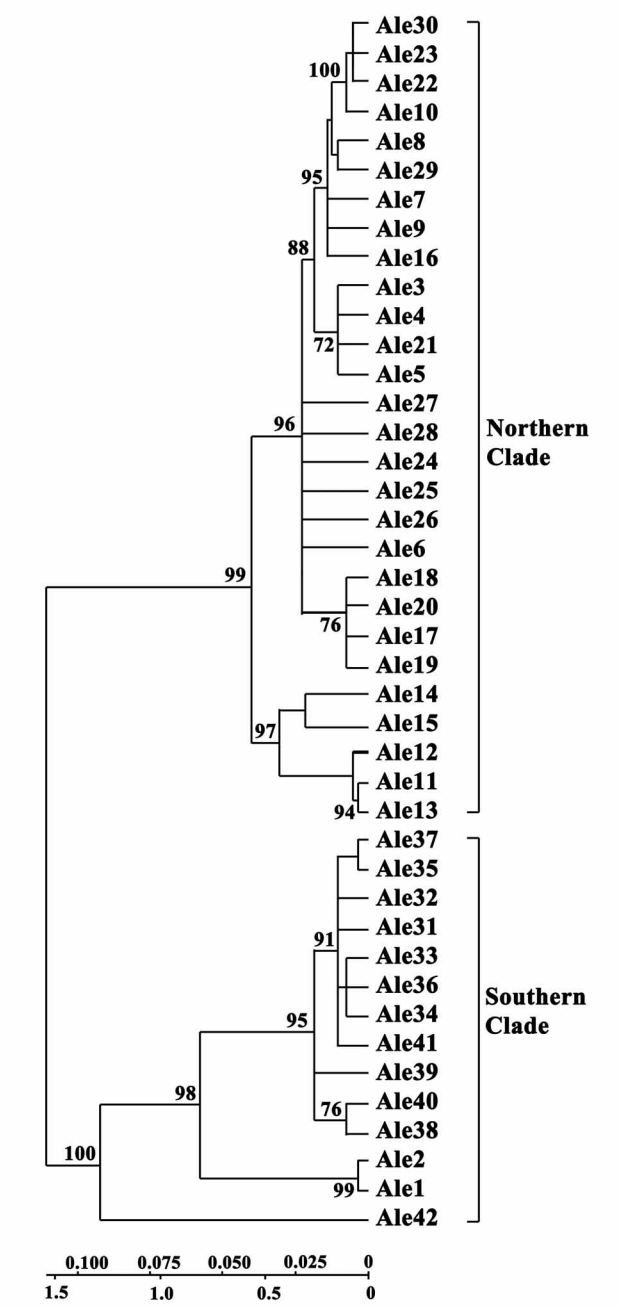

FIGURE 2. Maximum-likelihood tree based on the ITS-$_1$allelic sequences of *C. sinensis*. The numbers above branches indicate the percentage support among 1000 bootstrap replicates (>50%), the scale indicates genetic distance and estimated evolutionary time (in millions of years).

TABLE 2. The polymorphic sites and Accession numbers of the ITS1 haplotype in the populations of *C. sinensis*

Alleie and Accession numbers		Variable nucleotide sites	Total	Allelic sequence frequency JP	ZH	BDH	TG	LZ	QD	QID	HY	HD	HN	
		7891223344555667777999111111111111111111111111111111111111112222222222222222333333333334444445555566602808592691545786780001111222223333333445555556888889900122335667889900245556790111590117911211890578045782456789031234670146790136669686240021425665692438378054095807080	80	8	8	8	8	8	8	8	8	8	8
Ale1	DQ900891	CTGGGCCCGCGGACTGCAGAAGAAATGCGAACGCGCAAGAGAGAAGGAATCAACACGCACAGCTATTACCGCCCCCAGGGACGCGCGATACTGTTG	4	4	–	–	–	–	–	–	–	–	–	–
Ale2	EU979388	..........G..	2	2	–	–	–	–	–	–	–	–	–	–
Ale3	DQ900889	........C.....GATTAG.A..GC.....G.C.TGAG..A.GG.....A..	1	–	–	1	–	–	–	–	–	–	–	–
Ale4	EU979391	..........GATTAG.A..GC.....GGC.TGAG..A.GG.....A..	1	–	–	–	1	–	–	–	–	–	–	–
Ale5	EU979392	..........GATTAG.A..GC.....G.C.TGAG..A.GG.....A..C..	1	–	–	1	–	–	–	–	–	–	–	–
Ale6	EU979393	.A........GATTAG.A..GC.....G.C.TGAG..A.GG.....G.....A..	1	–	–	–	–	–	1	–	–	–	–	–
Ale7	DQ900885	..........GATTAG.AC.GC.....G.C.TGAG..A.GG.....A..	1	–	–	–	–	–	1	–	–	–	–	–
Ale8	DQ900884	..........GATTAG.A..GC.....G.C.TGAG..A.GG..	2	–	–	–	–	–	–	2	–	–	–	–
Ale9	EU979407	..........GATTAG.A..GC.....G.C.TGAG..A.GG.....G..G..	1	–	1	–	–	–	–	–	–	–	–	–
Ale10	EU979403	..........GATTAG.A..GC.....G.C.TGAG..A.GG.....C.....G..	5	–	2	1	1	1	–	–	–	–	–	–
Ale11	EU979404	..........GATTAG.A..GC.....G.C.TGAG...G.G..	1	–	–	–	–	–	–	–	1	–	–	–
Ale12	EU979405	..........GATTAG.A..GC.....G.C.TGAG.....G.....C	1	–	–	–	–	–	–	–	1	–	–	–
Ale13	DQ900883	..........GATTAG.A..GC.....G.C.TGAG....GG.....G..	3	–	–	–	–	–	1	1	1	–	–	–
Ale14	EU979402	.....G..C.....GATTAG.A..GC.....GGGC.TGA.G.AGGG..	1	–	–	–	1	–	–	–	–	–	–	–
Ale15	DQ900882	......G.C.....GATTAG.A..GC.....GGC.TGAG..AGGG..	1	–	–	–	–	1	–	–	–	–	–	–
Ale16	EU979406	........C.....GATTAG.AC.GCC.....G.C.TGAG..A.GGG....G..	2	–	–	–	–	–	–	2	–	–	–	–
Ale17	EU979394	..........GATTAG.AC.GC.....G.C.TGAGG.A.GG..	1	–	–	–	1	–	–	–	–	–	–	–
Ale18	EU979395	.A........AGATTAG.AC.GC.....C...GCC.TGAG..A.GG..	1	–	–	–	1	–	–	–	–	–	–	–
Ale19	EU979396	.A........GATTAG.AC.GC.....G....G.C.TGAG..A.GG..G.....T...G...C..	1	–	–	–	–	–	–	1	–	–	–	–
Ale20	EU979397	..........GATTAG.A..GC...G.....G.C.TGAG..A.GG..	1	–	–	–	–	–	1	–	–	–	–	–
Ale21	EU979389	A.A...G.....GATTAG.A..GC.....G.C.TGAG..A.GG..	1	–	–	–	1	–	–	–	–	–	–	–
Ale22	EU979401	..........GATTAG.AC.GC.....G.C.TGAG..A.GG..	15	–	2	4	2	2	2	1	2	–	–	–
Ale23	DQ900887	.A........GATTAG.A..GC.....G.C.TGAG..A.GG.....T....C..	1	–	1	–	–	–	–	–	–	–	–	–
Ale24	EU979408	..........GATTAG.AC.GC.....G.C.TGAG..A.GG.GC.G..TG.C.....T.G.....C..	1	–	1	–	–	–	–	–	–	–	–	–
Ale25	EU979409	..........GATTAG.A..GC.....G.CCTGAG..A.GG..C.....T.G..	1	–	–	–	–	–	–	–	1	–	–	–
Ale26	DQ900890	......G.TT.C.GATTAGCA.CGC.G.....GGC.TGCG..A.GG...G.....G..G.GTG..	3	–	1	–	–	–	2	–	–	–	–	–
Ale27	DQ900888	..........GATTAG.A..GC.....G.C.TGAG..A.GG.....A..	4	–	–	1	–	3	–	–	–	–	–	–
Ale28	EU979398	..........GATTAG.A..GC.....G..G.C.TGAG..A.GG.G.....G.....GG....G.....A..	1	–	–	–	–	1	–	–	–	–	–	–
Ale29	EU979399	..........GATTAG.A..GC...G.....G.C.TGAG..A.GG.....G.....AG..	1	–	–	–	–	–	–	1	–	–	–	–
Ale30	EU979400	..........GATTAG.A..GC.....G.C.TGAG..A.GG.....A.	2	–	–	–	–	–	–	–	2	–	–	–
Ale31	DQ900893	..........C.....G.AA.TA....GA.....C.....G.....T.GCTGCTAC...	3	–	–	–	–	–	–	–	–	2	1	
Ale32	EU979410	..........C.....G.AA.TA....G.....C.....G.....T.G.TGCTGC...	1	–	–	–	–	–	–	–	–	1	–	
Ale33	EU979412	.....C.....G.AA.TA....G.....C.....G.....T.G.TG..GC...	1	–	–	–	–	–	–	–	–	1	–	
Ale34	EU979413	...A.....C.....G.AA.TA....G.....C.....G.....T.GCTG..GC...	1	–	–	–	–	–	–	–	–	–	1	
Ale35	EU979411	..........C.....G.AA.TA....G.....C.....G.....T.G.TG..AC...	1	–	–	–	–	–	–	–	–	1	–	
Ale36	DQ900892	..........C.....G.AA.TA....G.....C.....G.....T.G.TG..GC...	1	–	–	–	–	–	–	–	–	1	–	
Ale37	EU979414	.....G.AA.TA....G.....C.....G.....T.G.TG..AC...	1	–	–	–	–	–	–	–	–	–	1	
Ale38	DQ900894	.....G.AA.TA....G.....C.....G.....T.GCTG..GC...	1	–	–	–	–	–	–	–	–	–	1	
Ale39	EU979415	.....G.AA.TA....G.....C.....G.....T.GCTG.TAC...	1	–	–	–	–	–	–	–	–	–	1	
Ale40	EU979416	.....G.AA.TA....G.....C.....G.....T.G.TG..GC...	1	–	–	–	–	–	–	–	–	1	–	
Ale41	EU979417	.A..C.....G.....G.AA.TA.C...G.....C.....G.....T.G.TG..GC...	1	–	–	–	–	–	–	–	–	–	1	
Ale42	DQ900895	.A.....T.....G..CAG.A..GC..C.....C.G.....G.AA.TA.....C...A.....G.....T.....C.GC...	5	2	–	–	–	–	–	–	–	1	2	

As shown in Table 3, average Kimura 2-parameter genetic distances among the populations ZH, QD, LZ, QID, BDH, TG and HY were between 0.020 and 0.060. The F_{ST} values were not significantly different among these populations. However, the genetic distances (ranging from 0.526 to 0.835 between these populations and populations HN and HD) representing the second clades were remarkably large (P<0.05).

TABLE.3. Pairwise distance matrix of the ITS1 sequences of the sampled populations?Below diagonal, average pairwise Kimura 2-parameter genetic distance; above diagonal, significant F_{ST} P values?Significance Level=0.05; P>0.05=-, P<0.05=+

Population	1	2	3	4	5	6	7	8	9	10
1 QID	0.000	-	-	-	-	-	-	+	+	+
2 QD	0.042	0.000	-	-	-	-	-	+	+	+
3 ZH	0.034	0.040	0.000	-	-	-	-	+	+	+
4 HY	0.020	0.023	0.084	0.000	-	-	-	+	+	+
5 LZ	0.056	0.074	0.060	0.035	0.000	-	-	+	+	+
6 TG	0.038	0.034	0.031	0.114	0.102	0.000	-	+	+	+
7 BDH	0.060	0.041	0.028	0.120	0.052	0.023	0.000	+	+	+
8 HN	0.790	0.760	0.780	0.780	0.786	0.786	0.803	0.000	-	+
9 HD	0.820	0.787	0.808	0.808	0.819	0.818	0.835	0.010	0.000	+
10 JP	0.638	0.604	0.632	0.618	0.634	0.637	0.655	0.526	0.582	0.000

AMOVA analysis (Table 4) showed that most of the variation stemmed from differences between the two major groups. For example, 67.6% of the total was attributable to between-group variations, while only 6.5% was due to variation between populations within groups**.** The results suggest there are low inter-population differences within each main clade but appreciable inter-individual variation within populations. Furthermore, the main source of genetic variation was groups which represented northern and southern China Sea, indicating that Chinese populations of *C. sinensis* should be considered as two distinct geographical groups.

TABLE.4. An analysis of genetic variation among the 10 sampled populations of *C. sinensis* using AMOVA.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation
Among groups	1	848.657	24.415	67.69
Among populations within groups	8	226.393	2.378	6.59
Within populations	70	649.375	9.276	25.72
Total	79	1724.425	36.069	100

* Fixation Index: F_{ST}=0.74281

Discussion

This study is the first to our knowledge to use ITS sequences to assess the genetic structure of a Chinese commercial marine bivalve. On the basis of our results, *C. sinensis* along the coast of China is separated into two basic clades. The first, comprising locations QD, ZH, LZ, QID, BDH, TG and HY (see Table.1), represented the temperate populations from the northern and middle parts of the China Sea (including the Bohai Sea, the Yellow Sea and the Dong Sea, latitudes 28.64°N to 39.78°N) while the second clade comprised the tropical locations (HD and HN) from the South China Sea (latitudes 18.20°N to 22.97°N). Inter-population genetic distances and F_{ST} values indicated a significant genetic differentiation between the two clades (Table 3, Fig 3). Moreover, the results of the AMOVA detected significant differences in the hierarchical levels among groups (Table 4) indicating significant population genetic structure. The spatial genetic heterogeneity for the ITS-1 allelic sequences in Chinese *C .sinensis* accords with the results of Pan *et al.* (2005) based on RAPD, Zhao *et al.* (2007) based on AFLP and Zhao *et al.* (2009) who used morphological variation and enzyme electrophores to analyze the genetic differentiation of all opatric populations of *C. sinensis.* The existence of two major lineages in Chinese *C. sinensis* may partly explain why the aquaculture of *C. sinensis* has experienced large-scale mortality following long distance stock translocation of seed clams in China since 2002. Our investigation suggests that exchange seed clams between the southern and northern groups of *C. sinensis* may be problematic.

Past geological and climatic events have probably played a major role in the differentiation of *C. sinensis* populations. Geographically, the marine regions of China extend vertically across tropical, subtropical and temperate regions with temperature the decisive factor. According to Zhang *et al*. (1963) and Liu *et al.* (1963), the Chinese marine molluscan fauna is made up of three components: (1) a rather depauperate boreal element occurring only in the Yellow Sea and the Bohai Sea; (2) an Indo-West-Pacific element composed of a rich fauna of southern species, some of which are widely distributed along the Chinese coast, while others are restricted to the Dong Sea and the South China Sea or to the South China Sea alone; (3) an endemic element of the Sino-Japanese region, which includes some temperate species occurring only in the Yellow Sea or the waters of

northern Japan, and warm-water species occurring in the Dong and South China Seas and in the waters of southern Japan. Xu (1997) suggested that the distribution of some broad-range marine bivalves such as *Modiolus elongata* (Swainson, 1821), *Atrina pectinata* (Linnaeuis, 1767), *Anomia chinensis* (Philippi, 1849) and *Cyclina sinensis* etc. can transgress boundaries between the above faunal regions. Our studies on the wide-ranging *C. sinensis*, distributed from Northeast China to the Far East of Russia, Japan, Korea and Southeast Asia, suggest that the boundaries may have complex effects. The present population genetic structure of a species may only be fully interpreted if one considers the influence of historical events and the complex interactions of biology, geography and climatic shifts (Hewitt 2000). Climatic shifts can create great changes in species geographical distributions and abundances, which can be expected to have detectable genetic consequences (Avise 2000; Hewitt 2000).

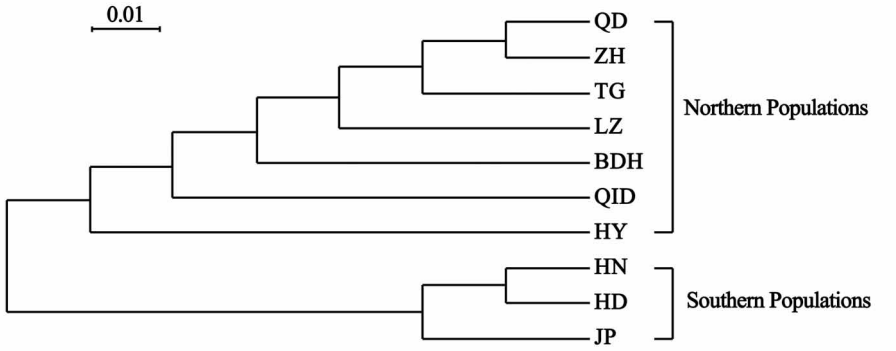

FIGURE.3. The Neighbour-joining tree showing the relationships between 10 populations of *C. sinensis* based on ITS-1 sequences.

The population genetic structures of marine species are often influenced by Pleistocene ice ages (Wang and Sun 1994; Benzie and Williams 1997; Briggs 1999). The two clades in *C. sinensis* may reflect isolation of marginal seas of the Northwestern Pacific during Pleistocene low sea-level stands. Some authors have suggested that historic barriers, such as sea level changes during the Pleistocene, may have played important roles in creating isolated populations by cutting off local sea basins from the Northwestern Pacific (Liu *et al*. 2006). Several marginal seas, the Sea of Japan, the Yellow Sea, the East China Sea and the South China Sea, separate East Asia from the northwestern Pacific Ocean. The marginal seas represent a unique tectonic and geographic feature in the Western Pacific region, and have a profound impact on regional climate and environment. 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. Land bridges were formed between present-day islands and the Asian continent as a result of the lower sea level, which would collectively isolate the South China Sea from the Pacific Ocean and the East China Sea-Yellow Sea. Similar genetic breaks have also been described in marine taxa between East China Sea and South China Sea populations of other marine species (Liu *et al*. 2007; Tzong 2007; Xu *et al*. 2009).

Another hypothesis concerning the geographical barrier between those areas was suggested by Xu (1997) who examined the affinities of bivalves from southern and northern China Seas. He found that the similarity of bivalve fauna between southern and northern China Seas was much less than the similarity of southern China Sea and Japan Sea faunas. Many Indian Ocean long shore bivalves such as *Vepricardium asiaticum* (Bruguiere, 1792), *Vepricardium coronatum* (Schröter, 1786) and *Vepricardium sinense* (Sowerby, 1841) etc. were abundant and widely distributed in the South China Sea and Japan Sea, but have never been reported from Chinese seas northward of the Taiwan Strait. On the other hand, large numbers of subtropical bivalves such as *Laevicirce soyoae* (Habe, 1951), *Bathy tellina citrocarnea* (Kuroda & Habe, 1958) etc. occur all around the Dong Sea, but have never been observed south of the Taiwan Strait, in the South China Sea. These observations are consistent with our finding that the ITS-1 allelic sequences in *C. sinensis* in Miyagi, Japan are most similar to those in the South China Sea region and differ considerably from those in northern China. Xu (1997) suggested that the freshwater-influenced sea coast cold region in the Zhejiang and Fujian provinces of China, with its winter minimum temperatures of about 8^{o}C and the intense annual freshwater upwelling from May to October, may act as isolating barriers preventing dispersal (Fig.1, B), decreasing colonization and the gene flow between regions. Zhao *et al*. (2009) found high genetic divergence at the enzyme level in *C. sinensis* from southern and northern China seas, and conjectured that this was due to extremely low gene flow between the two regions. Thus, the cold, low-salinity coastal current could be a mechanism for generating biodiversity and population differentiation, which might account for the present-day restricted larval dispersal of *C. sinensis* between southern and northern China Seas.

In summary we propose that, in *C. sinensis*, the genetic differences between the two geographical regions, the southern China Sea and the northern China Sea, may be a remnant of past geographical isolation during sea-level changes combined with present-day cold freshwater

upwelling. A molecular clock analysis of the observed ITS_1 allelic sequences suggests that the two groups of *C. sinensis* have experienced population isolation since the late Pleistocene ages (approximately between 0.35 and 1.91 MY ago). There has, however, been sufficient time since the last glacial maximum for genetic mixture between the populations if they are not in fact distinct species, unless the coastal fresh water upwelling has maintained the genetic differentiation.

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References

Avise, J.C. (2000) *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge, Massachusetts.

Barber, P.H., Palumbi, S.R., Erdmann, M.V. & Moosa, M.K. (2002) Sharp genetic breaks among populations of *Haptosquilla pulchella* (Stomatopoda) indicate limits to larval transport: patterns, causes, and consequences. *Molecular Ecology* 11, 659–674.

Benzie, J. & Williams, S.T. (1997) Genetic structure of giant clam (*Tridacna maxima*) populations in the West Pacific is not consistent with dispersal by present-day ocean currents. *Evolution* 51, 768–783.

Briggs, J.S. (1999) Coincident biogeographic patterns: Indo-West Pacific Ocean. *Evolution* 53, 326–335.

Caporale, D.A., Beal, B.F, Roxby, R. & Van Beneden, R.J. (1997) Population structure of *Mya arenaria* along the New England coastline. *Molecular Marine Biology and Biotechnology* 6, 33–39.

Chen, C.A., & Miller, D.J (1996) Analysis of ribosomal ITS1 sequences indicates a deep divergence between *Rhodactis* (Cnidaria: Anthozoa: Corallimorpharia) species from the Caribbean and the Indo-Pacific/Red Sea. *Marine Biology*126, 423–432.

Chen, D.P, Shen, H.S., Ding, Y.P., Yang, J.X. & Xu, P. (2004) Randomly amplified polymorphic DNA analysis of *Meretrix meretrix*, *Cyclina sinensis* and *Mactra vecerifermis*. *Marine Science Bulletin* P. R. China, 23, 84–87.

Chu, K. H., Li, C. P. & Ho, H.Y. (2001) The first internal transcribed spacer (ITS1) of ribosomal DNA as a molecular marker for phylogenetic and population analyses in Crustacea. *Marine Biotechnology* 3, 355–361.

Ding, X. L., He, M. X., Deng, F. J. & Zhang, X. Y., (2004) 18S-ITS1 sequence of rRNA in bivalves and its application in phylogenetic analysis. *Hereditas* 26, 319–324.

Eizadora, T.Y., Ma, A.J. & Virginia D.M., (2000) Sequence variation in the ribosomal DNA internal transcribed spacer of *Tridacna crocea*. *Marine Biotechnology* 2, 511–516.

Excoffier, L., Laval, G. & Schneider, S. (2006) Arlequin (version 3.1): an integrated software package for Population genetics data analysis, Computational and Molecular Population Genetics Lab (CMPG), Institute of Zoology, University of Berne Switzerland.

Feng, Y.W., Li, Q. & Kong, L.F. (2010) Twenty microsatellite DNA markers for the Venus clam (*Cyclina sinensis* Gmelin). *Conservation Genetics* 11, 1189–1192.

Gaffney, P.M., Orbacz, E.A. & Yu, Z. (1998) Using the D code system to identify DNA sequence variation for studies of population structure in marine organism. *Mutation Analysis* 2, 329.

Grewe, P.M., Krueger, C.C. & Aquadro, C.F. (1993) Mitochodrial DNA variation among lake trout (*Salvelinus namaycush*) strains stocked into Lake Ontario. *Fisheries and Aquatic Sciences* 50, 2397–2403.

Hasegawa, M., Kishino, H. & Yano, T. (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22, 160–174.

He, M.X., Huang, L.M., Shi, J.H. & Jiang, Y.P. (2005) Variability of ribosomal DNA ITS2 and its utility in detecting genetic relatedness of Pearl oyster. *Marine Biotechnology* 7, 40–45.

Hewitt, G.M. (2000) The genetic legacy of the quaternary ice ages. *Nature* 405, 907–913.

Källersjö, M., Proschwitz, T.V., Lundberg, S., Eldenäs, P. & Erséus, C. (2005) Evaluation of IST rDNA as a complement to mitochondrial gene sequences for phylogenetic studies in freshwater mussels: an example using Unionidae from north-western Europe. *Zoologica Scripta* 34, 415–424

Kenchington, E., Bird, C.J., Osborne, J. & Reith, M. (2002) Novel repeat elements in the nuclear ribosomal RNA operon of the flat oysters *Ostrea delis* C. Linnaeus, 1758 and *O.angasi* Sowerby, 1871. *Journal of .Shellfish Research* 21, 697–705.

King, T.L., Michael, S.E, Branimir, G. & Hoeh, W.R. (1999) Intraspecific phylogeography of *Lasmigona Subviridis* (Bivalvia Unionidae): conservation implications of range discontinuity. *Molecular Ecology* 18, 65–78

Kumar, S., Tamura, K., & Nei, M. (2005) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5, 150–163.

Lee, T. & Ó Foighil, D. (2005) Placing the Floridian marine genetic disjunction into a regional evolutionary context using the scorched mussel, *Brachidontes exustus*, species complex. *Evolution* 59, 2139–2158

Liu, R.Y., & Xu, F.S. (1963) Preliminary studies on the benthic fauna of the Yellow Sea and the East China Sea. *Oceanologia et Limnologia Sinica* 5, 306–321.

Liu, W.S., Ma, Y.H., Hu, S.Y. Miao, G.H. & Li, J.H. (2002) Rearing venus clam seeds, *Cyclina sinensis* (Gmelin), on a commercial scale. *Aquaculture* 21, 109–114.

Liu, X.Q., Fang, J.G., Bao, Z.M. & Wang, R.C. (2003) Progress in seed production and mariculture technology in economically significant species of clams in China. *Chinese Journal of Zoology* 38, 114–119.

Liu, J.X., Gao, T.X., Yokogawa, K. & Zhang, Y.P. (2006) Differential population structuring and demographic history of two closely related fish species, Japanese sea bass (*Lateolabrax japonicus*) and spotted sea bass (*Lateolabrax maculatus*) in Northwestern Pacific. *Molecular Phylogenetics and Evolution* 39, 799–811.

Liu, J.X., Gao, T.X., Wu, S.F. & Zhang, Y.P. (2007) Pleistocene isolation in the Northwestern Pacific marginal seas and limited dispersal in a marine fish, *Chelon haematocheilus*. *Molecular Ecology* 16, 275–288.

Mathews, L.M (2006) Cryptic biodiversity and phylogeographical patterns in a snapping shrimp species complex. *Molecular Ecology* 15, 4049–4063.

Mizukami, Y. & Kito, H. (1999) Nucleotide sequence variation in the ribosomal internal transcribed spacer regions of cultivated (cultivars) and field-collected thalli of *Porphyra yezoensis*. *Fisheries Science* 65, 787–789.

Nelson, T.S., Hoddell, R.J., Chou, L.M. Chan, W.K. & Phang,

V.P.E. (2000) Phylogeographic structure of false clownfish, *Amphiprion ocellaris*, explained by sea level changes on the Sunda shelf. *Marine Biology* 137**,** 727–736.

Page, T.J., & Linse, K. (2002) More evidence of speciation and dispersal across the Antarctic Polar Front through molecular systematics of Southern Ocean *Limatula* (Bivalvia: Limidae). *Polar Biology.* 25, 818–826.

Palumbi, S.R. & Baker, C.S. (1994) Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Molecular Biology Evolution* 11, 426–435.

Palumbi, S.R., Grabowsky, G. & Duda, T. (1997) Speciation and population genetic structure in tropical Pacific sea urchins. *Evolution* 51, 1506–1517.

Pan, B.P., Song, L.S., Bu, W.J. & Sun, J.S. (2005) Studies on Genetic Diversity and Differentiation between two Allopatric Populations of *Cyclina sinensis*. *ACTA Hydrobiologica Sinica* 29, 372–378.

Posada, D. & Crandall, K.A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.

Reece, K.S., Cordes, J.F., Stubbs, J.B., Hudson, K.L. & Francis, E.A. (2008) Molecular phylogenies help resolve taxonomic confusion with Asian *Crassostrea* oyster species. *Marine Biology* 153, 709–721.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X. & Rozas, R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19, 2496–2497.

Shilts, H.M., Pascual, M.S., & Ó Foighil, D. (2007) Systematic, taxonomic and biogeographic relationship of Argentine flat oysters. *Molecular Phylogenetics and Evolution* 44, 467–473.

Shulman, M.J. (1998) What can population genetics tell us about dispersal and biogeographic history of coral-reef fishes? *Australian Journal of Ecology* 23, 216–225.Shulman M.J. & Bermingham, E. (1995) Early life histories, ocean currents, and the population genetics of Caribbean reef fishes. *Evolution* 49, 897–910.

Stothard, J.R., Hughes, S. & Rollinson, D. (1996) Variation within the internal transcribed spacer (ITS) of Ribosomal DNA genes of intermediate snail hosts within the genus *Bulinus* (Gastropoda: Planorbidae). *Acta Tropica* 61, 19–29.

Swofford, D.L. (1998) *Phylogenetic analysis using Parsimony (PAUP), version 4.0*. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J.D., Gibson, T.J., & Plewniak, F. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.

Tzong, D.T. (2007) Population structure and historical demography of the spotted mackerel (*Scomber australasicus*) off Taiwan inferred from mitochondrial control region sequencing. *Zoological Studies.* 46, 656–663

Vane, W.R.I., Raheem, D.C. & Vogler, A.P. (1999) Evolution of the mimetic swallowtail butterfly *Papillo dardanus*: molecular data confirm relationship with *P. phorcas* and *P. constantinus*. *Biological Journal of the Linnean Society* 66, 215–229.

Vidigal, T. H.D.A, Spatz, L., Kissinger, J.C., Redondo, R.A.F, Pires, E.C.R, Simpson, A.J.G. & Cavvalho, O.S. (2004) Analysis of the first and second internal transcribed spacer sequences of the ribosomal DNA in *Biomphalaria tenagophila* complex (Mollusca: Planorbidae). *Memórias do Instituto Oswaldo Cruz* 99, 2153–2158.

Vierna, J., Martines-Lage, A. & Gonzalez-Tizon, A.M. (2010) Analysis of ITS_1 and ITS_2 Sequence in *Ensis Razor* Shells: suitability as molecular markers at the population and species level, and evolution of these ribosomal DNA spacers. *Genome* 53, 23–34

Vogler, A.P., & Salle, D.R. (1994) Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cicindela dorsalis*. *Molecular Biology and Evolution* 11, 393–405.

Wang, L.X., Xiang, J.H. & Zhou, L.H. (2001) Chromosome study of *Cyclina sinensis* Gmelin. *Journal of Northwest Science Technology University* 29, 94–96.

Wang, P. & Sun, X. (1994) Last glacial maximum in China: comparison between land and sea. *Catena* 23, 341–353.

Wilber, A.E., Arnold, W.S. & Bert, T.M. (2000) The genetic assessment of an "enhanced" bay scallop population: Do hatchery scallop, (*Patinopecten yessoensis*). *Journal of Shellfish Research* 19, 667–668.

Xu, F.S. (1997) *Bivalve Mollusca of China Seas*, *Science Press*, Beijing.

Xu, J.W., Chan, T.Y., Tsang. L.M. & Chu, K.H. (2009) Phylogeography of the mitten crab *Eriocheir sensu stricto* in East Asia: Pleistocene isolation, population expansion and secondary contact. *Molecular Phylogenetics and Evolution* 52, 45–56.

Xu J Z. (2000) The living habits of *Cyclina sinensis*. *Aquiculture* (in Chinese)1, 27–28

Yu, Y.S., Wang, H., Lu, P. & Wu, J.X. (1995) Habitat and growth of clam (*Cyclina sinensis* Gmelin). *Journal of Fisheries of China* 19, 276–279.

Yu, Y.S. & Zheng, X.D. (2001) A preliminary observation on the morphology of *Cyclina sinensis*. *Chinese Journal of Zoology* 36, 2–6.

Zhang, X., Qi, Z.Y. Zhang, F.S. & Ma, S.T. (1963) A preliminary study of the demarcation of marine molluscan faunal regions of China and its adjacent waters. *Oceanologia et Limnologia Sinica China* 5, 124–138.

Zhao, Y.M., Li, Q., Kong, L.F., Bao, Z.M. & Wang, R.C. (2007) Genetic diversity and divergence among clam *Cyclina sinensis* populations assessed using amplified fragment length polymorphism. *Fisheries Science* 73, 1338–1343.

Zhao, Y.M., Li, Q., Kong, L.F. & Mao, Y.Z. (2009) Genetic and morphological variation in the venus clam *Cyclina sinensis* along the coast of China. *Hydrobiologia* 635, 227–235.

Zhuang, Q.Q. (2001) *Fauna Sinica (Phylum Mollusca Class Bivalvia Family Veneridae*). Science Press China. Beijing.