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Population genetic diversity of marble goby (*Oxyeleotris marmoratus*) inferred from mitochondrial DNA and microsatellite analysis

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Introduction

The marble goby (*Oxyeleotris marmoratus*, Bleeker) also known as the sand goby is widely distributed in Southeast Asia, especially in Malaysia, Singapore, Thailand and Vietnam (Inger and Chin 1963; Mohsin and Ambak 1983; Cheah *et al*. 1994; Loo *et al*. 2015). It is an important commercial fish because of its good taste, rich nutrition, large size and potential in aquaculture. In China, the marble goby was introduced in 1986, and the comprehensive study was conducted in the artificial propagation and breeding. Nowadays, it is widely bred in southern China, such as Hainan, Guangdong and Fujian. In recent years, artificial culture of marble goby has suffered from germplasm degradation, such as earlier sexual maturity and reduced disease resistance which leads to a drastic reduction of the production (Lin and Kaewpaitoon 2000). Further, the number of wild marble goby was decreased dramatically due to habitat destruction and overfishing (Cheah *et al*. 1994). As a consequence, the marble goby may have the problem of genetic diversity, especially in Vietnam and China, the major breeding countries of marble goby.

Mitochondrial DNA is widely used to identify population genetic structure and variability due to its rapid evolutionary rate and complete maternal inheritance (Wilson *et al*. 1985; Sato *et al*. 2004). Most vertebrate mtDNAs contain a single noncoding segment called displacement-loop region (D-loop region). The D-loop region of the mtDNA is highly polymorphic and it has 5–10 times rate of nucleotide substitution than nuclear DNA (Aquadro and Greenberg 1983). Therefore, it is often used to study the phylogenetic relationship, to evaluate the population diversity, and to determine the phylogeographic structure in fish species (Zou *et al*. 2015; Chan *et al*. 2016; Zhao *et al*. 2016). As for nuclear molecular markers, simple sequence repeat (SSR) markers are more informative and powerful tools for population genetic study because they are multiallelic, codominant and abundant in eukaryotic genomes when compared with other molecular marker systems. Thus, both mtDNA control region and SSR markers are favoured as genetic tools for assessing genetic variation, genome mapping, population structure and intraspecific phylogenesis (Romana-Eguia *et al*. 2004; Sharma *et al*. 2015). In this study, 14 microsatellite and mitochondrial DNA markers were used to analyse the genetic diversity and population structure among three populations of marble goby. Our study will provide valuable information to facilitate the breeding programme and the genetic conservation of marble goby.

Materials and methods

Sample collection and DNA extraction

Fin tissues of marble goby were acquired from a total of 91 individuals at three locations including Mekong river of Ho Chi Minh city (N 106°63′, E 10 °76′), Vietnam (n = 28), Tianyuan aquatic seeding field of Zhuhai city (N 113°58′, E 22°27′), Guangdong, China (n = 31)

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Keywords. marble goby; genetic diversity; mtDNA control region; microsatellite; population structure.

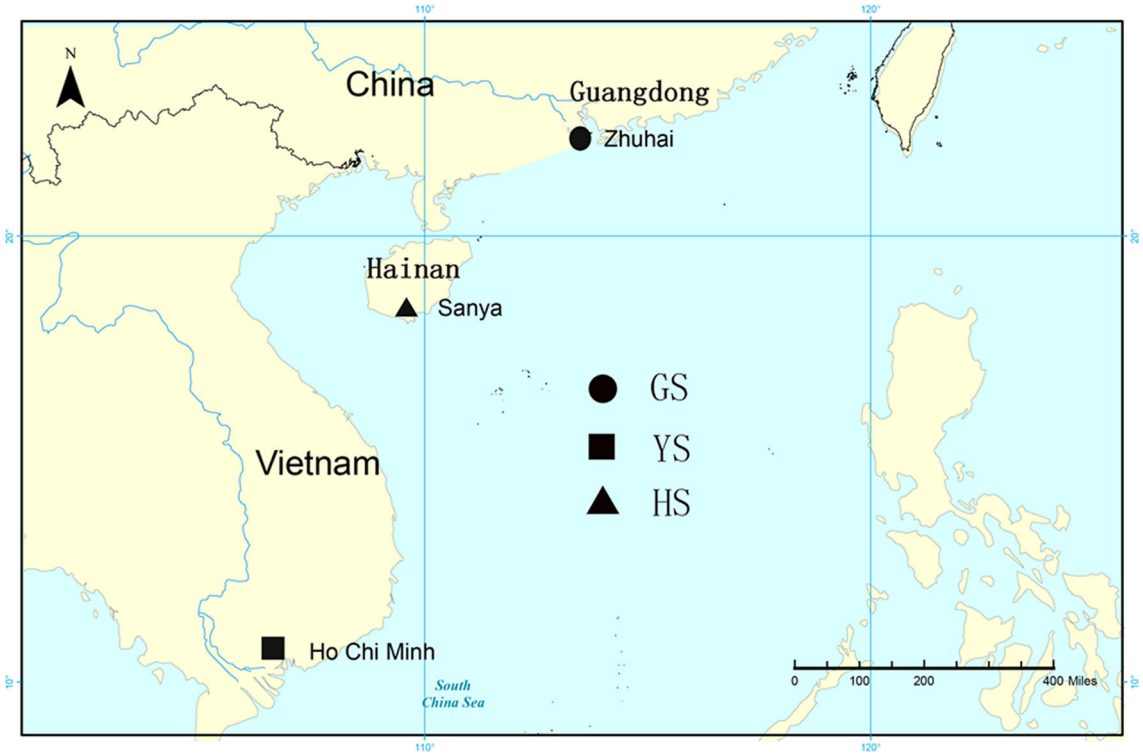

Figure 1. Three sampling locations of marble goby: Vietnam, Guangdong and Hainan are represented by square, circle and triangle dot, respectively.

Table 1. Information of sample collection.

Populations (abbreviation)	Latitude longitude	Sampling date	Sample number
Vietnam (YS)	N106°63′ E10°76′	Jan. 2011	28
Guangdong (GS)	N113°58′ E22°27′	Mar. 2011	31
Hainan (HS)	N109°51′ E18°31′	Apr. 2011	32

and Duanshan fry breeding base of Sanya city, Hainan (N 109°51′, E 18°31′), China ($n = 32$) (figure 1). Marble goby samples from three locations were designated as 'YS', 'GS' and 'HS', respectively. The details of the samples that we collected are listed in table 1. Total genomic DNA was extracted following standard proteinase K digestion and phenol–chloroform extraction method as described previously (Hayano *et al*. 2003).

Mitochondrial control region sequencing and analysis

Using Primer Premier 5.0 software, the primer pair of noncoding D-loop region was referred to its closely-related species, *Eleotris acanthopoma* (GenBank accession no. EU369677) (5′-CTGCCTCAAAGAAGGGAGATT-3′, 5′-TCACAGGGGTGCGGATACT-3′). PCR was carried out in 25 μL reaction mixture containing 2.5 μL 10× PCR buffer, 2 μL 25 mM $MgCl_2$, 2 μL 2 mM dNTP, 1 U *Taq* DNA polymerase, 0.5 μL each primer (10 μM), 16.3 μ L ultrapure water and 1 μL (200 ng/μL) DNA template. PCR reaction conditions were performed by an initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 52°C for 45 s and 72°C for 1 min with a final extension at 72°C for 8 min. The products were sequenced directly using an ABI 3730XL automatic sequenced by BGI-Premier Scientific Partner (Beijing, China). The sequences were identified as mtDNA control region via the comparison with the sequences existed in NCBI (accession numbers: EU177621–EU177628) based on nBLAST. The DNA sequences were aligned with ClustalX 2.0 multiple-alignment program. Selection of the best-fit nucleotide substitution models was performed in jmodeltest 2.0. The most appropriate nucleotide substitution model was GTR+I+G. The phylogenetic analyses were performed using the maximum likelihood (ML) with the PhyML 3.0 program. Bootstrapping was performed with 1000 replications. Nucleotide diversity (π) and haplotype diversity (h) and Tajima's D were estimated by using DnaSP 5.0. Population structure was evaluated by F_{ST}, which was calculated by an analysis of pairwise difference (distance method) with the analysis of molecular variance (AMOVA) in Arlequin ver. 3.1.

Table 2. Characterization of 14 marble goby (*O. marmoratus*) microsatellite loci.

Locus	Repeat motif	Primer sequences (5′–3′)	Size range (bp)	T_a (°C)	N_A	H_o	H_e	P (HWE)	Accession no.
H113	(CA)11	F: GGAAGCTGCTGACCTTGACTC R: CCTATGGTCCGTCCAGAGTGT	148–188	60	10	0.6604	0.7128	0.1519	JF264394
H137	(GT)9	F: GATCAGAGGGTTCAGAAAGCAG R: CATTACAGCACCGACAGAGGA	210–236	58	4	0.2075	0.2209	0.5780	JF264402
H27	(TG)21N105(AC)5	F: GATCAACAGTGTTTGCGTTAGG R: TCTCACCTGATGGAAAGATGG	242–302	56	13	0.7358	0.7686	0.2894	JF264376
H60	(GT)13N15(TG)6	F: GTTTGGCTGAAATGGTAGTGTG R: TGGAATGATGCTAGTGGCTGT	160–200	56	3	0.0943	0.1255	0.0854	JF264381
H167	(AC)9N73(AC)6	F: TCCATTACAGCACCGACAGAG R: GATCAGAGGGTTCAGAAAGCAG	220–232	58	3	0.2830	0.3245	0.5791	JF264409
H94	(TG)12	F: GAGGATTTCCCGCTTCTATG R: GCCGTCTTTCTGTTTGTCTTG	146–166	56	5	0.2452	0.3012	0.1256	JF264391
Y12	(CA)7N4(CA)6	F: ATTATGATCCCCCACCAGCT R: TGTGATTGTCCCCTCTCACAG	207–229	57	3	0.3208	0.3859	0.2631	JF419699
H117	(CA)8N111(CA)5	F: ATAGCTCTGCGACGTGATTGG R: GGACTTAGCTTTACCCTGTGGA	276–286	58	4	0.3962	0.4381	0.4815	JF264411
H138	(TC)5(AC)13	F: TAAGCCAGTGCCAGCAGAGT R: GCCCTGATTGTGACTGTGGAG	138–172	58	6	0.4717	0.4963	0.3953	JF264403
H191	(AC)11N51(GA)10N9(TG)4T2(TG)6	F: TGACATCTGTCCTGGCTTCG R: GCCTGCGTCTTTGACAACTC	254–288	58	15	0.7170	0.8033	0.2897	JF264413
H56	(AC)12	F: GCGAATTGCTGCAAGTGAGA R: GGTTGGGAGGAAGTGTTAGGA	258–272	56	6	0.2453	0.2424	0.5998	JF264379
H142	(CA)6G(AC)8N5(CA)7	F: GAAATTGGAACGGGAGGCA R: ATGGGAGCCACGACTCACA	93–123	58	11	0.7547	0.7175	0.3493	JF264405
H97	(CA)24	F: AATCTGGCTTGACGCACTCT R: TTCCGCACGGTATCCTCTT	191–263	56	20	0.8491	0.8927	0.0445*	JF264392
M351	(TTCAA)5	F: GATCCTTTGCTCTGTTTCAG R: TCCTGGGTCTGTTTAGTGTAG	247–293	54	5	0.6981	0.6759	0.1523	JF419693

N_A, allele number; H_o, observed heterozygosity; H_e, expected heterozygosity; T_a, annealing temperature; HWE, Hardy–Weinberg equilibrium. Statistically significant at $*P < 0.05$

Table 3. Genetic diversity estimation and Tajima's *D*-statistics on mtDNA control region sequences (855 bp).

Sample	*N*	No. of haplotypes	Haplotype diversity, *h* (%)	Nucleotide diversity, π (%)	Mean pairwise difference	No. of variable sites	Tajima's *D*
YS	28	5	43.4	0.127	1.085	11	−2.014*
GS	31	7	53.5	0.308	2.628	12	−0.405
HS	32	14	80.4	0.455	3.869	25	−1.415
Total	91	20	61.6	0.311	2.647	29	−1.696

*Significant deviation from beta-distribution at $P < 0.05$.

Table 4. Number of fish from three populations of marble goby according to haplotype distribution.

Haplotypes	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20
YS														1	2			3	1	21
GS												1	3			3	1	1	1	21
HS	1	1	1	2	1	1	1	1	2	1	1					3	2			14
Total	1	1	1	2	1	1	1	1	2	1	1	1	3	1	2	6	3	4	2	56

Microsatellite genotyping analysis

Sixteen microsatellite markers of *O. marmoratus* were developed by traditional method with constructing microsatellite-enriched library (Luo *et al*. 2013). Fourteen polymorphic microsatellite loci were selected, which show relative high allele number. These microsatellite loci were used for genotyping the marble goby from three different regions: Vietnam (YS), Guangdong (GS), and Hainan (HS). The primer sequences, microsatellite core sequences, product sizes and annealing temperature are listed in table 2. PCR amplification was conducted in a reaction volume of 20 μL containing 100 ng of genomic DNA, 1.5 mM Tris-HCl, 0.4 mM $(NH_4)_2SO_4$, 0.0375 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each of primer and 0.5 U *Taq* polymerase (Fermentas). The PCR was performed at the following conditions: an initial hot start at 94°C for 5 min, followed by 30 cycles with denaturation at 94°C for 30 s, annealing at locus-specific temperature for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. Fragment size of amplified DNA was determined on an AB 3500XL automatic sequencer. Popgene32 was used to calculate the number of alleles (*A*), observed heterozygosity (H_o), expected heterozygosity (H_e) and Wright's fixation index (F_{IS}). Hardy–Weinberg equilibrium (HWE), polymorphism information content (PIC), variance in allele frequencies and F_{ST} was achieved by Arlequin 3.1 software. Estimates of gene flow were derived by the equation: $N_m = [(1/F_{ST}) - 1]/2$.

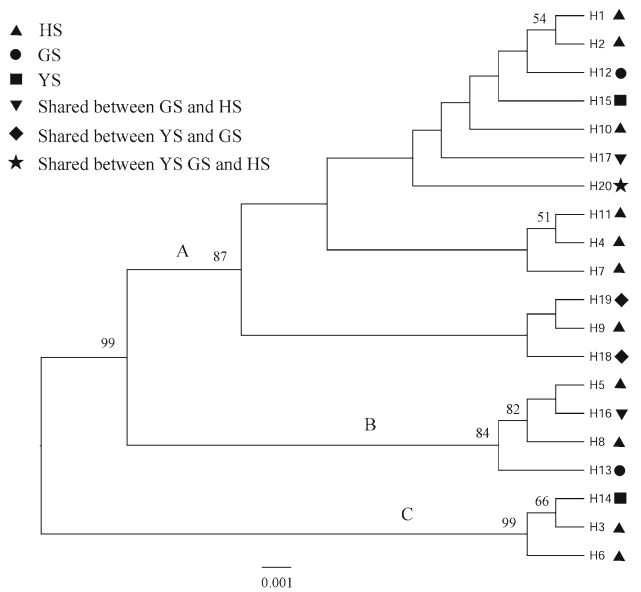

Figure 2. Maximum likelihood (ML) tree of the mtDNA control region haplotypes from *O. marmoratus*. The bootstrap proportions (>50%) in 1000 replications were shown as nodes.

Results and discussion

Sequence analysis of the 855-bp mtDNA control region revealed 29 variable sites in 91 marble goby individuals. The number of variable sites were 11, 12 and 25 in YS, GS and HS populations, respectively. Ninety-one sequences could be defined as 20 haplotypes based on the nucleotide variation. Haplotype and nucleotide diversities of the three populations ranged from 43.4 to 80.4% and 0.127 to 0.455%, respectively (table 3). The total nucleotide diversity (0.31%) and haplotype diversity (61.6%) were observed, which are similar to that of some fish species, such as shortnose sturgeon (*Acipenser brevirostrum*) (Grunwald *et al*. 2002) and chum salmon (*Oncorhynchus keta*) (Sato *et al*. 2004). A negative

Table 5. Genetic variation in 14 microsatellite loci in three breeding groups of marble goby.

Sample	Locus														Average across loci
	H113	H137	H27	H60	H167	H94	Y12	H117	H138	H191	H56	H142	H97	M351	
YS*N*	21	28	28	28	28	28	27	28	28	28	28	27	27	27	
A	6 (162–186)	3 (222–236)	11 (272–294)	1 (200–200)	2 (230–230)	3 (146–166)	5 (217–229)	3 (274–282)	6 (154–166)	7 (260–288)	4 (260–270)	9 (93–123)	15 (191–263)	4 (248–293)	5.642
H_e	0.753	0.260	0.786	0.000	0.036	0.260	0.575	0.519	0.678	0.646	0.260	0.805	0.798	0.687	0.504
H_o	0.762	0.071	0.821	0.000	0.036	0.000	0.148	0.607	0.500	0.250	0.250	0.704	0.519	0.926	0.400
PIC	0.690	0.240	0.747	–	0.035	0.240	0.504	0.397	0.620	0.607	0.242	0.762	0.770	0.611	0.497
F	− 0.037	0.720	− 0.064	–	− 0.018	1.000	0.738	− 0.192	0.249	0.606	0.022	0.109	0.338	− 0.373	
P	0.000*	0.000*	0.059	–	1.000	0.000*	0.000*	0.552	0.095	0.000*	0.449	0.000*	0.000*	0.005*	
GS *N*	31	31	31	31	31	31	31	31	31	31	31	31	31	31	
A	7 (148–184)	3 (210–236)	8 (242–298)	2 (180–200)	1 (232–232)	5 (146–166)	3 (207–229)	3 (276–282)	5 (138–162)	15 (252–284)	5 (258–270)	10 (93–123)	15 (203–263)	6 (247–293)	6.286
H_e	0.721	0.154	0.711	0.063	0.000	0.268	0.235	0.463	0.552	0.846	0.268	0.754	0.861	0.712	0.472
H_o	0.290	0.097	0.516	0.000	0.000	0.097	0.000	0.258	0.323	0.516	0.290	0.516	0.516	0.936	0.311
PIC	0.671	0.146	0.672	0.061	–	0.256	0.215	0.377	0.441	0.816	0.255	0.710	0.832	0.643	0.469
F_{IS}	0.591	0.361	0.262	1.000	–	0.633	1.000	0.434	0.406	0.380	− 0.103	0.304	0.391	-0.336	
P	0.000*	0.020*	0.000*	0.017*	–	0.000*	0.000*	0.002*	0.001*	0.000*	1.000	0.000*	0.000*	0.006*	
HS *N*	32	32	32	32	32	32	32	32	32	32	32	32	32	32	
A	7 (162–186)	3 (212–236)	11 (242–302)	2 (160–200)	3 (220–232)	3 (160–166)	2 (227–229)	3 (280–286)	7 (138–172)	10 (260–288)	5 (260–272)	9 (93–123)	16 (181–259)	4 (248–293)	6.071
H_e	0.658	0.298	0.789	0.062	0.253	0.301	0.310	0.441	0.549	0.755	0.233	0.732	0.874	0.652	0.493
H_o	0.562	0.281	0.656	0.000	0.156	0.031	0.000	0.344	0.531	0.281	0.188	0.375	0.688	1.000	0.364
PIC	0.605	0.265	0.755	0.059	0.229	0.272	0.258	0.363	0.500	0.715	0.222	0.687	0.849	0.575	0.454
F_{IS}	0.277	0.353	0.172	1.000	0.371	0.841	0.882	0.166	0.248	0.542	0.033	0.312	0.346	− 0.412	
P	0.001*	0.027*	0.005*	0.016*	0.009*	0.000*	0.000*	0.235	0.121	0.000*	0.050	0.000*	0.000*	0.000*	

N, sample size for successful PCR analysis; *A*, number of alleles (allele size range); H_e, expected heterozygosity; H_o, observed heterozygosity; PIC, polymorphism information content; F_{IS}, Wright's (1978) fixation index; *P*, *P* value of Hardy–Weinberg equilibrium; *Hardy–Weinberg equilibrium ($P < 0.05$); –, there is only one allele at the locus.

Table 6. AMOVA analyses of three marble goby populations, AMOVA-I obtained from mtDNA control region and AMOVA-II obtained from microsatellite loci.

	AMOVA-I			AMOVA-II		
Source of variation	Sum of squares	Variance components	Percentage of variation	Sum of squares	Variance components	Percentage of variation
Among populations	5.275	0.041Va	2.811	18.835	0.105Va	3.35
Within populations	123.714	1.406Vb	97.189	544.242	3.040Vb	96.65
Total	128.989	1.447		563.077	3.145	

Table 7. Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal).

Populations	YS	GS	HS
YS	–	0.9461	0.9464
GS	0.0554	–	0.9668
HS	0.0551	0.0338	–

Tajima's D value was observed in the YS populations ($P < 0.05$), which might be resulted from group expansion (Han *et al*. 2008). All haplotypes of mtDNA control region are available from GenBank under accession numbers of JN645822–JN645841. H20, comprising 61.5% of all samples, was the most widespread haplotype among three populations (table 4). It might derive from a single ancestor according to the theory of maternal inheritance of mtDNA. In the phylogenetic analyses, the maximum likelihood (ML) tree was constructed using the GTR+I+G model. Three clusters were supported by significant bootstrap proportions (node A: BP = 87%, node B: BP = 84% and node C: BP = 99%). However, ML tree could not clearly differentiate haplotypes of specimens from three different locations (figure 2). It could be explained by the occurrence of same haplotypes in different populations.

A total of 104 alleles at 14 microsatellite loci were detected from three different populations. Genetic heterozygosity which includes observed heterozygosity and expected heterozygosity. The average expected heterozygosity (H_e = 0.472–0.504) and observed heterozygosity (H_o = 0.311–0.400) at 14 loci were observed in our study (table 5), which is similar to the results of Ruzainah *et al*. (2009). YS populations (H_o = 0.400, H_e = 0.504) exhibited the highest value among all three samples, and this was consistent with the consequence of genetic diversity revealed by PIC value. Such results were ascribed to as if there is no wild marble goby resource in China. The population of marble goby of China was introduced from Southeast Asia. Through long-term artificial breeding, the genetic diversity of the cultured marble goby populations were relatively low compared to Vietnam population. Reduced genetic diversity in Chinese populations may result from founder effects. Founder effects could occur in artificial propagation when used in a small number of broodstock individuals or no wild individuals introduced, which can led to the loss of genetic diversity (Allendorf and Ryman 1987). HWE tests showed that most of the 14 loci deviated significantly from Hardy–Weinberg expectations ($P < 0.05$) and exhibited heterozygote deficiency. This might be caused by the presence of null alleles (Pemberton *et al*. 1995) or the Wahlund effect (Hartl and Clark 1997).

AMOVA was conducted to describe the variance components of marble goby populations. The results of AMOVA from mtDNA control region and microsatellite suggested that most of the variations occurred within populations (table 6), which is basically accordance with the population structure of marble goby in Southeast Asia (Chew *et al*. 2011). The pairwise genetic distance and genetic similarity were evaluated according to Nei's (1978) formula. GS and HS populations showed the lowest pairwise genetic distance (0.0338) and the highest pairwise genetic similarity (0.9668) (table 7), which suggested that the genetic distance between Chinese populations (GS–HS) was smaller than YS–GS and YS–HS. The results of mtDNA and microsatellite both indicated that higher genetic divergence and lower gene flow were found between Chinese and Vietnamese populations (table 8). F_{ST} value, in the present study, was much smaller when compared with marble goby based on the mtDNA (Chew *et al*. 2011).

Table 8. Genetic divergence (below diagonal) and gene flow (above diagonal) among three marble goby populations.

	mtDNA			Microsatellite		
Population	YS	GS	HS	YS	GS	HS
YS	–	7.585	10.164	–	11.605	12.546
GS	0.062*	–	–	0.041*	–	22.213
HS	0.047*	–0.004	–	0.038*	0.022*	–

*Significant deviation from beta-distribution at $P < 0.05$.

The difference may be related to the sampling locations and sample quantity. To further elucidate the genetic diversities of marble goby, more sampling locations, size and wild populations should be collected.

Our results demonstrated that the genetic diversity and genetic distance of Chinese populations were reduced when compared with the YS counterparts. The study, thus, suggests that the genetic conservation of marble goby populations in China is urgent. Moreover, it is important that the protection of wild stock and more wild resources should be introduced in artificial breeding to maintain the genetic diversity of cultivated populations. Our research provides valuable information for the management and conservation of the marble goby.

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