Aquaculture Research

Aquaculture Research, 2016, 1–7 doi: 10.1111/are.13041

Genetic diversity and population structure of *Sepiella japonica* (Mollusca: Cephalopoda: Decapoda) inferred by 16S rDNA variations

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Abstract

In order to describe the genetic diversity and phylogenetic relationship of five populations of cuttlefish (*Sepiella japonica*) along with China's coasts, partial 16S rDNA (510 bp in length) was amplified from 110 individuals. The five populations of cuttlefish inhabit Yellow Sea, East China Sea and South China Sea. In total, six haplotypes were identified and formed only one clade. Among the six haplotypes, one was shared by all populations, three appeared only in a single population, two appeared in two or three populations. Pair-wise FST were not proportional to the geographical distances. Haplotype diversity and nucleotide diversity were low, 0.3866 ± 0.067 and 0.00120 ± 0.00081 respectively. Among the five populations, Zhoushan population exhibited the highest genetic diversity which was suggested as the better select of germplasm resources for the reproduction and releasing of *S. japonica*.

Keywords: cephalopod, *Sepiella japonica*, artificial releasing project, mitochondrial 16S rDNA, genetic diversity

Introduction

The cuttlefish, *Sepiella japonica* (Mollusca: Cephalopoda: Sepioidea: Sepiidae) (Sasaki, 1929) is the only species among Sepiidae genus that inhabits the Chinese coastal waters (Li, Ye, Wu, Guo & Gul 2014). The cuttlefish is an economically valuable fishery resource and considered one of the four most famous sea products in China (Shen, Xie & Xu 2007). The cuttlefish stock was very strong in the East China Sea; the highest annual catching in Zhejiang Province reached 134 000 tons in 1957 (Ni & Xu 1986). However, population decline was largely due to over exploitation and habitat destruction. In present years, considerable work has been performed on the breeding of *S. japonica*, and substantial progress has been made (Wang, Jiang & Qiu 2006; Wu, Dong, Chi & Ding 2010). At the same time eggs and larvae-releasing program of *S. japonica* was launched in 2006 in Zhejiang Province and then continuously practiced (Tang, Zhong, Liu & Li 2005), which lead to a considerable increase in cuttlefish stock (Li 2012). With the development of breeding technology, some obvious problems arose, such as smaller adult size, mature faster sexually and albino eggs (Jiang, Wang, Xue & Zeng 2011). Breeding induction is urgently needed for this species (Chang, Wu, Lu, Zhu & Zhang 2008; Wu *et al.* 2010).

It is clear that knowledge about the population genetics and genetic structure of *S. japonica* is of significance in developing effective strategies for fishery management. Molecular markers are effective methods for detecting genetic diversity and population structure (Englbrecht, Freyhof, Nolte, Rassmann, Schliewen & Tautz 2000; Whitehead, Anderson, Kuivila, Roach & May 2003). Among all the molecular markers, mitochondrial DNA (mtDNA) markers are extensively used to evaluate genetic diversity and population structure

in freshwater and marine species (Qi, Guo, Xie, Wu, Lu, Duan & Zhou 2013). Li *et al.* (2014) described the genetic diversity of five geographical populations of *S. japonica* along the Chinese coast with mtDNA COI and determined their phylogenetic relationship with low levels of genetic variation in the populations. Zheng, Li, Wang, Yu, Tian and Wang (2005) reported genetic diversity in populations of *S. japonica* based on 16S rRNA sequence analysis with a conclusion of low levels of genetic variation in the populations. Also, 16S rRNA sequence had been documented to analyse phylogeny of Sepiidae (Yoshida, Tsuneki & Furuya 2006) and sepiids (Bonnaud, Lu & Boucher-Rodoni 2006). The use of multiple genetic marker systems could increase the resolving power of genetic studies, in contrast to the use of a single genetic marker (Gruenthal, Acheson & Burton 2007).

In this study, we employed partial mitochondrial ribosomal 16S subunit (16S rRNA) gene sequences to assess the genetic diversity and population structure of wild *S. japonica* inhabiting the Chinese coast, which would aid in the selection of strains for future broodstock development and improve management guidelines that aim to conserve diversity and minimize inbreeding.

Materials and methods

Source of cuttlefish

A total of 110 cuttlefish individuals from five populations along China's coastal areas, Yellow Sea (Qingdao, QD), East China Sea (Zhoushan, ZS; Wenzhou, WZ; Ningde, ND) and South China Sea (Zhanjiang, ZJ) (Fig. 1), were included in this study.

DNA extraction, 16S rDNA amplification and sequencing

Total DNAs were extracted from wrist tissues by using a standard phenol–chlorofom extraction. A complete fragment of the mtDNA COI gene was amplified using the primer pairs described by Folmer, Black, Hoeh, Lutz and Vrijenhoek (1994). The PCR reaction was performed in GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) and the following mixture consisted of 100 ng of template DNA, 2.5 μL dNTP (2.5 mM each), 2.5 μL 10× buffer, 2 μL $MgCl_2$ (20 mM), 1 μL primers (10 μM each) and 0.25 μL of *Taq* DNA polymerase (5 U μL^{-1}). Polymerase chain reaction cycling conditions were performed under the following thermal cycle condition: denaturing at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, annealing at 54°C for 50 s and 72°C for 1.5 min with a final extension step of 10 min at 72°C. Equal volumes of each product were quantified by gel electrophoresis method.

The PCR product was purified and subsequently sequenced.

Sequence analysis

The sequences were multiply aligned using Clustal W (Thompson, Higgins & Gibson 1994). The variation among sequences were analysed by the software of MEGA 4.0 version (Tamura, Dudley, Nei & Kumar 2007). The position and number of polymorphic sites and of the corresponding haplotypes were calculated using MEGA 4.0 (Tamura *et al.* 2007), haplotype diversity (h) as well as nucleotide diversity (π) were estimated with DnaSP 4.0 (Rozas, Sanchez-DelBarrio, Messeguer & Rozas 2003). The phylogenetic relationship among haplotypes was determined through UPGMA (Nei 1972) based on a matrix of Kimura two-parameter distance method calculated with parsimony analysis in MEGA 4.0 (Tamura *et al.* 2007). A median joining network (Bandelt, Forster & Röhl 1999) was constructed using NETWORK 4.611 software to classify the haplotypes. Analysis of molecular variance (AMOVA) and hierarchical were used to examine the amount of genetic variability panning within and between populations (Excoffier, Laval & Schneider 2005). Pair-wise F_{ST} was calculated to assess genetic differentiation between populations (Garcia, Manfredi, Fichera & Segura 2003). Testing the significance of pair-wise F_{ST} values consisted of randomizing multilocus genotypes between each pair of samples (10 000 permutations). We then got the value of Nm using the formula $F_{ST} = 1/(1/2Nm)$, which is specific for organelle genetic data (Takahata & Palumbi 1985; Goldberg & Ruvolo 1997).

Results

A total number of 14 variable sites were identified across all the aligned sequences, among which 10 sites were phylogenetically informative for parsimony analysis. And only six haplotypes were observed from all the samples (Tables 1 and 2).

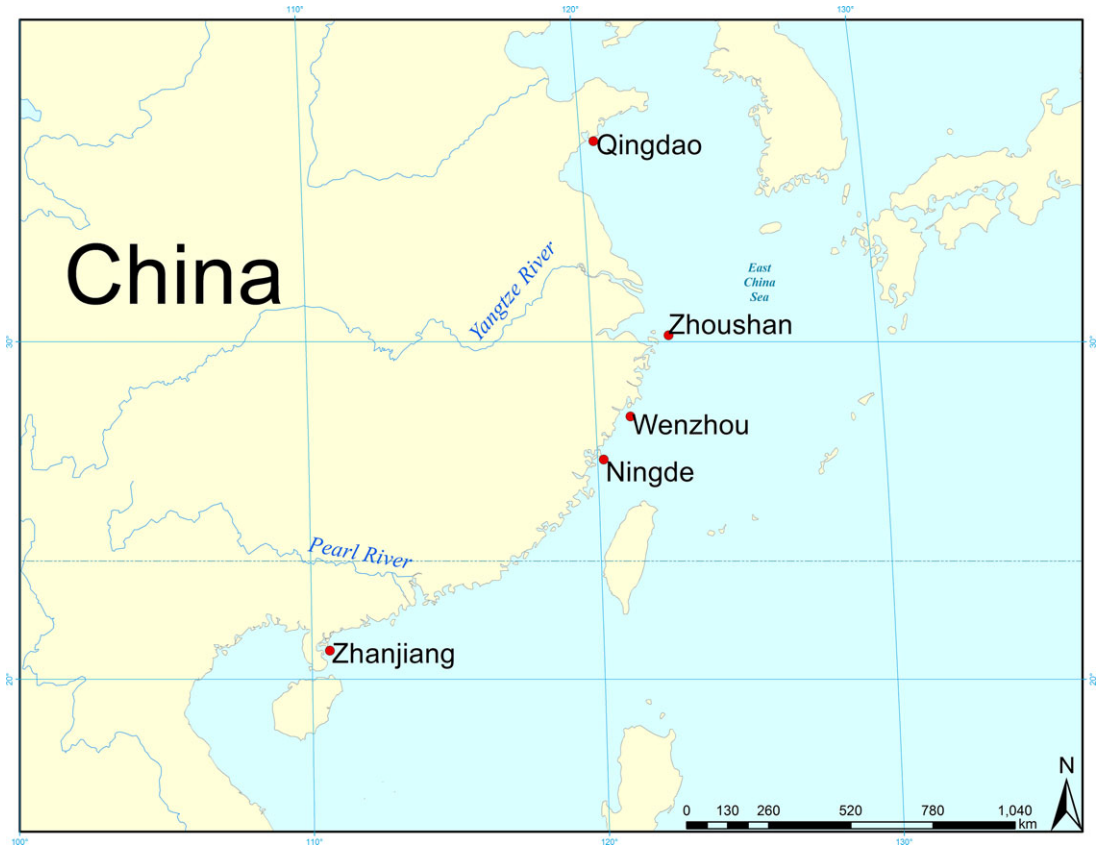

Figure 1 Map illustrating the locations of five *Sepiella japonica* populations in five geographical positions distributed in four provinces along the coast of China. QD: Qingdao population (Shandong Prov.), ZS: Zhoushan population and WZ: Wenzhou population (Zhejiang Prov.), ND: Ningde population (Fujian Prov.) and ZJ: Zhanjiang population (Guangdong Prov.).

The list of haplotypes used and the corresponding GenBank accession numbers are provided in Table 1. Three haplotypes were found only in one population, two were found to be shared by two or three populations and H1 was found to be shared by all five populations. The range of the nucleotide diversity (π) and haplotype diversity (h) for five populations varied from 0.00000 (QD) to 0.00350 (ZS) and from 0.0000 (QD) to 0.6838 (ZS) respectively. The Zhoushan population exhibited the highest level of genetic diversity in terms of value of above both diversities and Qingdao population showed the lowest genetic diversity (Table 1). Collectively, the distribution can be characterized by the coexistence of main locally restricted and minor widely distributed haplotypes, as shown in Fig. 2 there is no obvious characteristic of network among all the haplotypes.

The range of pair-wise F_{ST} varied from 0.00000 to 0.40541 with an average of 0.0775 ($P < 0.05$) (Table 3), suggesting a non-significant genetic variation among the five populations. Moreover, the AMOVA results revealed that 75.13% of genetic variance occurred within populations, whereas 24.87% of the genetic variance occurred among populations (Table 4), which also verified the results of F_{ST}. Furthermore, most pair-wise N_m values were >1 (Table 3), indicating high-level gene flow among populations as well.

Figure 3 showed the consensus UPGMA tree of the six haplotypes found from all tested populations. The haplotype 1 was found in all the five populations, the haplotype 2 and 3 were found in at least two populations, for instance, haplotype 2 was found in two populations: ZS and ZJ; haplotype 3 appeared in three populations: ZS, ND and ZJ. The other three haplotypes were found only in one population, for example, H4 in ZS, H5 in WZ and H6 in ZJ. In addition, most of the haplotypes clustered weakly (<50% bootstrap support),

Table 1 Collection sites, sample sizes (No.) and summary statistics of genetic variability for *Sepiella japonica*

Populations	Code	No.	Haplotypes						No. of haplotypes	Haplotype diversity (h ± SD)	Nucleotide diversity (π ± SD)
			H1	H2	H3	H4	H5	H6			
Qingdao	QD	22	22						1	0.0000 ± 0.000	0.00000 ± 0.00000
Zhoushan	ZS	23	7	3	11	2			4	0.6838 ± 0.080	0.00350 ± 0.00218
Wenzhou	WZ	20	18				2		2	0.1895 ± 0.108	0.00037 ± 0.00055
Ningde	ND	21	10		11				2	0.5238 ± 0.036	0.00103 ± 0.00055
Zhanjiang	ZJ	24	4	2	16			2	4	0.5362 ± 0.110	0.00108 ± 0.00078

Table 2 Variable position of 6 haplotypes of 16S rRNA DNA sequence for *Sepiella japonica*

Haplotype	Nucleotide position beginning from 5′ end														GenBank accession number
	174	222	226	231	233	234	235	243	285	306	336	447	458	494	
H1	G	G	T	G	–	–	T	T	G	A	T	C	A	G	KP699643
H2	*	*	*	A	A	T	*	*	*	*	*	*	*	*	KP699644
H3	*	*	*	A	–	–	*	*	*	*	*	*	*	*	KP699645
H4	A	A	–	A	A	–	A	–	A	T	A	T	T	*	KP699646
H5	*	*	*	*	–	–	*	*	*	*	*	*	*	C	KP699647
H6	*	*	*	*	–	–	*	*	A	*	*	*	*	*	KP699648

*Means the nucleotide is the same to the nucleotide of H1. – means the gap.

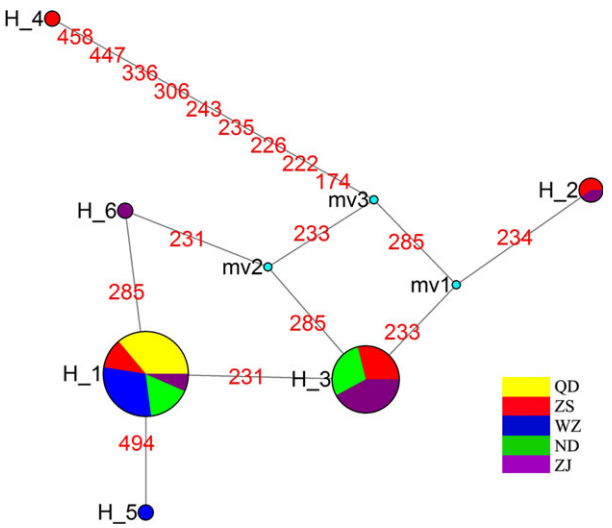

Figure 2 Median-joining network of six 16S rRNA gene haplotypes of *Sepiella japonica* from this study. On the connecting lines, red numbers present the variable sites between each haplotypes pairs. Different colours mean five populations in the network.

showed significantly less variation among these haplotypes. In brief, all the haplotypes fell into one clade, each covered individuals of all five populations.

Discussions

This study is the first report describing the genetic diversity and phylogenetic relationship of five different geographical populations of cuttlefish (*S. japonica*) along the Chinese coastal areas of Yellow Sea, East China Sea and South China Sea. Genetic variation among populations is the consequence of a balance between evolution and demographic process, leading to either heterogeneity or homogeneity among populations. The genetic variation within, between and among populations should record natural selection, fragmentation, genetic drift, migration and range of expansion as well as other evolutionary events (Slatkin 1985). With the releasing eggs and larvae of the cuttlefish to recover the treasure resource, it is important to reveal the

Table 3 The F_{ST} value and gene flow among 5 populations of *Sepiella japonica**

Populations	QD	ZS	WZ	ND	ZJ
QD	–	inf	1.48216	inf	inf
ZS	0.00000†	–	inf	8.75069	0.73332
WZ	0.25225†	0.00000†	–	inf	inf
ND	0.00000†	0.05405†	0.00000†	–	7.42896
ZJ	0.00000†	0.40541†	0.00000†	0.06306†	–

*The data above the diagonal are N_m; the data below the diagonal are F_{ST}.
†F_{ST} value's level of significance at $P < 0.05$; inf is infinite.

Table 4 Analysis of molecular variance of *Sepiella japonica* populations

Source of variation	d.f.	Variance components	Percentage (%)	*P*
Among populations	4	0.14720	24.87	<0.001
Within populations	105	0.44467	75.13	<0.001
Total	109	0.59187		

genetic diversity and population structure status of the cuttlefish. In this study, the low genetic diversity of the five geographical populations inhabiting China's coasts was detected by 16S rDNA, and was compared with the counterparts of the populations living ahead of artificial releasing (Zheng, Wang, Wang, Xiao & Chen 2001). We believe that such a low genetic diversity is not much associated with the artificial releasing, which is widely practiced in recent years. Zheng *et al.* (2001) reported the genetic variation in *S. japonica* using 661 bp COI gene sequences before the artificial releasing project was launched. Their findings strongly support our results as well as the fact that there were no considerable genetic differences between populations. On the other hand, somewhat different from our findings (22 haplotypes) they identified 11 haplotypes. However, the level of genetic diversity is in agreement with our study. Consequently, our findings were in agreement with the previous statement, in which the pelagic organism in the open ocean were commonly considered as low genetic variance and non-significant population differentiation, mainly because of probable ample opportunities of dispersal in different life history stages and the absence of physical barriers in the ocean system.

The haplotype diversity of all *S. japonica* individuals was similar to that of other cephalopods, for instance *Octopus vulgaris* (Teske, Oosthuizen, Papadopoulos & Barker 2007) and *Octopus variabilis* (Xu, Li, Guo, LÜ, Zhou & Wu 2011). Such a low level of diversity may associate with the overcatching of cephalopods, leading to a genetic bottleneck (Zhang 2007). The highest nucleotide diversity

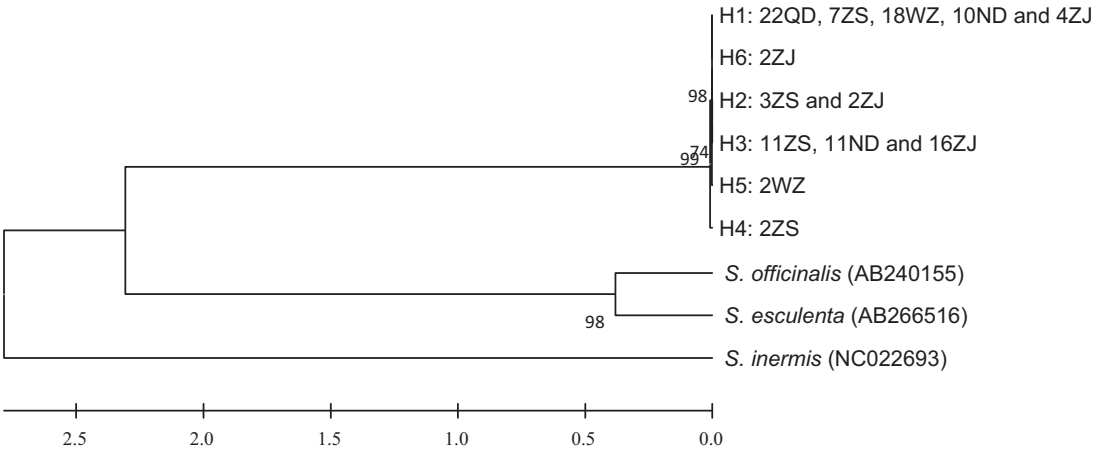

Figure 3 Molecular phylogenetic trees of *Sepiella japonica* using UPGMA method based on 16S rRNA gene sequence data using bootstrap test. Figures before the population codes which are behind haplotypes indicated the number of individuals from the population belonging to the haplotype. Figure indicated that all haloptypes fell into one branch and the samples of *S. japonica* analysed are monophyletic with respect to three outgroups, *S. inermis* (NC022693), *S. officinalis* (AB240155) and *S. esculenta* (AB266516) (100% bootstrap support).

($\pi = 0.00350$) and haplotype diversity ($h = 0.6838$) were documented for Zhoushan population, and the lowest were found in Qingdao population. Furthermore, the levels of haplotype diversity (h-value) of south populations were higher than that of Qingdao, indicating the possibility of bottleneck in the north population. The sea area of Zhejiang-Fujian was the traditional habitat of *S. japonica* and had the highest output of this species in China (Li 2012).

All the haplotypes fell into one clade according to the phylogenetic analyses for the individuals of five populations, and all populations could not be separated. Pairs of four south populations (except Qingdao) have no apparent divergence, furthermore, they share higher frequency of gene flow. Before the cuttlefish resource declined, Ni and Xu (1982) observed that the cuttlefish populations in East China Sea fell into two groups with significant genetic differentiation, which were Zhongjieshan–Yushan group (was equivalent to Zhoushan–Wenzhou group in this report) and Mindong–Beiji group (was equivalent to Ningde group in this report) with the bigger value of genetic distance between the two groups based on the morphological characteristic. In contrast, there is no apparent genetic differentiation between the corresponding populations in this study, although the resource recovered apparently since the releasing project launched (Li 2012). In addition, it is hard to form independent genetic populations for *S. japonica*, although many release activities for the cuttlefish have been practiced in a single or certain sea area for several years.

Higher value of F_{ST} indicates higher genetic differentiation among populations and a lower level of gene flow (N_m). F_{ST} values increase with a more pronounced geographical separation of the populations concerned, generally implying isolation by distance. However, in our study, genetic differentiation among populations is not very much associated with their geographic distribution. The UPGMA results suggested that most of the populations were clustered together, showing no clear phylogeographic structure (Fig. 3). We infer that this aforementioned characteristic of the releasing method could lead to the great gene flow among south populations without a significant phylogeographic structure. Regarding the genetic distance of *S. inermis,* it is the farthest among *S. japonica* and two species from *Sepia* as shown in Fig. 3, *S. inermis* has not been verified (WoMRS) as a scientific name of this species and the taxonomic status of this species possibly needs to be reconsidered.

In summary, low genetic variation and little divergence detected among the five geographical populations of *S. japonica* based on the complete 16S rDNA sequences were in agreement with the previous findings. However, the lower genetic variance of parent cuttlefish for the leasing larvae and eggs maybe is the key reason in comparison with the former research (Ni & Xu 1982), which suggests that the higher genetic diversity of parent cuttlefish for the leasing larvae and eggs is much more beneficial, for example, Zhoushan population in this study is a better choice as parent cuttlefish, meanwhile, it is good practice to conserve the genetic diversity and prevent it from declining any longer.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (grant no. 31101937), International S&T Cooperation Program of China (2014DFT30120), Zhejiang Provincial Natural Science Foundation of China (Y14C190008) and a Project of the Zhoushan City Science and Technology Bureau (2013C41001).

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