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Discordant patterns of genetic variation between mitochondrial and microsatellite markers in *Acanthogobius ommaturus* across the coastal areas of China

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Abstract

Acanthogobius ommaturus, which belongs to the family Gobiidae, is a euryhaline and demersal fish that is widely distributed in the coastal areas, harbors, and estuaries of China, D. P. R. Korea and Japan. In this study, the genetic diversity and genetic structure of five geographical populations of *A. ommaturus* was assessed using the mitochondrial hypervariable region gene and microsatellite markers. The results of the two genetic markers indicated that the *A. ommaturus* populations had a high level of genetic diversity. The mitochondrial marker detected weak genetic differentiation among populations, and the Neighbor-Joining tree showed that there was no obvious pedigree branches and geographic structure as well. However, population of Zhoushan showed significant genetic differentiation with other populations by microsatellite markers. The population of *A. ommaturus* has not experienced bottleneck effect recently. We speculated that the Pleistocene climate change and juvenile fish dispersal played an important role in the population differentiation of *A. ommaturus*.

Key words: mitochondrial markers, microsatellite markers, genetic diversity, genetic structure, *Acanthogobius ommaturus*

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1 Introduction

Population genetics is a genetic discipline that combines the research strategies and methods of genetics, statistics and mathematics to evaluate the level of population genetic diversity and analyze the genetic structure (Nei, 1975; Cavalli-Sforza, 1998). However, most of the life history of marine fish is completed in the ocean, with a vast range and unpredictable environment (Avise, 2000). Thus, it is very difficult to evaluate the population genetic structure of marine fish (Hewitt, 2000). China's offshore waters span three climatic zones: temperate, subtropical and tropical. Coupled with the impact of a large number of rivers entering the sea, China's offshore waters have become offshore fish habitats with significant differences in temperature, salinity and other environmental factors (Guo, 2018; Zhang et al., 2020, 2022). Therefore, when studying the population genetics of Chinese offshore fish, we should comprehensively consider the interaction of biology and geography, after that reasonably evaluate the population genetic diversity level and genetic structure of marine fish (Hewitt, 2000).

The appropriate genetic markers are critical for the accurate detection of genetic diversity and genetic structure of marine fish populations. Mitochondrial DNA (mtDNA) of fish is an important molecular marker because of its simple structure, strict maternal inheritance and lack of recombination (Guo et al., 2004; Dong et al., 2021; Kanakachari et al., 2020). As the largest non-coding region in the mtDNA genome, the control region (CR) has a high intraspecific polymorphism (Cann et al., 1984; Jaafar et al., 2020; Sharma et al., 2021). It is an ideal molecular marker for studying genetic diversity at population level. Microsatellite DNA, also known as short tandem repeats (STRs) or simple sequence repeats (SSRs), is widely distributed in the eukaryotic genome (Ellegren, 2004; Gharesouran et al., 2021; Susanti et al., 2021). Microsatellite DNA markers, as an efficient genetic molecular marker, have the advantages of co-dominance, high genetic polymorphism, good specificity, and conform to Mendel's law of inheritance (Wright and Bentzen, 1994; Ferreira et al., 2015; Li et al., 2020). These two marker methods are the most commonly used and effective molecular markers for the study of marine fish genetics.

Due to the limited ability of a single genetic marker to identify genetic information, the results were frequently one-sided and inaccurate (DiBattista et al., 2017; Meng, 2019). Hence, we could combine multiple genetic marker methods to study the level of genetic diversity and genetic structure among marine fish populations (Morgan et al., 2018). For example, Lane et al. (2016) have used mitochondrial DNA control region (mtDNA) sequences and microsatellite polymorphic DNA loci to detect the population genetic structure of *Polyprion oxygeneios* in the surrounding areas of New Zealand. The results showed that there was no significant difference within mtDNA data, while the results of microsatellite

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DNA markers showed that there were significant differences among samples from Hokitika and all other sites in New Zealand.

Acanthogobius ommaturus is a large goby fish and has high economic value, which belongs to Osteichthyes, Perciformes, Gobioidei, Gobiidae, *Acanthogobius*. It is a euryhaline, demersal fish, mainly distributed in China, the Korean Peninsula and Japanese coastal and harbor. The suborder Gobioidei is the largest group of fish species, with more than 2 000 species reported (Wu and Zhong, 2008). As a suitable species for offshore artificial culture and fishing, there were many studies on seedling raising, breeding technology, fecundity (Feng et al., 2004), morphology (Luo et al., 2008), feeding habits (Zhu et al., 2016) and ecology (Fan et al., 2005). Although there were some studies on the genetics of *A. ommaturus* (Wang and Zhao, 1994; Jin, 2013; Wang et al., 2016a), there was still a lack of systematic research on its genetic diversity and genetic structure. Song et al. ever detected a distinct pattern of geographic distance isolation in the Northwest Pacific Ocean by the partial sequence of the first hypervariable region of mtDNA CR (Song et al., 2010b). Conversely, only weak genetic differentiation among *A. ommaturus* populations was detected by AFLP markers (Song et al., 2010a). The above genetic information background hints us the importance and necessity of reevaluating the genetic structure of *A. ommaturus* population using multiple methods. Therefore, based on the mtDNA and SSR genetic markers, this study reinvestigated the genetic diversity of *A. ommaturus*, assessed the genetic structure and elucidated the historical population dynamics. The primary aims of the present study were to: (1) report on the real genetic diversity and genetic structure in *A. ommaturus* populations, as well as to elucidate its historical population dynamics at the mitochondrial and nuclear levels; (2) compare the results of the two genetic markers, in order to provide a reference basis for the selection of genetic methods in the subsequent genetic research of marine fish.

2 Materials and methods

2.1 *Sample collection and DNA extraction*

In this study, *A. ommaturus* individuals were collected from five locations (Fig. 1; Table 1). The fish were rapidly dissected, and muscle tissues were excised and preserved in 95% alcohol for subsequent experiments. The genomic DNA was extracted from *A. ommaturus* samples according to the lab standard phenol-chloroform extraction method (Sambrook et al., 1989).

2.2 *Sequencing of mtDNA*

Sequences of hypervariable regions in mitochondrial CR of 120 *A. ommaturus* individuals were amplified using the primers DL-x: 5′-CCCATCTCTAGCTCCCAAAGC-3′; DL-h: 5′-CTGTAGAGTGAACGCTTGGCATG-3′ (Song et al., 2011). The PCR was conducted in volumes of 25 μL reaction mixtures containing 17.25 μL ultrapure water, 2.5 μL 10×PCR buffer, 2 μL dNTPs, 1 μL each primer (5 μmol/L), 0.25 μL Taq polymerase (TakaRa, Japan), and 1 μL DNA template. The PCR reaction system referred to Song et al. (2010b). The PCR product was detected by electrophoresis with 2% agarose gel, and samples with good amplification effect were sent to biological company (TsingKe Biological Technology Co., Ltd., Qingdao, China) for sequencing. To ensure the accuracy of the sequences tested, each sample was sequenced in both directions. All sequences have been uploaded to Genebank (Genebank number: MZ869854–MZ869973).

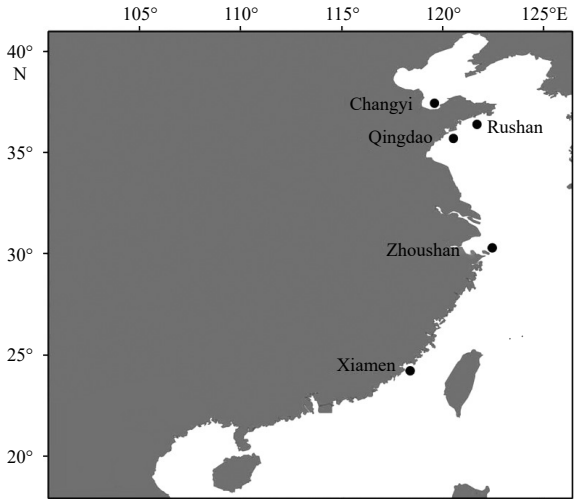

Fig. 1. Sampling sites of *Acanthogobius ommaturus*.

2.3 *Nuclear microsatellite genotyping*

Fourteen nSSR loci, previously developed for *A. ommaturus* by Song et al. (2020), were used in this study. The PCR were conducted in volumes of 25 μL reaction mixtures containing 17.25 μL ultrapure water, 2.5 μL 10×PCR buffer, 2 μL dNTPs, 1 μL each primer (5 μmol/L), 0.25 μL Taq polymerase (TakaRa), and 1 μL DNA template. The forward sequence of each primer pair was labeled with a fluorescent dye (FAM, HEX, TAMARA). The PCR reaction system referred to Song et al. (2020). Light avoidance is necessary during the whole process of experiment operation. PCR products were detected by 2% agarose gel. The qualified fluorescent products were sent to the biological company (Personal Biotechnology Co., Ltd., Shanghai, China) for STR scanning and genotyping.

2.4 *Data analysis for mtDNA*

All sequences were compared and analyzed by DNASTAR software package (DNASTAR, Inc., Madison, USA), and a series of related indices such as molecular polymorphism index, haplotype diversity and nucleotide diversity were calculated by Arlequin 3.5 software (Excoffier and Lischer, 2010). The haplotype Neighbor-Joining (NJ) tree was constructed with MEGA7.0 (Ku-

Table 1. Sample information, genetic statistics for the mitochondrial DNA (mtDNA) and smation for simple sequence repeat (SSR) of *Acanthogobius ommaturus*

Sample locality	Abbreviation	Sample time	Sample number for mtDNA	Haplotype number	Number of polymorphic sites	Haplotype diversity (h)	Nucleotide diversity (π)	Sample number for SSR
Zhoushan	ZS	2018.01	24	8	16	0.772±0.078	0.008±0.005	30
Changyi	CY	2007.09	24	10	10	0.746±0.091	0.003±0.002	28
Qingdao	QD	2018.10	24	8	10	0.681±0.091	0.005±0.003	24
Rushan	RS	2017.10	24	10	10	0.873±0.044	0.005±0.003	24
Xiamen	XM	2017.10	24	12	16	0.891±0.046	0.006±0.004	30
Total	/	/	120	36	38	0.793±0.070	0.006±0.004	136

mar et al., 2016), and its reliability was evaluated by 1 000 bootstrap resampling. Calculating pairwise genetic distance among population based on Kimura 2-parameter model. Arlequin 3.5 software (Excoffier and Lischer, 2010) was used to calculate pairwise F_{st} values to test population genetic structure. AMOVA analysis was used to evaluate population genetic structure of *A. ommaturus*. Arlequin 3.5 software (Excoffier and Lischer, 2010) was used to complete the historical dynamic analysis of the population, including neutral detection and nucleotide mismatch analysis. Multidimensional scaling analysis map based on F_{st} was drawn by SPSS Statistics 17.0 software (SPSS Inc., Chicago, USA). The correlation between geographic distance and genetic distance (F_{st} / (1–F_{st})) matrix was tested by using IBD 1.52 software (Bohonak, 2002; Jensen et al., 2005).The Bayesian skyline plot in Beast 1.8 software was used to detect the change of historical population size (Drummond and Rambaut, 2007).

2.5 *Data analysis for nSSR*

The software FSTAT-ver. 2.932 (Goudet, 2005) was used to calculate genetic diversity parameters, such as the number of allele (A), the observed genetic diversity within populations (H_o), the expected genetic diversity within populations (H_e). The pairwise genetic distance and the corresponding P-value were calculated by software Population 1.2 (Raymond and Rousset, 1995). The UPGAMA tree based on genetic distance was also constructed by Population 1.2. The three-dimensional factor correspondence analysis graph was drawn by software Genetix ver. 4.05 (Belkhir et al., 2004). The genetic structure of the populations was analyzed by using the software Structure ver. 2.2 (Pritchard et al., 2000). The most possible free mating clusters (K) were deduced and the probability of all individuals being allocated to each population was calculated (Evanno et al., 2005). The correlation between geographic distance and genetic distance (F_{st}/(1–F_{st})) matrix was tested by using IBD 1.52 software (Bohonak, 2002; Jensen et al., 2005). The software BOTTLENECK 1.2.02 (Piry et al., 1999) was used to analyze whether different geographical populations have experienced bottleneck effect.

3 Results

3.1 *Variation in mtDNA*

The total length of hypervariable region of mtDNA CR was 504 bp (including a 26 bp partial segment of the $tRNA^{pro}$ gene). All the following analyses were based on the 478 bp CR hypervariable region fragment. The sequence composition showed that the content of A+T (66.8%) was significantly higher than that of G+C (33.3%), which showed obvious base composition bias. A total of 38 polymorphic sites were detected within the 478 bp mtDNA CR fragment. There were 29 transition sites, 10 transversion sites and 1 insertion/deletion site. Among them, populations of of Xiamen and Zhoushan had the largest number of polymorphic loci (16), while other populations had the same number of polymorphic loci (10) (Table 1).

A total of 36 haplotypes were detected, of which 4 were shared. Hap6 was shared by five populations at most. The population of Zhoushan and Qingdao exhibited the fewest haplotypes (n=8), whereas the population of Xiamen has the most haplotypes (n=12; Table 1). High levels of haplotype diversity were detected within each population, and low levels of nucleotide diversity were observed (Table 1).

Based on 36 haplotypes, NJ tree was constructed with the *Acanthogobius flavimanus* as the outgroup (Fig. 2), which showed that the haplotypes of each population were randomly distributed on the NJ tree, and no phylogenetic structure corresponding to geography was detected (Fig. 3).

The among-population difference accounted for only 11.13% of the observed variation, with no significant differences (P>0.05), whereas the within-population difference accounted for 88.87% of the variation. The F_{st} index of five populations ranged from 0.031 to 0.184 (Table 2). The largest genetic differentiation occurred between populations of Zhoushan and Qingdao (F_{st} =0.183*). Most of the genetic differentiation occurred between population of Zhoushan and the other four populations, suggesting a pattern of weak genetic differentiation. According to Mantel test, the genetic distance (F_{st}/(1–F_{st})) was not significantly correlated with the geographical distance among populations (R^2=0.081, P=0.284).

In the neutral test, the results showed that the overall Tajima's D (Tajima's D= –1.727, P=0.013) and the Fu's Fs value (Fu's Fs= –25.659, P=0.000) were significantly negative value, indicating a recent expansion event. Both the sum of squared deviations (SSD=0.001 11) and raggedness index (0.013) tests were not statistically significant (P>0.05), suggesting that all the models could not reject the expansion hypothesis. The nucleotide mismatch distribution result obtained for *A. ommaturus* showed an overall unimodal pattern that was consistent with the expected distribution in the population expansion model (Fig. 4a).

According to the Bayesian skyline analysis, all populations of *A. ommaturus* had a stable growth rate (Fig. 4b). Concurrently, it showed a trend of significant expansion since about 15 000 a ago.

3.2 *Microsatellite variation*

Fourteen pairs of microsatellite primers were used in this study and 348 alleles were obtained (Table S1). The number of alleles per locus was 24.9 on average (range: 18–39). The allelic richness (R_s) of the five populations was 5.000–20.000, the observed heterozygosity (H_o) was 0.916 7–1.000 0, and the expected heterozygosity (H_e) was 0.706 8–0.920 0. The polymorphic information content ranged from 0.682 6 to 0.897 4, indicating that the five populations had high polymorphisms. Hardy Weinberg equilibrium test showed that most individuals of the five populations did not deviate from the equilibrium state after Bonferroni correction at 14 SSR loci (P>0.05).

The pairwise F_{st} values among populations ranged from 0.006 1 to 0.044 0 (Table 3). The F_{st} value between populations of Changyi and Xiamen was the lowest (0.006 1), and that between populations of Changyi and Qingdao was the highest (0.044 0). The overall differences were significant, only a few were not significant. The Mantel test showed that there was no significant correlation between F_{st}/(1–F_{st}) and geographical distance (R^2=0.275, P=0.119). The genetic distance $(\delta\mu)^2$ of the five populations ranged from 0.781 (between populations of Rushan and Changyi) to 8.726 (between populations of Zhoushan and Qingdao) (Table 3). The results of UPGMA tree based on genetic distance among five geographical populations showed that except population of Zhoushan, the other four populations were clustered together (Figure not shown).

The results of three-dimensional factor correspondence analysis (3D-FCA) showed that the three axes represented the three most important factors in factor correlation analysis, and accounted for 33.29%, 26.29% and 21.69% of the total genetic variation sources respectively (Fig. 5). Among them, the spatial distribution of populations of Zhoushan and Qingdao was relatively scattered, while populations of Rushan, Xiamen and Changyi overlapped together.

In the Structure analysis for all 136 individuals using the fourteen nSSR loci, the high values of ΔK were observed first at K=2, second at K=5 (Fig. 6). That is to say, there were two free groups

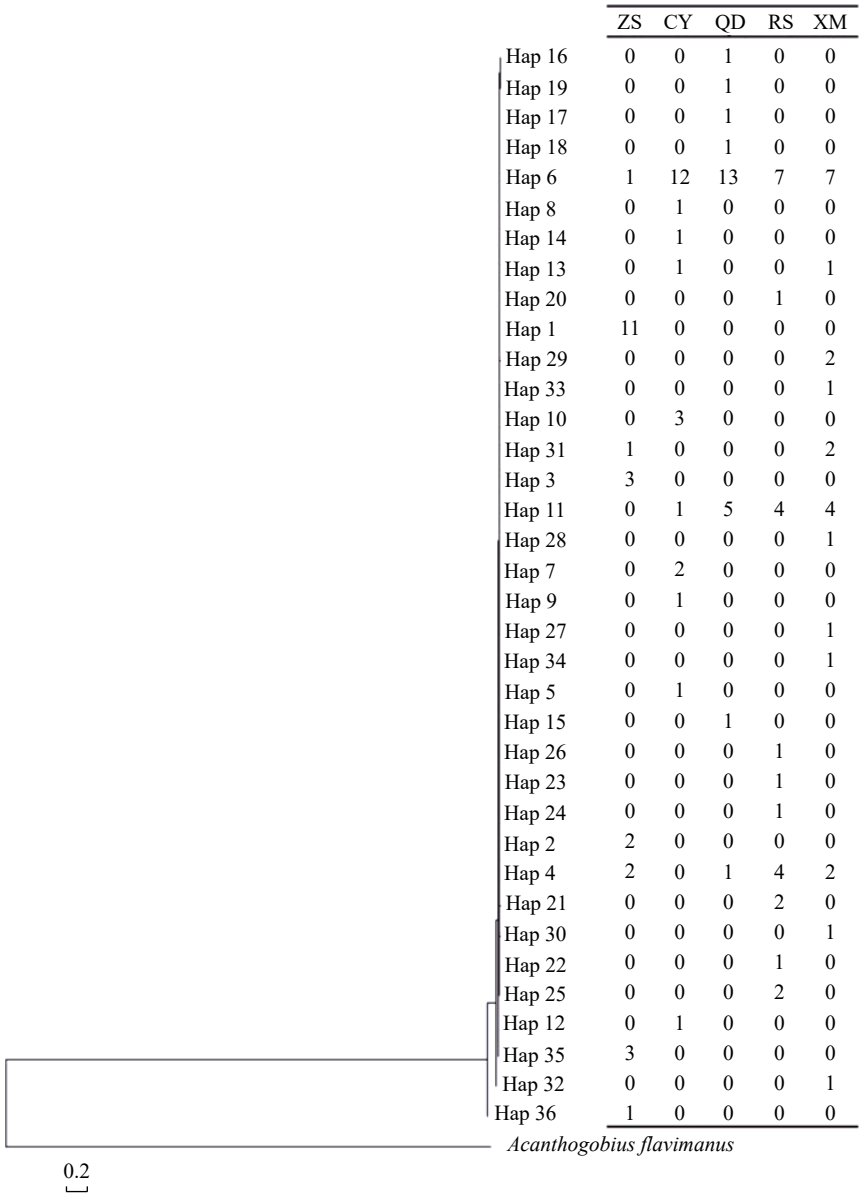

Fig. 2. Unrooted Neighbor-joining tree constructed using Kimura-2-parameter for 36 haplotypes of *Acanthogobius ommaturus* based on mitochondrial DNA hypervariable region analysis. On the right was the number of haplotypes in each population. ZS: Zhoushan; CY: Changyi; QD: Qingdao; RS: Rushan; XM: Xiamen.

in the most suitable distribution pattern of 136 individuals. And the proportion of individuals assigned to the two groups was similar (Fig. 6).

The Wilcoxon test was performed on the five *A. ommaturus* populations based on three different models: IAM, TPM and SMM. However, no heterozygosity excess was detected (P>0.05; Table 4). The results of Mode Shift Test showed that the allele frequencies of all *A. ommaturus* populations was normal L-type distribution. Therefore, this study suggested that *A. ommaturus* populations had not experienced bottleneck effect recently.

4 Discussion

4.1 *Genetic diversity analysis*

Based on the results of mtDNA and microsatellite markers, a high level of genetic diversity was detected in *A. ommaturus* populations. The large and stable effective populations, heterogeneous environmental conditions and suitable life habits are important for maintaining high genetic diversity of natural populations (Nei, 1987; Li et al., 2018). According to the four phylogeographic pattern types of marine fish classified by Grant and Bowen (1998), we speculated that *A. ommaturus* population may have experienced rapid population expansion, but they had not accumulated enough nucleotide variation. Therefore, mitochondrial results showed that all populations had high haplotype diversity and low nucleotide diversity. This phenomenon often occurred in marine fish, such as *Terapon jarbua* (Yang et al., 2018), *Trichiurus japonicus* (Wu et al., 2019), *Pholis fangi* (Gao et al., 2019), *Leiognathus equulus* (Gao et al., 2020), etc. Additionally, the results of nucleotide mismatch distribution and Bayesian skyline analysis also supported that the *A. ommaturus* population may have experienced recent expansion. Song et al. (2010b) ever reported that the Pleistocene sea level changes might lead to the *A. ommaturus* population expansion. Thus, the gradual growth of effective population caused by population expansion recently might be the reason for its population to maintain a high level of

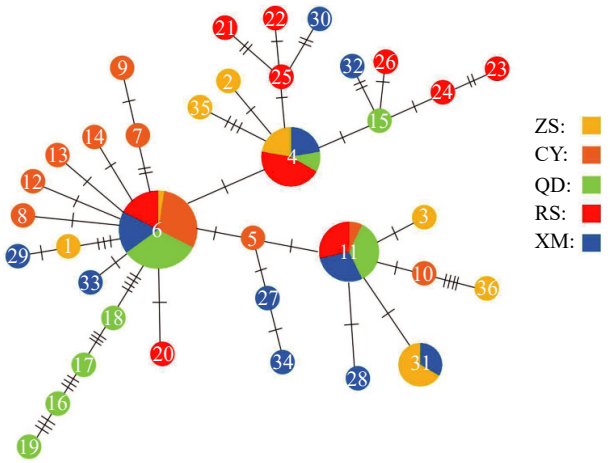

Fig. 3. Unrooted minimum spanning tree showing gene relationship among hypervariable region haplotypes of *Acanthogobius ommaturus*. ZS: Zhoushan; CY: Changyi; QD: Qingdao; RS: Rushan; XM: Xiamen.

Table 2. Pairwise F_{st} between *Acanthogobius ommaturus* populations

Population	ZS	CY	QD	RS
CY	0.175*			
QD	0.184*	0.042		
RS	0.160*	0.130*	0.087*	
XM	0.114*	0.031	0.037	0.052

Note: * significant at P<0.05. ZS: Zhoushan; CY: Changyi; QD: Qingdao; RS: Rushan; XM: Xiamen.

genetic diversity. The richness of genetic diversity of a species might be affected by population bottleneck events (Frankham et al., 2002). The genetic bottleneck effect test in microsatellite markers revealed that *A. ommaturus* populations have not experienced bottleneck events and thus retain more genetic variation. In summary, although *A. ommaturus* resources are declining and show decreased mean ages due to climate change, overfishing (Waelbroeck et al., 2002; He et al., 2014; Wu et al., 2019), their life history traits, environmental diversity (Wu and Zhong, 2008) and historical dynamics might ensure the accumulation of genetic mutations and abundant genetic diversity in *A. ommaturus* populations.

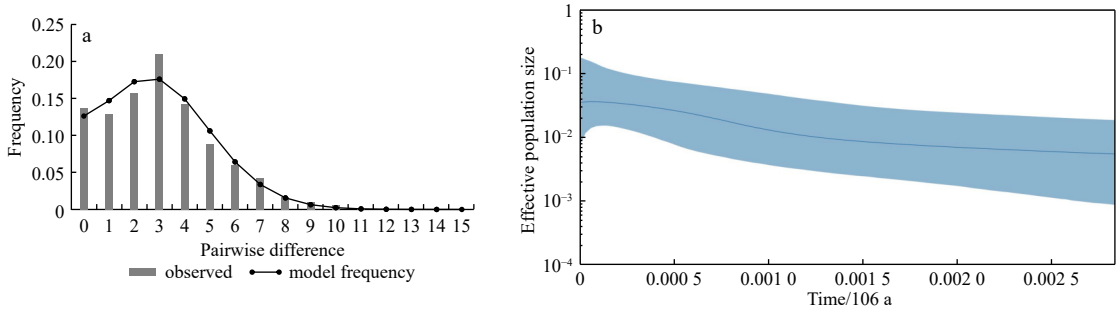

Fig. 4. Mismatch distributions (a) and Bayesian skyline (b) for *Acanthogobius ommaturus* based on control region sequences. Bayesian skyline plot of changes of effective population size through time for *Acanthogobius ommaturus*.

Table 3. Pairwise F_{st} (above diagonal) and $(\delta\mu)^2$ (below diagonal) among populations of *Acanthogobius ommaturus*

Population	RS	CY	XM	ZS	QD
RS	/	0.018 5*	0.008 3	0.023 5*	0.022 2*
CY	0.781	/	0.006 1	0.030 6*	0.044 0
XM	1.320	0.951	/	0.024 0*	0.028 3*
ZS	3.883	5.171	6.277	/	0.022 9*
QD	2.815	3.646	2.415	8.726	/

Note: * significant at P<0.05. ZS: Zhoushan; CY: Changyi; QD: Qingdao; RS: Rushan; XM: Xiamen.

4.2 *Weak and significant population genetic structure*

The results of mitochondrial marker indicated that there was gene exchange among 15 populations of *A. ommaturus*. The juvenile of *A. ommaturus* may disperse with ocean current, meanwhile, ocean currents were predicted to play an important role in promoting gene flow among populations (Hwang and Wong, 2005; Song et al., 2010a; Lu et al., 2018). There were relatively significant genetic differentiation between population of Zhoushan and other populations, which was similar with the results by Song et al. (2010b). The weak swimming ability of *A. ommaturus* larvae and a certain geographical distance would cause genetic differentiation of the Bohai Sea and East China Sea populations (Ovenden et al., 2004; Rocha et al., 2005).

The results of 3D-FCA showed that population of Zhoushan was obviously separated from the other four populations, while populations of Xiamen, Changyi and Rushan were overlapped and clustered together. Moreover, the results of UPGMA cluster tree based on genetic distance showed that population of Zhoushan was far away from other populations and clustered into a single branch. Therefore, based on the results of genetic structure analysis of two markers, we believed that there were some genetic differences between population of Zhoushan and other populations.

The geographical distance was thought to be the main reason for genetic differentiation of the Bohai Sea and the East China Sea populations (Song et al., 2010b). However, there was no significant positive correlation between geographical distance and $(F_{st} / (1–F_{st}))$ in the present study. In the vast ocean, the long distance was generally considered to be the main factor affecting genetic differentiation among different geographical populations of marine fish (Palumbi, 1994), which was particularly obvious by nuclear gene markers (Cunningham et al., 2009). Although ocean currents can promote gene exchange among different geographical populations of marine fish, resulting in genetic homogeneity, significant genetic differentiation can be induced among populations with a longer geographical distance (Palumbi, 2003). We speculated that the genetic differentiation between population of Zhoushan and other populations might be affected by special geographical location (Cao, 2016; Zheng, 2015). The Changjiang River diluted water has a wide influence area in the East China Sea, including the Changjiang River Estuary fishing ground,

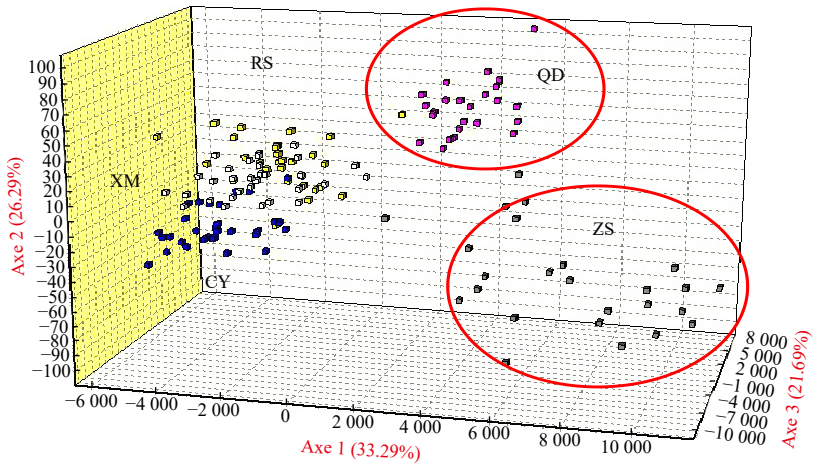

Fig. 5. Three dimensional factorial correspondence analysis (3D-FCA) showing relationships among *Acanthogobius ommaturus* populations based on fourteen microsatellite loci. On the right was the number of haplotypes in each population. ZS: Zhoushan; CY: Changyi; QD: Qingdao; RS: Rushan; XM: Xiamen.

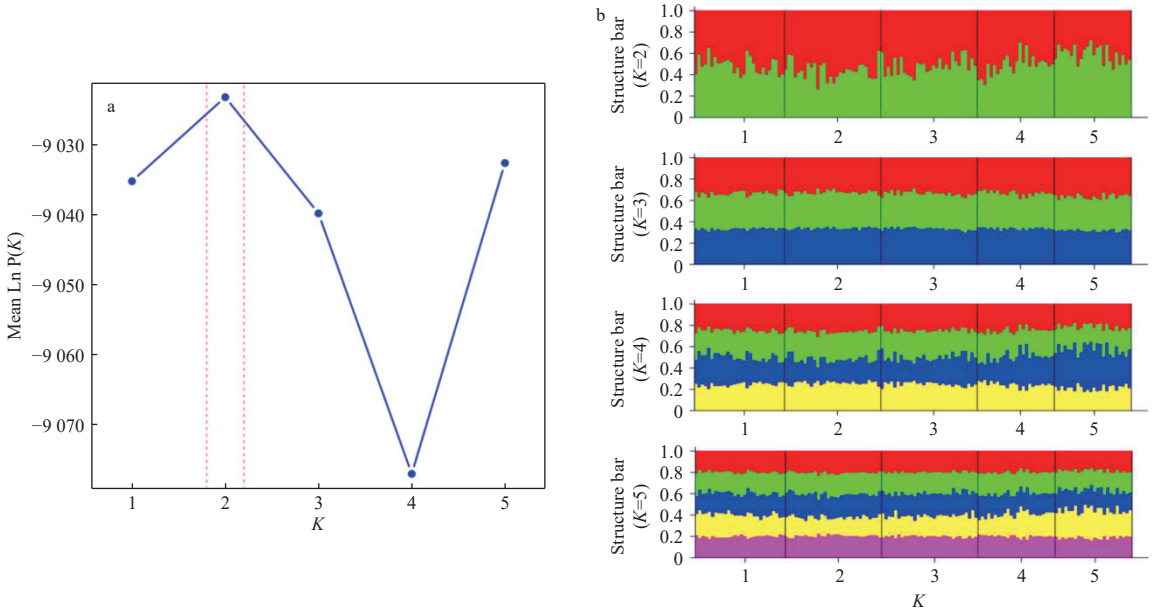

Fig. 6. The model choice criterion Ln P(K) (P(K): probability of K) of Structure analysis for each K value (a) and structure bar plots (K=2, 3, 4, 5) from fourteen microsatellite loci of five *Acanthogobius ommaturus* populations (b).

Table 4. Results of Wilcoxon's heterozygosity excess test, mode shift indicator for a genetic bottleneck in five populations of *Acanthogobius ommaturus*

Population	Wilcoxon sign-rank test			Mode shift
	IAM	TPM	SMM	
RS	0.991 70	1.000 00	1.000 00	L
CY	0.500 00	0.999 91	0.999 94	L
XM	0.619 57	0.999 94	0.999 94	L
ZS	0.500 00	0.999 79	0.999 94	L
QD	0.982 36	1.000 00	1.000 00	L

Note: RS: Rushan; CY: Changyi; XM: Xiamen; QD: Qingdao; ZS: Zhoushan.

Zhoushan fishing ground, etc (Zhang and Hu, 2005). Additionally, Zhoushan islands have complex environment (Yang, 2018), and the fish population nearby often showed different genetic characters, such as *Oplegnathus fasciatus* (Xiao et al., 2016), *Sebastiscus marmoratus* (Xu et al., 2017; Xu, 2018). Moreover, the population expansion and second connection after Pleistocene ice age from the refuge in the East China Sea may increase the peculiarity of population of Zhoushan (Liu et al., 2006; Song et al., 2010b; Wang et al., 2016b).

Through the results of the two genetic markers, we were more convinced that there were significant differences between population of Zhoushan and other populations. Moreover, the results of this study also indicated that microsatellite markers could detect more significant genetic differentiation for *A. ommaturus*. This fully illustrated the limitations of a single marker, thus the relevant genetic studies of other species should also be analyzed in combination with the results of multiple markers.

5 Conclusions

In this study, mitochondrial markers and microsatellite markers were combined to detect and analyze the genetic diversity

level and population genetic differentiation pattern of *A. ommaturus*. The results showed that the dispersal of juvenile fish and Pleistocene climate change may play an important role in the population differentiation of *A. ommaturus*. In addition, our results found high genetic diversity and the particularity of population of Zhoushan. Hence, more attention could be paid to population of Zhoushan in the follow-up.

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Supplementary information:

Table S1. Summary statistics for the variability 14 polymorphic microsatellite loci in five *Acanthogobius ommaturus* populations.

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