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GENETIC DIVERSITY OF *OCTOPUS MINOR* (SASAKI, 1920) INFERRED BY MITOCHONDRIAL NADH DEHYDROGENASE SUBUNIT 2 GENE.

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ABSTRACT

Octopus minor is an economically important resource commonly found in Chinese coastal waters. Mitochondrial DNA NADH dehydrogenase subunit 2 (ND2) gene were applied to assess genetic variation among eight populations collected from northern to southern part of China coast. Twenty four haplotypes were detected from 112 individuals of eight populations. Overall high haplotype (0.813) and low nucleotide diversity (0.044) was observed. AMOVA revealed (88.78%) variation among population and (11.22%) within population. The pairwise F_{ST} ranged between (-0.011 to 0.992. Gene flow (N_m) of Wenzhou and Dongshan population was much lower among others. Neighbour joining (NJ) tree clustered eight populations into three clades. Network analysis revealed that two (Hap1- Hap 9) haplotypes appeared in six and four populations respectively. The mismatch distribution showed unimodal pattern in calde two and three which suggested population expansion while clade one showed stability. The deviation might be result of several causes including low dissemination ability, influenced by late Pleistocene geological period or Changiang River outflow on population structure of O.*minor*. These results will enhance our understanding for better exploitation, protection and management of *O.minor.*

Key words: Cephalopod, *Octopus minor*, ND 2, Genetic diversity, Chinese waters.

INTRODUCTION

The Cephalopod group of organisms (squids, cuttlefish, Octopuses) belongs to phylum Mollusca and has cosmopolitan distribution (Norman, 2000). Octopuses have speedy growth, rapid maturity, excellence fertility and short life cycle (Boyle and Boletzky 1996; Ibanez *et al*., 2011). Nevertheless, the environmental fluctuations and adverse ecological conditions has immense impact on their migration pattern, distribution, population structure and genetic diversity (Dawe *et al*., 2007; Semmens *et al*., 2007; Car-denas *et al.,* 2009; Ibanez *et al*., 2011). Dispersal capacity and habitat (Daniels *et al*., 2002; Liu *et al.,* 2007) has influence on genetic structure of organisms where divergence in gene frequency depends on increased or concise dispersal competence. Restricted competence demonstrates greater gene flow isolation (Gyllensten 1985; Liu *et al*., 2007; Han *et al*., 2008; Riginos and Victor 2001).

Genetic research contributes for upraising and handling of resources where population history, genetic diversity and geographical partitioning throughout natural dispersal range can directly be analysed with genetic markers (Hutchings, 2000). Therefore, several population genetic investigations have been conducted in marine organisms, including cephalopod groups (Shaw 2003; Chang *et al*., 2010; Kang *et al*., 2012; Gao *et al*., 2016) using mitochondrial DNA genes which have high mutation and low recombination rates hence best projects population genetic structure, population differentiation and species relationships (Avise, 2000).

NADH dehydrogenase subunit 2 (ND2) proteins contribute to electron transfer and hydrogen to produce ATP through the progress of Phosphorylation. It has enormous contribution in proton trans-location across the inner mitochondrial membrane which involved in pH regulation in cells (Schauer *et al*. 2015). The ND2 gene evolved faster than other mtDNA genes. Therefore, widely used in molecular systematic and population genetic studies (Jin, *et al*., 2008; Dai *et al*., 2011).

Octopus minor (Sasaki, 1920) is an economically important species and often synonymies as *Octopus variabilis*. It is widely distributed in Korean Peninsula, Japanese archipelago and Chinese marine waters, (Okutani *et al*., 1987; Dong 1988; 1991; Qian *et al*., 2010; Lu *et al*., 2012). *O. minor* lays fewer eggs and attaches in selective places especially under shelter (Bo, *et al*., 2014; Bo *et al*., 2016). The sluggish lifestyle of *O. minor* slackens its migratory potential and curtails gene flow which leads to genetic differentiation (Dong, 1991). The profound aim of this study was to elaborate genetic diversity, population structure and enhance the existing knowledge about fishery management and conservation mechanisms of this species.

MATERIALS AND METHODS

In total, 112 specimens of *Octopus minor* were sequenced from eight geographic locations across Chinese coastal waters. The sampling sites were Dalian, Donghsan, Nantong, Qingdao, Shanghai, Wenzhou, Xiamen, and Zhoushan (Fig 1B, Table 1). Samples were collected using local fishing boats. Only adult individuals were chosen for experiments. Muscle tissues preserved in 90% ethanol and transported to Zhejiang Ocean University. Total genomic DNA was extracted using standard phenol-chloroform method (Sambrook *et al*., 1989). The concentrated DNA was further diluted with DEPC water to reach working concentration (20ng/ µl) for PCR reactions.

A fragment of ND2 gene was amplified using primers ND2F- 5′TCACTATCTTCCTCCCATTG 3′ and ND2R-5′ CCTAATATAGGAGGTAAACCTC3′, The pair of primers were designed using mtDNA gene sequence (accession number AB158363). The PCR mixture consisted of 1µl of each primer, 2.5 µl of 10X buffer, 2µl Mg^{2+} (20mmol/L), 2µl dNTPs (2.5mmol), 0.25 µl Taq DNA polymerase and 1µl of DNA template. The PCR conditions were as follows: denaturation at 94°C for 5 min, 35 cycles each of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72 °C for 30s, and the final extension at 72°C for 10 min. The PCR products were examined on a 1.5% agarose gel. Sequencing was performed on ABI 3730 automated sequencer. The DNA sequences were aligned using CLUSTALW. The base composition were estimated using software MEGA 6 (Tamura *et al*. 2013). The genetic parameters including polymorphic sites, haplotype diversity, nucleotide diversity and average pairwise differences (K) was estimated, using DnaSP (Librado and Rozas 2009). Analysis of molecular variance (AMOVA), mismatch distribution, Tajim's D, Fu's Fs and their corresponding *P*- values were obtained with software ARLEQUIN (Excoffier and Lischer 2010). The neighbour joining tree was constructed using MEGA 6 (Tamura *et al*. 2013). The median joining network was created using NETWORK software version 5.0.0.1 (Bandelt *et al*. 1999). Calculations of gene flow (N_m) were performed using formula $(1-F_{ST})/2F_{ST}$.

RESULTS

NADH dehydrogenase subunit 2 gene (ND2) data analysis performed based on one hundred twelve individuals of eight populations. Base composition of ND2 revealed T, 42.4%, C, 14.6%, A, 37.0%, G 6.0%. The composition characterizes unequal frequency T+A, were higher (79.4%), than G+C contribution.

Total of 24 haplotypes and 91 polymorphic sites were observed among them five were singleton and 86 were parsimony informatives. Eight InDel sites were observed which showed nine haplotypes with (0.447) diversity. Overall high (0.813) haplotype and low (0.044) nucleotide diversity was observed. The high haplotype diversity was observed in Shanghai (0.893) population while the lowest haplotype diversity was seen in Dongshan (0.318). The mean pairwise differences (K) ranged (0.333 to 2.571) Table 1. Analysis of Molecular variance (AMOVA) revealed that (88.78%), variation occurred among population while (11.22%) differences remained within population where fixation index F_{ST} was (0.887) (Table 2). The pairwise F_{ST} ranged between (-0.011 to 0.992). The Dongshan population showed lowest gene flow between groups. The details of gene flow were shown (Table 3). The phylogenetic analysis revealed three clades. The clade one consisted of (Dalian, Nantong, Qingdao, Shanghai, Xiamen and Zhoushan), clade two and three represented Wenzhou and Dongshan population respectively (Fig.2). The median joining network analysis revealed two frequent haplotypes (Hap 1 and Hap 9) which shared by six and four populations respectively whereas Wenzhou and Dongshan population parted similar to neighbor joining tree (Fig 1 A). The Tajima's D values were ranging between (-1.451 to1.459) where Dongshan population was significant ($P < 0.05$). The Fu's FS values were remained in between (-1.324 to 2.398). The Dongshan and Shanghai populations resulted negative values where Dongshan population was statistically significant ($P < 0.05$) (Table 4). The mismatch distribution revealed unimodal distribution in clade 2 and clade 3 whereas clade 1 showed stability with bimodal peak (Fig 3 A, B, C).

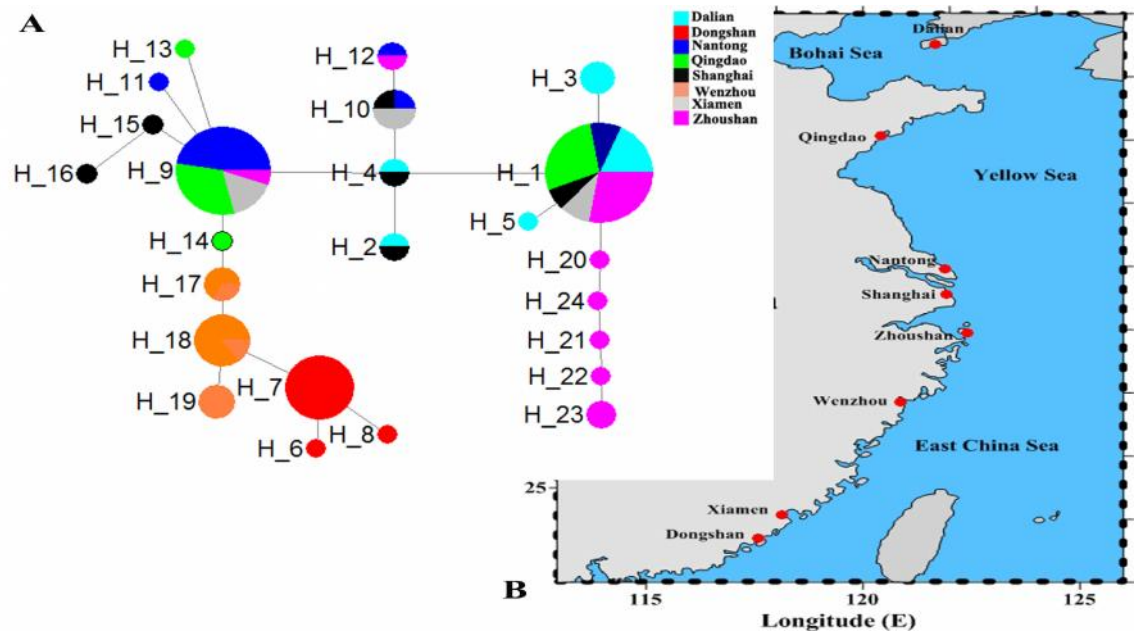

Fig 1 A & B. A. Median joining network for ND2 haplotypes. Different colours representing the populations in the network. B. Sample location of *Octopus minor* populations.

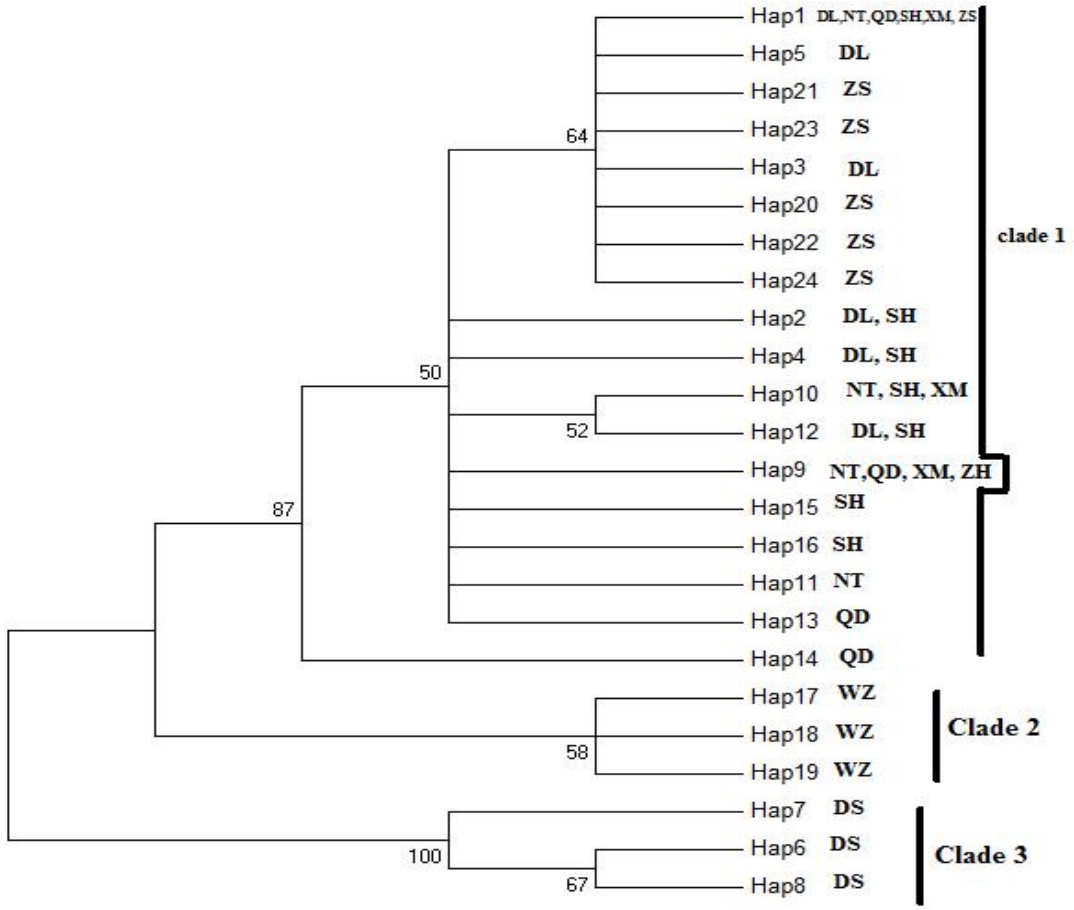

Fig 2. Neighbour Joining (NJ) tree of *O. minor* based on mtDNA ND2 gene haplotypes. The key demonstrates contribution of haplotypes and name of respective population. Key: DL= Dalian; DS= Donghsan; NT= Nantong; QD= Qingdao; SH= Shanghai; XM= Xiamen; ZS= Zhoushan

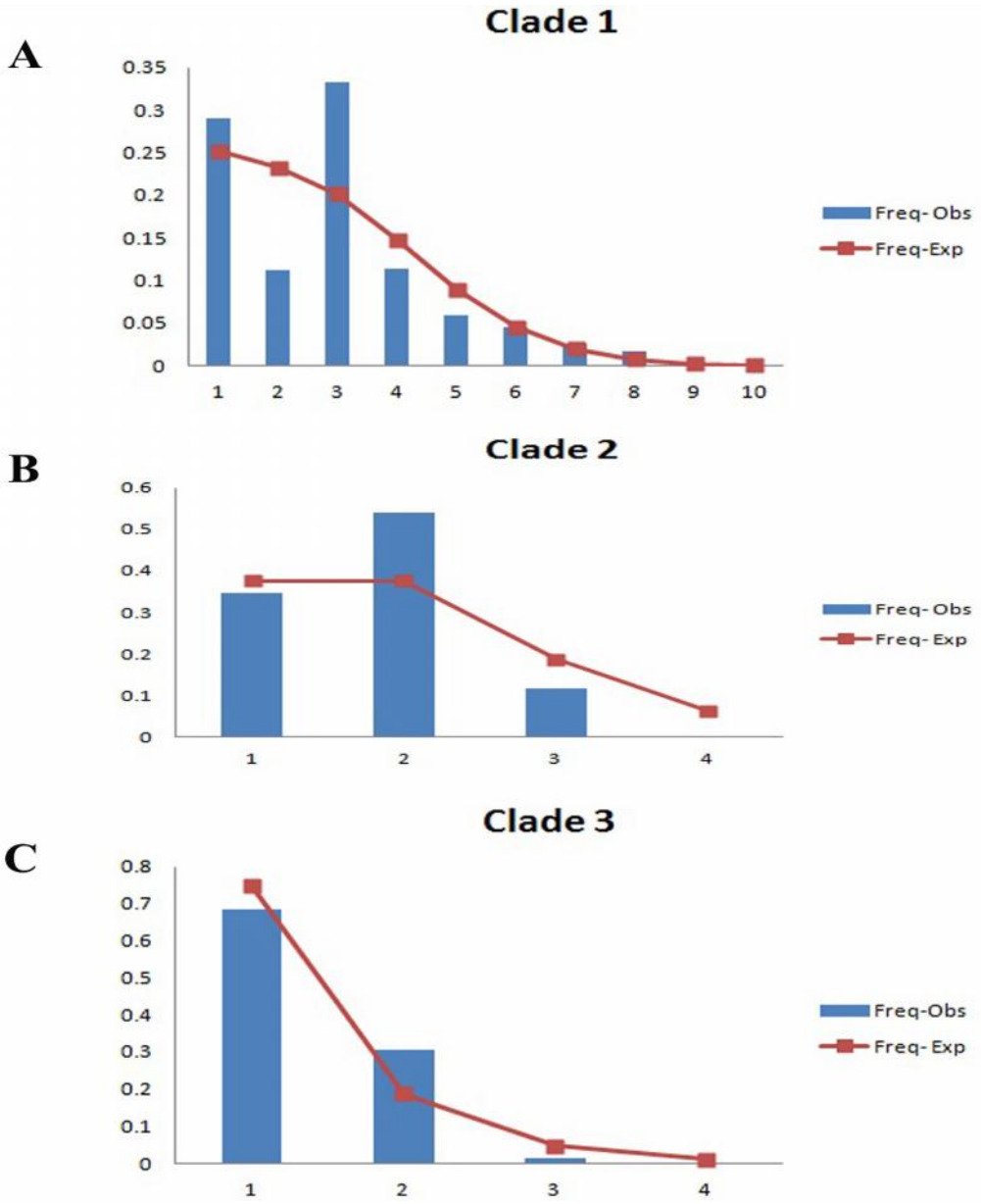

Fig 3. Mismatch distribution of three clades: (A) Dalian, Nantong, Qingdao, Shanghai, Xiamen, Zhoushan; (B) Wenzhou; (C) Dongshan.

Table 1. Sample location, Sample size, haplotype and nucleotide diversity of eight populations.

Sample Location	S. Size	No. of polymorphic sites	Haplotype (h)	Haplotype diversity (Hd)	Nucleotide diversity (π)	Average number of differences K
Dalian	14	4	5	0.659	0.0023	0.912
Dongshan	12	2	3	0.318	0.0008	0.333
Nantang	16	5	5	0.650	0.0035	1.383
Qingdao	20	4	4	0.574	0.0031	1.210
Shanghai	8	7	6	0.893	0.0065	2.571
Wenzhou	13	2	3	0.654	0.0019	0.769
Xiamen	9	3	3	0.722	0.0036	1.444
Zhoushan	20	10	8	0.647	0.0062	2.410

Table 2. Analysis of molecular variance of *Octopus minor* in eight populations.

Source of Variation	d.f	Sum of squares	Variance components	Percentage of variation
Among population	8	912.140	9.344 Va	88.78
Within Population	104	122.807	1.180 Vb	11.22
Total	112	1034.946	10.52492	
Fixation Index FST:	0.887			

Table 3. Pairwise F_{ST} and gene flow between eight populations of *O. minor*. Above diagonal are N_m values and below diagonal are pairwise F_{ST}

	Dalian	Dongshan	Nantong	Qingdao	Shanghai	Wenzhou	Xiamen	Zhoushan
Dalian	-	0.008	1.151	6.349	4.087	0.067	4.994	4.172
Dongshan	0.983*	-	0.008	0.010	0.014	0.004	0.009	0.018
Nantong	0.303*	0.981*	-	3.046	3.259	0.086	82.833	1.006
Qingdao	0.073*	0.980*	0.141*	-	10.369	0.082	30.75	2.948
Shanghai	0.109	0.972*	0.133*	0.046*	-	0.111	44.954	7.192
Wenzhou	0.882*	0.992*	0.857*	0.859*	0.818*	-	0.078	0.124
Xiamen	0.091*	0.983*	0.006	-0.016	-0.011	0.865*	-	2.605
Zhoushan	0.107	0.964*	0.332 *	0.145	0.065	0.801*	0.161	-

*($P < 0.05$).

Table 4. Tajima's D and Fu's FS tests, corresponding *p* value for eight population of *O. minor*.

Location	Tajima's D		Fu's Fs	
	D	*P*	Fs	*P*
Dalian	-0.292	0.420	0.033	0.518
Dongshan	-1.451	0.054	-1.324	0.008
Nantong	-0.047	0.512	0.542	0.662
Qingdao	0.665	0.766	2.077	0.869
Shanghai	0.138	0.533	-0.591	0.307
Wenzhou	0.544	0.737	0.249	0.497
Xiamen	1.459	0.942	2.398	0.888
Zhoushan	-0.314	0.429	0.168	0.546

DISCUSSION

Population genetic studies are effective mechanism to assess genetic diversity and to probe conservation genetics (Crandall *et al*. 1999; Divya *et al*., 2015). Generally, higher dispersal potential causes less genetic differentiation like in fish (Mandal, *et al*., 2012). *O.*minor has less dispersal capacity because of troglodytism (Yang, *et al*., 2015). Therefore, basic information of genetic variation and population structure is necessary for fisheries management and conservation of this species.

Present investigation based on mtDNA ND2 gene sequences revealed substantial differentiation among eight populations of *O.minor* found along coast of China (Fig 1 B). Nevertheless, high haplotype and low nucleotide diversity were detected which disclose rich genetic diversity and high evolutionary potential and consistent with previous workers (Lü *et al*., 2013; Li *et al*., 2010; Xu *et al*., 2011) but Dongshan population showed relatively less haplotype diversity (0.318). Analysis of molecular variance (AMOVA) showed 88.78% variation among population and fixation index (0.887) was statistically significant ($P < 0.05$). This is in agreement with early approaches of investigation where significant differences reported in *O. minor* (Chang *et al*., 2010; Guo *et al*., 2011; Li *et al*., 2010) and *O. ocellatus* (Lü *et al*., 2011). In addition, it is inconsistent with results of AFLP (Yang, *et al*., 2015). The least gene flow (Nm) values were shown by Dongshan and Wenzhou populations which distinctly consistent with statement of higher F_{ST} values describe lower gene flow (Nm) and higher differentiation among populations (Hedrick, 2005). The F_{ST} values are meaningful for population genetics. Wright (1978) had given certain values of differentiation. His given values (0 to 0.05) describe the less differentiation, (0.05 to 0.15) defines moderate differentiation, (0.15 to 0.25) mean as high differentiation, above 0.25 values describes very high

genetic variations (Gao *et al*., 2016). The pairwise F_{ST} values were described in (Table 3).

The phylogenetic analysis revealed three clades. It is in agreement with previous investigations (Li, *et al*, 2010, Xu, *et al*., 2011) but partly differ with CO11 results, in which, Zhoushan population clustered separately (Lü, *et al*., 2013) and disagree with Li *et al*, (2010) and Chang *et al*., (2010) where Xiamen population clustered separately. Furthermore, Sun *et al*, (2010) and Yang *et al* (2015) separated five populations (Dalian, Yantai, Qingdao, and Lianyungang) into two clades which differ with present results. The network pattern of genealogy illustrated that Hap1 was shared by six populations; Hap9 was shared by four populations while Wenzhou and Dongshan population shared none of their haplotypes with counterpart populations (Fig 1 A). It is in agreement with Chang, *et al* (2010) where Wenzhou population separated while disagree with reference to Xiamen population. In present result the haplotypes of Xiamen population shared with other six populations (Fig 1 A). The mismatch distribution suggested historical population expansion in clade 2 and clade 3 while clade 1 showed stability.

The suitable gene exchange needs high dispersal potential during life history stages, such as planktonic egg and active migration. The *O. minor* has lethargic lifestyle, meagre dispersal potential; juveniles develop directly, benthic eggs (Dong, 1991) and benthic environment. These attributes hamper this commercially important species for frequent gene flow. In addition, various factors can probably be reasons for genetic differentiation 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, Changjiang River might influence in genetic structure of populations (Lü *et al*, 2011).

Present investigation enhances the existing knowledge of *O.minor* population genetics and recommend separate management unit for Dongshan and Wenzhou population with reference to conservation and management perspective.

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