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Full Length Research Paper

Population genetic structure of coral reef species *Plectorhinchus flavomaculatus* in South China Sea

Zhi Qiang Han[1#], Yong Zhen Li[2#], Guo Bao Chen[2] and Tian Xiang Gao[1]*

[1]The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao. 266003 P. R. China.
[2]Marine Fishery Resources Division, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou. 510300 P.R. China.

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The population genetic structure and the dispersal ability of *Plectorhinchus flavomaculatus* from South China Sea were examined with a 464 bp segment of mtDNA control region. A total of 116 individuals were collected from 12 coral reefs in Xisha, Zhongsha and Nansha archipelagos and 22 haplotypes were obtained. Two lineages were detected, which might have diverged in different glacial refuge during Pleistocene low sea levels. Contrary to homogenization expectation, AMOVA and pairwise F_{ST} revealed significant genetic structure between Nansha and other two archipelagos (Xisha and Zhongsha). Pattern of isolation by distance was observed in this species, suggesting that mean dispersal distance of *P. flavomaculatus* is no more than 300 km. The different environment in glacial ages may cause the different pattern of population demography in lineages (stable model in lineage A and sudden expansion model in lineage B).

Key words: *Plectorhinchus flavomaculatus*, genetic structure, mitochondrial DNA.

INTRODUCTION

Understanding fish stock structure is an important component of successful and sustainable long-term management and has attracted considerable interest, because of a fundamental interest in biotic evolution (Tudela et al., 1999). Determination of population genetic structure provides essential information to underpin resource recovery and to aid in delineating and monitoring populations for fishery management. Molecular genetic techniques offer the ability to identify and delineate fish stock structure where it may not be apparent from phenotypic or behavioural characteristics (Shaklee and Currens, 2003). Such techniques have been used successfully to understand the structure of marine fish species (Liu et al., 2006; Kochzius and Blohm, 2005).

The commercially important coral reef species gold-spotted sweetlips, *Plectorhinchus flavomaculatus*, a member of family Haemulidae, is widely distributed in tropical and some temperate marine waters of the Indo-West Pacific, ranging from East and South Africa to South China Sea, southern Japan and Australia (Masuda et al., 1984). It is predominate coral reef species in Xisha, Zhongsha and Nansha archipelagos in South China Sea (Chen et al., 2007). However, there is no population genetic study about this species. There is only limited information about characteristics of this species, which can give a clue to its population genetic structure. The species inhabits coastal waters near reefs and weedy areas, or in current channels occasionally inshore; juveniles enter estuaries and harbors. The length of pelagic larval stage is about three weeks (Neira et al., 1998). Most coral reef fishes are thought to be highly sedentary with movements limited to a few kilometers for even the most mobile species (Holland et al., 1996). However, long distance (about 180 km) movements have been reported in this species (Arara and Rose, 2004). These early life-history characteristics and long movements indicate that potential dispersal of larva and adult in this species is high. These may ensure connectivity between stocks and cause genetic homogenization between islands.

Coral reef ecosystems are ecologically diverse and a major focus of marine conservation, so they represent an excellent system in which to study marine population

*Corresponding author. E-mail: gaozhang@ouc.edu.cn. Tel: 86-532-82032063. Fax: 86-532-82032076.

#These authors contributed equally to this work.

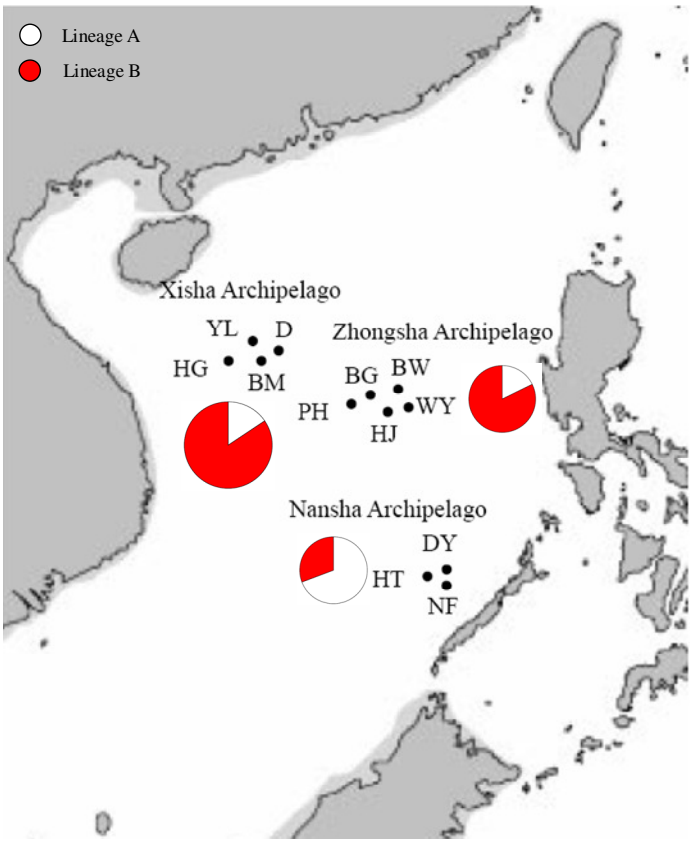

Figure 1. Sampling sites and haplotype frequencies in three groups. The area of the circle is proportional to sample size.

structure (Danilowicz, 1997). South China Sea is a marginal sea of Western Pacific, which was an enclosed inland sea connected to the Pacific through the Bashi Strait between Taiwan and Luzon (Wang, 1999). It represents an area of globally significant marine shallow-water, tropical biodiversity and is one of the centers of coral reef diversity in the world. The location of the South China Sea at the junction between the Pacific and Indian Ocean basins has resulted in it becoming a center of aggregation of marine species from both Oceans. Xisha, Zhongsha and Nansha archipelagos are groups of low coral islands and reefs in South China Sea. There are small and large scales distances among coral reefs in these archipelagos (40 – 860 km). So, coral reef-dependent fish *P. flavomaculatus* in these archipelagos provide an excellent opportunity without obvious physical barriers to examine the connectivity of populations and estimate the dispersal ability on both fine and broad spatial scales. In the present study, we sequenced the 5' end of the mtDNA control region of *P. flavomaculatus* collected from the Xisha, Zhongsha and Nansha archipelagos in South China Sea to reveal the population structure of *P. flavomaculatus* and estimate the dispersal ability of this species.

MATERIALS AND METHODS

Sampling and sequencing

A total of 116 adults of *P. flavomaculatus* were collected in 12 coral reefs from Xisha, Zhongsha and Nansha archipelagos in South China Sea in 2004 through a scientific survey (Figure 1 and Table 1). Muscle samples were preserved in 70 - 90% ethanol before DNA extraction.

Genomic DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method. The first hypervariable segment of the mtDNA control region was amplified with fish primers DL-S and DL-R. The primer sequences are DL-S: 5'- CCC ACC ACT AAC TCC CAA AGC -3' (forward) and DL-R: 5'- CTG GAA AGA ACG CCC GGC ATG -3' (reverse) (Lee et al., 1995).

Each PCR reaction was performed in a volume of 50 µL containing 20 - 50 ng template DNA, 5 µL of 10×reaction buffer, 5 µL of $MgCl_2$(25 mM), 1 µL of dNTPs (10 mM), 10 pM of each primer and 2.5 units of *Taq* DNA polymerase (Promega) in an Eppendorf Mastercycler 5333. Initial denaturation was for 3 min at 94°C, followed 40 cycles of 45 s at 94°C for denaturation, 45 s at 50°C for annealing, and 45 s at 72°C for extention; and a final extention at 72°C for 10 min. All sets of PCR included a negative control reaction tube in which all reagents were included, except template DNA. PCR product was separated on a 1.5% agarose gel and purified with the Gel Extraction Mini Kit (Watson BioTechnologies Inc., Shanghai). The purified product was used as the template DNA for cycle sequencing reactions performed using BigDye Terminator Cycle Sequencing Kit (ver. 2.0, Applied Biosystems, Foster City, California), and sequencing was conducted on an ABI Prism 3730 (Applied Biosystems) automatic sequencer with both forward and reverse primers. The primers used for sequencing were the same as those for PCR amplification.

Data analyses

Sequences were edited and aligned using Dnastar software (DNASTAR, Inc., Madison, USA). Molecular diversity indices such as number of haplotypes, polymorphic sites, transitions, transversions, and indels, were obtained using the program ARLEQUIN (Ver. 2.0) (Schneider, 2000). Haplotype diversity (h), nucleotide diversity (π) and their corresponding variances were calculated after Nei (1987) as implemented in ARLEQUIN.

Genetic relationships among haplotypes were reconstructed using the neighbour-joining method (Saitou and Nei, 1987) implemented in MEGA 2.0. Genetic distances were generated for phylogenetic reconstruction using Kimura's two-parameter substitution model. We used bootstrap analysis with 1000 replicates to evaluate support for phylogenetic relationships. A minimum-spanning haplotype network was estimated using the program TCS 1.18 (Clement et al., 2000). This program was used to obtain a 95% confidence limit for parsimony (Templeton et al., 1995), as well as to construct a cladogram that showed the nested structure of the haplotypes. The nested haplotype network was used in the nested clade analysis (NCA) developed by Templeton et al. (1987) and Templeton and Sing (1993) in order to infer historical and ongoing processes responsible for the observed patterns of significant association of geography and nested haplotypes. The NCA takes into account gene genealogies, haplotype frequencies and geographical distribution of haplotypes to discriminate between historical events (such as fragmentation or range expansion events) and ongoing process (such as gene flow). Program GEODIS 2.2 (Posada et al., 2000) was used to calculate NCPA distance measures and tests for significant association between genealogy and geography. The interpretation of the results followed the updated inference key for the nested haplotype tree analysis of geo-

Table 1. Sampling information of *P. flavomaculatus.*

ID	Sampling site	Date of collection	Group	Sample size	Coordinates	
HT	Houteng Reef, Nansha archipelago	May 2004	Nansha	1	116°11′E	10°38′N
NF	Nanfang Reef, Nansha archipelago	May 2004	Nansha	11	116° 33′E	10° 19′N
DY	Dayuan Reef, Nansha archipelago	May 2004	Nansha	1	116° 07′E	11° 10′N
HG	Huaguang Reef, Xisha archipelago	June 2004	Xisha	2	111° 39′E	16° 16′N
YL	Yingle Reef, Xisha archipelago	June 2004	Xisha	20	112° 12′E	16° 46′N
D	Dong Island, Xisha archipelago	June 2004	Xisha	12	112° 41′E	16° 38′N
BM	Binmei Reef, Xisha archipelago	June 2004	Xisha	24	112° 30′E	16° 19′N
PH	Paihong Reef, Zhongsha archipelago	July 2004	Zhongsha	1	113° 43′E	15° 39′N
BG	Bengu Reef, Zhongsha archipelago	July 2004	Zhongsha	6	114° 02′E	16° 00′N
BW	Biwei Reef, Zhongsha archipelago	July 2004	Zhongsha	21	114° 28′E	16° 06′N
WY	Wuyong Reef, Zhongsha archipelago	July 2004	Zhongsha	3	114° 48′E	15° 52′N
HJ	Haijiu Reef, Zhongsha archipelago	July 2004	Zhongsha	13	114° 29′E	15° 36′N

graphical distance (Templeton, 2004).

Population structure was measured with an analysis of molecular variance (AMOVA) incorporating sequence divergence between haplotypes. To estimate gene flow among coral reefs, we first conducted AMOVA analysis with three groups representing the Xisha, Zhongsha and Nansha archipelagos. The significance of the covariance components was tested using 1000 permutations. In addition, pairwise genetic divergences between sample sites were estimated using the fixation index F_{ST}, which included information on mitochondrial haplotype frequency and genetic distances (The sites Huaguang reef, Paihong reef, Houteng reef and Dayuan reef were excluded, because of small sample size). The significance of the F_{ST} was tested by 1000 permutations for each pairwise comparison. To test for isolation by distance (Slatkin, 1993), pairwise values of $F_{ST}/(1-F_{ST})$ were plotted against geographical distance (one-dimensional stepping-stone model) between sample sites of *P. flavomaculatus.* The strength and significance of the relationship between genetic distances and geographic distances was assessed using reduced major axis (RMA) regression and Mantel tests using IBDWS (Isolation by distance web service at http://phage.sdsu.edu/~jensen/) (Bohonak, 2002). The sites Huaguang reef, Paihong reef, Houteng reef and Dayuan reef were excluded in the IBD analysis, because of small sample size.

Historical demographic/spatial expansions were investigated by two different approaches. First, the *D* test of Tajima and F_S test of Fu were used to test for neutrality (Tajima 1989; Fu 1997). Second, historic demographic expansions were also investigated with the distributions of pairwise differences between sequences (mismatch distribution) (Rogers and Harpending, 1992), 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 under the sudden-expansion model of Rogers was tested by means of a least squares approach. Various expansion parameters (θ_0, θ_1 and 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 $\tau = 2ut$, where u 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.

Owing to the paucity of fish fossils, there are few references to correlate mutation in DNA sequence and time among fish. However, evolution of the control region seems to be much faster in some bony fishes including Arctic charr (Brunner et al., 2001; 5 – 10%/MY), Japanese anchovy (Liu et al, 2006; 5 - 20%) and crimson snapper (Zhang et al., 2006). In the present study, sequence divergence rate of 10%/MY was applied for the control region sequences.

RESULTS

A 464 bp segment of the 5' end of the control region was obtained from 116 specimens (58 from Xisha archipelago, 34 Zhongsha archipelago and 14 from Nansha archipelago). Sequence comparison of this segment revealed 22 polymporphic sites with 20 transitions and 5 transversions. These polymorphic sites defined 22 haplotypes, giving an over all haplotype diversity of 0.6655 ± 0.0468 and nucleotide diversity of 0.0054 ± 0.0032. The haplotype diversity and nucleotide diversity were high in Nansha populations, but the haplotype diversity and nucleotide diversity in Xisha and Zhoushan were generally low (Table 2).

The intraspecific relationship among three geographic regions revealed two haplotype lineages, which were supported by moderate bootstrap values (Figure 2). The net divergence between two lineages was 0.89%. Applying sequence divergence rate in control region, the divergence of lineages A and B occurred about 89,000 years ago. There was clear geographical pattern in the distribution of haplotypes (Figure 1 and Table 2). Lineage A comprised 9 haplotypes, which were mainly distributed (8 haplotypes) in Nansha groups, but two haplotypes were detected in Xisha and Nansha groups. This lineage displayed a star-like shape, with a dominant haplotype H1, including several closely related singletons, but also some relatively divergent haplotypes (Figure 3). The haplotype H1 had an extensive distribution in three geographic regions. While other haplotypes except H21 in lineage A were geographically confirmed to Nansha group. Another lineage (Lineage B) contains most Xisha and Zhongsha group individuals, including all individuals from Benggu reef, Wuyong reef and Huaguang reef (Table 3).

Table 2. Measures of genetic diversity within groups: number of samples (n), number of haplotypes, haplotype diversity, nucleotide.

Groups	n	Number of haplotypes	Haplotype diversity	Nucleotide diversity	Mean pairwise difference	Number of individuals in lineage A (proportion %)	Number of individuals in lineage B (proportion %)
Xisha	58	9	0.5729±0.0709	0.0042±0.0027	1.96±1.13	9(15.52%)	49(84.48%)
Zhongsha	45	9	0.6192±0.0765	0.0044±0.0028	2.06±1.17	8(17.78%)	37(82.22%)
Nansha	13	11	0.9744±0.0389	0.0104±0.0061	4.81±2.51	9(69.23%)	4(30.77%)
Total	116	22	0.6655±0.0468	0.0054±0.0032	2.51±1.36	26(22.41%)	90(77.59%)

Diversity and number of individuals in two lineages within each group.

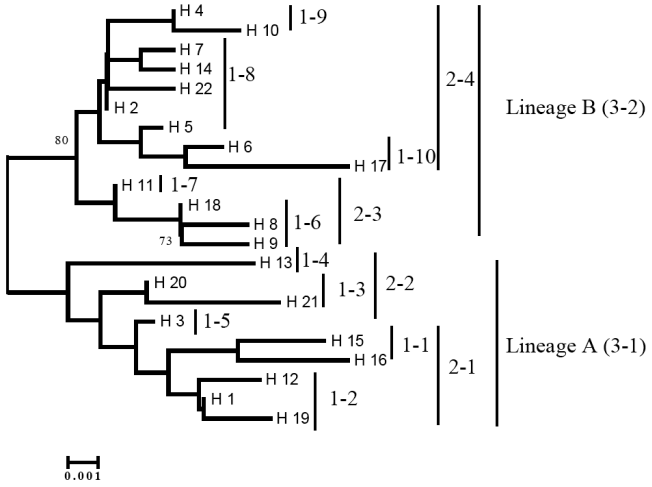

Figure 2. NJ tree for control region mtDNA sequence data of *P. flavomaculatus* based on K-2P substitution model. Bootstrap values >70% are showed at nodes, 1000 replicates. Nested clades are also presented.

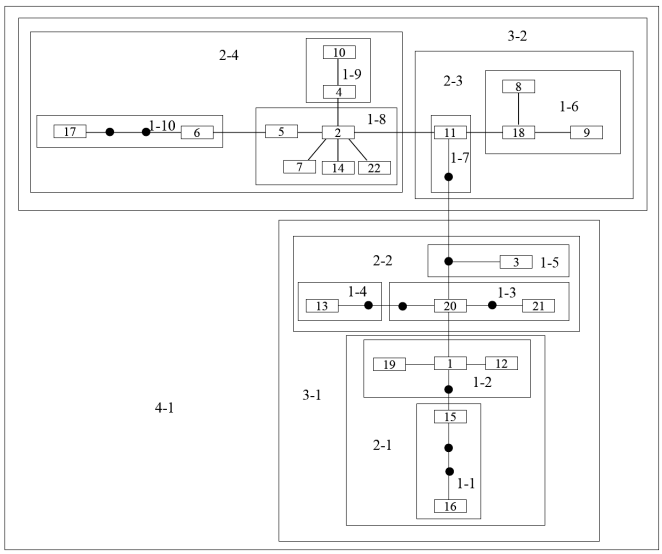

Figure 3. Haplotype network and nested clade design of the 22 haplotypes detected in *P. flavomaculatus.* Numbers in panes represents haplotypes. Filled circles represent intermediate hypothesized haplotypes between observed haplotypes, and lines between haplotypes represent a one-step mutational change.

The minimum-spanning network for *P. flavomaculatus* gave two 3-step clades, four 2-step clades and ten 1-step clades (Figure 3). Overall, this network closely matched the haplotype NJ tree (Figure 2). The NCA revealed no significant association between genetic variability and geographical distribution at most of nested clades, but one step clade 1 - 8 and high-level clade 4 - 1 showed significant levels of geographical association. Based on the inference key of Templeton (2004), two inference patterns were obtained in two nested clades (Table 4). The clade 1 - 8 suggested restricted gene flow or dispersal but with some long distance dispersal among three groups. The high-level categories clade 4 – 1 showed restricted gene flow with isolation by distance between Nansha and other two groups.

Significant genetic structure among three groups were revealed by AMOVA, with 11.84% of genetic variation was found among three groups (P = 0.004). A small (2.88%) and no significant (P = 0.320) amount of genetic diversity was found among sampling sites within groups (Table 5). The one factor AMOVA for Xisha and Zhongsha groups revealed no significant genetic structure between sampling sites within two groups (P = 0.953 in Xisha, P = 0.197 in Zhongsha). Most of F_{ST} between samples in Xisha and Zhongsha groups were low and not significant (Table 6). However, most of F_{ST} between Nanfang reef in Nansha group and other samples from Xisha and Zhongsha groups were generally high and significant. Among sample sites for *P. flavomaculatus*, a Mantel test indicated a significant relationship (P = 0.04, r = 0.804) between $F_{ST}/(1-F_{ST})$ and geographic distance indicating isolation by distance (Figure 4), with geographic distance explaining 80% of the variation in genetic differentiation for species.

The mismatch distribution of all *P. flavomaculatus* haplotype sequences were clearly bimodal; one mode corresponded to the number of differences between lineage A and B, and the other to differences among individuals within both lineages (Figure 5). To obtain more precise estimates, the neutrality tests and mismatch distribution analysis were performed for each lineage. As

Table 3. Distribution of haplotypes among locatities.

Lineage A											Lineage B														
	H1	H3	H12	H13	H15	H16	H19	H20	H21	Total		H2	H4	H5	H6	H7	H8	H9	H10	H11	H14	H17	H18	H22	Total
HT				1						1	NF	1									2	1			4
NF	1	1	1		1	1	1	1		7	HG	1				1									2
DY						1				1	YL	11		1		2		2						1	17
YL	3									3	D	8			1								1		10
D	2									2	BM	17				2							1		20
BM	3								1	4	PH						1								1
BW	4									4	BG	5		1											6
WY	3								1	4	BW	14	1							2					17
											WY	2							1						3
											HJ	6	2			2									10

expected from the star like statistical parsimony network, the mismatch distribution for lineage B fitted the predicted distribution under a model of sudden expansion and did not differ significantly ($P > 0.05$) from expectation.

Furthermore the neutrality tests resulted in negative Tajima's D (-1.77, $P = 0.015$) and Fu's F_S values (-9.81, $P = 0.000$), and they were significantly different from zero in both tests. However, the sudden expansion model was rejected in the mismatch analysis for lineage A ($P = 0.001$), indicating a stable model. The neutrality tests in lineage A resulted in negative but no significant values. The tau value (τ), which reflects the location of the mismatch distribution crest, provides a rough estimate of the time when rapid population expansion started. The observed value of the age expansion parameter (τ) was 0.808 units of mutational time in lineage B. The estimate of time of expansion for lineage B, based on the rates mentioned above for control region, was 17 kyr years ago.

DISCUSSION

Marine fishes generally show low levels of genetic differentiation among geographic regions due to higher dispersal potential during planktonic egg, larval, or adult history stages coupled with an absence of physical barriers to movement between ocean basins or adjacent continental margin (Grant and Bowen, 1998). *P. flavomaculatus* with long time of pelagic larval stage and long movement might show low levels of genetic differentiation among coral reefs. However, this was not the case in our present result. Contract to our hypothesis, two distinct lineages and limited gene flow were identified in *P. flavomaculatus* sampled from Nansha and other two groups. Different glacial refuge during Pleistocene low sea-level stands may cause the divergence of two lineages. The provisional molecular clock yields an estimate of late Pleistocene divergence between the two lineages. Previous studies have revealed that Pleistocene glaciations was the factor causing strong genetic divergence in Southeast Asia (Liu et al., 2006).

Significant genetic differentiation and strong gene frequency observed over distances as long as 600 km showed highly limited genetic exchange between populations of *P. flavomaculatus* in the absence of contemporary dispersal barriers. However, pairwise F_{ST} and AMOVA indicated high gene flow among coral reefs within Xisha and Zhongsha regions. Therefore, *P. flavomaculatus* in study area is divided into two major stocks: a south

Table 4. Nest contingency analysis of geographical associations for mtDNA control region sequence data from *P. flavomaculatus,*

Nested Clade	χ^2 (permutation test)	*P*	Inference chain	Inferred pattern
1-8	71.96	0.009	1-2-3-5-6-7-Yes	Restricted Gene Flow/Dispersal but with some Long Distance Dispersal
4-1	22.90	0.013	1-2-3-4-No	Restricted Gene Flow with Isolation by Distance

Clades without geographic association are not shown.

Table 5. Analysis of molecular variance (AMOVA) for the control region haplotypes of *P. flavomaculatus*.

Source of variation	Variance components	Percentage of variance	F/φ-statistics	*P*
All populations				
Among groups	0.1582	11.87	0.117	0.004
Among sites within groups	0.0383	2.88	0.033	0.320
Within sites	1.1362	85.25	0.147	0.002
Xisha group				
Among sites	-0.0488	-5.02	-0.050	0.953
Within populations	1.0207	105.02		
Zhongsha gropu				
Among sites	0.0678	6.39	0.064	0.187
Within sites	0.9932	93.61		

Table 6. Pairwise F_{ST} (below) and *P* (above) values among samples of *P. flavomaculatus.*

	NF	YL	D	BM	BG	BW	HJ
NF	-	0.002	0.024	0.003	0.015	0.017	0.073
YL	0.221	-	0.905	0.874	0.374	0.607	0.472
D	0.179	-0.055	-	0.865	0.374	0.819	0.556
BM	0.249	-0.035	-0.050	-	0.262	0.717	0.496
BG	0.320	-0.001	-0.001	0.011	-	0.213	0.129
BW	0.209	-0.027	-0.054	-0.031	0.058	-	0.596
HJ	0.102	-0.014	-0.033	-0.020	0.078	-0.031	-

stock including samples from Nansha archipelago, and a north stock including samples from Xisha and Zhongsha archipelagos. Strong association with geography and genealogy was showed at two nested clades 1 - 8 and 4 - 1. Restricted gene flow or dispersal but with some long distance dispersal and restricted gene flow with isolation by distance were the inference patterns in two clades (Templeton, 2004). These inferences were consistent with the biology of this species. Relatively long movement and potential dispersal of larva might be responsible for the genetic homogenization among coral reefs in Xisha and Zhongsha with distance ranging from 40 to 275 km. Long distance between the Nansha and other two groups (more than 700 km) was responsible for the population subdivision.

The plot of $F_{ST}/(1-F_{ST})$ and geographic distance revealed a strong pattern of isolation by distance in *P. flavomaculatus* (Slatkin, 1993), supporting the population subdivision between the south and north stocks. Marine environments are often seen as open habitats in which isolation by distance is the main mechanism that may promote genetic differentiation. Patterns of isolation by distance are often established over long periods through equilibrium between gene flow and drift (Slatkin, 1993). Isolation by distance in *P. flavomaculatus* indicated this species is at genetic equilibrium under dispersal and genetic drift. Studies of genetic structure that focus on isolation by distance model have the potential of allowing more rigorous ecological conclusions by using geographic comparisons to look for significant signals. Comparisons of close and distant population could provide information about the dispersal ability of species (Palumbi, 2003). In our study, isolation-by-distance comparisons among coral reefs suggested mean dispersal distance of *P. flavoma-*

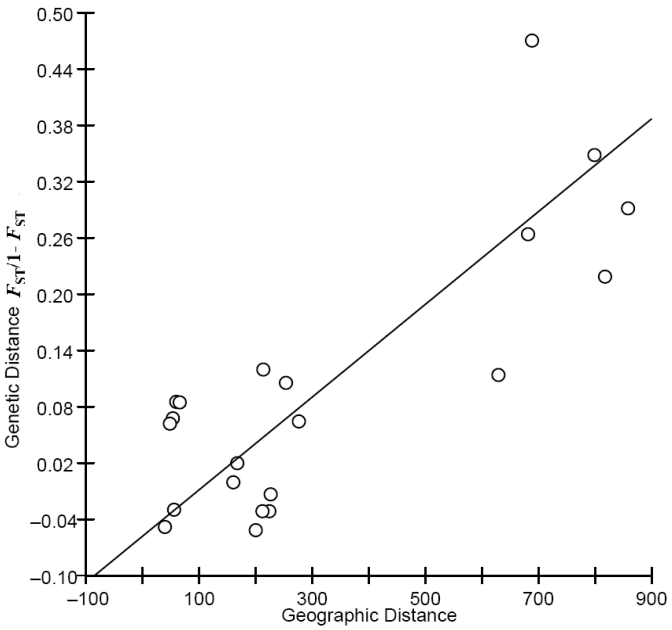

Figure 4. Plot of pairwise estimates of $F_{ST}/(1- F_{ST})$ and geographic distance between samples of *P. flavomaculatus*.

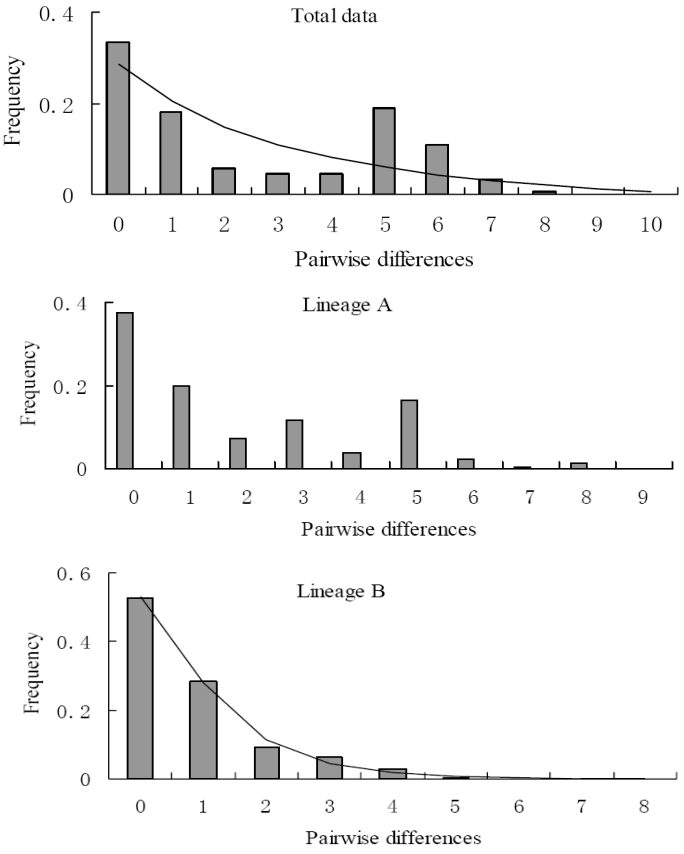

Figure 5. The observed pairwise difference (bars), and the expected mismatch distributions under the sudden expansion model (solid line) of control region haplotypes in *P. flavomaculatus*. The sudden expansion model in lineage A was rejected.

culatus is no more than 300 km. This is similar to estimate from tagged re-capture data in coastal Kenya (180 km). Tropical Pacific sea urchins also show a pattern of isolation by distance, in which F_{ST} measured with mitochondrial sequence data increases markedly with geographic distance between pairs of populations (Palumbi et al., 1997). Isolation by distance was also detected in Atlantic cod (*Gadus morhua*) and coral reef species Australian barramundi (*Lates calcarifer*) (Pogson et al., 2001; Chenoweth et al. 1998).

P. flavomaculatus with large values of *h* and *π* probably are attributed to long evolutionary history in a large stable population (Grant and Bowen, 1998). Furthermore, the neutrality tests and mismatch distribution for lineage A are also consistent with this population demography. However, both the Tajima's and Fu's tests are statistically significant for lineage B, supporting the demographic expansion in this lineage. Estimates of population expansion time indicated extensive population expansion 17,000 years ago, indicating population expansion after Last Glacial Maximum (LGM). During the LGM, changes in area and configuration were much more drastic in the Xisha and Zhongsha archipelagos than in the Nansha archipelago (Voris 2000). This different environment may cause the different pattern of population demography in lineages (stable model in lineage A and sudden expansion model in lineage B).

The significant population structure in Xisha, Zhongsha and Nansha archipelago revealed limited gene flow and pattern of isolation by distance among coral reefs. The dispersal ability of *P. flavomaculatus* was estimated in this study. These results have important implication in fishery management of *P. flavomaculatus* and implied that many local stocks may exist in this widely distributed species. Different stocks with unique genetic structure should be managed as distinct units, and such units require separate monitoring and management owing to the different levels of gene flow. More rigorous studies on life history, tagging, and advanced genetics are recommended to gain better understanding of the biology and essential requirements of this important species.

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