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Genetic Diversity Comparison of *Pampus minor* between Chinese and Malaysian Populations Inferred from mtDNA Cytb

Yuan Li[1], Liyan Zhang[2], Karhoe Loh[3], Ji Feng[1], Xinqing Zheng[1], Puqing Song[1] and Longshan Lin[1,*]

[1]*Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, China*
[2]*Fujian Institute of Oceanography, Xiamen 361012, China*
[3]*Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur 50603, Malaysia*

Yuan Li and Liyan Zhang have contributed equally to this paper.

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YL and LL conceived and designed experiments. YL, XZ, PS and JF performed all experiments and wrote the manuscript. LZ and KL analyzed the data.

Key words
***Pampus minor*, Genetic diversity, Population structure, Population expansion, Cytb.**

ABSTRACT

Pampus minor is often mistakenly identified as the larva of *Pampus argenteus* or *Pampus cinereus* because of its small size. Despite its importance, studies on the population genetics of *P. minor* are not yet available. In the present study, the mitochondrial Cytb gene was employed to investigate the population genetics of *P. minor* collected along the coasts of China and Malaysia. The genetic diversity of all *P. minor* populations was moderate, and two major haplotype lineages were detected that were differentiated approximately 0.3 million years ago. These two haplotype lineages differed significantly in frequency distribution of Chinese and Malaysian populations, showing an imperfect geographical pedigree structure. Results of AMOVA also showed that the genetic differentiation was mainly among populations. According to the distribution of the haplotypes, an ancestral haplotype existed in both the Chinese population and the Malaysian population, further confirming that the Chinese and Malaysian *P. minor* populations originated from the same refuge in the South China Sea. A historical demographic analysis indicated that *P. minor* experienced a recent population expansion during the late Pleistocene period. Due to the need of *P. minor* to adapt to the diverse habitats, unique haplotypes were ultimately formed under the differing pressures of natural selection. This study is expected to provide a basis for future research of the population genetics of *P. minor*.

INTRODUCTION

Pampus minor Liu and Li, 1998 belongs to the class Actinopterygii, order Perciformes and family Stromateidae. It is a newly discovered warm-water *Pampus* species, distributed mainly in the offshore to the south of the Taiwan Strait (Liu and Li, 1998; Liu *et al*., 2002). Due to the similar external characteristics and small size (adult fish are generally less than 150 mm long), *P. minor* has often been mistaken for *P. argenteus* or *P. cinereus* (Cheng, 1962; Liu and Li, 1998). Zhang (2011) suggested that there is introgressive hybridization between *P. argenteus* or *P. cinereus* in the South China Sea, whereas our studies demonstrate that this species is *P. minor* (Li *et al*., 2013). There are few studies on *P. minor*, and those available are limited to morphological (Liu and Li, 1998) and phylogenetic (Guo *et al*., 2010; Cui *et al*., 2010, 2011) studies. Basic surveys on the status of fishery resources and the distribution of *P. minor* have not been reported, l*et al*one studies on its population genetic diversity.

Based on morphology and DNA barcoding studies on a large number of *P. minor* samples collected, we summarized the identifying morphological characteristics of *P. minor* as follows (Liu and Li, 1998; Li, 2015): oval body; eye diameter more than 1/2 of the head length; dorsal fins VII-IX 34-39, pectoral fins 22-24, anal fins V-VII 35-39, and caudal fins 18-20; transverse occipital canals and the dorsal branches of the lateral-line canal on top of the head with a truncated rear edge; ventral transverse occipital canals sparse and slightly longer than or equal in length to the dorsal branches; gill rakers, thin (delicate), sparse, 3-4+8-10=11-14; and vertebrae 29-31.

P. minor is also distributed in Malaysian waters, where it has been recorded recently. If the same species lives in heterogeneous habitats, different genetic diversity often emerge to allow for adaptation to local environments,

* Corresponding author: linlsh@tio.org.cn
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ultimately shaping different genetic structures. The habitats of the northern South China Sea and Malaysian waters are significantly different with different surface sea temperatures, salinity and substrate environment (See *et al*., 2016). Hence, it is of interest to determine whether there is a significant difference in the genetic diversity of the *P. minor* populations in these waters and whether a significantly divergent genetic structure has formed. Only a few studies on the genetic diversity and genetic structure of pomfret fish can be found, and these focused mainly on the study of *P. argenteus* (Peng *et al*., 2009; Zhao *et al*., 2011a; Archangi *et al*., 2013) without any relevant study on *P. minor*. Sequence analysis is a widely used technique that is considered suitable for testing the genetic diversity of some species. In the present study, based on the accurate identification of *P. minor*, the mitochondrial DNA cytochrome b gene (Cytb), which has a moderate nucleotide mutation rate, was used as a molecular marker (Abbas *et al*., 2017) to comparatively analyze the genetic diversity, genetic structure, and historical demographics of this species along the coasts of China and Malaysia, thereby increasing the understanding of the status of its genetic diversity and clarifying the species' geographical distribution pattern. This study is of great practical significance for the protection of *P. minor* and for the promotion of its sustainable utilization.

MATERIALS AND METHODS

Sample collection

Four different geographical populations (96 individuals) of *P. minor* were collected from China (Beihai, Haikou) and Malaysia (Kuala Selangor, Mukah) in 2016 (Fig. 1; Table I). To ensure the accuracy of the taxonomy, all specimens were identified according to their morphological characteristics (Liu and Li, 1998). Back-muscle tissues were excised and preserved in 95% alcohol for subsequent molecular experiments.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from muscle tissue by proteinase *K* digestion and extracted with a Qiagen DNeasy kit. The extracted DNA was assessed by 1.5% agarose gel electrophoresis and stored at -20°C for PCR amplification. The mtDNA cytochrome b (Cytb) was amplified with the primers L14734: 5′-AACCACCGTTGTTATTCAACT-3′(Inoue *et al*., 2001) and H15149: 5′-CTCAGAATGATATTTGTCCTCA- 3′ (Ohdachi *et al*., 1997). Each PCR was performed in a 25-μL reaction mixture containing 17.5 μL of ultrapure water, 2.5 μL of 10×PCR buffer, 2 μL of dNTPs, 1 μL of each primer (5 μM), 0.15 μL of Taq polymerase, and 1 μL of DNA template. The PCR was performed under the following conditions: 4 min of initial denaturation at 94°C; 32 cycles of 30 s at 94°C for denaturation, 30 s at 50°C for annealing, and 30 s at 72°C for extension; and a final extension at 72°C for 10 min. After purification of the PCR products, both strands were sequenced. The newly determined Cytb sequences were deposited in GenBank under the accession numbers MF616364–MF616380.

Fig. 1. *Pampus minor* Liu and Li, 1998.

Data analysis

All Cytb sequences were aligned and edited manually using DNASTAR software. Molecular diversity indices such as polymorphic sites, haplotype number, transitions and transversions were calculated by ARLEQUIN version 3.5 (Excoffier *et al*., 2005). Genetic relationships among haplotypes were reconstructed using the neighbor-joining (NJ) method implemented with 1000 replicates in MEGA 5.0 (Tamura *et al*., 2011). Analysis of molecular variation (AMOVA) was performed using ARLEQUIN to

Table I.- Information and molecular indices of *P. minor*.

ID	Populations	n	Date	NH	NUH	*h* ± SD	*л*± SD	*k* ± SD
TB	Beihai	24	2016.11	5	2	0.6377±0.0606	0.0019±0.0016	0.7717±0.5842
TH	Haikou	24	2016.12	5	2	0.5290±0.1042	0.0014±0.0013	0.5942±0.4921
TKS	Kuala Selangor	24	2016.05	7	3	0.6341±0.0973	0.0048±0.0032	2.0109±1.1731
TSK	Mukah	24	2016.05	7	4	0.5036±0.1226	0.0028±0.0021	1.1558±0.7732
Total		96	-	17	-	0.7818±0.0224	0.0054±0.0033	2.2493±1.2490

NH, numbers of haplotypes; NUH, numbers of unique haplotypes; *h*, haplotype diversity; *л*, nucleotide diversity; *k*, average number of pairwise differences.

investigate the partition of genetic variation among the populations. Population pairwise F_{ST} and exact-*P* test were also performed using ARLEQUIN. A minimum spanning tree was constructed via the MINSPN*Et al*gorithm as implemented in ARLEQUIN to show the relationship among haplotypes and subsequently drawn by hand.

Historical demography/spatial expansions were inferred by neutrality testing and mismatch distribution analysis, as implemented in ARLEQUIN. Deviations from neutrality were evaluated using Fu's F_S and Tajima's *D*. Nucleotide mismatch distributions were applied to evaluate the population growth and spatial range expansion. The values of τ were transformed to estimates of real time since expansion with the equation $\tau=2\times\mu\times t$, where μ is the mutation rate for the whole sequence under study, and *t* is the time since expansion. In the present study, a sequence divergence rate of 0.2×10^{-7}/site/year was applied to the Cytb sequences of *P. minor* (Avise, 1994; Sun *et al*., 2012). Bayesian skyline plots were created with BEAST v.8 (Drummond and Rambaut, 2007).

RESULTS

Genetic diversity

The length of the Cytb sequence was 415 bp after manual proofreading and comparison. A total of 14 mutations, 10 parsimony informative sites, and four singleton sites were found in the target fragment. There were 14 transitions, three transversions and no insertions/deletions. The ratio of transitions to transversions was 4.7, indicating that the mutations in the Cytb sequence of *P. minor* had not reached saturation. The A+T content (62.34%) was higher than the G+C content, indicating a significant AT preference.

In the coding gene, most of the mutations were synonymous substitutions, which occurred mostly at the third locus of the codon. All mutations in the *P. minor* sequence occurred at the third codon due to the relatively slight pressure of natural selection on the nucleotide mutation of the third codon in the protein-encoded gene. No mutations were detected at the first or second codon due to the strongly limited functionality of these codons. The sequences of all nucleotides encoded 138 amino acids, and no mutated amino acid was detected, showing that all mutations are synonymous substitutions.

The 96 collected *P. minor* samples represented 17 haplotypes. The Beithai population had five haplotypes, of which two were unique haplotypes; the Haikou population had five haplotypes, of which two were unique haplotypes; the Kuala Selangor population had seven haplotypes, of which three were unique haplotypes; the Mukah population had seven haplotypes, of which four were unique haplotypes (Table I); the Chinese population had seven haplotypes, of which three were common haplotypes and four were unique haplotypes; and the Malaysian population had eleven haplotypes, of which three were common haplotypes and seven were unique haplotypes. Only Hap_2 was common in the three populations, and it was also the only haplotype commonly found in both the Chinese and the Malaysian populations, being identified in 30 individuals. It is possibly an ancestral haplotype. In the *P. minor* populations of this study, the haplotype diversity (*h*) was 0.782±0.022, and the nucleotide diversity (π) was 0.005±0.003 (Table I), indicating that for the characteristics of high *h* and low π, the genetic diversity was moderate.

Genetic structure

The neighbor-joining (NJ) tree was constructed based on the 17 Cytb haplotypes of pomfret. The results showed two major haplotype lineages in the *P. minor* populations, though the bootstrap values were not high. However, pedigree structure that strictly corresponded to the geographical location was not detected (Fig. 2). Lineage A consisted of seven haplotypes (62 individuals), six of which were all in the Chinese group and Hap_2 from 14 Malaysian individuals and 16 Chinese individuals; Lineage B consisted of 10 haplotypes (34 individuals), which were all in the Malaysian group (Table II).

Table II.- Distribution of haplotypes among four *P. minor* populations in lineages A and B.

Haplotype	Total	TB	TH	TKS	TSK
Lineage A					
Hap_1	26	10	16		
Hap_2	30	11	5	14	
Hap_3	1	1			
Hap_4	1	1			
Hap_5	2	1	1		
Hap_6	1		1		
Hap_7	1		1		
Lineage B					
Hap_8	22			5	17
Hap_9	1			1	
Hap_10	2			1	1
Hap_11	1			1	
Hap_12	1			1	
Hap_13	2			1	1
Hap_14	2				2
Hap_15	1				1
Hap_16	1				1
Hap_17	1				1

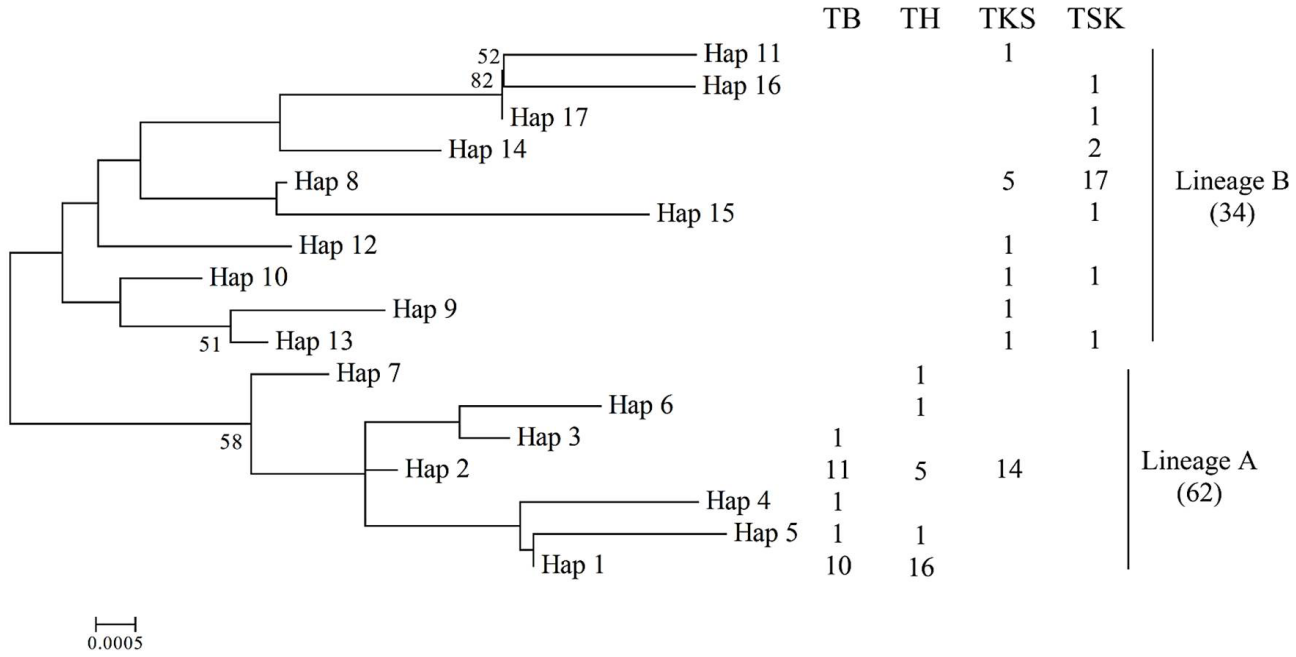

Fig. 2. NJ tree of Cytb haplotypes of *P. minor*. Bootstrap supports >50 in 1,000 replicates.

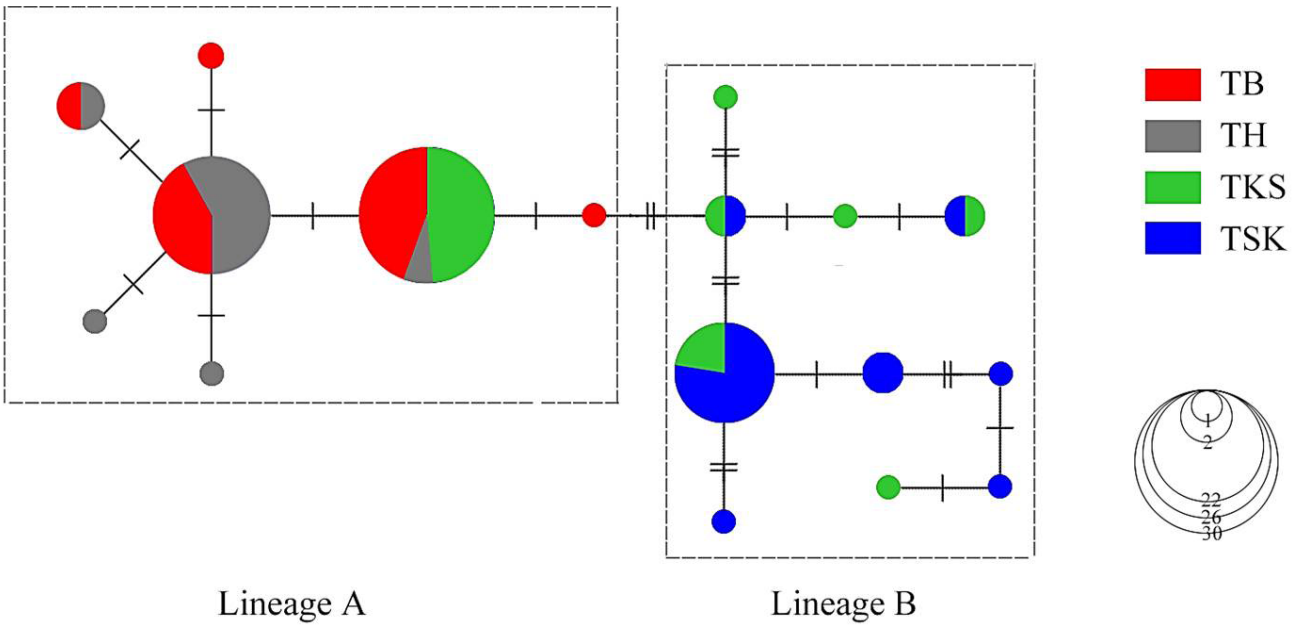

Fig. 3. Unrooted minimum spanning tree depicting the genetic relationship among the Cytb haplotypes of *P. minor*. Circle sizes are proportional to the haplotype frequency. Perpendicular tick marks on the lines joining the haplotypes represent the number of nucleotide substitutions.

Based on the 17 haplotypes of *P. minor*, the haplotype network diagram was constructed by minimum spanning tree (MST) methods (Fig. 3). The results showed a significant pedigree structure in the topology of the network, which is consistent with the results of the NJ tree. The frequency distribution of the two haplotype lineages in the *P. minor* populations was calculated (Table II). The results showed that Hap_1, Hap_2, and Hap_8 had a

relatively high distribution frequency and were the main haplotypes. Except for Hap_2, the frequency distributions of the two haplotype lineages in the geographical groups were significantly divergent; in other words, an imperfect geographical structure was present.

The net genetic distance between the two haplotype lineages was calculated to be 0.0059, and Lineages A and B differentiated approximately 0.3 million years ago based on a mitochondrial Cytb differentiation rate of 2% per million years.

The results of the population pairwise F_{ST} show that except for the moderate differentiation between the North Sea population and the Haikou population, the F_{ST} values of the remaining populations were higher than 0.25, exhibiting great differentiation (Wright, 1965), and the statistical test between all groups showed significant differences (Table III). The results of the exact-*P* test showed that except for the North Sea population and the Haikou population, which yielded an insignificant *P* value in the statistical test, the *P* values between all other group pairs in the statistical test were significant (Table III). This result suggested significant genetic differentiation between all group pairs except for the North Sea population and the Haikou population, which showed only weak genetic exchange.

Table III.- Results of the pairwise F_{ST} (below diagonal) and exact-*P* tests (above diagonal) among four populations of *P. minor*.

Populations	TKS	TSK	TB	TH
TKS		0.0000*	0.0000*	0.0000*
TSK	0.3952*		0.0000*	0.0000*
TB	0.4211*	0.7877*		0.1334
TH	0.3019*	0.7535*	0.0809*	

*, Significant at $P<0.05$ by the permutation test.

Table IV.- AMOVA of *P. minor* populations based on mitochondrial Cytb sequences.

Source of variation	Sum of squares	Percentage	*F* statistic	*P*
One gene pool				
Among populations	54.719	56.52	F_{ST}= 0.5652	0.000
Within populations	52.125	43.48		
Two gene pools (TB and TH) (TSK and TKS)				
Among groups	40.448	45.23	F_{CT}=0.45233	0.346
Among populations within groups	14.271	17.84	F_{SC}=0.32573	0.000
Within populations	92	36.93	F_{ST}=0.63072	0.000

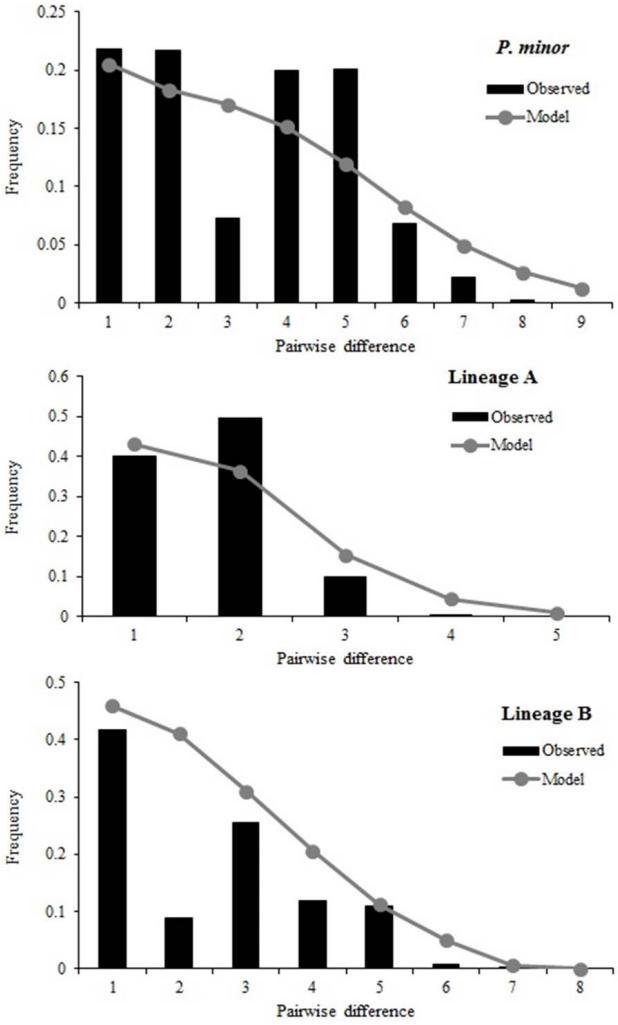

Fig. 4. Mismatch distributions of Cytb haplotypes of *P. minor*.

The genetic structure (Table IV) of the *P. minor* populations was detected using analysis of molecular variance (AMOVA). First, all *P. minor* groups were analyzed as one gene pool. The results showed that the genetic differentiation index (F_{ST}) between the *P. minor* populations was large, with a genetic difference of 56.52%, and the statistical test result was significant. In contrast, the genetic difference within a population was only 43.48%. Second, to further confirm the genetic structure of the *P. minor* population, the four populations were grouped into two gene pools according to the geographical distribution of *P. minor*: the Chinese group (TB and TH) and the Malaysian group (TSK and TKS). The results showed that the genetic variation between the two lineages was 45.23%, but the statistical test was not significant. The genetic variation within a population between groups

was only 17.84%, and the statistical test was significant. The genetic variation within a group was 36.93%, and the statistical test was significant.

Historical demographics

Although two lineages were detected in the *P. minor* populations, no obvious geographical structure existed in the distribution of the different populations, which may be the result of non-equilibrium in genetic variation after isolation and differentiation. Because of the significant differentiation existed, the two lineages were analyzed separately for their historical demographics. Both the distribution of mismatched nucleotides in all sequences of *P. minor* and the two lineages showed a single peak, respectively.

The results of the neutrality test showed (Table V) that the values of Fu's F_S were negative, and the statistical tests were significant ($P<0.05$), indicating that *P. minor* experienced a population expansion event. Furthermore, the values of the *D* test were negative, but the statistical tests were not significant. However, the sum of squared deviations (SSD) and Harpending's raggedness index (*HRI)* statistical tests were not significant ($P>0.05$), indicating no significant deviation from the expected distribution under the population expansion model. Therefore, the expected distribution can be used for the historical demographic analysis of population (Fig. 4).

The τ value for the distribution of the unmatched nucleotide provides a rough estimated time of population expansion. The τ of Lineage A was larger than that of Lineage B, at 0.844 (95% CI: 0.279-4.184) and 0.125 (95% CI: 0.0488-2.361), respectively (Table V). According to the differentiation rate of 2% per million years and the τ value, the time of population expansion in *P. minor* for Lineages A and B was estimated to be 2.01×10^5 and 1.51×10^5 years ago, respectively, which were in the late Pleistocene period. The ratio of the θ_0 after and before the expansion was infinite, indicating that the size of the effective maternal population of *P. minor* after the expansion experienced a sharp increase.

The Bayesian skyline plots revealed a detailed demographic history of population size changes, from which we could see that both lineages A and B had undergone population expansion in the late Pleistocene. The effective population size of lineage A increased sharply after the last glacial maximum (LGM) approximately 5.5×10^4 years before the present, and the effective population size of lineage B increased slowly from 1.7×10^5 years ago (Fig. 5).

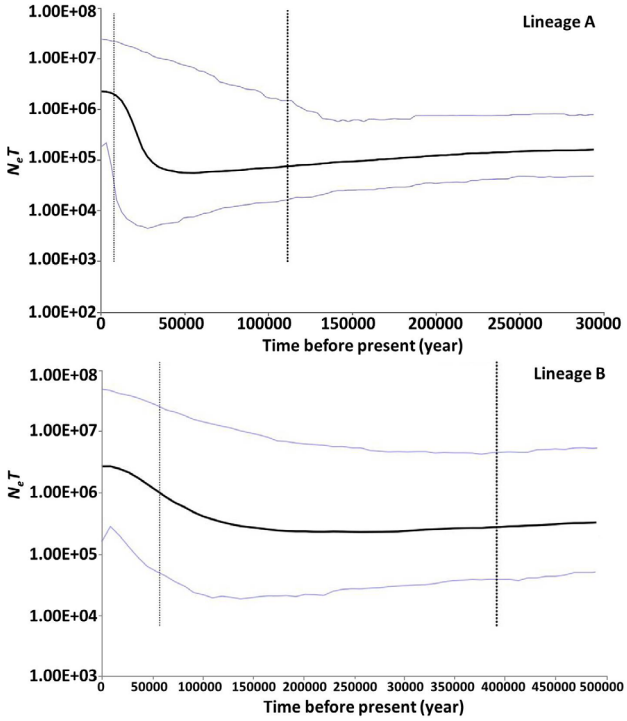

Fig. 5. Bayesian skyline plots showing N_{ef}T (N_{ef}, effective population size; T, generation time) changes over time for *P. minor* based on Cytb sequences. The upper and lower limits of the blue line represent the 95% confidence intervals of highest posterior densities (HPD) analysis. The black line represents median estimates of N_{ef}T.

Table V.- Summary of the molecular diversity, neutral test and goodness-of-fit test for *P. minor*.

	n	NH	*h* ± SD	*π*± SD	*k* ± SD	Tajima's *D*		Fu's *Fs*		Goodness-of-fit test				
						D	*P*	*Fs*	*P*	*τ*	θ_0	θ_1	*SSD*	*HRI*
Lineage A	62	7	0.5976± 0.0350	0.0017± 0.0014	0.6996± 0.5360	-0.796	0.262	-3.013	0.046	1.67	0.000	99999	0.024ns	0.178ns
Lineage B	34	10	0.5829± 0.0996	0.0035± 0.0024	1.4510± 0.9041	-1.254	0.090	-4.377	0.004	1.25	0.000	99999	0.439ns	0.165ns
All	96	17	0.7818± 0.0224	0.0054± 0.0033	2.2493± 1.2490	-0.480	0.369	-5.783	0.033	--	0.000	4.082	0.022ns	0.057ns

NH, numbers of haplotypes; *h*, haplotype diversity; *π*, nucleotide diversity; *k*, average number of pairwise differences; ns, $P>0.05$.

DISCUSSION

Genetic diversity is the basis of species diversity and ecological diversity. Species diversity and genetic diversity form the basis of ecosystem diversity. The research of genetic diversity is increasing attention from scholars in China and other countries. The genetic diversity of species directly affects the adaptability of the species to the environment. The higher the diversity is, the greater the potential for the evolution of the species and the stronger the ability of the species to adapt to environmental changes. In contrast, the possibility of degradation or extinction of the species also exists (Rosel *et al*., 1995).

Compared to the genetic diversity of the Cytb gene sequence in *Trachidermus fasciatus* (h=0.97±0.011) (Gao *et al*., 2013) and *P. argenteus* (h=0.775±0.041) (Zhao *et al*., 2011b), the genetic diversity of Chinese and Malaysian *P. minor* populations was moderate. From the perspective of historical evolution, for marine fish, the large number of populations explains the maintenance of higher h in natural populations. According to the research findings, the overall genetic diversity of the *P. minor* population (h=0.7818±0.0224; π=0.0054±0.0033) belongs to the second of the four types of marine fish, which was proposed by Grant and Bowen (1998), *i.e.,* a high h and a low π. A fish population of this type may have experienced a historical expansion event, *i.e.,* a rapid expansion from a small effective group into a large group in a short period of time. In the process of group expansion, with the increase in the number of groups, the h was improved, but not enough time has passed to accumulate the variants produced in the nucleotide sequence, resulting in a genetic diversity pattern with a high h and a low π (Grant and Bowen, 1998).

Stepien (1999) believes that fish along the continental shelf maintain long-term stable numbers and that a large effective population is responsible for the high h. Although pomfret resources have declined due to overfishing (Jin *et al*., 2005), the large amount of resources and the large number of effective populations have maintained moderate genetic diversity in *P. minor*. In addition, *P. minor* is widely distributed and highly adaptable to prey, whose spawning grounds exhibit different characteristics according to the different waters of the distribution; the environmental conditions are also not homogeneous (Wu *et al*., 2012). These life history features and the environmental heterogeneity of *P. minor* can promote rapid population growth, resulting in slight selective pressure on the population, which may lead to the accumulation of more genetic mutations and a rich genetic diversity.

The distribution of genetic diversity will be affected not only by evolutionary forces (*e.g.,* migration) but also by historical events, habitat discontinuities and an unstable number of populations can cause differentiation among species groups (Stepien, 1999). Both the NJ tree and haplotype network showed significant genetic differentiation in *P. minor* populations. The results of AMOVA also showed that the genetic differentiation was mainly attributable to the genetic variation among populations. The two lineages differentiated approximately 0.3 million years ago, and this differentiation may have been affected by the Pleistocene ice age. Many studies have shown that the Pleistocene ice age had a huge impact on the genetic structure of marine fish populations (Liu *et al*., 2007; Shen *et al*., 2011; Zhao *et al*., 2011a, b; Wu *et al*., 2012; Gao *et al*., 2013).

At the end of the Quaternary Period, the global climate experienced a series of glacial and interglacial changes. Climate fluctuations occurred over a period of approximately 100 kyr in the past 800 kyr (Lambeck *et al*., 2002). The end of the fourth glacial period was approximately 420 kyr ago (Petit *et al*., 1999), which is consistent with the differentiation time of the two haplotype lineages of *P. minor*. With the arrival of the fourth ice age, the sea level dropped approximately 120-140 m, and the *P. minor* population may have been isolated in a refuge in the South China Sea; after the ice age, as the sea level rose, the *P. minor* populations in the refuge would have expanded toward the coasts of China and Malaysia. The NJ tree and haplotype network showed that Hap_2 was the only common haplotype in the Chinese and Malaysian populations, found in the highest number of individuals and identified as the ancestral haplotype, which further verified that the Chinese and Malaysian *P. minor* populations cohabitated the same refuge in the South China Sea. After the population expansion event, the *P. minor* populations resided in the different sea areas and adapted to the diverse habitats resulting in many new mutations, the accumulation of rich haplotype diversity, and the formation of a unique haplotype. However, time was not sufficient for mutations to occur in the nucleotide. According to the distribution of haplotypes, except for the ancestral haplotype, all remaining haplotypes observed in the Chinese and Malaysian *P. minor* populations were significantly differentiated, and different geographical regions shaped their own unique haplotypes, respectively. The results of the F_{ST} and exact P tests also showed significant genetic differentiation between the Chinese and Malaysian *P. minor* populations, which further verified the accumulation of genetic variation that allowed adaptation to the living environments. As Malaysia is at low latitude, with high water temperatures, more diversified habitats, and ocean currents bringing rich sources of plankton by monsoon, the Malaysian *P. minor* population was under

slight selective pressure and accumulated more genetic variation, thus showing higher genetic diversity.

Based on the above results, we conclude that the expansion period of *P. minor* from the refuge to the coasts of China and Malaysia was short. With the passing of time, the Chinese and Malaysian *P. minor* populations will accumulate enough genetic variation to achieve complete differentiation. Similar genetic structure have been detected from *P. chinensis* in the similar distribution areas. Due to the lack of the basic biological information on *P. minor* and insufficient understanding of its floating time and spawning characteristics, the real cause of this genetic structure in *P. minor* cannot be accurately speculated. In future studies, more *P. minor* specimen will be collected from Vietnam, the Malay Peninsula, and the Strait of Malacca to investigate its exactly genetic diversity and genetic structure with more mtDNA markers and/or genomic approaches such as SNPs survey by using RAD-seq method.

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Statement of conflict of interest

The authors declare no conflicts of interest including in the implementation of the research experiments and the writing of this manuscript.

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