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Mitochondrial COI sequences revealed shallow but significant divergences among *Amphioctopus aegina* (Octopoda, Octopodidae) populations in coastal waters of China

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Abstract

Amphioctopus aegina is an important fishery resource in the coastal waters of China. In the present study, the genetic diversity and population genetic structure among four populations of *A. aegina* throughout its distributional range in China were assessed using the mitochondrial cytochrome oxidase 1 (COI) sequences. The results revealed a generally low genetic diversity (Hd: 0.2842–0.6670; Pi: 0.0007–0.0015) in *A. aegina* populations. The neighbor-joining (NJ) phylogenetic tree and the haplotype networks, as well as the results of the molecular variance (AMOVA) analyses, indicated a shallow phylogeographic structure among the four populations. However, pairwise Φ_{ST} statistics and genetic distance analyses revealed significant ($p<0.01$) genetic differentiation among Qinzhou and the rest three populations of Zhanjiang, Huizhou, and Dongshan. The demographic history analyses indicated a population expansion in *A. aegina,* and the role of Leizhou peninsula isolation in shaping the population differentiation. These results would largely enhance our understanding of the genetic structure and hence promote the scientific management of *A. aegina* fishery resources in coastal waters of China.

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Introduction

The marbled octopus, *Amphioctopus aegina* (Gray, 1849), is a moderately sized benthic octopus inhabiting muddy substrates in the coastal zone of the Indian and Western Pacific Oceans. Capture fishing of octopuses in these regions includes this species (Nabhitabhata., 2014). In China, *A. aegina* also represents an economically important fishery species distributed in coastal waters, including south of the East China Sea and the South China Sea (Dong, 1988). It possesses high protein, low-fat content, and abundant mineral elements with critical nutritional values (Lei et al., 2006). Therefore, it has long been the target for commercial fishing throughout its distribution range. The annual fisheries for this species yield hundreds of tons in Hongkong coastal waters alone (Dong, 1988). However, during the last two decades, the wild population of *A. aegina* was rapidly decreasing because of overexploitation, and the aquaculture activities instead has been attempted (Promboon et al., 2011; Nabhitabhata, 2014). The rapid decline of wild populations calls for immediate steps forward for resource management and conservation for this species. Understanding the population genetic structure is a crucial component of the successful and sustainable management of fishery resources (Öztürk and Altınok, 2021). Unfortunately,up to date, no population genetic study has been investigated for this specie in China and throughout the world. There is only limited information concerning the basic biology and ecology of this species (Ignatius et al., 2011; Promboon et al., 2011; Osman et al., 2015) that would provide clues for their population genetic structure. For example, matured females of *A. Aegina* reproduce small eggs and planktonic hatchlings (Villanueva and Norman, 2008; Promboon et al., 2011). The planktonic phase of larva would last long (20-30 days) before they settle into the substrate and become juveniles (Promboon et al., 2011; Nabhitabhata et al., 2014). Such a reproductive strategy would predict the high dispersal potential of individuals (Villanueva and Norman, 2008) and hence weak subdivision among populations (Peres et al., 2020; Tang et al., 2021). The exact phylogeographic divergence among *A. aegina* populations still remains to be explored to underpin scientific management and conservation of their fishery resources. To investigate the phylogeography of *A. aegina*, we examined the partial sequences of the mitochondrial cytochrome oxidase1 (*COI*) gene in 71 individuals collected from four localities across their full distributional ranges in China. The obtained sequences were analyzed to determine their genetic diversity and population genetic structure, which would provide useful information for the management and conservation of these critical fishery resources in the coastal waters of China.

Materials and Methods

Sample collection and DNA isolation

A total of 71 specimens of adult *A. aegina* were collected from four localities along the coast of China, as shown in **Figure 1**, from September 2017 to May 2019 through bottom trawling in marine fishery surveys. The muscle tissues of each specimen were removed and stored in 95% ethanol and then transported to the laboratory. The total genomic DNA was isolated from the tissues using the standard phenol-chloroform method (Sambrook et al., 1989).

Figure 1 The sampling locations of *A. aegina* along the coast of China. The sampling localities were Dongshan, Huizhou, Zhanjiang, and Qinzhou, as shown on the map.

Mitochondrial DNA amplification and sequencing

The partial sequences of the mitochondrial *COI* gene were used to determine the genetic variation and population genetic structure of *A. aegina* in coastal waters of China. The DNA amplifications were carried out using a set of primer (F5′-TAAACTTGAGGGTGACCAAAAAAT-3′; R 5′-GGTCAACAAATCATAAAGATAT TG-3′) designed specifically for the locus for *A. aegina* according to the previous study (Lin et al., 2004; Zhang et al., 2017). The PCR assay was performed in 20μl volumes containing 100 ng template DNA, 1×reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM each primer, 4.0 units Taq DNA polymerase (Promega, USA) using a PTC-200(BIORAD, USA) PCR machine. The PCR amplification was conducted under the following conditions: 5 min initial denaturation at 94 ℃, 40 cycles of 1 min at 94 ℃ for denaturation, 1 min at 51 ℃ for annealing, and 1 min at 72 ℃ for extension, and a final extension at 72 ℃ for 5 min. All PCR amplifications included a negative control reaction in which all reagents were included, except for the template DNA. PCR products were verified by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The Gel Extraction Mini Kit (Watson Bio Technologies, China) was used to purify the PCR products. Afterward, the PCR products were sequenced in both directions using the Sanger Sequencing Technology procedure at Invitrogen Ltd., China. The obtained sequences were submitted into the GeneBank of NCBI database with the accession numbers OM283649-OM283719 (https://www.ncbi.nlm.nih.gov/search/all/?term=OM28 3649-OM283719).

Data analyses

The sequences obtained from 71 specimens were edited and aligned using MEGA 6.0 software (Tamura et al., 2013). The molecular diversity indices, such as the number of haplotypes (n), haplotype diversity (Hd), nucleotide diversity (Pi), and the mean number of pairwise differences (k) as well as their corresponding variances, were analyzed using DnaSP 5.10.01 (Librado and Rozas, 2009). The net average genetic distance was calculated for phylogenetic tree reconstruction with MEGA6.0 using the model of Tamura (2013). A neighbour-joining (NJ) phylogeographic tree was constructed to determine the genetic relationships among the populations using the Kimura-2-parameter (K2P) model (Kimura, 1980) with 1000 bootstrap replicates implemented in MEGA 6.0 software (Tamuraet al., 2013). In addition, a haplotype network was generated to examine the genealogical relationships using a reduced median network approach using POPART software (Leigh and Bryant, 2015). The population structure was further measured with a molecular variance analysis (AMOVA) by determining the genetic variability within and among populations using ARLEQUIN 3.5

(Excoffier and Lischer, 2010). The significance of the covariance components was tested using 1000 permutations. Pairwise genetic differentiation coefficient (Φ_{ST}) values were calculated to examine the genetic differentiation between populations using the computed pairwise distances model (Nei and Li, 1979) with 10,000 permutations in ARLEQUIN 3.5 (Excoffier and Lischer, 2010). Pairwise genetic distances between populations were also generated to examine the population subdivision and calibrate the divergence time among populations using MEGA 6.0 (Tamura et al., 2013). Appropriate nucleotide substitution rates for mitochondrial *COI* have not been calibrated for Cephalopod species. Generally, a divergence rate of 0.7–2.4% per million years (Mya) has been calibrated for *COI* locus for multiple mollusk species (Hellberg and Vacquier, 1999; Marko, 2002). Using such a divergence rate for the mitochondrial *COI*, the divergence time was retro-calculated based on the net genetic distance between *A. aegina* populations using the formula: $t = D/2\alpha$. In the formula, t represents the divergence time between the populations, and D and α, respectively, represent the net genetic distance between the populations and the nucleotide substitution rate of the mitochondrial locus (Nei, 1987).

The population demographic history was examined using two different approaches. Firstly, the *D* test of Tajima and the *FS* test of Fu were used to test if neutrality holds (Tajima, 1989; Fu, 1997). Large negative *D*-values in Tajima's *D*-test or negative *FS* values in Fu's *FS* test would usually be good indicators of a population expansion (Tajima, 1989; Fu, 1997). Secondly, the 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 of q0, q1 (q before and after population growth), and t (time since expansion). The concordance of the observed with the expected distribution under the sudden expansion model of Rogers and Harpending was tested using a least-squares approach.

Results

Genetic diversity in populations

A 620 bp fragment of the mitochondrial *COI* gene (**Figure 2**) was analyzed based on 71 sequences from the four populations of *A. aegina*. Sequence comparisons of the segment revealed 15 polymorphic sites, including 12 singleton and three parsimony-informative variable sites (**Table1**). These polymorphic sites defined 15 haplotypes among the 71 sequences, giving a haplotype diversity (Hd) of 0.2842-0.6670, nucleotide diversity (Pi) of 0.0007-0.0015, and the mean number of pairwise differences (k) of 0.4000-0.9070 for each population (**Table 2**). Among the 15 haplotypes (Hap1-Hap15), 12 (80 %) haplotypes were represented by a single sequence in the samples, and only 2 (13%) haplotypes were shared among the populations (**Table1**).

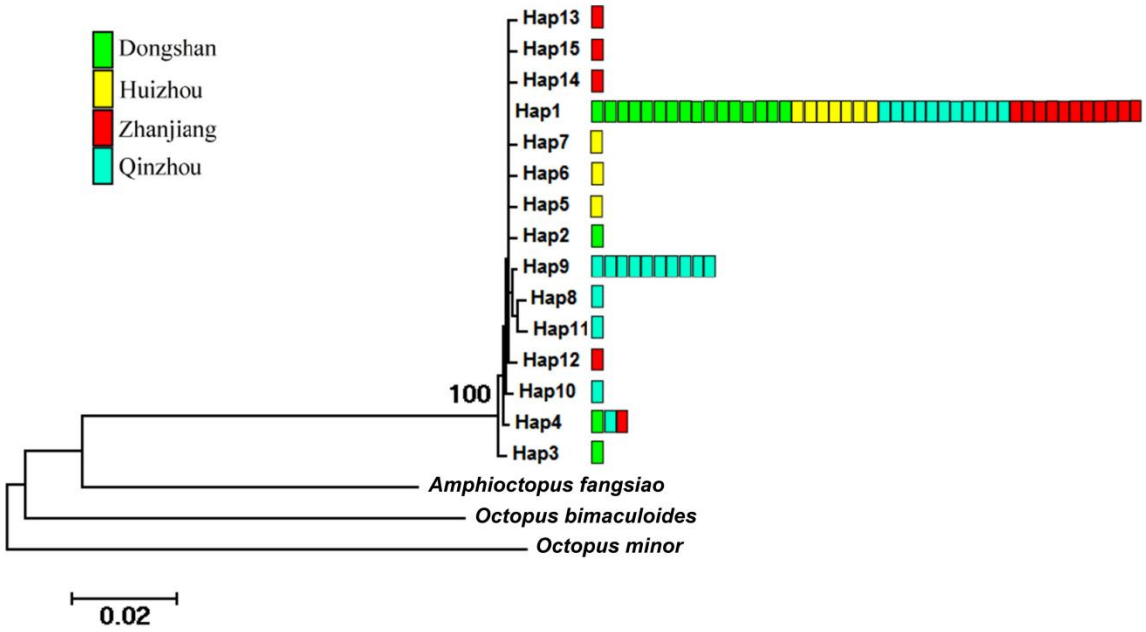

Figure 2 Neighbor-joining phylogenetic tree constructed from the haplotypes of mitochondrial *COI* sequences; Bootstrap supports of ≧70% in 1000 replicates are shown. *Amphioctopus fangsiao* (GeneBank accession number: NC_007896.1), *Octopus bimaculoides* (GeneBank accession number: KF225006.1) and *O.minor* (GeneBank accession number: NC_038213.1) were used as the outgroups when the NJ trees were constructed.

Table1 Haplotypes of *COI* sequences and their frequency observed in the four populations of *A. aegina*

	133344 1136818908 0693651686	44555 89089 73584	*Dongshan*	*Huizhou*	*Zhanjiang*	*Qinzhou*
H1	AAGTATACTT	GCTAT	17	7	11	11
H2	. . . . .	 C	1			
H3	. . . . .	. T C . .	1			
H4	. . A	. . . . .	1		1	1
H5	. . . . .	. . . G .		1		
H6	. . . . . G	. . . . .		1		
H7	. . . . . C .	. . . . .		1		
H8	. . . . . G . . .	. . . . .			1	
H9	. . . . G	. . . . .			1	
H10	. . . C	. . . . .			1	
H11	. . . . . C	. . . . .			1	
H12	G	A				1
H13	. . . . .	A				10
H14	. . . . . T . .	. . . . .				1
H15	G T	. . . . .				1

Table 2 Sample collections and genetic variations among *A. aegina* populations revealed by the mitochondrial COI sequences

Populations	*Sample Size (no)*	*Number of polymorphic site(no)*	*Number of Haplotypes (Hap)*	*Haplotype diversity (Hd)*	*Nucleotide diversity (pi)*	*Average number of differences (k)*
Dongshan	20	4	4	0.2842	0.0007	0.4000
Huizhou	10	3	4	0.5333	0.0010	0.6000
Zhanjiang	16	5	6	0.5417	0.0010	0.6250
Qinzhou	25	5	6	0.6670	0.0015	0.9070
Total	71	15	15	0.5640	0.0012	0.7410

Population genetic structure and phylogeography

The neighbor-joining (NJ) trees constructed from the 15 haplotypes revealed no distinct lineages of haplotypes, hence inferring a shallow genealogical structure among populations. The short scale bar generally observed in the phylogenetic tree indicates their close relatedness among these haplotypes. Similar to the phylogeographic tree, the constructed median-joining network is also star-like, with 15 haplotypes closely mixing with no haplogroups could be identified (**Figure 3**). Such a shallow population structure was again supported by the AMOVA analyses because the majority (85.46%) of the total genetic variations were detected within the populations. In comparison, only 14.54% of the variations were detected among populations (**Table 3**).

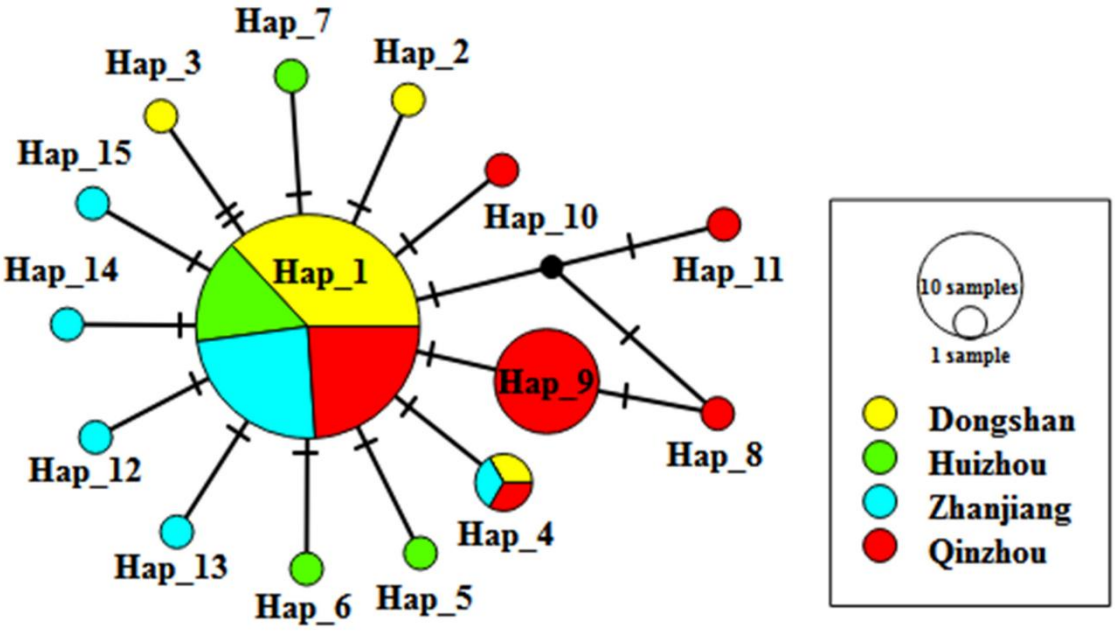

Figure 3 Median-joining network constructed from the haplotypes of the mitochondrial *COI* sequences. Different colors represent different populations analyzed. The size of the circle was proportional to the haplotype frequency observed in populations.

Table 3 Molecular variances within and among *A. aegina* populations as revealed by the mitochondrial COI sequences

Source of variations	*df*	*Sum of squares*	*Variance component*	*Percentage (%)*
Among populations	3	3.8760	0.0560 Va	14.5400
Within populations	67	22.0680	0.3294 Vb	85.4600
Total	70	25.9440	0.3854	
Fixation Indices	0.1454			

However, pairwise Φ_{ST} analyses revealed significant (0.1772-0.2087, $p<0.01$) differentiation among Qinzhou and all the rest three populations, inferring a restricted gene flow between them (**Table 4**). The pairwise genetic distance between populations also supported such differentiation because the genetic distances among populations from Qinzhou and the other three regions were much higher than those within these three regions. The largest genetic distances were observed between Qinzhou and Dongshan &Huizhou populations (**Table 4**), and the net genetic distance between them was 0.0003, suggesting a very recent differentiation. Using a nucleotide substitution rate of 0.7-2.4% per Mya usually applied for the mitochondrial *COI* sequences for mollusk species, the calibrated divergence time between them was 6.25-21.43 ka BP.

Table 4 The values of pairwise Φ_{st} and genetic distance among four populations of *A. aegina* inferred from mitochondrial COI sequences

Populations	*Dongshan*	*Huizhou*	*Zhanjiang*	*Qinzhou*
Dongshan	-	0.0008	0.0008	0.0014
Huizhou	0.0110	-	0.0010	0.0015
Zhanjiang	-0.0097	-0.0008	-	0.0015
Qinzhou	0.2087**	0.1772**	0.1827**	-

Note: The values of pairwise Φ_{st} and genetic distance were respectively given in below and above diagonal; ** represents *P* value < 0.01.

Demographic history and neutrality

The demographic history of *A. aegina* was investigated using mismatch distributions, which are the distribution of pairwise genetic differences between pairs of haplotypes according to a sudden expansion. The mismatch distributions appeared to be unimodal in *A. aegina*, which matched the expected distributions under the sudden expansion model (**Figure 4**). This interpretation was also supported by the Tajima's (*D*= -2.2100 and P = 0.0000) as well as Fu's (*Fs* = -15.2612 and P = 0.0000) neutrality tests which resulted in negative values with statistically significant ($p<0.01$).When each population was analyzed individually, the majority of the populations still showed significant negative values in both neutrality tests except for the Qinzhou population, in which only Fu's *Fs* test displayed significantly negative (**Table 5**).

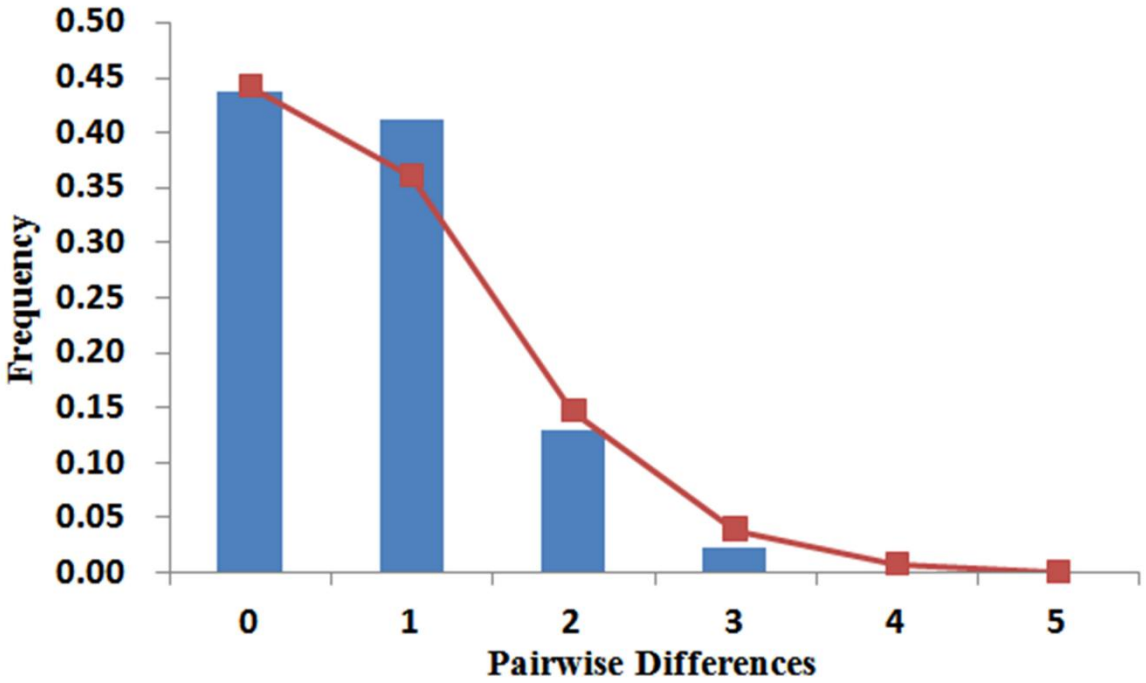

Figure 4 The Observed pairwise difference (bars), and the expected mismatch distributions under the sudden expansion model (solid line) of *COI* haplotypes in *A. aegina.*

Table 5 The neutral test based on the Tajima's D and Fu's Fs statistics in each *A. aegina* population

Populations	*Tajima's D*	*P*	*Fu's Fs*	*P*
Dongshan	-1.8679	0.0070	-2.0737	0.0080
Huizhou	-1.5622	0.0470	-1.9637	0.0080
Zhanjiang	-1.9286	0.0120	-4.2535	0.0000
Qinzhou	-0.9032	0.2130	-2.2067	0.0380
Total	-2.2100	0.0000	-15.2612	0.0000

Discussion

In the present investigation, we examined the genetic diversity and the population genetic structure in *A. aegina* sampled throughout its full distribution range in China based on the mitochondrial *COI* sequences. Following what has been previously revealed for many Cephalopod species (Strugnell et al., 2017; Roura et al., 2019), generally low genetic diversity has also been observed in *A. aegina* populations, as indicated by the generally low haplotype diversity (Hd = 0.2842–0.6670), nucleotide diversity (Pi = 0.0007–0.0015), and the mean number of pairwise differences (k = 0.4000-0.9070). Zheng et al. (2001) have partly attributed such low genetic diversity in Cephalopoda to the more fragility to bottleneck effect because of overfishing due to their semelparous life strategy. Such interpretation may hold particularly true for *A. aegina* because overfishing has long been noticed for this species and many other octopuses' species in coastal waters of China (Lü et al., 2012; Gao et al., 2016; Muhammad et al., 2019). These results highlighted the necessity of immediate steps forward to conserve this important fishery species.

Both the neighbor-joining (NJ) tree and median-joining network constructed from the haplotypes in four populations revealed no distinct lineages of haplotypes, hence inferring a shallow genealogical structure. Such shallow genealogical structure may partly be attributed to the close relationships between all the haplotypes because only one or two substitutions were usually observed between haplotypes. This inference was also supported by our AMOVA

analyses, in which the majority (85.46%) of the total genetic variations were detected within the population, and only 14.54% were detected among populations. Such interpretation seems to correspond to our previous inference of low differentiation among *A. aegina* populations due to their reproductive strategy of reproducing small eggs and planktonic paralarvae. However, significant ($p<0.01$) genetic differentiation was revealed among Qinzhou and all the rest three populations by both our pairwise Φ_{ST} and genetic distance analyses. Such results may indicate a restricted gene flow among them, possibly due to the isolation of the Leizhou Peninsula. The Leizhou Peninsula is the third-largest peninsula located at the southernmost tip of China. Combined with Hainan island, they form a natural geographic barrier between the Gulf of Tonkin and the rest of the South China Sea, with only a long and narrow Qingzhou strait flowing through (Sun and Tang, 2018). Such natural geographic isolation also represents a barrier to gene flow among populations, and substantial population differentiation was usually detected in marine species dwelling on both sides of the peninsula (Sun and Tang, 2018; Wang et al., 2019; Yi et al., 2021).

Nevertheless, such genetic differentiation between the population of Qinzhou and the rest of the three populations was obviously in their infancy, as indicated by the week bootstrap support for their differentiation in the tree and the generally low values of genetic distance among the populations. Based on the average net genetic distance (0.0003) between Qinzhou and the rest three populations, the calibrated divergence time between them was 6.25-21.43 ka BP. That falls into a time scope of post-last-glacial-maximum (LGM) (~20 ka BP) when Gulf of Tonkin become isolated by the Leizhou peninsula through marine transgression (Yao et al., 2009). Such a time calibration and scenario may point to a differentiation by post-LGM isolation of Leizhou peninsula in *A. aegina* populations. Nevertheless, such a differentiation scenario may also predict substantial population expansion, especially for the Qinzhou population, when they were re-established in the Gulf of Tonkin through marine transgression during post-LGM. Our historical demography analysis seemed to support this inference by revealing that most populations showed significant negative values in both neutrality tests, and the mismatch distribution appeared to match the expected distributions under the sudden expansion. These results provided further support for our inference of differentiation by post-LGM isolation in *A. aegina* populations in coastal waters of China. However, such an assumption was only based on the sequences of a single mitochondrial *COI* gene with a length of 620 bp, which may not provide sufficient genetic information for an accurate assessment of the population history. Further studies involving more molecular markers are recommended to support our inference and understand the driving forces that shaped the population genetic structure of *A. aegina* in coastal waters of China.

Conclusions

Our results revealed low genetic diversity and shallow but significant genetic differentiation in *A. aegina* populations in coastal water of China. The generally low genetic diversity observed in *A. aegina* calls for immediate steps forward for the conservation of this fishery species. The shallow but significant genetic differentiation observed among populations suggests that future conservation management efforts should include both populations across the Leizhou Peninsula. Our results would largely enhance our understanding of the genetic structure and hence promote the scientific management of *A. aegina* fishery resources in coastal waters of China in the future.

Acknowledgments

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