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The Clam *Cyclina sinensis* (Gmelin) Phylogeography Study with 28S rRNA Gene and Potential of Nuclear rRNA Genes in Genetic Assessments of Molluscs

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Abstract Intraspecific diversity of molluscan species is usually studied based on maternally inherited mitochondrial DNA, from which only part of the evolutionary history can be reflected. Some nuclear ribosomal RNA genes such as 28S rRNA represent potential candidates that can be easily applied in phylogeography because of lacking intraindividual variation. However, considering their low polymorphism, genetic appraisals on whether and how they can be used in population studies are necessary. Here, we applied a short 28S rRNA to assess genetic patterns of the clam *Cyclina sinensis* along the coast of China and compared the results with a former study based on COI and ITS-1 analyses. The results revealed the 28S rRNA data set was characterized by an extremely low level of variation, with only seven haplotypes defined for 93 individuals. Haplotype and nucleotide diversity for each population was almost the lowest when compared with the other two markers. However, the distribution of two dominant haplotypes showed clear geographic patterns, and significant population differentiation was revealed between the East China Sea and the South China Sea. These patterns were highly concordant with findings of the former study that populations of *C. sinensis* were historically separated by land bridges among sea basins. Our study suggested that although the nuclear rRNAs have shortcomings such as low variation, they have advantages including lack of intraindividual variation and high amplification rates. Applying rRNA genes can enrich the toolbox of nuclear markers in molluscan phylogeographic studies.

Key words marine phylogeography; molluscs; nuclear gene; genetic break

1 Introduction

The basic level of marine biodiversity is genetic diversity, which provides a natural variation of the raw material of evolution and ensures the survival of populations withstanding environmental changes (May and Godfrey, 1994). Studies on genetic diversity can provide valuable biological and evolutionary information that is essential for the successful conservation and/or management of marine species (Xiao *et al.*, 2009).

Phylogeography is a powerful tool for using genetic data to understand intraspecific diversity patterns both in space and time (Avise, 2009). In recent decades, massive studies have been carried out in diverse taxonomic groups (see Hickerson *et al.*, 2010; Shafer *et al.*, 2010; Turchetto-Zolet *et al.*, 2013). However, research effort on marine species, especially on the largest marine phylum Mollusca, is still not comparable to that on terrestrial and freshwater groups (Beheregaray, 2008). Moreover, the previous studies were mainly based on some universal, partial sequences of mitochondrial DNA (mtDNA) (Cutter, 2013). Although mtDNA alone can provide valuable insights on phylogeography, it can only reflect part of the evolutionary history because of maternal inheritance, belying the complex amalgam of processes that shaped genetic diversity (Hare, 2001). Independent nuclear genes, with different patterns of evolution and modes of inheritance from mtDNA, can provide a comprehensive perspective on phylogeographic history (Funk and Omland, 2003; Avise, 2009).

At present, a state-of-the-art approach in nuclear phylogeography is a genomic scan based on next-generation sequencing, which can offer extremely high resolution on population differentiation (Emerson *et al.*, 2010). However, in practice, the cost for sequencing hundreds of specimens, which is typically required in phylogeographic studies, is a heavy burden for small research projects. Traditional nuclear markers like ribosomal RNA (rRNA) genes are still useful in phylogeographic studies, especially for

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numerous marine invertebrates that have not been studied thoroughly. The rRNA genes tend to evolve in a concerted way, which is known as 'concerted evolution' that result in nucleotide homogeneity among multigene families (Dover, 1982). Specific rRNA regions can be selectively amplified by universal markers followed by direct DNA sequencing (Freeland *et al.*, 2011). Compared with other nuclear markers such as microsatellites, this approach does not require species-specific development for species that are never examined before (Zane *et al.*, 2002). The transferable markers also facilitate comparisons of evolutionary patterns among different species (Schlötterer, 2004). However, to employ this method, it is important to know whether a conserved nuclear gene comprises sufficient polymorphic sites to reflect the evolutionary history of a species.

In this study, we performed a phylogeographic study on the bivalve *Cyclina sinensis* (Gmelin) using a partial 28S rRNA gene. *C. sinensis* is a common bivalve with a wide distribution in China and Japan, and is usually found from the muddy sand bottom of the intertidal zone (Qi, 2004). This species has a short planktonic larval duration of about 7 days on average in laboratory conditions (Zeng and Li, 1991), indicating there is limited gene flow among distant populations. The indication was verified by a former study on populations along the coast of China (Ni *et al.*, 2012), in which significant population differentiation was revealed by both COI and ITS-1 genes between populations of the East China Sea (ECS) and the South China Sea (SCS). This background provides a chance for us to compare the performance of the 28S rRNA gene in illustrating phylogeographic break within the *C. sinensis* populations. According to our results, we also discussed the potential and pitfalls of using rRNA genes in molluscan phylogeographic studies.

2 Materials and Methods

2.1 Sample Collection

Specimens of *C. sinensis* used in this study are a subset of populations from a former study of Ni *et al.* (2012). They are selected to represent both the ECS and SCS lineages according to former results. A total of 93 individuals from 11 localities were used here, with a range of 5–10 individuals per site (Fig.1 and Table 1). Samples were

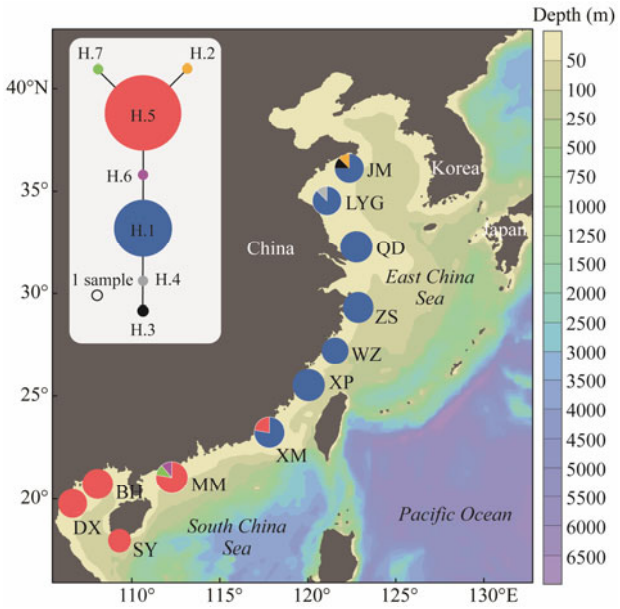

Fig.1 Sampling localities for the clam *C. sinensis* along the coast of China and the distribution of haplotypes in each population. Haplotype network is shown (top left) with the sizes of circles being proportional to their frequencies.

Table 1 Sampling information and diversity indices for the 11 populations of *C. sinensis*

Sampling site	Latitude, longitude	Individuals	Haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Jimo (JM)	36°20′N, 120°43′E	8	3	0.464	0.004209
Lianyungang (LYG)	34°37′N, 119°31′E	8	2	0.250	0.000847
Qidong (QD)	31°52′N, 121°40′E	10	1	0.000	0.000000
Zhoushan (ZS)	30°01′N, 122°04′E	9	1	0.000	0.000000
Wenzhou (WZ)	27°57′N, 120°54′E	7	1	0.000	0.000000
Xiapu (XP)	26°50′N, 120°02′E	10	1	0.000	0.000000
Xiamen (XM)	24°25′N, 118°12′E	9	2	0.389	0.002619
Maoming (MM)	21°29′N, 111°06′E	10	3	0.378	0.001347
Beihai (BH)	21°25′N, 109°07′E	9	1	0.000	0.000000
Dongxing (DX)	21°27′N, 107°58′E	8	1	0.000	0.000000
Sanya (SY)	18°13′N, 109°38′E	5	1	0.000	0.000000

collected from October 2005 to January 2010 from public access areas without specific permit. The adductor muscle was either incised and stored in 95% ethanol or frozen at −30℃ immediately for DNA extraction in the near future. Total genomic DNA was extracted from about 50 mg muscle tissue following a phenol-chloroform purification procedure described by Li *et al.* (2002).

2.2 Sequence Acquisition

A fragment of the 28S rRNA gene was amplified using the primer pair 28SMIDF (5'-CTTGAAACACGGACCAAGG-3' forward) and 28SD6R (5'-CCAGCTATCCTGAGGGAAACTTCG-3' reverse) (Mikkelsen *et al.*, 2006). Each polymerase chain reaction (PCR) was carried out in 50-μL volumes including 2 U Taq DNA polymerase (Takara Co.), about 100 ng template DNA, 0.25 μmol L^{-1} of each primer, 0.2 mmol L^{-1} dNTPs, 1×PCR buffer, and 2 mmol L^{-1} $MgCl_2$. The PCR amplification was conducted on a GeneAmp® 9700 PCR System (Applied Biosystems), and the cycling conditions were as follows: initial denaturation

at 94℃ for 3 min, followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 56℃ for 1 min, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min. The products were checked using 1.5% TBE agarose gel electrophoresis stained with ethidium bromide. The target fragments were purified with EZ Spin Column PCR Product Purification Kit (Sangon) and sequenced using BigDye chemistry (Applied Biosystems Inc., USA) on an ABI PRISM 3730 (Applied Biosystems) automatic sequencer. We did not pursue cloning of the gene because multiple peaks indicating the intraindividual variations were not observed in all specimens.

2.3 Data Analyses

Sequences were edited and aligned using the DNASTAR software (DNASTAR, Inc.) and then refined manually. Haplotypes were defined using the DnaSP 5 under the setting of sites with gaps considered (Librado and Rozas, 2009), and deposited in GenBank with accession numbers HQ881576-HQ881582. Their phylogeny was inferred using a maximum parsimony network in the TCS 1.21 package (Clement *et al.*, 2000). The gaps were treated as a fifth state because we think that haplotypes separated by an indel event are different. We used ARLEQUIN 3.5 (Excoffier and Lischer, 2010) to calculate molecular diversity indices including haplotype diversity (h) and nucleotide diversity (π). Spatial analysis of molecular variance (SAMOVA) implemented in the software SAMOVA 1.0 (Dupanloup *et al.*, 2002) was performed to define groups of populations (K) that were spatially homogeneous. The group number was tested from $K=2$ to $K=10$ with 100 simulated annealing processes. Partitioning of the genetic variation was accomplished by a hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) in ARLEQUIN 3.5 with 10000 permutations. Pairwise Φ_{ST} was also calculated in the same software under the setting of pairwise difference with 10000 permutations followed by a standard Bonferroni correction (Rice, 1989).

3 Results

We successfully amplified and sequenced the partial 28S rRNA gene for all 93 individuals. As predicted, the data set was characterized by an extremely low level of sequence variation. The final alignment was 297 bp long, including two polymorphic sites and four gaps, defining a total of seven haplotypes. Seven of the 11 populations were dominated by only one haplotype, and haplotype and nucleotide diversities for each of them were zero (Table 1). The rest populations had two or three haplotypes. JM showed the highest variation with haplotype diversity (h) of 0.464 and nucleotide diversity (π) of 0.004209, while the LYG was the lowest with $h=0.250$ and $\pi=0.000847$ (Table 1). The parsimony network of the haplotypes showed a simple topology with shallow genetic divergence. The haplotype H.1, as the most abundant one with 56 copies (accounting for 60.2%), showed significant geographic distribution in seven ECS populations. The second common haplotype H.5 had 32 copies (34.4%), with major distribution in four SCS populations, but also appeared in a neighbouring ECS population (XM) with 2 copies (Fig.1).

For SAMOVA analysis, the method determined the optimal grouping K, for which the among-group component (F_{CT}) of the total genetic variance was the highest. In this study, the highest value was observed at two groups, group 1 including JM, LYG, QD, ZS, WZ, XP and XM, and group 2 including MM, BH, DX and SY. Under this grouping strategy, hierarchical analyses of AMOVA indicated a significant level of differentiation among groups ($\Phi_{CT}=0.873$, $P=0.003$; Table 2), explaining 87.32% of the overall genetic variation. Variation within populations accounted for 12.29% ($\Phi_{ST}=0.877$), while variation among populations within groups ($\Phi_{SC}=0.031$) only accounted for 0.39% of the total variation. Pairwise analyses revealed uniformly large and significant Φ_{ST} values between populations in different seas after a standard Bonferroni correction, while none of the values between populations within the ECS or SCS was significant (Fig.2).

Table 2 Result of analysis of molecular variance (AMOVA)

Grouping		Source of variation	*d.f.*	Φ-statistics	% of variation	*P* value
Group 1	JM, LYG, QD, ZS, WZ, XP, XM	Among groups	1	$\Phi_{CT}=0.873$	87.32	**0.003**
Group 2	MM, BH, DX, SY	Among populations within groups	9	$\Phi_{SC}=0.031$	0.39	0.145
		Within populations	82	$\Phi_{ST}=0.877$	12.29	**0.000**

Note: Significant differences (P-value < 0.05) are indicated in bold.

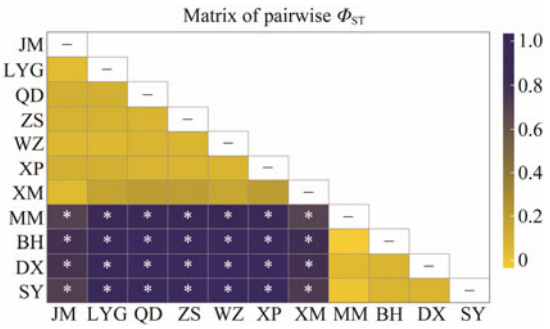

Fig.2 Heatmap visualizing pairwise Φ_{ST} values between the *C. sinensis* populations. Asterisk (*) indicates the significant P value after a standard Bonferroni correction.

4 Discussion

4.1 Low Variation of the 28S rRNA Gene in *C. sinensis* Populations

C. sinensis is one of the most widely distributed bivalves in China, and its genetic pattern has been studied using diverse genetic markers including amplified fragment length polymorphism (Zhao *et al.*, 2007) and sequence data (Ni *et al.*, 2012). In this study, we accessed its genetic patterns using a partial conserved 28S rRNA gene. Unsurprisingly, the data set was characterized by an extremely low level of polymorphism. Only seven haplotypes were defined and no more than five mutations were required to

connect them. When compared with the COI and ITS-1 analyses results in the former study of Ni *et al.* (2012), the 28S rRNA gene showed the lowest haplotype diversity in each population except for XM and MM (Fig.3), and so was the case for comparison of nucleotide diversity results (figure not shown).

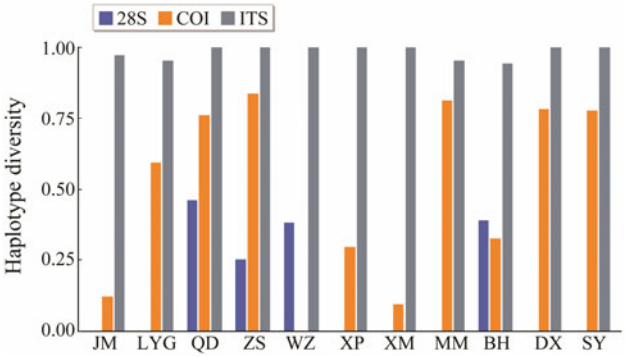

Fig.3 Haplotype diversity compared with three genes, ITS-1 (grey), COI (orange), and 28S rRNA (blue) in each population. The ITS-1 and COI data are from a former study by Ni *et al.* (2012).

4.2 Phylogeographic Split Between the East and the South China Seas

Although the 28S rRNA data set has low variation, significant population differentiation was revealed between the ECS and SCS populations. Multiple evidences from the haplotype distribution, pairwise Φ_{ST}, and AMOVA analyses all support the conclusion. This finding was concordant with the results of COI and ITS-1 analyses in the former study (Ni *et al.*, 2012), in which the significant population structure was also indicated between the two seas. The pattern can be explained by the 'vicariance, then secondary contact' hypothesis which represents a general phylogeographic rule for coastal species in the seas of China (Ni *et al.*, 2014). During the glacial periods, the sea level in the ECS and SCS declined about 120 m (Wang and Sun, 1994). A large land bridge exposed from eastern China to Taiwan Island, serving as a barrier to the gene flow of marine species between the two sea basins (Voris, 2000). When sea levels rose as glaciers melted, the barrier disappeared and the isolated populations recolonized the coastline and met at borders of the sea basins. The same evolutionary scenario was also revealed in other coastal species, including clam *Coelomactra antiquata* (Kong and Li, 2009), mitten crab *Eriocheir sensu stricto* (Xu *et al*., 2009), and two fishes *Chelon haematocheilus* (Liu *et al.*, 2007) and *Bostrychus sinensis* (Qiu *et al.*, 2016).

4.3 Application of Conserved Nuclear rRNAs in Molluscan Phylogeography

The resolution of phylogeography is closely linked to the molecular methods generated to detect the variation. Different genetic markers may have different inheritance patterns and mutation rates, providing different information about genetic diversity and population differentiation (Freeland *et al.*, 2011). There are no standard criteria to evaluate which one is superior to the others because each of them has inherent strengths and weaknesses.

In this study, with the short and conserved 28S rRNA gene, we found that although the alignment was with low variation, it could reveal the significant population differentiation between the ECS and SCS. This finding makes us believe that nuclear rRNAs are valuable in studying molluscan phylogeography. They have advantages and can be applied in some cases. First, rRNA genes can be directly amplified and sequenced, while cloning is usually required prior to sequencing for other nuclear genes. Some studies have revealed there were no putative heterozygotes in the 28S rRNA datasets (*e.g.*, Zhang *et al.*, 2006; Hurry *et al.*, 2014). Second, inner primer pairs can be easily designed for the conserved rRNA genes. This approach enables nuclear sequence acquisition from shelled molluscs with high DNA degradation due to an inadequate fixation of tissues in ethanol. Third, due to the phenotypic plasticity in shell morphology, molluscs contain high levels of cryptic species diversity (Lemer *et al.*, 2014). Significant divergence in rRNAs can be treated as strong signal for the existence of cryptic species or long-term isolation of populations. Of course, this kind of genes shows low variation, and can not be employed to resolve fine-scale genetic patterns and detailed evolutionary processes in a short time scale. Markers with higher resolution such as microsatellites and SNPs are needed in such cases.

5 Conclusions

In this study, we applied a short and conserved 28S rRNA gene to reveal the genetic patterns of a clam *C. sinensis*, and discussed the potential and pitfalls of using nuclear rRNAs in molluscan phylogeography. Although the 28S rRNA gene here can reflect the genetic break in *C. sinensis*, it does not mean that rRNAs should be considered in all studies. Instead, by demonstrating the possibility of using short rRNAs in some specific situations, this study enriches the toolbox of nuclear markers in genetic assessments of molluscs. Since no marker is perfect in all cases, it is worthy to know their strengths and limitations. It is no doubt that the genomic approach based on next-generation sequencing will become more popular in future phylogeographic studies, and more molluscan species will be benefited from this advanced biotechnology revolution.

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