Genetics and Molecular Research

Characterization of novel expressed sequence tag-simple sequence repeat markers and analysis of genetic diversity in four geographic populations of *Thais luteostoma*

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Genet. Mol. Res. 15 (4): gmr15048702
Received April 8, 2016
Accepted August 9, 2016
Published October 24, 2016
DOI http://dx.doi.org/10.4238/gmr15048702

ABSTRACT. In this study, the genetic diversity in four geographic populations (Yushan Island, Zhoushan, Wenzhou, and Xiamen) of *Thais luteostoma* was analyzed using 21 microsatellite loci. The results of this study showed that the alleles obtained from different populations ranged from 2 to 8. The average number of alleles and effective alleles were 4.59 and 3.16, respectively. The observed heterozygosity and expected heterozygosity values were in the range 0.338-0.372 and 0.452-0.495, respectively. The polymorphism information content ranged from 0.273 to 0.785. We observed a high level of genetic diversity at 9 of the 21 microsatellite markers in these populations. The genetic differentiation indices of the four geographic populations ranged from 0.0312 to 0.0565, showing a medium level of genetic differentiation.

The genetic distances among populations ranged from 0.158 to 0.465. The UPGMA tree indicated that the Yushan Island and Zhoushan populations clustered first, and these subsequently clustered with the Wenzhou and Xiamen populations, indicating that the Xiamen shellfish population was least related to the other populations. The information regarding the shellfish population structure obtained in this study would facilitate the genetic breeding and conservation of *T. luteostoma*.

Key words: *Thais luteostoma*; Microsatellite; Geographic populations; Genetic diversity

INTRODUCTION

Thais luteostoma is an important marine shellfish species that inhabits the southern and northern coastal regions of China; this species is generally located in the lower intertidal zone, 20 m within canals between rocks and gravel. *T. luteostoma* mostly feeds on costive miniature gastropods, oysters, barnacles, and bryozoans (Zhang and Zhang, 2005; You and Chen, 2010). *T. luteostoma* shells are believed to possess medicinal value; moreover, its meat is considered a delicacy. Therefore, this shellfish has a high economic value. However, overfishing of this shellfish owing to the strong demand has led to a serious decline in *T. luteostoma* numbers over the past few years. In fact, *T. luteostoma* is no longer available in many of the coastal areas. Current research on *T. luteostoma* is mainly focused on the toxicity, nutritional values, imposex, and radula of this species (Guan and Han, 2004; Lu et al., 2007; Zhu et al., 2008; Huang et al., 2013). However, *T. luteostoma* has hitherto not been subjected to microsatellite genetic analysis.

Microsatellites, or simple sequence repeats (SSRs), are rich in polymorphic sites and are inherited co-dominantly; advantages of SSR sequencing include its repeatability and simplicity of method. Therefore, this method has been widely used to evaluate genetic diversity, genetic linkage, map construction, and molecular marker-assisted breeding, among others (Somers et al., 2004). The presence of SSRs in marine shellfish has facilitated the analysis of genetic diversity in various shellfish species, including *Crassostrea gigas*, *Chlamys farreri*, *Meretrix meretrix*, *Sinonovacula constricta*, and *Tegillarca granosa* (Zhan et al., 2008; Bai et al., 2011; Li et al., 2011; Lu et al., 2011; Wang et al., 2011; Zhang et al., 2012; Dong et al., 2013; Wu et al., 2014).

In this study, the genetic diversity of wild *T. luteostoma* populations collected from four different regions of China (Yushan Island, Zhoushan, Wenzhou, and Xiamen) was analyzed using 21 microsatellite markers. Additionally, the potential genetic differentiation in Chinese populations of *T. luteostoma* was assessed.

Yushan Island is the name given to a group of islands located in the East China Sea. It is a part of Shipu Town, Xiangshan County, Zhejiang Province. Yushan Island is composed of 13 islands and 41 reefs, and is located approximately 27 miles from the mainland. The transparency of seawater around this island extends up to 10 m; thus, it is a typical oceanic island. In addition to its unique geographical environment, the *T. luteostoma* from this island show the characteristics of ecological isolation. This project provides useful information for the protection and sustainable development of the Yushan Island *T. luteostoma* germplasm resource, as well as a theoretical basis for improving the genetic diversity of *T. luteostoma*.

MATERIAL AND METHODS

Sample collection and genomic DNA extraction

One hundred and sixty *T. luteostoma* samples were obtained from four locations on the Eastern coast of China (Yushan Island, Zhoushan, Wenzhou, and Xiamen) in 2015. The sample information is summarized in Table 1. The collected samples were analyzed in the Key Laboratory of Aquatic Germplasm Resources of Zhejiang Province. Gastropods from the samples were preserved in 100% ethanol at the sampling site and transported to the laboratory for DNA extraction. Genomic DNA was extracted from foot muscles using a modified phenol-chloroform system-based procedure (Taggart et al., 1992) and the DNA fragments were detected on 1% agarose gels.

Table 1. *Thais luteostoma* sampling sites.

Populations	Latitude/longitude	Sampling date	Sampling number
Yushan Island (YS)	N 28°53.3'2.20" E 122°15'7.49"	March 2015	40
Zhoushan (ZS)	N 30°43'1.68" E 122°46'3.18"	May 2015	40
Wenzhou (WZ)	N 27°29'37.44" E 121°05'15.62"	August 2015	40
Xiamen (XM)	N 24°28'55.96" E 118°05'4.12"	July 2015	40

Amplification of microsatellite loci

The extracted DNA sequences were amplified by polymerase chain reaction (PCR) using 21 microsatellite markers developed by our laboratory. The primer sequences, microsatellite core sequences, and optimum PCR amplification conditions are listed in Table 2. The 25-μL PCR mixtures comprised 80-100 ng genomic DNA, 0.8 μM each primer, 1X PCR buffer (20 mM Tris-HCl, 20 mM KCl, and 10 mM $(NH_4)_2SO_4$; pH 8.4), 0.2 mM dNTP mix, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (TaKaRa, Otsu, Japan). The samples were amplified using the following thermal cycling conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at temperatures as shown in Table 2 for 40 s, extension at 72°C for 45 s, and a final extension at 72°C for 8 min. The amplified sequences were visualized on 8% polyacrylamide gels and analyzed using a Bio-Rad Mycycler (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The number of alleles per locus and the range of allele sizes, as well as the observed heterozygosity (H_O) and expected heterozygosity (H_E), were calculated using the Cervus software v.3.0 (Kalinowski et al., 2007). Conformance with the Hardy-Weinberg equilibrium (HWE), allele number (N_A), effective allele number (N_E), and F-Statistics were assessed using PopGene32 (Yeh et al., 2000). All results were corrected for multiple simultaneous comparisons using the Bonferroni correction (Rice, 1989).

Table 2. Characteristics of 21 pairs of microsatellite primers of *Thais luteostoma*.

Locus	Primer sequence (5'-3')	Repeat mode	Annealing temperature (°C)	Product size
TL01	F:TACAGGCCTCTGCCAAGTCT R:CACAGGGGAAAACTGGCTAA	$(CTGC)_5$	49.8	210-225
TL02	F:CCAGTTTGGGGTCTGTCTGT R:TATCACGACCATCCTCCCTC	$(CCTG)_5$	55.2	155-180
TL06	F:TACCAGATGAAACGCCATGA R:AATGGTGTGGCCAAATAAGG	$(GTGA)_5$	59.6	265-280
TL07	F:GAGTCGTTGCTTCCATAGGC R:AAAAACCATTCTGGCAGACG	$(CCTT)_5$	59.6	135-145
TL08	F:TGGTAATCCCAAAACTGCAA R:GGACCCCCACAGCTATTTTT	$(TTAA)_6$	57	210-230
TL12	F:CCACACCACACCTTTCCTCT R:CAGACGTGTATTCATGCGCT	$(GCT)_7$	62	250-270
TL13	F:TATCAGCCCGTTCTTCCAAC R:CTCCTCGCCAGTAACGAGTC	$(GTT)_7$	62	210-225
TL15	F:ACAGGAGCTGGTGCTGTTCT R:GCTGAGGTATGCTGTTCCGT	$(AGG)_7$	64.6	230-245
TL16	F:GCAACCAAGTCTCCAAAAGC R:CTTTGGCTGTGTCTGGATCA	$(GCT)_7$	62	185-205
TL18	F:CGTTCCCTTCTTTGTTTCCA R:GCGATTTTATCGCTCGCTAC	$(GTG)_6$	56.6	210-225
TL19	F:TGTCCATCTATCCCTCTGGC R:AGCCGCTAGTCCTACAGCAG	$(CAG)_6$	56.6	240-260
TL20	F:ATTGGGCACAGGAGATTCAG R:GAGAGAAGGAGGACGACGTG	$(GCA)_6$	58.4	215-235
TL22	F:GATGCAACAGATGCTGGCTA R:GGTGGTGGAAGTGGTAGTGG	$(CAC)_6$	58.4	250-265
TL24	F:GACTGAAGGTGGTGTGAGCA R:TCCAACTGTAGGCAGTGCAG	$(CTG)_6$	58.4	190-205
TL25	F:TGTCAAGAACTCCCCGTACC R:CTGACAGTGGCCTCACACAC	$(TGC)_6$	58.4	160-180
TL26	F:TGCCTGAGACATTGAGGATG R:GACGAGCCATTCTTTGTTCC	$(TG)_9$	58.4	210-220
TL29	F:ACGCATGTCAATTCGTCAAA R:TGAGCTGTGGTTGAAAAACG	$(CAAA)_5$	57	140-155
TL32	F:CACAAGACAAGCAAGGCAAA R:TCAGGAAAACGACCCACTTC	$(GCT)_6$	55.2	225-240
TL33	F:CCCATCCTCTGTCTGGTCAT R:GTCCAGTTCCGTGATGAGGT	$(CCA)_6$	48	160-225
TL35	F:AGAGGACTGAGCACCGACAT R:CTGCCTGGCAAAATCTTGTT	$(TGA)_7$	62	235-250
TL37	F:GGCTGTGTTTGGGAAGTCAT R:TCATCAGCTACAGCAGCACC	$(TGT)_7$	62	170-185

RESULTS

Genetic diversity

One hundred and thirty-four different alleles were observed across all loci of all samples (Table 3). A majority of the loci detected in the four populations showed a high degree of polymorphism. The average observed and expected values of heterozygosity for all populations ranged from 0.338 to 0.372 and from 0.452 to 0.495, respectively. These results suggested that 21 loci were polymorphic in the four wild populations of *T. luteostoma.*

The N_A per locus (total: 21 loci) ranged from 2 to 8 in each population, and the N_E was between 0.96 and 6.56 (Table 3). The Xiamen (XM) population showed the highest N_A (mean = 5.1), N_E (mean = 3.38), H_E (mean = 0.495), and polymorphism information content (PIC) (mean = 0.484), while the Yushan Island (YS) population presented the lowest values (mean

Table 3. Twenty one-site specific recombination analysis of 4 populations in *Thais luteostoma.*

Population	Microsatellite loci										
	TL01	TL02	TL06	TL07	TL08	TL12	TL13	TL15	TL16	TL18	TL19
YS											
N_A	4	5	5	4	5	2	5	3	5	3	5
N_E	3.74	4.36	4.21	3.56	3.79	1.28	4.22	1.68	4.32	1.76	4.57
H_O	0.400	0.400	0.200	0.150	0.550	0.000	0.800	0.450	0.750	0.350	0.550
H_E	0.400	0.501	0.523	0.359	0.597	0.389	0.476	0.301	0.519	0.386	0.621
PIC	0.535	0.523	0.424	0.354	0.515	0.372	0.503	0.343	0.515	0.329	0.503
P value	0.0021	0.0000	0.0019	0.2224	0.0024	0.0013	0.0030	0.0004	0.0000	0.0084	0.0022
ZS											
N_A	5	6	5	4	6	2	6	3	5	4	6
N_E	4.37	4.16	3.18	2.11	4.58	0.96	5.03	1.34	3.55	2.19	4.68
H_O	0.472	0.433	0.239	0.230	0.540	0.100	0.574	0.489	0.643	0.344	0.632
H_E	0.605	0.523	0.547	0.389	0.612	0.474	0.627	0.374	0.577	0.406	0.674
PIC	0.612	0.601	0.433	0.365	0.597	0.273	0.508	0.349	0.606	0.377	0.581
P value	0.0098	0.5410	0.0030	0.0109	0.0230	0.1279	0.0119	0.0139	0.0026	0.0000	0.3554

	TL20	TL22	TL24	TL25	TL26	TL29	TL32	TL33	TL35	TL37	
YS											
N_A	4	4	4	3	4	3	3	4	5	4	
N_E	3.22	3.66	2.96	1.73	3.25	2.73	2.16	3.13	4.13	3.16	
H_O	0.050	0.153	0.052	0.350	0.150	0.150	0.450	0.200	0.550	0.400	
H_E	0.347	0.410	0.438	0.452	0.305	0.230	0.561	0.543	0.491	0.407	
PIC	0.350	0.384	0.306	0.350	0.317	0.402	0.573	0.535	0.518	0.379	
P value	0.0002	0.1399	0.1012	0.0034	0.0000	0.2251	0.0031	0.0103	0.0031	0.0016	
ZS											
N_A	5	4	4	3	3	3	4	5	6	3	
N_E	4.20	2.07	3.06	1.21	2.75	2.07	2.05	3.15	3.03	1.97	
H_O	0.133	0.216	0.130	0.388	0.210	0.177	0.388	0.132	0.437	0.345	
H_E	0.374	0.477	0.342	0.274	0.276	0.367	0.578	0.543	0.564	0.324	
PIC	0.388	0.342	0.346	0.334	0.298	0.389	0.544	0.598	0.574	0.354	
P value	0.0004	0.0079	0.5310	0.0310	0.0112	0.0134	0.1369	0.0129	0.0156	0.0000	

	TL01	TL02	TL06	TL07	TL08	TL12	TL13	TL15	TL16	TL18	TL19
WZ											
N_A	6	6	4	3	7	3	6	4	6	4	7
N_E	4.18	3.95	2.16	1.52	4.95	2.01	5.17	1.76	4.09	2.23	5.01
H_O	0.450	0.401	0.302	0.245	0.520	0.128	0.554	0.453	0.574	0.275	0.577
H_E	0.602	0.493	0.556	0.345	0.572	0.375	0.495	0.275	0.612	0.367	0.689
PIC	0.616	0.567	0.446	0.352	0.577	0.295	0.543	0.347	0.569	0.345	0.578
P value	0.2965	0.0805	0.0000	0.0037	0.6225	0.3418	0.1093	0.0006	0.0053	0.6225	0.3418
XM											
N_A	6	6	5	3	8	3	6	4	6	4	7
N_E	4.13	4.16	3.27	1.84	6.56	1.87	4.12	2.12	3.19	2.18	5.33
H_O	0.472	0.377	0.321	0.276	0.456	0.203	0.495	0.475	0.563	0.305	0.561
H_E	0.595	0.610	0.528	0.388	0.594	0.508	0.586	0.383	0.594	0.355	0.601
PIC	0.627	0.580	0.432	0.354	0.785	0.275	0.579	0.356	0.552	0.365	0.662
P value	0.1093	0.0036	0.0034	0.1671	0.5932	0.0402	0.2272	0.0000	0.2792	0.2246	0.0013

	TL20	TL22	TL24	TL25	TL26	TL29	TL32	TL33	TL35	TL37	
WZ											
N_A	5	4	5	4	4	4	5	5	6	4	
N_E	3.28	2.31	3.11	2.76	3.01	2.02	2.21	3.11	4.01	2.09	
H_O	0.234	0.234	0.175	0.301	0.264	0.185	0.401	0.237	0.495	0.411	
H_E	0.389	0.452	0.348	0.421	0.341	0.344	0.588	0.468	0.588	0.395	
PIC	0.395	0.353	0.324	0.327	0.302	0.357	0.560	0.578	0.558	0.365	
P value	0.0001	0.3451	0.2356	0.0816	0.0000	0.0006	0.6215	0.3412	0.1091	0.0002	
XM											
N_A	5	4	5	4	4	4	6	6	7	4	
N_E	3.18	2.76	3.42	2.88	2.12	2.35	4.12	4.11	5.15	2.19	
H_O	0.246	0.346	0.289	0.349	0.278	0.199	0.399	0.308	0.505	0.398	
H_E	0.406	0.458	0.345	0.406	0.350	0.450	0.645	0.593	0.573	0.421	
PIC	0.345	0.377	0.349	0.397	0.366	0.423	0.588	0.595	0.683	0.426	
P value	0.0006	0.0763	0.0401	0.2275	0.0016	0.2385	0.2262	0.0014	0.1457	0.0036	

N_A = 4, mean H_E = 0.452, mean PIC = 0.430); the locus TL02 was a highly polymorphic locus in all four populations of *T. luteostoma* (Figure 1).

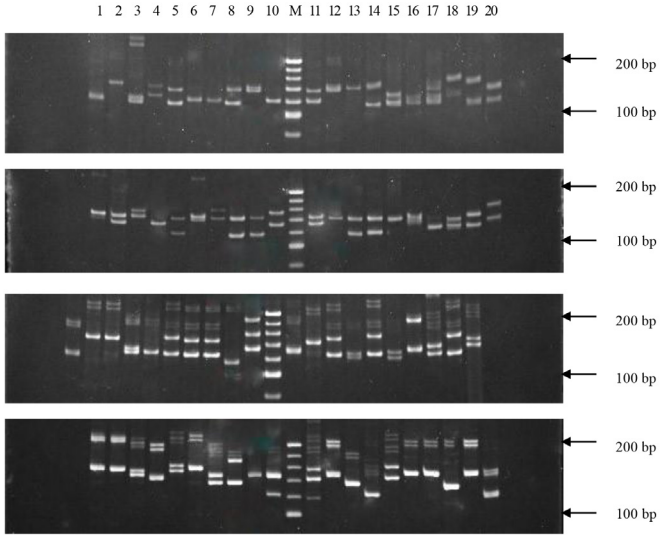

Figure 1. Amplified allele of loci TL02 in partial populations of *Thais luteostoma* from Yushan Island, Zhoushan, Wenzhou, and Xiamen.

Significant deviations from HWE occurred at five loci (TL02, TL15, TL16, TL20, and TL26) in the YS population, three loci (TL18, TL20, and TL37) in the Zhoushan (ZS) population, six loci (TL06, TL15, TL20, TL26, TL29, and TL37) in the Wenzhou (WZ) population, and two loci (TL15 and TL20) in the XM population after the Bonferroni correction. Additionally, no significant genotypic linkage disequilibrium was detected between the loci (P > 0.05/40) in each case.

Genetic differentiation among populations

The genetic differentiation index (F_{ST}) is a guideline for scaling the genetic variance of a population. Populations with F_{ST} values >0.25 (compared to other populations) showed a high level of genetic differentiation; however, populations with 0.15 < F_{ST} < 0.25 and F_{ST} < 0.15 indicated medium and low levels of genetic differentiation (Hartl and Clark, 1997). F_{ST} between the four groups of *T. luteostoma* was 0.0312-0.0565 (Table 4); the F_{ST} was highest (0.0565) between the ZS and XM populations and lowest (0.0312) between the YS and ZS populations.

Table 4. Coefficient of gene differentiation (F_{ST}) in four geographic populations of *Thais luteostoma.*

Population	YS	ZS	WZ	XM
YS	-			
ZS	0.0312	-		
WZ	0.0431	0.0449	-	
XM	0.0512	0.0565	0.0473	-

Cluster analysis

The unbiased genetic distance and unbiased genetic similarity indices among the four groups were calculated with PopGene, using the method described by Nei (1987) (Table 5). The results showed that the genetic distance and genetic similarity index of *T. luteostoma* among the four groups were 0.158-0.465 and 0.692-0.781, respectively. The genetic distance between the shellfish from YS and ZS was the lowest (0.158), while the genetic similarity index was the highest (0.781). However, the ZS and XM groups exhibited the highest genetic distance and lowest genetic similarity index (0.465 and 0.692, respectively).

Table 5. Nei's genetic identification (above diagonal) and genetic distances (below diagonal) between four geographic populations of *Thais luteostoma.*

Population	YS	ZS	WZ	XM
YS	-	0.781	0.743	0.701
ZS	0.158	-	0.727	0.692
WZ	0.234	0.346	-	0.734
XM	0.421	0.465	0.405	-

The genetic distance (UPGMA) was calculated on the MEGA 4.0 platform using a clustering analysis method (Figure 2). In this method, shellfish from Yushan Island were first clustered with those from Zhoushan, and subsequently with shellfish from Wenzhou and Xiamen.

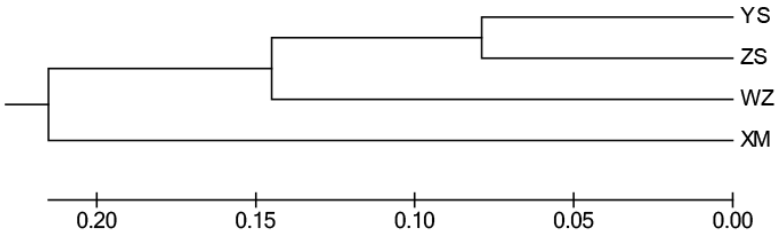

Figure 2. Cluster dendrogram of four geographic populations of *Thais luteostoma.*

DISCUSSION

Genetic diversity comprises the degree of population genetic variation and its different geographical patterns, that is, the genetic structure of the population, including all the genetic information. This diversity is required for a species to adapt to a variety of environmental conditions in order to survive and evolve, and is the basis for population evaluation. The ability of a population to adapt to different environments, and its evolutionary potential, increases with the genetic variation in the population (Zhang et al., 2003; Ma et al., 2009).

Microsatellite markers have several advantages, including a high degree of polymorphism, stability of results, high heterozygosity, convenient detection, and codominant inheritance; therefore, they can be used to distinguish between heterozygous and homozygous individuals. Consequently, these markers can be used to study population genetic diversity, phylogenetic relationships, and evolution. In this study, a PIC ranging from 0.273 to 0.785 was observed in 21 microsatellite loci, with shellfish from Yushan Island exhibiting the lowest PIC (0.430). The selected markers provided abundant genetic information; nine pairs showed

high polymorphism and could be used to analyze the genetic diversity in other *T. luteostoma* populations. Heterozygosity, which reflects the richness of the allele in populations and the degree of genetic variation in individuals, plays a major role in the measurement of genetic variation within populations. A high degree of genetic variation, indicated by a high numerical value, is indicative of rich genetic information. On the other hand, low genetic variation is indicative of poor genetic diversity. Liu et al. (2009) reported an average expected heterozygosity of 0.678-0.7017 in different seedling sources of *Mizuhopecten yessoensis* in Japan and China. Qu et al. (2009) reported an average expected heterozygosity of 0.590-0.615 in three breeding groups of nacre. In this study, an analysis of genetic variations using microsatellites revealed a higher degree of heterozygosity in wild shellfish compared to bred shellfish. The observed heterozygosity in *T. luteostoma* obtained from four geographical regions was in the range 0.338-0.372 (compared to an expected heterozygosity of 0.452-0.495). Yushan Island showed the lowest expected heterozygosity (0.452). Therefore, *T. luteostoma* from Yushan Island has low species diversity, necessitating the urgent implantation of protective protocols.

The Hardy-Weinberg equilibrium law states that a group in a state of balance shows no significant differences between the observed and expected heterozygosity; moreover, the frequency distribution of all alleles in the group should be relatively stable. Significant differences between the observed and expected heterozygosity are correlated with a higher range of allele frequency. Analysis of the obtained P values for conformance with the HWE revealed that 5, 6, 3, and 2 microsatellite loci in shellfish obtained from Yushan Island, Wenzhou, Zhoushan, and Xiamen, respectively, deviated from the HWE, which was attributed to human disturbance, mutations, or migration; however, the overall genetic structure of *T. luteostoma* was in equilibrium.

In this study, the genetic differentiation index of the four *T. luteostoma* groups was in the range 0.0312-0.0565, which indicates a moderate genetic differentiation among the four groups; the largest differentiation was observed between the Xiamen and Zhoushan groups (0.0565). This could be attributed to the geographic region (far away from the coast). Moreover, the wild-type group is less affected by artificial selection, probably because of the geographical isolation, thus rendering a medium level of genetic differentiation. The genetic distance is generally defined as a measure of evolutionary divergence of genes originating from a common ancestor (Nei, 1978). Under this assumption, the genetic distance is usually defined as a function of gene frequency, showing differences between the groups; therefore, the genetic relationship among groups can be determined according to their size. The genotypic differences are positively correlated with the genetic distance among groups, which indicates that the genetic diversity reduces with an increase in genetic distance of a related group. In this study, the genetic distance was analyzed by 21 microsatellite markers. Previously, Zhang et al. (2005) analyzed the genetic distance among species using different microsatellite makers under random conditions. Therefore, at least 15 microsatellite markers must be used to analyze the genetic relationship between groups, to ensure the accuracy of the genetic distance and the results. The genetic distances among the four sample groups of *T. luteostoma* were in the range 0.158-0.465; the genetic distance between Yushan Island and Zhoushan was the smallest, while samples from Xiamen and Zhoushan showed the largest genetic distance.

The results of this study indicated that the genetic diversity of shellfish species from Yushan Island was lower than that seen in the other three groups; the samples from Xiamen showed a certain degree of genetic differentiation from the other three groups. The preservation and protection of shellfish from Yushan Island require the selection of healthy

and mature wild individuals as parents, as well as hybridization with Xiamen shellfish (which presents the largest genetic distance from Yushan shellfish). The genetic exchange among different geographic groups of shellfish and the increased hybridization advantage would allow the offspring to better adapt to the isolated-island-sea environment. This would also avoid inbreeding, which leads to a lack of heterozygosity.

Sustainable development of *T. luteostoma* involves standardization of the breeding mode, stringent supervision, setting and monitoring of bottom stream standards, and the development of a set of reasonable and scientific protection measures. This will prevent the degradation of wild germplasm resources and deletion of excellent characteristics. Additionally, it will facilitate better breeding and conservation to ensure the reasonable and sustainable utilization of *T. luteostoma* resources, and promote the healthy and harmonious development of *T. luteostoma* aquaculture.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by grants provided by the Zhejiang Province Public Technology Applied Research Project (#2016C33090) and the Ningbo Agricultural Science and Technology Research Project (#2014C10018).

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