

Full Length Research Paper

Genetic population structuring and demographic history of red spotted grouper (*Epinephelus akaara*) in South and East China Sea

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Climatic oscillations during the Pleistocene ice ages in the Northwestern Pacific produced great changes in species' geographical distribution and abundance, which could be expected to have genetic consequences in marine fishes. In order to estimate the demographic history and genetic structure of *Epinephelus akaara* in South and East China Sea, 87 individuals were sampled from 7 localities throughout the distribution range of the species. Mitochondrial DNA (mtDNA) variations were analyzed using DNA sequence data from the 5' end of the control region. Nucleotide diversity was moderate in *E. akaara* (0.011). The demographic history of *E. akaara* was examined using neutrality test and mismatch distribution analyses and results indicated late Pleistocene (162,000 ~ 542,000 years ago) population expansion occurred in the species. Molecular variance analysis (AMOVA) and conventional population statistic F_{ST} revealed statistically significant levels of genetic structuring existed in *E. akaara*.

Key words: Climatic oscillations, *Epinephelus akaara*, mtDNA, population expansion, population genetics.

INTRODUCTION

Due to periodic climatic oscillations over the Pleistocene, range contractions and expansions are thought to have greatly influenced the amount and distribution of intra-specific genetic variation in many species (Avice, 2000; Hewitt, 2000). The late Quaternary period (the past one million years) was characterized by a series of large glacial-interglacial changes (Imbrie et al., 1992). The major climatic oscillations occurred during the past ~800,000 years (800 kyr) with a ~100 kyr dominant cycle. During glacial maxima, declines in sea levels of 120 – 140 m have been noted (Lambeck et al., 2002). Severe climatic shifts can produce great changes in species' geographical distribution and abundance (Dynesius and Jansson, 2000; Hewitt, 2000). Range changes can be expected to have genetic consequences and the advent of DNA technology provides suitable markers to examine the genetic effects of these changes (Hewitt, 2000).

In the modern oceans, most of (> 75%) the marginal

basins are concentrated in the Western Pacific continental margin (Tamaki and Honza, 1991). During late Quaternary glacial cycles, the sea-level-induced environmental signal was amplified in the marginal seas of the Western Pacific, giving rise to drastic changes in areas and configurations of these seas (Liu et al., 2006; Wang, 1999). For example, during the Last Glacial Maximum (LGM), when the sea level was at its lowest, about 130 m below the present sea level, an extensive area of the continental shelf of the East China Sea was exposed (Xu and Oda, 1999) (Figure 1). Under the most severe environmental conditions in glacial periods, *Epinephelus akaara* might have become extinct over large parts of its range and survived in glacial refugium, which was likely to have been located in the basin of East China Sea and South China Sea. The closure and opening of the seaways between the marginal seas and the Pacific Ocean led to reorganization of the sea circulation (Wang, 1999). For example, the path of the Kuroshio Current was forced to migrate eastward during Last Glacial Maximum due to the emergence of the Taiwan-Ryukyu land bridge (Ujiié et al., 2003; Ujiié and Ujiié, 1999). It is generally believed that the consequences of glaciations were much

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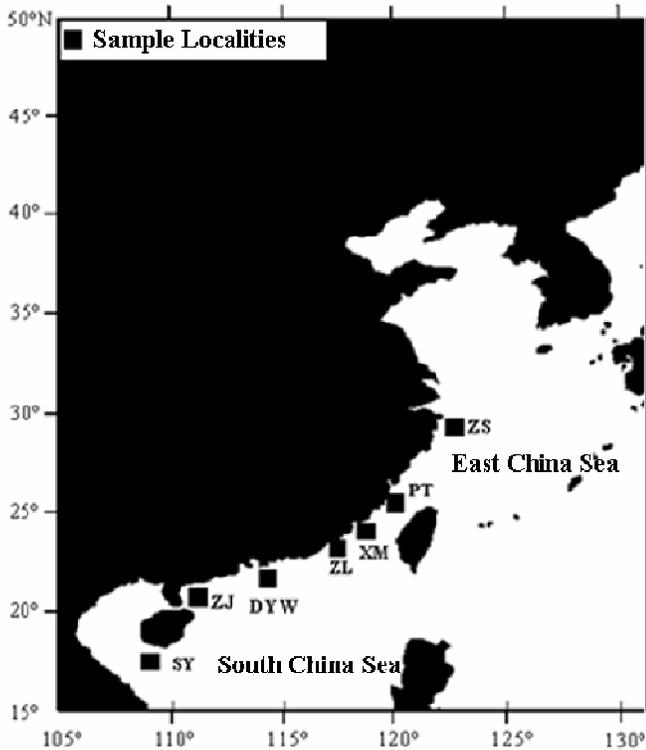


Figure 1. Map showed sample locations of *E. akaara*. Details of the sampling locations are described in Table 1. SY = SanYa population, ZJ = ZhanJiang population, DYW = DaYaWan population, ZL = ZhiLin population, XM = XiaMen population, PT = PingTan population, ZS = ZhouShan population.

more dramatic in the marginal seas than in open sea systems. For these reasons, the Northwestern Pacific appears to provide one of the best natural settings to study how colonization events, population bottlenecks, long term isolation, and subsequent mixing have affected the lineage structure and geographical differentiation of marine species.

The species of the red spotted grouper, *E. akaara*, is a reef fish with fragmentation habit and early life history (Ukawa et al., 1966), sex change (Tanaka et al., 1990), which widely distributed in tropical and subtropical regions of Southeast Asia, especially in the East and South China Sea (Xie, 1999). A lot of literatures existed on the aquaculture and biology of *E. akaara*, and Deng (2004) and Yang and Liu (2006) have done some researches on the different tissue of the red-spotted group by isozyme analysis and RFLP analyses of mitochondrial genome. But little research has been done on genetic structure and genetic diversity for the species (Kusaka et al., 1994; Hamamoto et al., 1986; Fukuhara and Fushimi, 1984). With the development of aquaculture of red-spotted grouper in China, especially in East and South China Sea, it is necessary to understand the population structure and genetic diversity of *E. akaara*.

In the present study, populations of *E. akaara* were

collected throughout their natural range to investigate the evolutionary history of the species. Haplotype sequences for the mitochondrial DNA (mtDNA) non-coding control region were surveyed. The fast evolutionary rate of mtDNA control region coupled with its maternal inheritance has made it an extremely suitable marker with which to study population level phenomena (Avice et al., 1987). The control region was the most utilized region of the mitochondrial genome for population genetic studies of fishes based on sequencing (Grunwald et al., 2002; Bowen and Grant, 1997; Lee et al., 1995). Patterns of the distribution of observed number of differences between haplotypes have been utilized to delineate population processes (Excoffier, 2004; Ray et al., 2003; Rogers, 1995; Rogers and Harpending, 1992). In addition, several methods have been applied to estimate population parameters and to test biological hypotheses (e.g. Fu, 1994, 1997; Tajima, 1983, 1989a,b). The population history of *E. akaara* was inferred to illuminate how these species had responded to the severe climatic oscillations in Pleistocene ice ages. These data were also used to characterize population structuring of this commercially important species throughout its range. Such information may assist the species' fisheries management.

MATERIALS AND METHODS

Sample collection

The red spotted grouper were collected over much of its range in China during 1998-2005 (Table 1, Figure 1). A total of 87 specimens were sampled from 7 localities (Figure 1). Muscle tissues sampled from SanYa, ZhanJiang, DaYaWan, ZhiLin, Xiamen, PingTan and ZhouShan, specimens were preserved at -70°C before DNA extraction.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from muscle tissue using proteinase K digestion followed by a standard phenol-chloroform method (Sambrook et al., 1989). DNA was subsequently resuspended in 80 μ L of TE buffer. PCR primers specific to the red spotted grouper were used. The fish primers L16528 (5'-TCACCC CTG GCT CCC AAA GCC AG -3') and H427 (5'-TGC ATA TAA AAG AAT GCY CGG CAT G-3'), which targeted a portion of transfer RNA (tRNA)-pro and the central conserved region of the control region (Kong et al., 2003) was used in a polymerase chain reaction (PCR) to amplify the first hypervariable segment of the mtDNA control region. PCR was carried out in 50 μ L volume containing 1.25U *Taq* DNA polymerase (Takara Co., Dalian, China), 10 - 100 ng DNA template, 200 nmolL^{-1} forward and reverse primers, 200 molL^{-1} of each dNTPs, 10 mmolL^{-1} Tris, pH 8.3, 50 mmolL^{-1} KCl, 1.5 mmolL^{-1} MgCl_2 . The PCR amplification was carried out in a Biometra thermal cycler under the following conditions: 3 min initial denaturation at 95°C, 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 5 min. All sets of PCR included a negative control reaction tube in which all reagents were included, except DNA template. PCR product was purified with Gel Extraction Mini Kit (Watson BioTechnologies Inc., Shanghai). The purified product was used as the DNA template for cycle sequencing reactions perform-

Table 1. Sampling data of *E. akaara* including sample abbreviation (ID), sample name, sample size, number of haplotypes and date of collection.

ID	Sample	Sample Size	Date of collection	No. of haplotypes	<i>h</i>	π	<i>S</i>	<i>k</i>
SY	SanYa	14	4 March 1999	14	1.00 ± 0.03	0.012 ± 0.007	10	3.85 ± 2.06
ZJ	ZhanJiang	15	7 April 2000	11	0.93 ± 0.05	0.010 ± 0.006	13	3.39 ± 1.84
DYW	DaYaWan	5	8 April 2001	4	0.90 ± 0.16	0.007 ± 0.006	6	2.40 ± 1.56
ZL	ZhiLin	13	1 March 2001	6	0.72 ± 0.13	0.006 ± 0.004	9	1.97 ± 1.91
XM	XiaMen	10	6 April 2001	9	0.98 ± 0.05	0.017 ± 0.010	18	5.69 ± 2.98
PT	PingTan	15	September 2005	9	0.89 ± 0.07	0.008 ± 0.005	12	12.79 ± 1.56
ZS	ZhouShan	15	January 1998	10	0.94 ± 0.04	0.011 ± 0.006	17	3.58 ± 1.93

Several diversity indices for control gene were also indicated.

h = gene diversity (Nei, 1987), π = nucleotide diversity, *k* = mean pairwise nucleotide differences (Tajima, 1983), *S* = number of polymorphic sites.

ed using BigDye Terminator Cycle Sequencing Kit (ver. 2.0, PE Biosystems, Foster City, California), and sequencing was conducted on an ABI Prism 3700 (Applied Biosystems) automatic sequencer with both forward and reverse primers. The primers used for sequencing were the same as those for PCR amplification.

Data analysis

Sequences were edited and aligned using Dnastar software (DNASTAR Inc., Madison, USA). Molecular diversity indices such as haplotype diversity (*h*), nucleotide diversity (π) and the mean number of pairwise differences (*k*), and their corresponding variances were calculated following Nei (1987) implemented in the program ARLEQUIN (ver. 2.000; Schneider et al., 2000). The gamma distribution with shape parameter (Γ) for the rate heterogeneity among sites was calculated using the program Modeltest Version 3.06 (Posada and Crandall, 1998).

A neighbor-joining tree (Saitou and Nei, 1987) of the haplotypes was also constructed under the model of Tamura and Nei (1993) with a gamma correction (TrN + Γ , Γ = 3.14) using MEGA2.0 (Kumar et al., 2001) and evaluated with 1000 bootstrap replicates. Genealogical relationships were also examined based on the whole dataset, by constructing haplotype networks using median-network approach (Bandelt et al., 1995, 2000).

Population genetic structure was evaluated with F_{ST} statistics. The significance of the F_{ST} was tested by 1000 permutations in ARLEQUIN. Population structure was further investigated using the molecular variance software package (AMOVA) in ARLEQUIN (Excoffier et al., 1992). The significance of the variance components of genetic structure was tested using 1000 permutations.

The diversity index $\theta = 2N_e u$, where N_e is the effective female population size and *u* is the mutation rate per sequence per generation, was estimated by two methods: Tajima's (1983) statistic and Fu's (1994) UPBLUE statistic, Tajima's estimator (θ_{π}) is equal to the mean number of pairwise differences between two sequences in the sample and Fu's UPBLUE (θ_u) estimator was based on generalized linear regression with genealogy estimated using the UPGMA method. Tajima's estimator was calculated in ARLEQUIN and Fu's UPBLUE estimator was calculated at Dr. Fu's Website (<http://hgc.sph.uth.tmc.edu/cgi-bin/upblue.pl>).

Historical demographic patterns of the red spotted grouper were examined by two approaches. First, we evaluated whether the control region evolved under strict neutrality. Several widely used statistical tests were applied, including Tajima's *D* (Tajima, 1989a, b), and Fu's F_S (Fu, 1997). Significantly negative *D* values can be due to factors such as selection, population expansion and bottle-

necks (Tajima, 1989b). Fu (1997) found that the F_S was sensitive to population demographic expansion, which generally led to large negative F_S values. The significance of the neutrality statistics were tested by generating random samples under the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation algorithm adapted from Hudson (1990).

Second, historical demographic expansions were investigated by examination of frequency distributions of pairwise differences between sequences (mismatch distribution; Excoffier, 2004; Ray et al., 2003; Rogers and Harpending, 1992). Mismatch distributions can be used to test the hypotheses about the population demographic history and selection (Rogers and Harpending, 1992). The parameters of the demographic expansion τ , θ_0 , and θ_1 were estimated by a generalized nonlinear least-square approach and confidence intervals of the parameters were computed using a parametric bootstrap approach (Schneider and Excoffier, 1999). The estimator τ (tau, an estimate of the mode of the mismatch distribution) was an index of time since expansion was expressed in units of mutational time (Rogers and Harpending, 1992). The values of τ were transformed to estimates of real time since expansion with the equation $\tau = 2ut$, where *u* was the mutation rate for the sequence under study per generation and *t* was the time measured in generations since expansion. θ_0 and θ_1 corresponded to the mutation parameter before and after population growth. The mismatch analysis was performed in ARLEQUIN, and the deviation from the sudden population model was further tested using the Harpending's raggedness index (*Hr*) (Harpending, 1994).

RESULTS

In red spotted grouper, a 331 bp segment at the 5' end of control region (including a 66 bp partial segment of the tRNA^{Pro} gene) was sequenced in the 87 specimens. Three intraspecific polymorphic sites were found in the 66 bp partial segment of the tRNA^{Pro} gene. Sequence comparisons of the 265 bp segment of control region revealed 35 polymorphic sites with 28 transitions, 7 transversions and 3 indels, defining 47 haplotypes in 87 individuals. Fifteen of the 35 polymorphic sites were singletons. Two conserved regions of 5' end control region at positions 1 - 12 and 52 - 93 including 12 and 42 bp, respectively. 81% of the 47 haplotypes were exclusive, and six of the left were shared among populations.

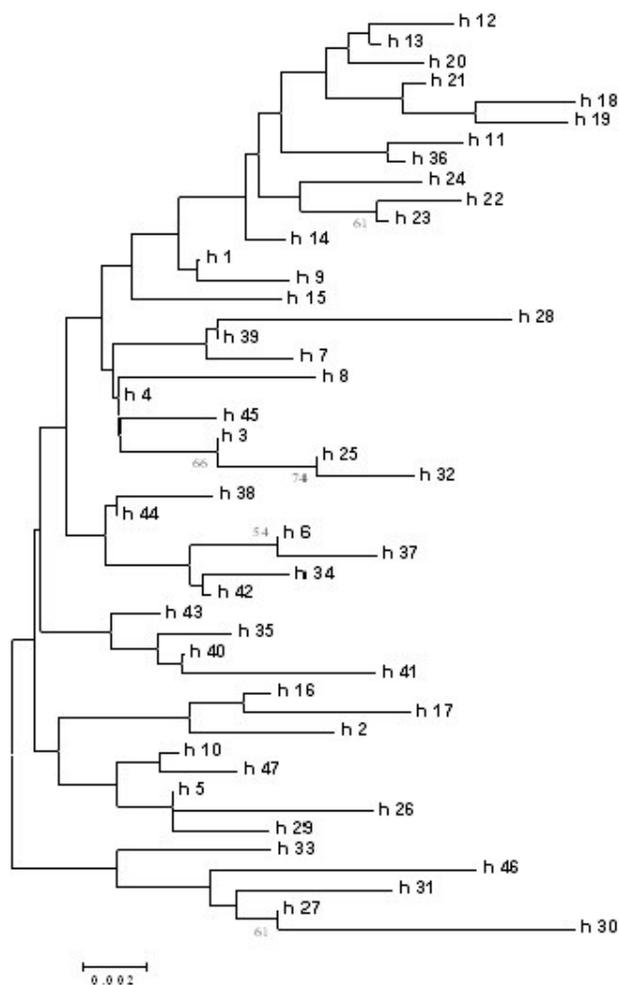


Figure 2. Neighbor-joining tree constructed using Tamura and Nei distances with a gamma shape parameter of 3.14 for 47 control region haplotypes of *E. akaara*. Bootstrap supports of > 50% in 1000 replicates are shown.

SanYa population had no common haplotypes with other population (Table 4). The gamma distribution shape parameter was 3.14, indicating single mutation rate heterogeneity among sites. All the analyses in this work were based on the 265 bp segment of control region. All haplotypes of control region sequences have been deposited in the Genbank database under Accession Nos. EU744254 – EU744300.

Evidences from the mitochondrial control region indicated no obvious genealogy among the red spotted grouper (Figure 2). Nucleotide diversity of the red spotted grouper ranged from 0.006 to 0.017 which was moderate to compare with those of other marine fishes (Teske et al., 2003; Grunwald et al., 2002).

The topology of the neighbor-joining tree of red spotted grouper haplotypes was shallow, and there were no significant genealogical branches or clusters of samples corresponding to sampling localities (Figure 1). Typically, haplotypes in a sample were scattered throughout the

tree. The shallow phylogeny is consistent with a population expansion after a bottleneck (Slatkin and Hudson, 1991). Haplotype network for red spotted grouper was also constructed (Figure 3) with median-network approach. Network of the red spotted grouper was star-like with a frequent haplotype shared by all samples (Table 4), which indicated signature of population expansion for the species.

Haplotype diversity (h) was high for all populations, ranging from 0.72 to 1.00. Nucleotide diversity (π) varied among populations, ranging from 0.006 (ZL) to 0.017 (XM) (Table 1). Pairwise comparisons of F_{ST} indicated significant genetic differentiation across the range of the red spotted grouper, especially for SanYa population which had significant genetic differentiation with other population ($P < 0.003$) (Table 2). And some of the population pairwise F_{ST} statistics were negative, which indicated remarkable gene flow existed among some populations (Table 2). AMOVA showed that the genetic variation among populations was 14.94% ($P = 0.000$).

Estimates of θ were shown in Table 3. There was a considerable range in the estimates of θ . Fu's UPBLUE estimator was higher than the Tajima's estimator ($\theta\pi$). The Tajima's estimator for the red spotted grouper is insensitive to low-frequency mutations. Since mutations at low frequency are more likely to be new, $\theta\pi$ is a useful measure of persistent genetic variation. Fu's UPBLUE estimator puts heavy emphasis on recent mutations, thus reflecting relatively recent population events (Fu, 1997).

The results of the neutrality tests for the red spotted grouper are shown in Table 3. Fu's F_S test of neutrality was significant at 5% level (1000 permutations) for most of locations and for the total samples except for DYW and ZL populations. Tajima's D statistics values were all negative but not significant at 7 sites and nearly significant in pooled samples. Therefore, we conclude that the control region under study has not evolved according to the neutral Wright-Fisher model.

The mismatch analysis produced a unimodal distribution of pairwise differences (Figure 4) consistent with the sudden population expansion model, and the Harpending's raggedness index ($H_{ri} = 0.009$, $P > 0.05$) was low, indicating a significant fit of the observed and expected distribution to further evidence of population expansion in the species (Harpending, 1994).

Molecular clock for the control region has not been determined with precision for marine fishes, and seems to vary among major taxonomic groups. In some bony fishes, the control region seems to mutate at about the same rate as protein-coding mtDNA regions. This has been demonstrated for some groups of fishes including salmonids (Shedlock et al., 1992), East African cichlids (Sato et al., 2003; 2.2 - 4.5% divergence per million years), Australian rainbow fishes (Zhu et al., 1994; 3%/MY) and snooks (Donaldson and Wilson, 1999; 3.6%/MY). However, evolution of the control region seems to be much faster in some other bony fishes including Lake Malawi cichlids (Sturmbauer et al., 2001; 6.5 -

Table 2. The conventional population *Fst* statistics (below the diagonal) and corresponding *P*-value (above the diagonal).

Population	SY	ZJ	DYW	ZL	XM	PT	ZS
SY		< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001
ZJ	0.217		0.528	0.017	0.002	0.311	0.964
DYW	0.256	-0.010		0.553	0.500	0.912	0.330
ZL	0.379	0.091	-0.018		0.020	0.181	0.018
XM	0.344	0.126	-0.005	0.097		0.023	0.004
PT	0.299	0.007	0.078	0.022	0.086		0.224
ZS	0.251	-0.037	0.025	0.094	0.126	-0.017	

SY = SanYa population, ZJ = ZhanJiang population, DYW = DaYaWan population, ZL = ZhiLin population, XM = XiaMen population, PT = PingTan population, ZS = ZhouShan population.

Table 3. Tajima's *D* and Fu's *F_s* statistics, corresponding *P*-value, mismatch distribution parameter estimates and estimates of θ .

Population	Tajima's D		Fu's <i>F_s</i>		Mismatch Distribution			θ_H	θ_U
	<i>D</i>	<i>P</i>	<i>F_s</i>	<i>P</i>	τ	θ_0	θ_1		
SY	-0.87	0.84	-12.60	0.00	3.66	0.13	66658.75	3.75	3.18
ZJ	-0.60	0.32	-5.10	0.01	3.63	0.00	3415.00	3.47	4.05
DYW	-1.15	0.06	-0.70	0.18	5.12	0.00	4.56	2.46	2.94
ZL	-0.99	0.16	-1.63	0.12	5.89	0.01	2.45	1.69	2.22
XM	-0.49	0.30	-3.17	0.03	6.31	0.01	58.20	5.69	6.39
PT	-0.83	0.21	-3.28	0.02	3.03	0.00	42.93	2.66	3.32
ZS	-1.20	0.12	-3.54	0.02	2.27	1.67	60.56	3.55	4.95
Total	-1.25	0.10	-26.01	0.00	4.31	0.03	20.98	3.79	6.33

SY = SanYa population, ZJ = ZhanJiang population, DYW = DaYaWan population, ZL = ZhiLin population, XM = XiaMen population, PT = PingTan population, ZS = ZhouShan population.

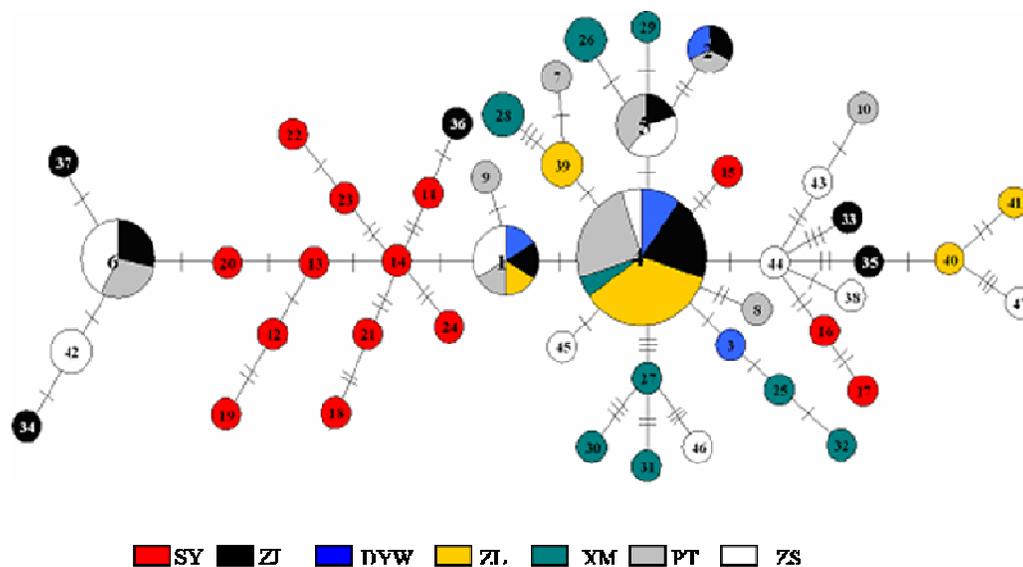
**Figure 3.** Unrooted minimum spanning trees showing genetic relationship among control region haplotypes for *E. akaara*. The sizes of circles are proportional to haplotype frequency. Perpendicular tick marks on the lines joining haplotypes represent the number of nucleotide substitutions. SY = SanYa population, ZJ = ZhanJiang population, DYW = DaYaWan population, ZL = ZhiLin population, XM = XiaMen population, PT = PingTan population, ZS = ZhouShan population.

Table 4. Sample abbreviations (ID), sample size (N) and distribution of haplotypes of all sequenced individuals of red-spotted grouper (*Epinephelus akaara*).

ID	N	h1	h2	h3	h4	h5	h6	h7	h8	h9	h10	h11	h12	h13	h14	h15	h16	h17	h18	h19	h20	h21	h22	h23	h24
SY	14											1	1	1	1	1	1	1	1	1	1	1	1	1	1
ZJ	15	1	1		4	1	2																		
WYW	5	1	1	1	2																				
ZL	13	1			7																				
XM	10				1																				
PT	15	1	1		5	2	2	1	1	1	1														
ZS	16	2			1	2	3																		
Total	87	6	3	1	20	5	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ID	h25	h26	h27	h28	h29	h30	h31	h32	h33	h34	h35	h36	h37	h38	h39	h40	h41	h42	h43	h44	h45	h46	h47		
SY																									
ZJ									1	1	1	1	1	1	1										
WYW																									
ZL														2	1	1									
XM	1	2	1	1	1	1	1	1																	
PT																									
ZS																			2	1	1	1	1	1	1
Total	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	2	1	1	1	1	1	1

SY = SanYa population, ZJ = ZhanJiang population, WYW = DaYaWan population, ZL = ZhiLin population, XM = XiaMen population, PT = PingTan population, ZS = ZhouShan population.

8.8%/MY), Arctic charr (Brunner et al., 2001; 5 - 10%/MY) and butterfly fishes (McMillan and Palumbi, 1997) with as much as 33 - 100%/MY at selected hotspots. According to red spotted group morphologic and physiological characteristics correlated with molecular clocks in other vertebrates (Martin and Palumbi, 1993), a moderate molecular clock may be more appropriate for *E. akaara*. In the present study, sequence divergence rate of 3 - 10%/Myr was applied for the control region sequences of *E. akaara*. Occasionally, pronounced rate heterogeneity is reported even among closely related lineages (Zhang and Ryder, 1995), so large variance may exist in the divergence rate used in the present study. However, a molecular

clock-based time estimate provides an approximate time frame for evaluating phylogeographical hypotheses. If we assumed that the unimodal distributions reflect population expansion, the timing of this expansion estimated from $\tau = 4.31$ began at about 162,000 ~ 542,000 years ago.

DISCUSSION

Pleistocene ice ages and patterns of genetic diversity

As expected, range contractions and expansions played a central role in shaping the genetic diversity of the species. High nucleotide diversity

can be found in SanYa population of South China Sea and in ZhouShan population of East China Sea (Table 1). Changes in area and configuration were much more drastic in the marginal basins like the East China Sea and South China Sea than in the open area of Pacific side. During the LGM when the sea level was at its lowest, about 130 m below the present sea level, an extensive area of the continental shelf of the East and South China Sea was exposed (Xu and Oda, 1999). During glacial periods, the displaced populations had to survive along the glacial refugium in a compressed biome, inevitably leading to reduced genetic diversity (Hewitt, 1996, 2000). Similar result was found in coral reef fish species with

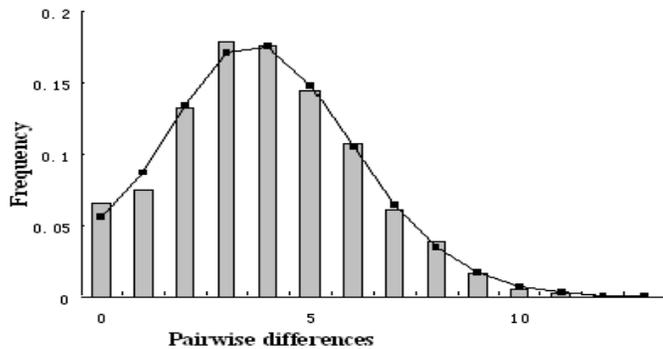


Figure 4. The observed pairwise difference (bars), and the expected mismatch distributions under the sudden expansion model (solid line) of control region haplotypes *E. akaara*

different habitat preferences (lagoon or outer slope) in French Polynesia, species inhabiting lagoons demonstrated reduced mtDNA diversity compared to species inhabiting stable environments (the outer slope) because lagoons dried out during Holocene sea-level regression (Fauvelot et al., 2003). Postglacial expansion into new territory was suggested to be important in the geographic distribution of population and species genomes (Hewitt, 2000). If an ancestral population and a derived population are compared, the genetic diversity is expected to be higher in the ancestral population (Savolainen et al., 2002). When haplotype diversity is close to 1, it could not discriminate among different populations and is therefore no longer an informative measure of polymorphism (Li, 1997). This is the case in the present study, so we consider the more appropriate measure of genetic diversity, nucleotide diversity (π). In South China Sea, nucleotide diversity was highest in the southern population of SanYa (0.012) and lowest in the northern population of ZhiLin (0.006). In contrast, nucleotide diversity was highest in the northern population of ZhouShan (0.011) and lowest in the southern population of PingTan (0.008) in East China Sea. In general, northern populations of *E. akaara* showed higher nucleotide diversities than southern ones in East China Sea (Table 1). In the South China Sea, the northern populations showed lower nucleotide diversities, which was consistent with the hypotheses that the glacial refugia of *E. akaara* were located in the basin of East China Sea and South China Sea.

Population genetic structure

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 margins (Grant and Bowen, 1998; Hewitt,

2000). According to our results, *E. akaara* did not conform to this pattern and showed differentiation throughout its range. Result of AMOVA detected significant differences at all population levels, and most of the conventional population F_{ST} statistics were significant, especially for SanYa population, indicating that significant population genetic structure existed throughout the range of the red spotted grouper. Cold and Richardson (1994) found that habit fixation could give rise to genetic structure in *E. morio* by mtDNA analysis (Cold and Richardson, 1994), and the red spotted grouper was also reef fish, which might illuminate the significant population structure in *E. akaara*. The differentia of propagation and sex change along the distribution range of the red-spotted grouper also may prevent gene flow among populations. The results also have important implications for fisheries management of the species. The significant genetic structure in *E. akaara* throughout its range points out the need to take into account self-recruitment to avoid local over exploitation and decline.

Population history—crashes and expansions

Both the neutrality tests and the mismatch distribution analysis indicated population expansion in the species. Estimate of population expansion time indicated an extensive population expansion ~162 kyr ago in *E. akaara*. Additionally, simulations demonstrated that range expansions may lead to a molecular signature quite similar to that observed after a pure demographic expansion (Ray et al., 2003). Population range expansion must have occurred after the Last Glacial Maximum for *E. akaara*. So, the past population demographic expansion and geographical range expansion could explain the observed mitochondrial diversity in *E. akaara*. Pleistocene climatic oscillations produced changes in temperatures, current patterns, upwelling intensity, and the displacement, or eradication of coastal habitats (Bond et al., 1997; Kennett and Ingram, 1995; Kotilainen and Shackleton, 1995). Deterioration of rocky reef habitats for *E. akaara* must have occurred when sea levels lowered (120 – 140 m below present sea level) during glacial maxima, which may be the reason given the population expansion.

For the uncertainty on the estimates of population expansion time, it is difficult to link population expansion of the species to any particular Pleistocene paleo-climatic event in the present study. However, it is evident that Pleistocene ice ages had great effect on the demographic history of the species. Similar conclusions have been reached on other marine fishes. Historical population expansions were detected in mackerel *Scomber scombrus* and chub mackerel *Scomber japonicus* (Zardoya et al., 2004), and Pleistocene population expansions were also detected in Japanese sea bass (*Lateolabrax japonicus*) and spotted sea bass (*Lateolabrax maculatus*) in Northwestern Pacific (Liu et al., 2006a).

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