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Population Genetic Structure and Genetic Diversity of Coral Reef Species *Lethrinus olivaceus* in the South China Sea

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ABSTRACT

Lethrinus olivaceus is an important coral reef fish in the South China Sea. To reveal the genetic structure and genetic diversity of *L. olivaceus*, a 495 bp segment of mitochondrial DNA control region was sequenced. We collected a total of 65 individuals from three archipelagos (Xisha, Zhongsha and Nansha) in the South China Sea and 107 polymorphic sites were obtained defining 39 haplotypes. Based on the NJ tree, two distinct lineages were detected, with strong frequency differences in the geographical distribution. Both lineages were found in Xisha and Zhongsha archipelagos, but only one lineage (lineage B) was detected in Nansha archipelago. Contrary to homogenization expectation, AMOVA and pairwise F_{ST} values showed that the genetic differences among three archipelagos were all significant. The pattern of population demography showed sudden expansion model in lineage A and stable model in lineage B. These results indicated that there might be three different fishery management units of *L. olivaceus* in the South China Sea. Each of archipelagos should be treated as one independent management unit.

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Authors' Contribution
ZH conceived and designed the work. YL and NS collected the specimens. ZD and NS performed the experiments. ZD analysed the data and wrote the manuscript. NS, ZH and TG revised the manuscript.

Key words
***Lethrinus olivaceus*, Genetic structure, Genetic diversity, Control region, Coral reef fish.**

INTRODUCTION

It is believed that coral reefs are the regions with high biodiversity and abundant resources, which have attracted a great interesting to study (Bellwood *et al*., 2004; Zhao *et al*., 2006; Wilkinson, 1999). Understanding fish genetic structure is an important component of successful and sustainable long-term management (Liu *et al*., 2017). Molecular markers have been conducted to reveal the genetic structure and genetic diversity of coral reef fish species. Such techniques have been used successfully to understand the structure of coral reef species, such as Pomacentridae (Bay *et al*., 2006), *Chromis margaritifer* (Underwood *et al*., 2012) and *Caesio cuning* (Ackiss *et al*., 2013).

The South China Sea, an important tropical marine area, locates in the junction of the Indian Ocean and the western Pacific. *Lethrinus olivaceus*, one important coral reef species, belonging to family Lethrinidae, is widely distributed in the South China sea and Indo-West Pacific, and some temperate marine waters (Carpenter *et al*., 1989; Chen *et al*., 2015; Greenfield, 2006; Li, 2010). The adult size of this carnivorous fish commonly reaches 70 cm, and they always dwells on the sandy shores, reef slopes and lagoons (Philippe *et al*., 2013). However, there was no population genetic study on this species and the genetic structure of this species in the South China Sea is unknown. The length of larval stage could usually give a clue to population genetic structure of marine species (Purcell *et al*., 2006). The larval stage of *L. olivaceus* is longer than 20 days (Neira *et al*., 1998; Liu, 2007; Li *et al*., 2008; Lu *et al*., 2011), indicating that the potential larval dispersal of *L. olivaceus* is high.

Most coral reef fishes are thought to be highly sedentary (Herwerden *et al*., 2003). Nevertheless, a long larval stage could increase the passively disperse distances by ocean currents. The sedentary of adults limits the genetic exchange, but the dispersal of larval boosts it. Xisha, Zhongsha and Nansha islands are three major coral reefs in the South China Sea. Previous studies revealed different population genetic patterns for coral reef fish among these three regions. There were no genetic structures in some coral reef species, such as *Priacanthus macracanthus* (Xiong *et al*., 2015) and *Plectorhynchus gaterinus* (Sun *et al*., 2010) among these regions. However, significant population differentiation of *Plectorhinchus flavomaculatus* was detected in these regions (Han *et al*., 2008). The genetic structure in *L. olivaceus* among Xisha, Zhongsha and Nansha islands is unknown.

In the present study, we sequenced the 5'end of the

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mtDNA control region of *L. olivaceus* collected from the Xisha, Zhongsha and Nansha islands in the South China Sea to reveal the population structure and genetic connectivity among three coral reefs. We also discussed the historical demography of *L. olivaceus*. The study will provide theoretical basis for fishery management and be helpful for the protection of coral reef species.

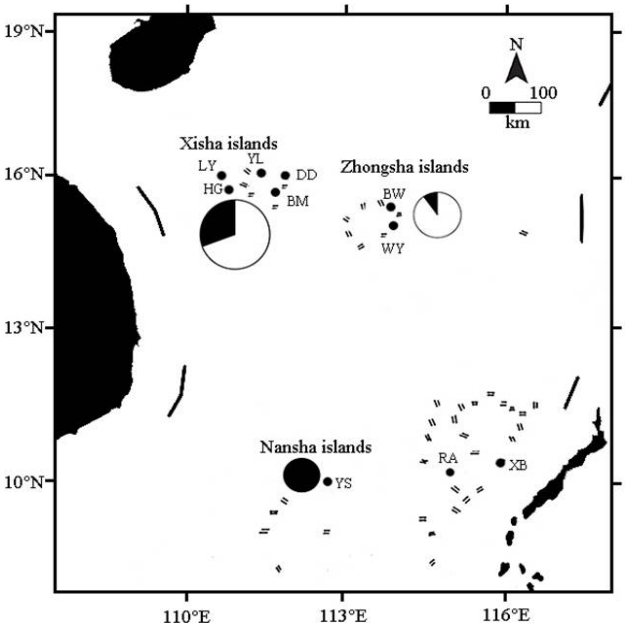

Fig. 1. Sampling sites and haplotype frequencies in three groups. The area of the circle is proportional to sample size. White of the circle represents lineage A and black represents the lineage B.

MATERIALS AND METHODS

Sample collection and DNA extraction

Through a scientific fishery resources survey conducted by the South China Sea Fisheries Research Institute , sixty five individuals were collected in 10 locations from Xisha, Zhongsha and Nansha islands in the South China Sea during May to July, 2004 (Fig. 1; Table I). All individuals were identified based on morphological characteristics, and muscle tissues were preserved with 95% ethanol, stored at -20°C. Genomic DNA was extracted from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method (Sambrook *et al.*, 1982).

MtDNA control region amplification and sequencing

A fragment of mtDNA control region was amplified using forward primer DL-S: 5′-CCCACCACTAACTCCCAAAGC-3′ and reverse primer DL-R: 5′-CTGGAAAGAACGCCCGGCATG-3′ (Lee *et al.*, 1995). Each PCR reaction was performed in a volume of 50 µL containing 20-50 ng template DNA, 5µL of 10×reaction buffer, 5 µL of $MgCl_2$ (25 mM), 4 µL of dNTPs (2.5 mM), 10pM of each primer and 2.5 units of *Taq* DNA polymerase (Promega). Sterile distilled H_2O was added to reach a total volume of 50 µL. The PCR amplification was carried out with an initial denaturation at 94°C for 3 min, and 40 cycles of 45 s at 94°C for denaturation, 45 s at 50°C for annealing, and 45 s at 72°C for extension, and a final extension at 72°C for 10 min. Negative controls were conducted with all reagents included, except template DNA. PCR product was separated on a 1.5% agarose gel. Then clear PCR products were selected to purify with the Gel Extraction Mini Kit (Watson BioTechnologies Inc., Shanghai) and both strands were sequenced at last. Control region sequences have been deposited in the GenBank database under Accession Nos. MH346334- MH346372.

Table I.- Sampling information of *L. olivaceus*.

Sites	Date of collection	Groups	Size	Coordinates
RA	2004.05	Nansha	1	115°52`E 9°43`N
XB	2004.05	Nansha	1	116°09`E 9°43`N
YS	2004.05	Nansha	4	112°58`E 9°27`N
HG	2004.06	Xisha	10	111°39'E 16°16'N
YL	2004.06	Xisha	4	112°12'E 16°46'N
D	2004.06	Xisha	2	112°41'E 16°38'N
BM	2004.06	Xisha	16	112°30'E 16°19'N
LY	2004.06	Xisha	17	111°35'E 16°28'N
BW	2004.07	Zhongsha	1	114°28'E 16°06'N
WY	2004.07	Zhongsha	9	114°48'E 15°52'N

Data analyses

Sequences were edited and aligned using DNASTAR software (DNASTAR, Inc., Madison, USA). We used software MEGA (6.0) (Tamura *et al.*, 2013) to select the best DNA sequence mutation model. Molecular diversity indices such as number of haplotypes, polymorphic sites, indels, transitions and transversions, were obtained using the program ARLEQUIN (Ver.3.5) (Excoffier *et al.*, 2010). Based on Tamura and Nei (1993) model, haplotype diversity (h), nucleotide diversity (π) and their corresponding variances were calculated.

The Neighbor-joining tree (NJ) (Saitou and Nei, 1987) and the minimum spanning tree were constructed to analyze the genetic relationships among haplotypes. The NJ tree was implemented with 1000 replicates in MEGA, and *Lethrinus miniatus* (EU835280, EU835281) downloaded from GenBank was used as the outer group. The minimum spanning tree was drawn by hand, based on the output of the number of nucleotide differences. The homologous sequences of the mtDNA control region from

genus *Lethrinus* were downloaded from GenBank, which were used to construct NJ tree. They were *L. olivaceus* (EU983095, EU983096, EU983097, EU983098) and *L. miniatus* (EU835280, EU835281).

Population structure was measured with an analysis of molecular variance (AMOVA) by using ARLEQUIN. We first conducted AMOVA with three groups representing the Xisha, Zhongsha and Nansha archipelagos. Additionally, five sample sites within Xisha archipelago were measured to verify the significance of genetic variance within group. Their significance of the covariance components was tested using 10,000 permutations. Pairwise genetic divergences between sample sites were tested by the fixation index F_{ST}, which was also performed in ARLEQUIN. The values of F_{ST} and geographical distance between sample sites were used to test for isolation by distance (Weir *et al*., 1984; Wright, 1943). The reduction major axis (RMA) regression and Mantel tests were performed in IBD 1.52 (Bohonak, 2002), which were used to assess the significance of the relationship between genetic distances and geographic distances.

The historical demographic pattern of *L. olivaceus* was investigated by using neutrality tests (Fu, 1997; Tajima, 1989) and mismatch distribution analysis (Rogers and Harpending, 1992). The *D* test of Tajima and F_S test of Fu were used to test for neutrality to examine historic demographic expansions. Historic demographic expansions were also investigated with mismatch distribution, which is based on three parameters: θ_0, θ_1 (θ before and after the population growth) and τ (time since expansion expressed in units of mutational time. The concordance of the observed with the expected distribution in the sudden expansion model was tested by a least-squares approach (Schneider *et al*., 1999). Various expansion parameters (θ_0, θ_1, τ) were estimated by a general nonlinear least-squares approach. The values of τ were transformed to estimates of real time since expansion with the equation $\tau = 2\mu t$, where μ is the mutation rate for the whole sequence under study and t is the time since expansion. Both mismatch analysis and neutrality tests were performed in ARLEQUIN.

The lack of fish fossils had resulted in a lack of references of correlate mutation in DNA sequence and time among fish. Sequence divergence rate of the control region of *Arctic charr* seemed to be 5-10%/MY (million years) (Brunner *et al*., 2001), and the rate estimate for Cichlid Fishes amounted to 6.5–8.8%/MY (Sturmbauer *et al*., 2001), but *Sardine* seemed to be much faster than other fishes (Bowen and Grant, 1997; 15-20%/MY). As a result, sequence divergence rate of 10%/MY was applied for the control region sequences in our study.

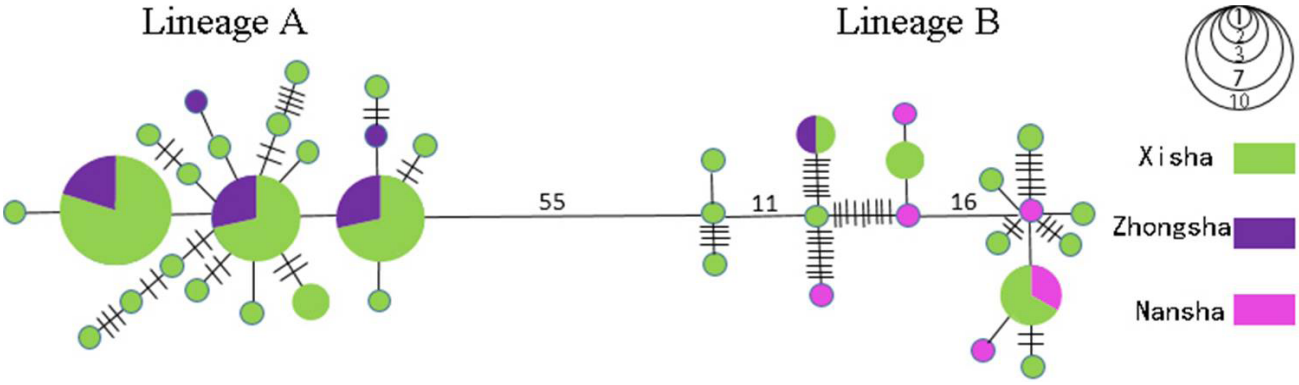

Fig. 2. Unrooted minimum spanning trees showing genetic relationship among control region haplotypes for *L. olivaceus*. The sizes of circles are proportional to haplotype frequencies. Perpendicular tick marks and numbers on the lines joining haplotypes represent the number of nucleotide substitutions.

Table II.- Molecular diversity indices of *L. olivaceus*.

Groups	n	Number of haplotypes	Haplotype diversity	Nucleotide diversity	Mean pairwise difference	No. of individuals in lineage A (proportion %)	No. of individuals in lineage B (proportion %)
Xisha	49	31	0.9566±0.0169	0.0619±0.0305	31.02±13.77	34(69.39%)	15(30.61%)
Zhongsha	10	7	0.9333±0.0620	0.0266±0.0148	13.24±6.52	9(90%)	1(10%)
Nansha	6	6	1.0000±0.0962	0.0256±0.0156	12.87± 6.77	0(0%)	6(100%)
Lineage A	43	21	0.9025±0.0282	0.0052±0.0031	2.556±1.399		
Lineage B	22	18	0.9784±0.0213	0.0301±0.0156	15.16±7.048		
Total	65	39	0.9553±0.0143	0.0642±0.0315	32.38±14.30	43(66.15%)	22(33.85%)

RESULTS

Sequence variation and genetic diversity

A 495 bp segment of the 5' end of the control region was obtained from 65 individuals (49 from Xisha archipelago, 10 from Zhongsha archipelago and 6 from Nansha archipelago). Sequence comparison of this segment revealed 107 polymorphic sites and 76 of them were parsimony informative sites. These polymorphic sites defined 39 haplotypes and five of them were shared among three archipelagos (Fig. 2). There were 70 transitions, 26 transversions, and 15 indels. The C, T, A and G composition of the sequence were 21.24%, 30.70%, 32.43% and 15.64%, respectively, and the composition of A+T was richer than G+C. The overall nucleotide diversity was 0.0642±0.0315 and haplotype diversity was 0.9553±0.0143. The haplotype diversity was high in Nansha groups, but the nucleotide diversity was low as same as that of Zhongsha groups. The nucleotide diversity in Xisha was highest among the three groups (Table II). The nucleotide substitution model used in molecular diversity indices was Tamura and Nei (1993), which was identified as the best DNA sequence substitution model. This model was also used to the pairwise population F_{ST}.

Genetic structure

The NJ tree was constructed based on haplotypes, including the downloaded sequences of this species from Genbank. *L. miniatus* was chosen as outgroup. The NJ tree revealed two haplotype lineages among three geographic regions (Fig. 3). The net genetic distance between two lineages was 0.120. Applying sequence divergence rate in mtDNA control region, the divergence of lineages A and B occurred about 1.2 million years ago (Ma).

There were obvious geographical differences in the distribution of haplotypes (Fig. 1; Table II). Lineage A included 18 haplotypes, and all of them were in Xisha and Zhongsha groups. However, lineage B contained 17 haplotypes, and it existed in the three groups and occupied the 100% of Nansha group. The obvious geographic structure for two lineages was also supported by the minimum spanning tree (Fig. 2). In lineage A, the dominant haplotypes formed the center of a star-like network, which suggested that this branch had experienced demographic expansions. On the contrary, the center of star-like network did not be appeared in lineage B, and the most haplotypes of it were incompact. The network revealed a strong genetic difference between lineage A and lineage B.

Significant genetic differentiation among three groups were revealed by AMOVA, with 33.95% of genetic variation was found among three groups ($P = 0.008$) (Table III). A small (3.34%) and no significant (P=0.138) of genetic variation was found among sampling sites within groups. Additionally, we conducted AMOVA on the Xisha group alone, and there was no significant genetic structure in Xisha group (P=0.436). Due to the small sample size of the RA reef, XB reef and BW reef (Table I), the AMOVA analysis was not carried out independently in Zhongsha and Nansha groups.

Table III.- Results of AMOVA analysis of *L. olivaceus* populations.

Source of variation	Variance components	Percentage of variance	F/φ-statistics	P
All populations				
Among groups	12.7315	33.95	0.340	0.008
Among sites within groups	1.2520	3.34	0.050	0.138
Within populations	23.5147	62.71	0.373	0.002
Xisha group				
Among sites	-0.0905	-0.31	-0.003	0.436
Within populations	28.8881	100.31		

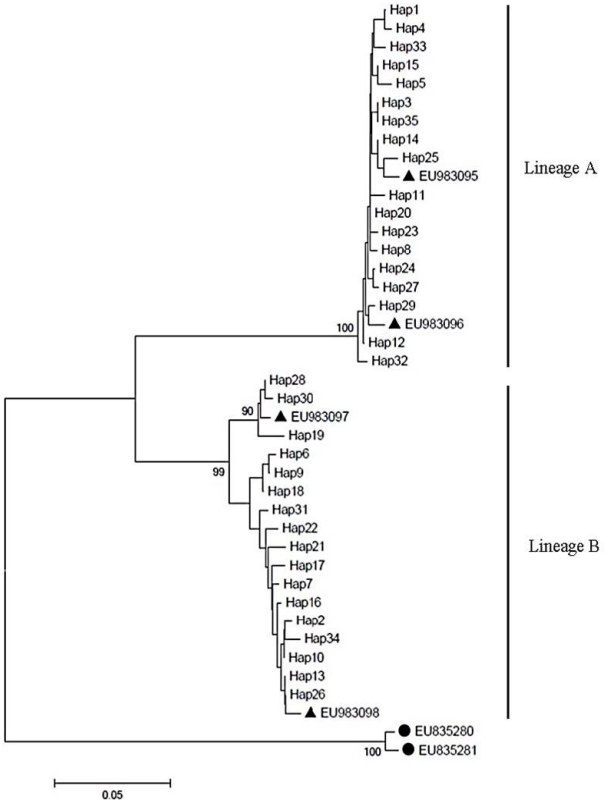

Fig. 3. Neighbor-joining tree constructed using Tamura-Nei model for control region haplotypes of *L. olivaceus*. The congener *L. miniatus* was chosen as out-group. Bootstrap supports of > 90% in 1000 replicates are shown.

The results of pairwise population F_{ST} (ranging from -0.167 to 0.286) showed that genetic differences between sample sites in Xisha islands were insignificant (P>0.05) (Table IV). However, the values of F_{ST} between YS reef from Nansha islands and other reefs were higher (ranging from 0.341 to 0.967) and most of them were significant. Most of the values of F_{ST} between WY reef from Zhongsha islands and other reefs were also significant. When the Mantel test was analyzed for them, the genetic distance was significantly related to geographical distance (P=0.019, r=0.838) (Fig. 4). Due to the small sample size, RA reef, XB reef and BW reef were excluded.

Table IV.- Pairwise F_{ST} (below) and P (above) values among islands of *L. olivaceus*.

	YS	BM	D	HG	LY	YL	WY
YS	-	0.008	0.045	0.009	0.066	0.037	0.002
BM	0.444	-	0.233	0.482	0.684	0.252	0.030
D	0.927	0.013	-	0.409	0.451	0.144	0.026
HG	0.620	-0.024	-0.167	-	0.362	0.573	0.426
LY	0.341	-0.048	0.033	0.001	-	0.249	0.049
YL	0.950	0.110	0.286	-0.056	0.136	-	0.155
WY	0.967	0.227	0.459	0.069	0.246	0.067	-

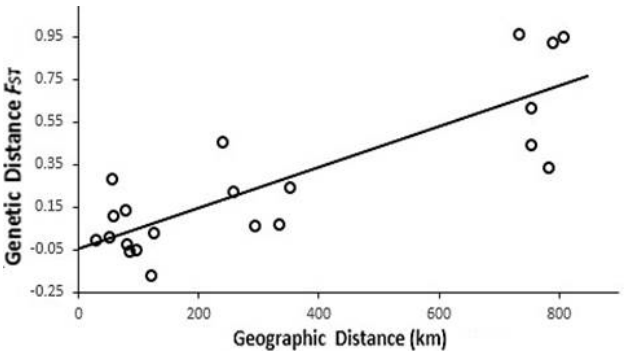

Fig. 4. Plot of pairwise estimates of F_{ST} and geographic distance between islands of *L. olivaceus*.

Historical demographics

The mismatch distribution of all *L. olivaceus* haplotype sequences and lineage B was multimodal, but lineage A was strongly unimodal and closely matched the expected distributions under the sudden-expansion model (Fig. 5). Although the overall sequences and the lineage B were not ideal when matched the expected distributions under the sudden-expansion model, both of them did not deviate significantly (P>0.05). As a result, all of them could be used to analyze historical demographics. To obtain more precise estimates, the neutrality tests were performed for each lineage. The results of neutrality tests for lineage A were negative (Tajima's D= -1.89, P = 0.007; Fu's FS= -14.93, P = 0.000), but the results of lineage B were different (P>0.05) (Table V). Both of mismatch distribution and neutrality tests revealed that the lineage A had experienced demographic expansions, while the lineage B was relatively stable.

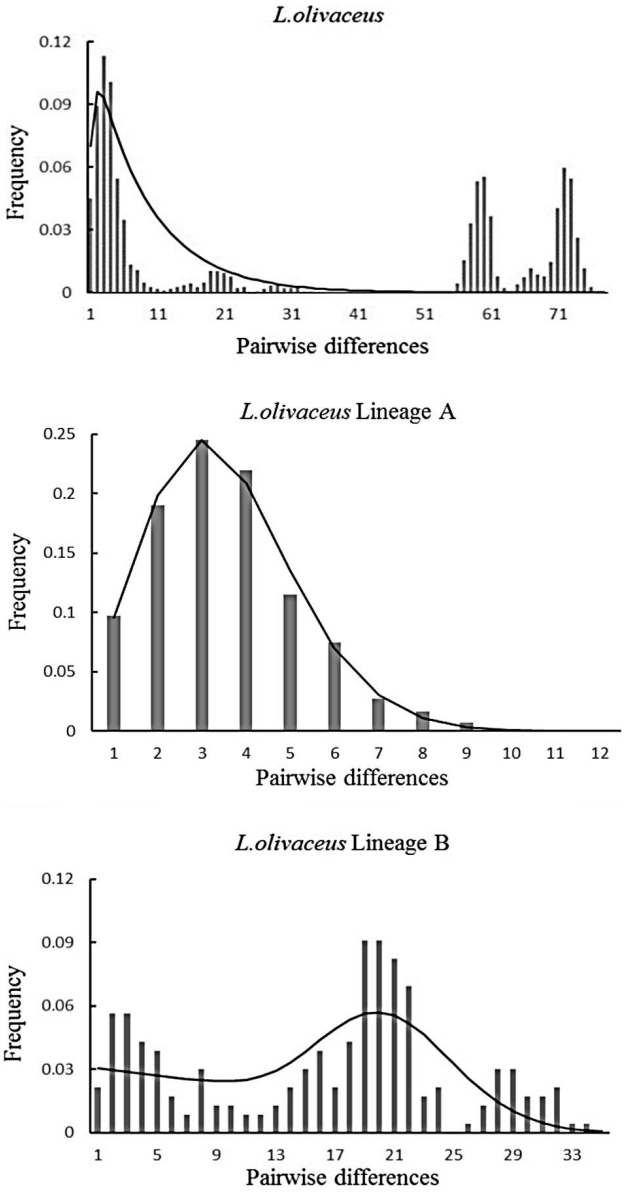

Fig. 5. The observed pairwise difference (bars), and the expected mismatch distributions under the sudden expansion model (solid line) of control region haplotypes in *L. olivaceus*.

The observed value of the age expansion parameter (τ) in lineage A was 2.52 (95%CI: 1.17 ~3.82). Based on

the sequence divergence rate mentioned above for control region, we estimated that the population expansion of the lineage A was 50,900 years ago (95%CI: 23,600~77,200 years ago). The ratio of effective female population sizes after expansion and before expansion (θ_0/θ_1) was 346 for lineage A.

Table V.- Tajima's *D* and Fu's *Fs*, corresponding *P*-value, and mismatch distribution parameter estimates.

Species	Tajima's *D*		Fu's *Fs*		Mismatch distribution		
	D	P	Fs	P	τ (95%CI)	θ_0	θ_1
L. oliv	1.68	0.97	-1.10	0.41			
A	-1.89	0.007	-14.93	0.000	2.52 (1.17-3.82)	0.095	32.89
B	-0.40	0.38	-3.16	0.096			

L. oliv., *L. olivaceus*; A, *L. olivaceus* lineage A; B, *L. olivaceus* lineage B.

DISCUSSION

For a long time, it was believed that the lack of obvious physical barriers and the ability of larvae passively disperse great distances by ocean currents, led to the low level of genetic differentiation among geographic regions of marine species (Morrison *et al*., 2004). However, this is not the case in our study. We found that there were two haplotype lineages among three geographic regions, and significant genetic structure was found among three archipelagos. The results of pairwise population F_{ST} showed that genetic differences among groups were significant. There were obvious geographical differences for the distribution of haplotypes. Lineage A were only detected in Xisha and Zhongsha groups. However, lineage B existed in all three groups.

The present study revealed limited gene flow of *L. olivaceus* among three archipelagos. In order to test the dispersal ability of this species, the Mantel test analysis was performed. The results showed a strong pattern of isolation by distance. Comparisons of close and distant population genetic variation could provide information about the dispersal ability of species (Palumbi, 2003). In our study, we found that there were no significant genetic differences under 150 km (Fig. 4). On the contrary, when the distance was farther than 240 km, the pairwise F_{ST} were significant. Hence, we hypothesized that long distance might limit the dispersal of *L. olivaceus*. This hypothesis was in line with the conclusion that dispersal distance of *P. flavomaculatus* was no more than 300 km in the South China Sea (Han *et al*., 2008), and was consistent with the view that larvae of many coral reef species were more likely to inhabit around their birthplace (Almany *et al*., 2007; Kingsford *et al*., 2002; Planes *et al*., 2001).

The formation of lineage A and lineage B might be related to the Pleistocene ice period. During the Pleistocene ice age, the decline of sea level in the South China Sea caused most of the continental shelves to be exposed (Wang *et al*., 2004), which lead to the southern part of the South China Sea to be isolated from other areas. This may influence the distribution of the lineages in this species. Generally speaking, the genetic diversity of the ancestral population was higher than that of the derived one (Savolainen *et al*., 2002). Since the genetic diversity of the lineage B was significantly higher than that of the lineage A (Table II), we speculated that the lineage B was the ancestral population. Following the rise of sea level, both lineages began to spread. In our study, the lineage B was found in both the north and south of the South China Sea, but lineage A was only found in the north. Based on these, we hypothesized that the lineage A originated from north and might spread from north to south. However, there was a trench with a thousand of meters depth between Nansha islands and the north of the South China Sea (Feng, 1982), which might block the spread of lineage A. This was the reason why lineage A was absent in Nansha group. Certainly, the limited dispersal ability of *L. olivaceus* should be taken into consideration. In addition, we also need more individuals from Nansha islands and the north area of Xisha and Zhongsha islands to support our assumptions.

The haplotype diversity of three groups was high, but the nucleotide diversity of Xisha group was obviously higher than Zhongsha and Nansha groups. Compared to the genetic diversity of mtDNA control region in *P. flavomaculatus* (h=0.665, π=0.005) (Han *et al*., 2008) and *Acanthopagrus schlegeli* (h=0.994, π=0.0075) (Cao, 2016) in the surrounding area, the genetic diversity of *L. olivaceus* was higher. Higher haplotype diversity and nucleotide diversity were attributed to a long evolutionary history in a large stable population (Grant and Bowen, 1998). On the contrary, the lower haplotype diversity might be due to inadequate numbers of population, and the lower nucleotide diversity might be due to the "founder effect" or a rapid expansion from a small effective group into a large group in a short period of time. Accordingly, we hypothesized that the population of *L. olivaceus* in the South China Sea should be a relatively stable one and had a relatively long evolutionary history. The neutrality test and the mismatch distribution of lineage B strongly supported this hypothesis, while the case of lineage A (the derived one) was absolutely different and supported that it had experienced demographic expansions. During the late Pleistocene period, there was a series of glacial-interglacial changes (Imbrie *et al*., 1992), which might be the reason

why lineage A experienced demographic expansions. This phenomenon was similar to the historical demographics of many marine fishes (Liu *et al*., 2006).

Based on the genetic structure of *L. olivaceus*, there might be three different fishery management units in the South China Sea. Each of archipelagos should be treated as one independent management unit. In the present study, we also assessed the dispersal ability of *L. olivaceus*, which was also helpful for fishery management. However, with the power of fishing pressure increasing in the South China Sea, the size of its population must be affected. As a result, we suggested that the conservation for *L. olivaceus* was necessary. With no doubt, the lack of individuals and single molecular marker will bring incomprehensive analyses. Consequently, we will collect more specimens in Zhongsha and Nansha islands and the surrounding sea of Taiwan Strait, using various molecular markers, and attempt to reveal the population genetic structure and genetic diversity comprehensively in our future study.

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Statement of conflict of interest

The authors declare no conflicts of interest.

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