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Genetic Diversity and Population Structure of Five *Meretrix lamarckii* Populations Along the Southeast China Sea

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ABSTRACT

The clam *Meretrix lamarckii* is an ecologically and economically important species in the coastal regions of China. In order to characterize the genetic diversity and population structure of *M. lamarckii*, a 750 bp region of mitochondrial COIII gene and an 822 bp region of 12S rRNA gene were sequenced and analyzed for 118 and 105 individuals from five populations (ZS, WZ, ZP, ST and ZJ) in the Southeast coastal areas of China, respectively. Results revealed that 33 haplotypes were defined in COIII gene, and 30 haplotypes were defined in 12S rRNA gene. The pairwise F_{st} values between the ZS population and other four populations were range from 0.122~0.154 (COIII, $P< 0.05$) and 0.052~0.228 (12S rRNA, $P<0.05$), respectively. The revealed that ZS population was significant divergence from other four populations, and no significant genetic divergence among WZ, ZP, ST and ZJ populations. Moreover, results from the median-joining network, plot of STRUCTURE and the UPGMA trees were similar to pairwise F_{st} values. Results from AMOVA indicated that the genetic variation of *M. lamarckii* populations was mainly from the variation within populations (COIII: 93.27%, 12S rRNA: 92.32%). The results of neutrality tests combined with the mismatch distribution indicated recent population expansion of *M. lamarckii* on large spatial scales in the period of late Pleistocene (0.1-0.12 Ma).

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Authors' Contribution
YY conceived and designed the experiments. JF, XW and YG performed the experiments. YY and JL analyzed the data. BG provided analysis tools. YY wrote the paper. JF and XW collected field material and processed the samples.

Key words
***Meretrix lamarckii*, 12S rRNA, COIII, Genetic diversity, Genetic structure**

INTRODUCTION

The hard clam *Meretrix lamarckii* belongs to the Veneridae (Mollusca, Bivalvia, Eulamellibranchia, Venerida, Veneridae). It is an ecologically and economically important in the coastal regions of China, Korea and Japan, which usually inhabits in the shallow water with a sandy seabed in the intertidal zones (Ma, 2001). *M. lamarckii* is an important marine bivalve molluscs with abundant nutrition and valuable medical properties, which has been widely considered as a delicious seafood (Zhang *et al*., 2014). It has a brief pelagic phase of about 5-6 days with the adult is benthic and relatively immobile (Shao *et al*., 2017). In recent decades, the wild stocks of *M. lamarckii* have been harvested by fishermen because of their high commercial value and it is widely cultured in China. From 2007 to 2011, Zhang *et al*. (2011) carried out artificial breeding experiments of *M. lamarckii*. On the basis of successfully bred in 2007, 3 million *M. lamarckii* seedlings with an average shell length of more than 1 mm were bred in 2011. However, with the rapid increasing in demands, the natural stocks of *M. lamarckii* have declined dramatically due to environmental pollution, over exploitation and habitat destruction (Shao *et al*., 2017). Therefore, the genetic information of *M. lamarckii* is critical for the sustainable management of natural resources and to increase the production of the clam.

At present, genetic information of *M. lamarckii* has been carried out by 13 microsatellite loci in previous study (Teng *et al*., 2015). The results show that the genetic diversity of the four geographical populations is in middle level, the variation among populations is high level, and the genetic differentiation level is significant. Genetic diversity and genetic structure of *M. lamarckii* population by mitochondrial molecular markers has not been reported previously.

Mitochondrial DNA (mtDNA) sequence is extensively used to evaluate genetic diversity in marine species due to its small molecular weight, maternal inheritance, relatively rapid substitution rate, and lack of recombination (Guo *et al*., 2004). The mtDNA is well established as a molecular marker in a wide range of taxonomic, phylogenetic, population and evolutionary investigations in animals (Liu *et al*., 2018). COIII gene, as one of mitochondrial cytochrome oxidase subunits, it has a moderate rate of evolution. The 12S rRNA gene is a highly conserved evolutionary marker and it is an effective genetic marker commonly used to explore the molecular

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phylogenetics and classification of aquatic animals (Zhou *et al*., 2015; Wang *et al*., 2017). In the present study, we used the gene sequences of partial mitochondrial cytochrome oxidase subunit III (COIII), and ribosomal 12S subunit (12S rRNA) to assess the genetic diversity and population structure of five natural populations of *M. lamarckii* in Southeast China Sea. It provides theoretical data for the genetic structure of natural resources of *M. lamarckii* along the coast of China and provides research basis and reference for the breeding of its seedlings. The results of this study will provide details about the status of genetic diversity within cultured populations and support future genetic management and germplasm identification.

MATERIALS AND METHODS

Sample collection and DNA extraction

Wild adult specimens of *M. lamarckii* were collected from 5 coastal localities in the Southeast China Sea (Zhoushan [ZS], Wenzhou [WZ], Zhejiang Province and Zhangpu [ZP], Fujian Province and Shantou [ST], Zhanjiang [ZJ], Guangdong Province). Geographic locations and sample sizes of all the examined populations are provided in Figure 1 and Table I. All the samples were collected from September 2016 to April 2017. Tissues from the adductor muscle were dissected from fresh specimens, preserved in absolute ethanol before DNA extraction.

The genomic DNA of *M. lamarckii* was extracted by using the improve salting-out method (Rivero *et al*., 2006), and electrophoresis in a 1.5% agarose gel. The concentration and purity of DNA was determined on the Nano Drop 2000c UV-Vis Spectrophotometer (Thermo Scientific). The extracted DNA was dissolved in 1×TE buffer and stored frozen at -20 ^{0}C.

Amplification and sequencing

Primers were designed based on the complete mitochondrial genome in NCBI database (GenBank accession: KP244452) by using the Primer-premer 6.0 (Singh *et al*., 1998). The primers of COIII gene were amplified using forward primer COIII-F: 5'-ACAAGCAGTTCGACTCTG-3', and reverse primer COIII-R: 5'-GACCTACATAAGCCTCAATCT-3'. The primers of 12S rRNA gene were amplified using forward primer 12S-F: 5'-GCTTAGATAGTCGTGTTG-3', and reverse primer 12S rRNA-R: 5'-CGCCTAGACCCCACAA-3'. Each PCR reaction was performed in a final volume of 50μL containing 40ng template DNA, 6 pM of each primer, 2 ×Taq PCR Master Mix (Com Win Biotech Co., Ltd, Beijing, China) and plus ddH2O to 50μL. The PCR amplification was performed in a thermocycler (BIO-RAD, S1000TM, USA) under the reaction conditions: he reaction conditions were as follows: initial denaturation at 95^{0}C for 3 min; 35 cycles of 95^{0}C for 30 s (denaturation), 51^{0}C for 30 s (annealing), 72^{0}C for 1.5min (elongation), final elongation at 72^{0}C for 10 min and stored at 4^{0}C. The quality of extracted PCR products was assessed using electrophoresis on 1.5% agarose gel in TAE buffer and observed under UV light. Fragments were DNA sequenced using both forward and reverse primers by Hangzhou TSINGKE ZiXi Biotechnology Co. Ltd., Hangzhou, China.

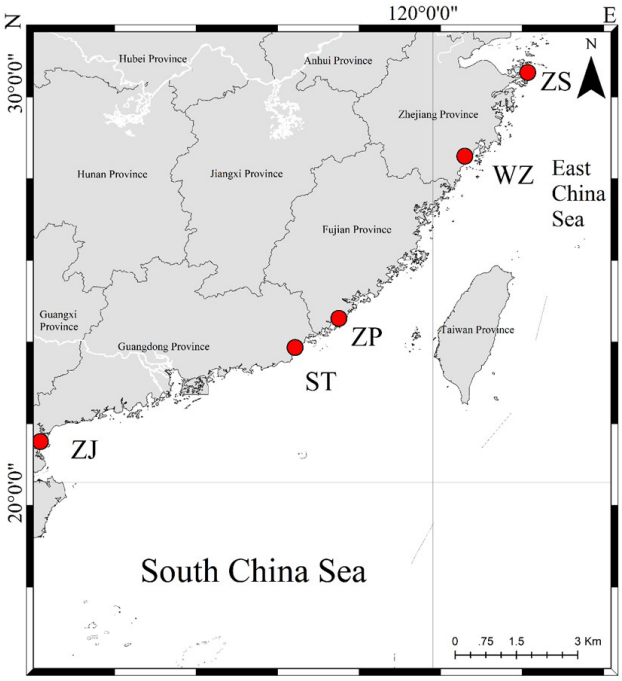

Fig. 1. Map shows the five geographical locations of *M. lamarckii* samples collected in this study along the coast of China Sea, i.e. Zhoushan and Wenzhou (Zhejiang Prov.), Zhangpu (Fujian Prov.), Shantou and Zhanjiang (Guangdong Prov.).

Sequence analysis

For all sequence analyses, genetic similarities were evaluated using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to identify the origin. Sequence alignments were performed with CLUSTAL W (Thompson *et al*., 1994) using default parameters, and manual adjustments were made in MEGA7.0 (Kumar *et al*., 2016). DNASP 6.0 (Rozas *et al*., 2017) was used to estimate the total number of haplotypes (N). All of the haplotypes for each locus were deposited in the Genbank database (accession numbers: MG888543-MG888575). Molecular diversity indices such as haplotype diversity (h), nucleotide diversity (π) for each population. Analysis of Molecular Variance (AMOVA), genetic differentiation coefficient (F_{st}) and neutrality tests

Table I. The details of populations and the genetic parameters of COIII and 12S rRNA genes in *M. lamarckii*.

Sample site (Abbr.)	Sampling Date	Latitude, longitude	COIII				12S rRNA			
			N	n	h	π	N	n	h	π
Zhoushan (ZS)	2016.09	122°31′30°04′	24	5	0.754	0.00267	16	10	0.917	0.00282
Wenzhou (WZ)	2016.12	120°52′23°52′	23	7	0.715	0.00239	23	12	0.842	0.00231
Zhangpu (ZP)	2017.01	117°36′24°07′	24	10	0.783	0.00244	22	7	0.636	0.00116
Shantou (ZT)	2017.04	116°42′23°39′	23	14	0.822	0.00295	23	7	0.522	0.00084
Zhanjiang (ZJ)	2017.04	110°33′21°07′	24	12	0.844	0.00303	21	6	0.495	0.00090

N: NO. of samples *n*: NO. of haplotypes *h*: Haplotype diversity *π*: Nucleotide diversity.

(Tajima's *D* and Fu's *Fs*) were performed using Arlequin 3.5 software (Excoffier and Lischer, 2010). The UPGMA tree of five samples was constructed based on the Nei standard genetic distance with 1,000 bootstrap replicates using the program MEGA 7.0. The software Network 5.1 (Bandelt *et al*., 1999) was utilized to construct the haplotypes network based on the Median-Joining method. The genetic structure of the population was analyzed by using the STRUCTURE v2.3 software (Pritchard *et al*., 2009). The calculated results were analyzed by using the STRUCTURE HARVESTER (Earl, 2012), it is the theoretical population number. Ka/Ks values of COIII and 12S rRNA gene sequences of *M. lamarckii* were calculated by using KaKs Calculator 2.0 software (Wang *et al*., 2010) in gMYN model, and Ka/Ks box plot was drawn by R program.

RESULTS

Genetic diversity

Sequences of the five populations obtained 750 base pair COIII gene sequences from 118 individuals and 33 haplotypes were detected (GenBank accession number MG888543-MG888575). Haplotype 2 was shared by five populations with the largest number (38.14% in total). Haplotype 5 was found to be shared by four populations. Haplotype 7, haplotype 9 and haplotype 14 each were shared by three populations. Haplotype 15 and haplotype 17 were shared by two populations. The other haplotypes each was found in a specific population (Table II). The haplotype diversity (*h*) and the nucleotide diversity (*π*) within populations ranged from 0.715 (WZ) to 0.844 (ZJ) and from 0.00239 (WZ) to 0.00303 (ZJ), respectively (Table I).

Sequences of the 822bp 12S rRNA gene were obtained from 105 specimens, and 30 haplotypes were detected among all samples (GenBank accession number MG888513-MG888542). Haplotype 1 was shared by five populations with the largest number (52.38% in total). Haplotype 8 was found to be shared by four populations. Haplotype 7 was shared by three populations. Haplotype 3, haplotype 16 and haplotype 18 each were shared by two populations. The other haplotypes each was found in a specific population (Table II). The haplotype diversity (*h*) and the nucleotide diversity (*π*) within populations ranged from 0.495 (ZJ) to 0.917 (ZS) and from 0.00084 (ST) to 0.00282 (ZS), respectively (Table I).

Genetic variation

The pairwise F_{st} values of five populations of *M. lamarckii* showed that (Table III), in the COIII gene, the F_{st} values ranged from - 0.016 (ZP-ST) to 0.168 (ZS-ZP). ZS showed great genetic divergences when compared to the other four populations (WZ, ZP, ST and ZJ populations), with significantly F_{st} values ($P<0.05$). No significant divergence was found among WZ, ZP, ST and ZJ populations ($P>0.05$). Similar result of F_{st} values was found in the 12S rRNA gene. The F_{st} values ranged from - 0.015 (ST-ZJ) to 0.228 (ZS-ZJ) in 12S rRNA gene.

The AMOVA test of *M. lamarckii* based on haplotype frequencies revealed that the genetic variation occurred within populations was 93.27% for COIII gene and 92.32% for 12S rRNA gene, among populations was 6.73% for COIII gene and 7.68% for 12S rRNA gene (Table IV). The haplotype networks of COIII and 12S rRNA genes (Fig. 2) showed a radial structure centered on the haplotype shared by five populations (COIII: Hap2, 12S rRNA: Hap1). The haplotypes of the ZS population (yellow) were different from other haplotypes. The haplotypes of the four populations except ZS did not show a significant trend of division, suggesting that there might be greater genetic differentiation between the ZS population and the other four populations. The results of COIII and 12S rRNA gene calculation were consistent with those of UPGMA phylogenetic tree (Nei, 1972), indicating that the five populations were obviously divided into two branches, of which ZS was an independent branch and the other four populations were clustered into one branch (Fig. 3).

The structure analysis indicated that K=2 is the most likely number of clusters (Fig. 4). The results further confirm the similarity between groups: ZS and other groups are assigned to one cluster, and the other four groups are assigned to another cluster. The results are also consistent with the F_{st} value and haplotype networks.

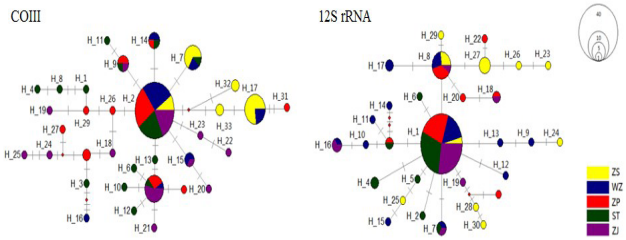

Fig. 2. The haplotypes network of five populations of *M. lamarckii* (Zhoushan (ZS), Wenzhou (WZ), Zhangpu (ZP), Shantou (ST) and Zhanjiang (ZJ)).

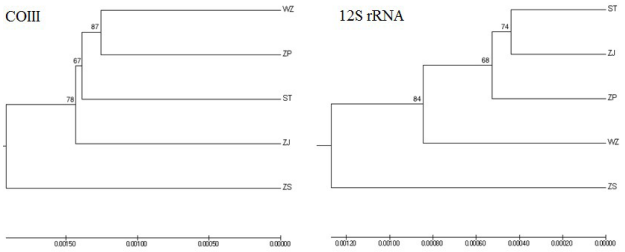

Fig. 3. UPGMA dendrogram of five *M. lamarckii* populations based on *Nei's* genetic distance in COIII and 12S rRNA gene (Zhoushan (ZS), Wenzhou (WZ), Zhangpu (ZP), Shantou (ST) and Zhanjiang (ZJ)).

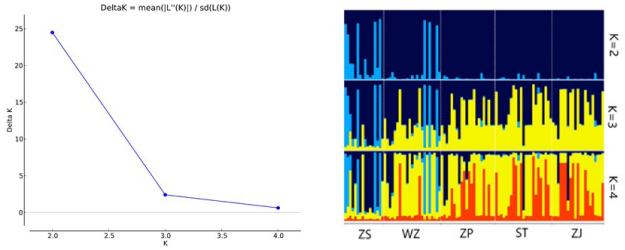

Fig. 4. The STRUCTURE analysis of five populations based on COIII and 12S rRNA (Zhoushan (ZS), Wenzhou (WZ), Zhangpu (ZP), Shantou (ST) and Zhanjiang (ZJ); DeltaK mean the best K value).

Population dynamics analysis

Tajima' *D* and Fu's *Fs* tests were performed to test whether the COIII and 12S rRNA fragments evolved under neutrality or not. Both Tajima' *D* and Fu's *Fs* tests resulted in negative values in most of the populations (Table V), while no value was statistically significant ($P > 0.05$) in

Table II. Distribution of haplotypes in COIII and 12S rRNA genes in *M. lamarckii*.

COIII						12S rRNA				
	ZS	WZ	ZP	ST	ZJ	ZS	WZ	ZP	ST	ZJ
Hap1				1		Hap1	2	9	13	16
Hap2	4	12	11	10	8	Hap2				1
Hap3				1		Hap3			1	1
Hap4				1		Hap4				2
Hap5		1	3	1	6	Hap5				1
Hap6				1		Hap6				1
Hap7	6	2		1		Hap7		1		1
Hap8				1		Hap8	3	3	4	
Hap9			2	1	1	Hap9		1		
Hap10				1		Hap10		1		
Hap11				1		Hap11		1		
Hap12				1		Hap12		1		
Hap13				1		Hap13		1		
Hap14		2	1	1		Hap14		1		
Hap15		2			1	Hap15		1		
Hap16		1				Hap16		1		
Hap17	10	3				Hap17		2		
Hap18					1	Hap18			1	
Hap19					1	Hap19				
Hap20					1	Hap20			1	
Hap21					1	Hap21			1	
Hap22					1	Hap22			1	
Hap23					1	Hap23	1			
Hap24					1	Hap24	1			
Hap25					1	Hap25	1			
Hap26			1			Hap26	1			
Hap27			1			Hap27	4			
Hap28			2			Hap28	1			
Hap29			1			Hap29	1			
Hap30			1			Hap30	1			
Hap31			1							
Hap32	2									
Hap33	2									

COIII gene and all values were statistically significant ($P < 0.05$) in 12S rRNA gene. The sum of the square deviations (SSD) per locality of ST and ZJ population were 0.00000 and 0.00741, respectively, and range from 0.00043 to 0.00730 for the 12S rRNA gene. *P*-values of SSD between the observed and expected mismatch distributions were

all statistically insignificant (P > 0.05), indicating the presence of non-equilibrium and a population expansion event in *M. lamarckii*. The results suggested very little population change in the past in *M. lamarckii*. The distribution curves of base mismatch under population expansion were calculated by DnaSP 6.0 software (Fig. 5). The overall mismatch curves of the five populations showed a single peak distribution, which accorded with Poisson distribution. The parameters of population expansion are estimated by Arlequin software. Under the 95% confidence interval, estimating population expansion events based on generalized nonlinear minimum variance. The expansion time of the group is 0.1-0.12 Ma, according to the formula $t=\tau/2\mu k$ conversion (where t is the time since expansion; μ is the mutation rate for the whole sequence under study, which is 2.0% ~ 2.4% per million years; k is the sequence length). According to the Ka/Ks box plot (Fig. 6), the mean values of Ka/Ks of each population in COIII fragments were relatively close, and no population was obviously selected by purification. In the 12S rRNA fragment, the Ka/Ks mean of ZJ and ZS populations was significantly higher than that of other populations, which may be that the two populations were more significantly affected by population purification selection.

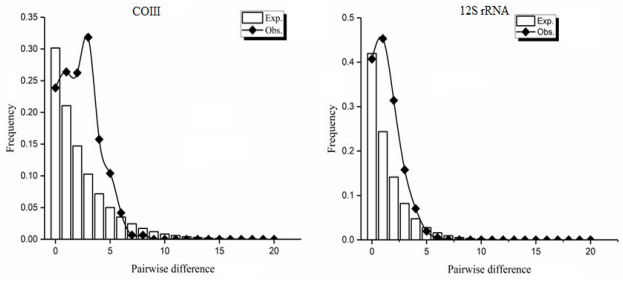

Fig. 5. Nucleotide mismatch distribution of populations of *M. lamarckii* based on *COIII* and 12S rRNA genes sequences.

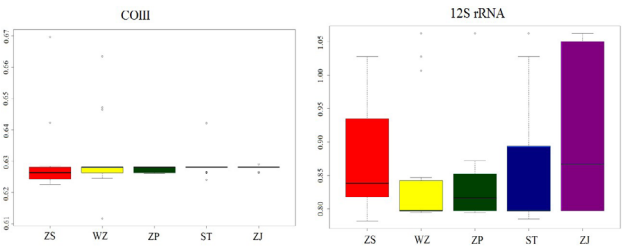

Fig. 6. Box plot of *M. Lamarckii* based on COIII and 12S rRNA genes sequences (Zhoushan (ZS), Wenzhou (WZ), Zhangpu (ZP), Shantou (ST) and Zhanjiang (ZJ).

DISCUSSION

Genetic diversity

This study showed that the genetic diversity of the five populations of *M. lamarckii* in the southeastern coast of China was in the middle level. The average haplotype diversity (h) was 0.784 (COIII) and 0.682 (12S rRNA), and the average nucleotide diversity (π) was 0.00270 (COIII) and 0.00161 (12S rRNA), respectively. It is slightly lower than that of other marine organisms, such as *Mytilus galloprovincialis* h = 0.946, π = 0.0207 (COIII) (Zhou *et al.*, 2015a). *Octopus variabilis* h = 0.909, π = 0.034 (COIII), h = 0.984, π = 0.028 (12S rRNA) (Xu *et al.*, 2011). The results were consistent with that of the four geographical populations in the southeastern coast of China based on microsatellite markers (Teng *et al.*, 2015). In addition, the results of two mitochondrial gene analyses were slightly different, which may be related to the difference of evolution rate and conservation (Gao *et al.*, 2007).

Genetic structure and differentiation and dynamic analysis

F_{st} is the most widely used parameter to measure the degree of genetic differentiation among populations. In this study, the genetic differentiation coefficients of ZS population and other populations ranged from 0.122 to 0.168, $P < 0.05$ (COIII) and 0.052 to 0.228, $P < 0.05$ (12S rRNA) ($0.05 < F_{st} < 0.25$), indicating that there was moderate significant genetic differentiation between ZS population and other populations. However, the F_{st} values of the other four populations (WZ, ZP, ST and ZJ) were less than or close to 0.05 and were no significant ($P > 0.05$), indicating that the genetic differentiation among these four populations was not obvious, and there was frequent gene flow among the four populations. Structure analysis, haplotype networks and the UPMGA cluster analysis all supported the conclusions.

The main reason for this result may be the formation of ocean current pattern, lack of effective barriers in the marine environment (Gu *et al.*, 2015). From the geographical distance, short distance between ZS and WZ, but there was a significant genetic differentiation. This may be because ZS is located in the Qiantang River estuary with Hangzhou Bay, which affects the gamete exchange of *M. lamarckii*. Lv and Song (2007) found that all the year round in the ZS area are affected by the Yangtze River diluted water and the Yellow Sea and East China Sea mixed water mass, with sufficient water exchange capacity. In summer, during the breeding season of *M. lamarckii* (late July to the end of August), the Yangtze River diluted water moved southeastward and then quickly turned to the direction of northeastern Jeju Island between

Table III. The pairwise F_{st} values based on COIII (below) and 12S rRNA (above).

Sample (Abbr.)	Zhoushan	Wenzhou	Zhangpu	Shantou	Zhanjiang
Zhoushan		0.05224*	0.13544*	0.22477*	0.22752*
Wenzhou	0.12229*		0.00766	0.06072*	0.05418
Zhangpu	0.16836*	0.00364		0.01369	0.00201
Shantou	0.14067*	-0.00664	-0.01581		-0.01497
Zhanjiang	0.15409*	0.03895	-0.00111	0.01092	

Note: Values with superscripts* are significantly different (P<0.05).

Table IV. Analysis of molecular variance (AMOVA) of *M. lamarckii* populations.

Gene	Source of variation	d.f.	Sum of squares	Variance components	Percentage variation%
COIII	Among populations	4	4.237	0.02828Va	6.73
	Within populations	113	44.288	0.39193Vb	93.27
	Total	117	48.525	0.42021	100
12S rRNA	Among populations	4	3.672	0.02786Va	7.68
	Within populations	100	33.509	0.33509Vb	92.32
	Total	104	37.181	0.36295	100

Table V. The analysis of neutrality test for five populations.

Test	COIII					12S rRNA				
	ZS	WZ	ZP	ST	ST	ZS	WZ	ZP	ST	ZJ
Tajima's *D*	2.37154	1.36170	1.47731	0.61726	0.77487	0.53571	1.01077	1.30582	2.01499	1.74190
p-value	0.99460	0.07430	0.05040	0.29170	0.23340	0.32470	0.16720	0.09090	0.00440	0.02160
F_S	0.98022	1.25853	4.31609	9.58212	5.79893	4.98830	7.21931	3.51993	4.73107	3.12876
p-value	0.73150	0.23360	0.00600	0.00000	0.00140	0.00230	0.00000	0.00310	0.00000	0.00230
SSD	-	-	-	0.00000	0.00741	0.00468	0.00043	0.00425	0.00091	0.00730
p-value	-	-	-	0.81330	0.47790	0.67950	0.97400	0.58390	0.78130	0.40600

122°10’ ~ 122°30’E. WZ, ZP, ST and ZJ are all located in the southwest direction of ZS. The influx of water from the Yangtze River prevents the gene flow between ZS and other four populations. Secondly, the coastal current of Zhejiang and Fujian distributes in the coastal waters, south of the Yangtze Estuary, and its flow direction is from south to north, strong in winter and gradually weakening in spring. In May, from the north to the sea area near Pingtan island in Fujian Province (Ma, 2009), it was difficult for the ZS population to genetic exchange with the four southern populations by means of ocean currents during breeding period. In addition, the coastal current of Eastern Guangdong is strong in summer, and flows northeast, and passes through the Taiwan Strait. The South China Sea warm current flows from southwest to northeast from the coastal areas of Eastern Guangdong and the deep-water areas outside Guangdong. There are currents in the Taiwan Strait flowing to the northeast, and the flows in the Strait connect with those in the East China Sea, East Guangdong, South China Sea. The presence of these currents enables frequent gene exchange among WZ, ZP, ST and ZJ populations through perennial flow exchange. The results of this study were different from those obtained by Teng *et al*. (2015) using microsatellite markers. This study only collected samples in or near South China Sea area, and the variation among populations was significant, which may be related to the location of sample collection and use different marker. Consistently, marine species with pelagic larvae stages, ocean currents are known to be involved in the formation of population genetic structure (Hedgecock, 1994). With the influence of current, these characteristics of the short larval stage of *M. lamarckii* cannot facilitate gene flow via the distance transmission. In marine environments lacking adequate reproductive

barriers, ocean currents, environmental factors, and the duration of planktonic stages all affected gene exchange and genetic distribution among populations (Arnaud *et al*., 2000; Vadopalas *et al*., 2004; Zhan *et al*., 2009; Xi *et al*., 2011). Population dynamics analysis suggested that the *M. lamarckii* had experienced population expansion in the southeastern coast of China. The population expansion time was about 0.1-0.12 million years ago, and it was in the late Pleistocene. Late Pleistocene is about 0.01-0.126 million years ago. With the intense global climate change, the sea level periodically rises and falls, with the maximum amplitude of 120-140 m, and frequent glacial-interglacial alternations, many species experienced significant contraction and expansion during this period (Liu *et al*., 2017).

Diversity protection and sustainable utilization

At least two distinct geographic populations of *M. lamarckii* in the southeastern coast of China were identified, which should be divided into management units in fisheries. Relevant management departments should strengthen protection of the wild population of *M. lamarckii*. Sampling should be done locally due to genetic structure of the local wild population will be affected by long-distance breeding or stock enhancement. In the breeding process, number of parents and effective population size should be increased, and the reduction of genetic diversity caused by inbreeding should be avoided. On this basis, the relevant departments should formulate a scientific and reasonable genetic diversity monitoring program to protect the germplasm resources of *M. lamarckii*. In addition, we should effective and proper management, protection of species habitats, reduce environmental pollution and improve the reproduction of species, thus ensuring the sustainable development of the resources of *M. lamarckii* in China.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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