A mitogenomic phylogeny and genetic history of *Amphioctopus fangsiao* (d'Orbigny 1839-1841) from China

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Abstract

Phylogeny and genetic diversity of *Amphioctopus fangsiao* were assessed by sequence analysis of complete mitochondrial genomes, sequenced from 15 individuals of nine populations. The whole mtDNA genomes size were ranging from 15977 to 15990 bp. Data revealed 1642 polymorphic sites and 1023 parsimony informative sites. The phylogenetic analysis based on neighbor joining tree disclosed two clades. It consisted of four (Dalian, Yantai, Qingdao and Nantong) and five populations (Shanghai, Zhoushan, Xiamen, Dongshan and Zhanjiang). Genetic differentiation coefficient (FST) was recorded higher i.e 0.61476. While, the AMOVA analysis showed that 61.48% of the genetic variation existed between the two clades. However, only 38.52% of the genetic variation existed within each clade. In further, the net genetic distance between the two groups was 0.030. The possible reason of differentiation is quaternary glacial period and Yangtze River.

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Introduction

Amphioctopus fangsiao belongs to family Octopodidae. It distributes widely in west Pacific Ocean, especially in Yellow Sea and Bohai Sea (Dong, 1988; Lu *et al*., 2012). *A. fangsiao* considered as a potential species for aquaculture (Wang *et al*., 2015), due to its rapid growth rate and high nutritional value. Tons of its fisheries have annually been harvested alone in Shandong province. However, it has been reported that its natural population is decreasing because of over-exploitation (Zhang *et al*., 2009). Therefore, it is intuitive to have its resource management and conservation. Until now, few studies are available to elucidate *A. fangsiao* genetic aspects (Gao *et al*., 2002; Zhang *et al*., 2009, and Lü *et al*., 2010, Muhammad *et al*, 2019). Gao *et al*. (2002) and Zhang *et al*. (2009) used allozyme and amplified fragment length polymorphism (AFLP). Lü *et al*. (2011, 2010) examined several *A. fangsiao* populations using mitochondrial 16S rRNA and the COI gene and Muhammad *et al*. (2019), investigated using three mtDNA genes (ATPase 6, ND2 and ND5). All the earlier studies showed certain level of differentiation. The whole mtDNA genome approach of investigation was not carried earlier.

A. fangsiao is one of the marine species that spawn life time in marine ecosystem. The fluctuating physical properties of marine ecosystem influence organisms which restrict gene flow over large geographic distances (Maltagliati *et al*., 2002, Hellberg *et al*., 2002, Fernandez *et al*., 2011). Unlike other marine organisms, having high range of dispersal capacity, large population sizes, high fecundity and planktonic mood of dispersal which helps in extensive gene flow (Bohonak 1999, Zid *et al*., 2012), the Octopus species like *Octopus minor*, *O. vulgaris* and *A. fangsiao* show limited dispersal capacity both at larval and adult stages (Oosthuizen *et al*., 2004; Kim *et al*., 2009; Kang *et al*., 2012; Lü *et al*., 2013, De Luca *et al*., 2016, 2015, Melis *et al*., 2018). *A. fangsiao* adults are either slow mover or like sessile, crawling away on the sea bed or burrowing deep in mud. It has benthos-attached eggs, spent only a few days as planktonic larval stage. Therefore, it has weak dispersal and limited gene flow, resulting in the genetic differentiation among populations.

Assessment of genetic diversity within and among populations has immense importance, which deliver a possible genetic source for future adaptation (Xu, 2012) and in area of evolutionary biology (Hao *et al*., 2015, Ortego *et al*., 2015). In natural population its level is determined by the interplay of mutation, migration, drift and selection (Harrison, 1991, Vellend and Geber 2005, Wethington *et al*., 2007). The role of each of these forces depends on the life history of the species. It's mating system and dispersal abilities, as well as extrinsic factors such as the landscape matrix, its history and anthropogenic actions (Gow,

et al., 2004; Husemann *et al*., 2012). In the present study, we analyzed data of fifteen complete mitochondrial genes gained, from fifteen individuals collected from nine localities across the Chinese coast. The sequences were examined to understand the genetic diversity and phylogeny of *A. fangsiao* and reveal the possible issues impacting the phylogeographic pattern. The results of the present investigation provide profound information for the future management and conservation of this species.

Materials and methods

Samples were collected from nine locations (Fig. 1). Thereafter, they were preserved in 95% ethanol and transported to the laboratory. The total genomic DNA was isolated from muscle tissues using a standard protocol. A set of 20 PCR primers were used to amplify overlapping fragments that covered the whole mitochondrial genome of the *Amphioctopus fangsiao*. Primarily, primers were designed on the basis of complete mtDNA sequence Gene Bank under accession number NC_007896.1 (Akasaki *et al*., 2006). Later on, specific primers were designed according to newly obtained sequences to facilitate primer walking. Thermocycler conditions were as follows: denaturation at 94 $^{\circ}$C for 5 min, 35 cycles 94°C for 30s, annealing at 50°C for 30s, extension at 72°C for 30s, and the final extension at 72°C for 7 min. Electrophoresis was performed on a 1.2% agarose gel and was sequenced

using the same PCR primers. Sequences were aligned using CLUSTALX 1.83, Thompson *et al*. (1997) was used for alignment and editing of sequences and BioEdit 7.0.1, (Hall, 1999). Using CG View Server to draw the mtDNA ring diagram. The neutrality test of haplotype and nucleotide diversity indices their variances as well (Tajima's D, Fu and Li's D*, Fu and Li's F*) were calculated using DnaSP version 5.0 software package, (Librado and Rozas, 2009). Genetic distance calculation and cluster analysis were carried out by using Mega 6.0 software. The phylogenetic tree was constructed by UPGMA model and the bootstrap (repetition number 1000) was used to check the branch trust. *Octopus Aegina* (NC_029702.1), (Zhang *et al*., 2015), *Octopus variabilis* (NC_015896.1), (Cheng *et al*., 2012) *Octopus vulgaris* (NC_006353.1), (Yokobori *et al*., 2004), *Octopus bimaculoides* (NC_028547.1), Domínguez *et al*. (2015) mtDNA were used as the out group in the Phylogeography analysis. The haplotype networking was created using NETWORK software version 5.0.0.1 (Bandelt *et al*., 1999). The genetic structure of the population was assessed using the FST statistic, and its significance was repeated using Arlequin 3.5 for 1 000 cycles. The relationship between (1-FST) / 2-FST and the geographical distance of the sample is plotted to determine whether the population genetic structure conforms to the geo-isolation model (Slatkin, 1993).
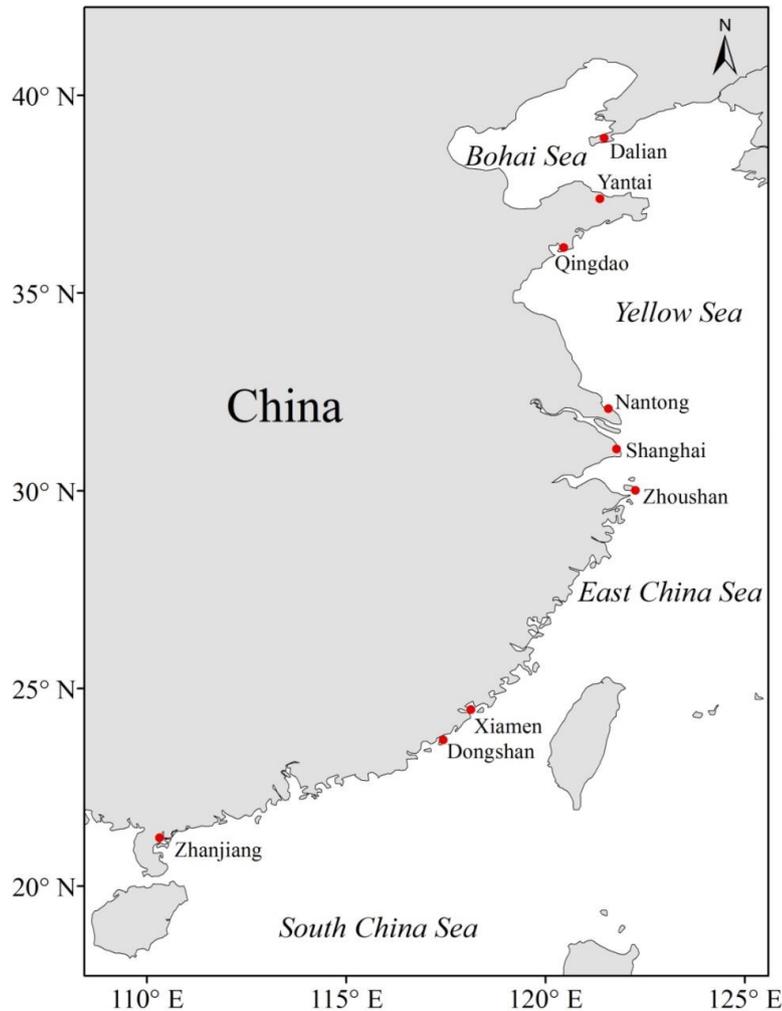
Figure 1: Map showing collection sites of *Amphioctopus fangsiao* specimens.

The genetic structure of the population was further detected using the molecular variation analysis AMOVA method in Arlequin 3.5 software. Homoplasic sites were exposed by the use of the phylogeny network investigation as applied in the Network 4.6 program.

The historical dynamics of the population was carried out using two methods: (1) using Tajima's D and Fu's Fs test to determine whether the neutral hypothesis was established, Tajima's D and Fu's Fs neutral test results were negative and significantly deviated from neutral, (2) The use of nucleotide mismatch distribution (mismatch distribution) analysis to test the existence of group expansion; unpaired distribution test and neutral hypothesis test were used Arlequin3.01 software. The differentiation time of the CO1 gene and cytochrome b gene were calculated by molecular clock theory. The evolution rate of CO1 gene was calculated by 0.5% to 1. 4%/ million years (MY), and the evolution rate of

cytochrome b gene were calculated by 2.15% to 2.6% / MY, Zhao *et al*. (2013).

Results

A total of 15 complete mtDNA genomes of *Amphioctopus fangsiao* were characterized (Gene Bank Accession No (MF 029678–MF 029691). The mtDNA genome sizes range from 15977 to 15990 bp, with the G+C content ranging from 22.39% to 22.93%. The mtDNA genome contains all 37 genes as typically present in mollusks, which include cytochrome c oxidase subunits I–III (COI, COII, COIII), ATPase subunit 6 and 8 (ATP6, ATP8) and NADH dehydrogenase subunits 1–6 and 4L (ND1– 6, ND4L); 22 tRNA genes, two rRNA genes. Eight

of 22 tRNA genes (tRNA-Lys, tRNA-Ala, tRNA-Arg, tRNA-Asn, tRNA-Ile, tRNA-Ser, tRNA-Asp, and tRNA-Thr) were located in the light chain and the remainders were found in the other chain (Fig. 2). In the *A. fangsiao* mtDNAs, 1642 positions were variable and 1023 were parsimony-informative. The complete mitochondrial genomes of *A. fangsiao* and other related species such as *O. bimaculatus*, *O. conispadiceus*, *A. fangsiao* and *O. vulgaris* were compared to estimate intraspecific nucleotide diversity (Pi) (Librado and Rozas, 2009). The analysis showed that gene by gene Pi values were highly variable (Table 1). Nucleotide diversity in *A. fangsiao* varies from ~0.2% in ATP8 genes to 6.5% in the ATP6 genes, with highest among protein-coding genes value of Pi (~1%) detected in ATP6 gene (Table 1).
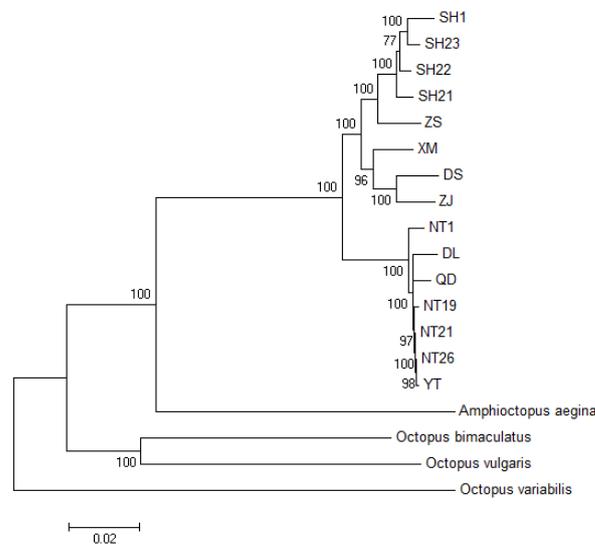

Figure 2: Neighbor-joining tree inferred from complete mitochondrial genomes of *Amphioctopus fangsiao*, *Octopus bimaculatus*, *Octopus variabilis*, *Octopus vulgaris* and *Amphiotopus aegina*. The representatives of other four related species were used as outgroups. The highly significant statistical supports are listed in the order ML/NJ (≥95%/N75%). Statistically supported mtDNA clades are designated by Latin letters. Scale bar indicates replacements per site. Key: SH: Shanghai, ZS: Zhoushan, XM: Xiamen, DS: Dongshan, ZJ: Zhanjiang, QD: Qingdao, NT: Nantong, YT: Yantai.

Table 1: Nucleotide diversity (Pi) of mtDNA genes in *Amphioctopus fangsiao*.

Locus	Length (bp)	Position	Pi
CO3	780	1-780	0.02862
ND3	351	1115-1465	0.03305
ND2	1038	1533-2570	0.03035
CO1	1533	2542-4074	0.02865
CO2	687	4078-4764	0.02756
ATP8	156	4831-4986	0.01600
ATP6	705	4989-5681	0.06513
ND5	1719	5729-7465	0.03964
ND4	1344	7533-8858	0.04104
ND4L	237	8873-9124	0.01584
CYTB	1140	9314-10445	0.02484
ND6	513	10438-10950	0.04702
ND1	942	11026-11967	0.03907

There were 15 haplotypes among all the 15 mtDNA genomes. The genetic distances across the mtDNA genomes ranged from 0.007 to 0.059 (Table 2). The phylogenetic trees were generated using NJ and ML methods, where *O. aegina*, *O. variabilis*, *O. vulgaris*, *O. bimaculoides* used as outgroup (Fig. 3) it revealed two clades A (Dalian, Yantai, Qingdao, Nantong populations) and B (Shanghai, Zhoushan, Xiamen, Dongshan and Zhanjiang populations) (Table 3). The haplotype network analysis also revealed two cladded and none of the haplotype was shared (Fig. 4). The AMOVA analysis showed that 61.48% of the genetic variation existed between the two clades, while only 38.52% of the genetic variation existed within each clade. FST of the two clades reached 0.61476. The net genetic distance between the two groups was 0.030.

The molecular clocks showed that the genetic distances between the two clades of COI gene were 0.027, and the genetic distances between the two clades of Cytb gene was 0.215. The differentiation time was estimated to be about $119 \times 104 \sim 540 \times 104$ million years ago before the Tertiary period (according to the fish and shrimp and crab's COI gene 0.5% to 1.4% per million years of evolution rate inferred (Knowlton and Weigt, 1998), the evolution rate of the cytochrome b gene was calculated by 2.15% to 2.6%/ million years).

In the present study we projected the ratio of the number of non-synonymous substitutions per non-synonymous sites (KA) to the number of synonymous substitutions per synonymous sites (KS) of (CYTB), and (COX3) gene, (NADH3) gene, (NADH2) gene, (COX1) gene, COX2) gene, ATP synthase F0 subunit 8 (ATP-8) gene, ATP synthase F0 subunit 6 (ATP-6) gene and found low KA/KS values in both clades (KA/KS =0.0126 and 0.9827 in clades A and B, respectively), representing the influence of negative selection.

Table 2: Genetic distances between pairwise populations of *Amphioctopus fangsiao*.

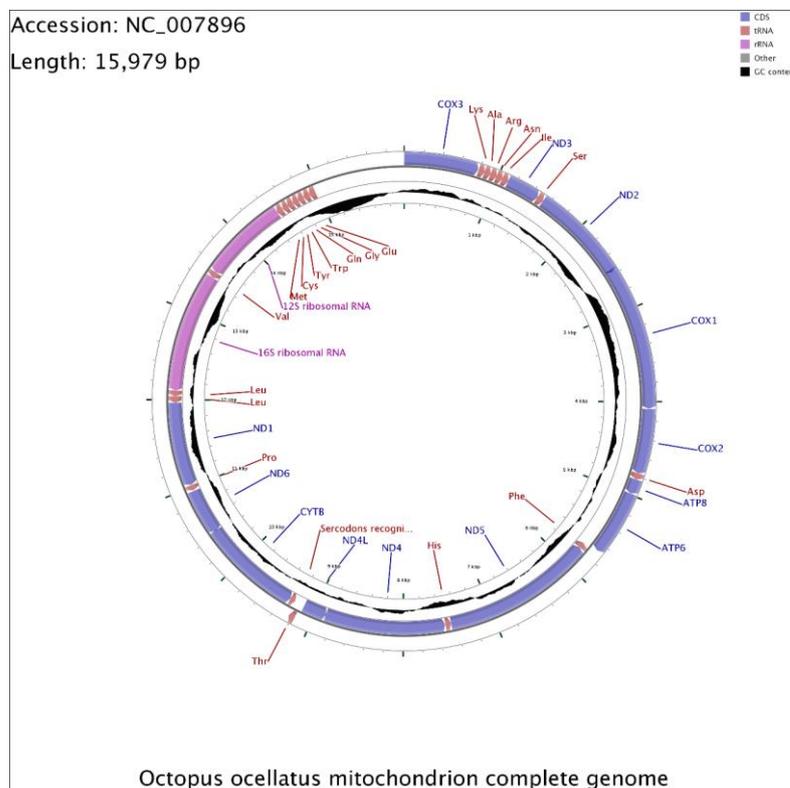|	DL	QD	YT	NT	SH	ZS	XM	DS	ZJ
DL									
QD	0.012								
YT	0.008	0.007							
NT	0.007	0.006	0.001						
SH	0.044	0.043	0.038	0.037					
ZS	0.053	0.051	0.047	0.045	0.019				
XM	0.049	0.048	0044	0.043	0.030	0.037			
DS	0.059	0.057	0.053	0.050	0.031	0.041	0.031		
ZJ	0.054	0.052	0.048	0.046	0.029	0.037	0.031	0.026	

Figure 3: Mitochondrial genome map of *Amphioctopus fangsiao*.

Table 3: Geographical distribution of *Amphioctopus fangsiao* samples used in this study.

| Sequence ID	Site ID	Province	Country	Clade	GenBank Accession No
DL	Dalian	Liaoning	China	A	MF029678
QD	Qingdao	Shandong	China	A	MF029684
YT	Yantai	Shandong	China	A	MF029690
NT1	Nantong	Jiangsu	China	A	MF029680
NT19	Nantong	Jiangsu	China	A	MF029681
NT21	Nantong	Jiangsu	China	A	MF029682
NT26	Nantong	Jiangsu	China	A	MF029683
SH1	Shanghai	Shanghai	China	B	MF029685
SH21	Shanghai	Shanghai	China	B	MF029686
SH22	Shanghai	Shanghai	China	B	MF029687
SH23	Shanghai	Shanghai	China	B	MF029688
ZS	Zhoushan	Zhejiang	China	B	MF029692
XM	Xiamen	Fujian	China	B	MF029689
DS	Dongshan	Fujian	China	B	MF029679
ZJ	Zhanjiang	Guangdong	China	B	MF029691
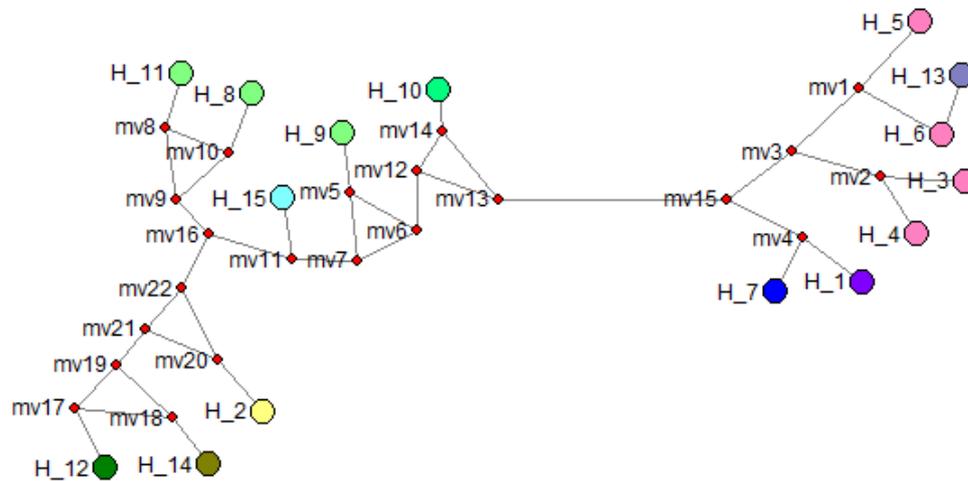
Figure 4: Median-joining network of mtDNA haplotypes observed in 15 *Amphioctopus fangsiao* specimens. This network illustrates the relationships between two major clades A and B. Median vectors are shown by red dots.

Discussion

Amphioctopus fangsiao is a potential species for aquaculture (Wang *et al*., 2015; Feng *et al*., 2017) and over-exploitation causes its population decline (Zhang *et al*., 2009). Therefore, basic information of phylogeny and population history counts valuable. A few investigations are in account for its population genetics, by utilizing AFLP , microsatellite and Allozyme (Gao *et al*., 2002, Zhang *et al*., 2009, and Feng *et al*., 2017) and detailed genetic structure (Muhammad *et al.,* 2019). These earlier investigations excluding Muhammad *et al.* (2019) are limited to northern part of the coast and do not provide complete profile of population genetic of this economically important species. However, our previous prediction of population differentiations of *A. fangsiao* be validated by present investigation (Lü *et al*., 2010, 2011). Here, we are describing the phylogeny

and population history of nine populations of *A. fangsiao* (Fig. 1) using complete mtDNA from 15 individuals. Molecular markers separated north (Dalian, Nantong, Qingdao, Yantai) and south populations (Huizhou, Shanghai, Xiamen and Zhenjiang), which is in accordance with our previous results of CO1 and 16S rDNA (Lü *et al*., 2010, 2011), and differ with AFLP results where northern populations Dalian, Qingdao were placed in one clade while Yantai and Lianyungang were reported to be another clade. This pattern of separation is like *Octopus minor* populations where south and north populations are distinctly distributed into two clades (Lü *et al*., 2013).

The genetic distance between the two clades was 0.030, and the genetic differentiation index F_{ST} reached 0.61476. The higher F_{ST} values illustrate lower level of gene flow (N_m)

and higher genetic differentiation among populations (Hedrick 2005; Ye *et al*., 2015). The standard values of F_{ST} are illustrated as 0.05 defines negligible genetic differentiation while greater than 0.25 defines high genetic differentiation within the analyzed population (Weir and Cockerham, 1996). Based on given standard obtained results showed greater differentiation among *A. fangsiao* populations. The higher genetic diversity was also reported in other species of the area, such as; genetic diversity between seven populations of *Octopus variabilis* was (0.91) based on the 12S rRNA and COIII gene (Xu *et al*., 2011). Kang *et al*. (2012) reported variations in *Octopus minor* populations of different localities ranging from (0.109 to 0.999). The genetic divergence of *Maoricolpus roseus,* found less than (1%) using mtDNA 16S and CO1 gene, Kirsten *et al*. (2015). Muhammad *et al*. (2018) investigated eight populations ranged (0.011 to 0.992). Comparison to said studies, present investigation showed moderate level of differentiation. There are two concepts to illustrate the genetic variations in *A. fangsiao* populations. It might be due to life style such as lack of planktonic larval stages and limited migration range (Lv *et al*., 2013). These factors might limit the dispersal ability and resulting reduction of gene flow. Nevertheless, this explanation does not support in case of clade A where no signification differentiation found within the group. The probable reasons

for genetic differentiation are results of various factors including geographic isolation, current, life history characteristics, (Gao *et al*., 2016), Islands and Gulfs also contribute in gene flow complications, the early glacier activities, where sea level encountered climatic fluctuations during the Pleistocene period and caused gene flow restriction in marine organisms (Imbrie *et al*., 1992). Besides it, the Changjiang River might influence in genetic structure of *A. fangsiao* populations along China coast (Lü *et al*., 2011). It is generally believed that the marine hydrological conditions, the ecological characteristics of the species and the life history can cause the differentiation of marine life (Muss *et al*., 2001; Yong *et al*., 2009). An intriguing possibility is that this type of environmentally driven genetic structure in Ocean species promotes allopatric speciation, whereby genetically different population divergence during glacial cycles (France and Kocher 1996, Zardus *et al*., 2006, Schüller 2011). A number of divergent forces effect genetic variation, including geographic separation, existing and life history features (Gao *et al*., 2016).

Estimation of the differentiation time of the octopus group using different molecular clocks showed that the genetic distances between the two clades of COI gene was 0.027, and the genetic distances between the two clades of Cytb gene was 0.215, so the differentiation time was estimated to be

about 119×104~ 540×104 million years ago (MYA) before the Tertiary period (according to the fish and shrimp and crabs' COI gene 0.5% to 1.4%/ MYA of evolution rate inferred (Knowlton and Weigt, 1998), the evolution rate of cytochrome b gene was calculated by 2.15% to 2.6%/ MYA), corresponding to the early Pleistocene period the genetic differentiation between both populations has been attributed to the repeated Pleistocene glaciations (1.25-0.7 MYA), (Mark *et al*., 2005, Clark *et al*., 2006).

There were 15 haplotypes among all the 15 mitochondrial DNA genomes. The genetic distances across the mtDNA genomes ranged from 0.007 between NT and YT, to 0.059 between north-south region (Dongshan located in the south of China and Dalian located in the north of China), with an average genetic distance of 0.057. To test whether there is a signature selection in the *A. fangsiao* mitochondrial genome, we assessed the ratio of the number of non-synonymous substitutions per non-synonymous sites (KA) to the number of synonymous substitutions per synonymous sites (KS) and found low KA/KS values in both clades (KA/KS = 0.0126 and 0.9827 in clades A and B, respectively), representing the influence of negative selection. Negative selection plays an important role in maintaining the long-term stability of biological structures by removing deleterious mutations, Ana M Pérez O'Brien *et al*. (2014). However,

further studies are required to confirm differentiation of *A. fangsiao*.

Conclusion

Among populations main divergence factor is the ice age quaternary glacial period. Yangtze River acts as a physical barrier to larval dispersal and variation between both populations. This lays the foundation for the future development and utilization of the octopus resources in China.

Acknowledgements

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