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Genetic Diversity and Structure Analysis of *Phascolosoma esculenta* in the Coastal Zone of South-eastern China Based on Mitochondrial *Cyt b* Gene

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Abstract

Phascolosoma esculenta is a newly developed mariculture species in south-eastern China. However, its wild populations are diminishing because of marine environmental pollution, beach reclamation, and overexploitation. To investigate the genetic diversity and genetic structure of the wild populations of *P. esculenta*, the entire sequence (1136 bp) of its mitochondrial cytochrome b (*Cyt b*) gene were sequenced and analyzed in 80 organisms sampled from four areas. A total of 59 variable sites and 49 haplotypes (61.25% of the total samples) were detected from the sequence, indicating a high level of polymorphism. High haplotype diversity (0.976±0.007) and low nucleotide diversity (0.00439±0.00027) were detected. Topologies of neighbor-joining trees were shallow and the clustering of haplotypes was not correlated with their geographical distributions. Both AMOVA and pairwise Fst analyses showed that the genetic differentiation between populations was relatively weak, which might be influenced by its life history, paleoclimate, and recent human activity. The neutrality test and mismatch-distribution analysis revealed that the expansion of *P. esculenta* populations occurred approximately 0.046 million years ago.

Keywords: *Phascolosoma esculenta*, *Cyt b* gene, genetic diversity, genetic differentiation.

Introduction

Phascolosoma esculenta, a species restricted to the coastal zone of south-eastern China, belongs to the phylum Sipuncula and family Phascolosomatidea (Li 1988). It burrows in muddy and sediment beaches along the high tide areas of the intertidal zone and feeds on the benthic diatoms and organic debris. During its breeding season in summer, the viripotent *P. esculenta* adults release their sperms or eggs into the water to complete fertilization with the stimulation of the spring tides (Zhu, Wang, Xu, & Zeng, 2007). Its free-swimming larvae, which can flow with ocean currents, last for approximately 15 days (Jin, Zhu, Xu, & Wang, 2011), likely facilitating gene flow among different populations within a wide geographic range. Notably, as "a replacer of the Chinese caterpillar fungus in sea", *P. esculenta* possesses a high dietary and

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medicinal value. Thus, it has become a newly developed mariculture species in China, and its aquaculture has provided remarkable economic results in the recent years. However, in the condition of immature artificial breeding, the cultured seedlings mainly come from wild populations. In addition, with marine environmental pollution, beach reclamation, and excessive commercialization of wild adults, the wild *P. esculenta* populations have shown rapid depletion, which, undoubtedly, have influenced its sustainable fisheries resources utilization. Fortunately, the resource protection and artificial breeding of *P. esculenta* have been valued greatly. To date, its alimentary canal and nephridium structure(Lei, Lu, Ding, & Zhu, 2013; Long, Lu, & Zhu, 2014a; Long, Sheng, Lu , Ding, & Zhu, 2014b), gonadogenesis and gametogenesis(Zhu et al., 2007; Ying et al., 2009; Long, Sheng, & Zhu, 2015a), fertilization cytology (Zhu et al., 2008; Long, Sheng, Xu, Wang, & Zhu, 2015b), embryonic development(Jin et al., 2011), nutritional ingredients of muscle(Zhou, Ding, Xu, Li, & Yan, 2007) and partially functional genes (Wang, Su, Li, Jun, & Li, 2010; Liu et al. 2013) have been reported, whereas the population genetics data necessary for the resource protection of *P. esculenta*, such as population diversity and genetic structure among different geographical populations, are still scarce. However, the population genetics information of *Sipunculus nudus*, which also belongs to the phylum Sipuncula, has been widely reported (Wang, Du, & Li, 2006; Song et al., 2011; Du, Chen, Deng, & Wang, 2009). Therefore, it is necessary to investigate the genetics of the wild *P. esculenta* populations that can reveal the degree of population depression, and based on which we can protect and exploit the *P. esculenta* populations effectively.

Characterized by a simple structure, strict maternal heredity, lack of recombination and relatively rapid evolutionary rates, mtDNA has enabled us to study molecular phylogenesis, population genetics, and conservation aspects (Huang et al., 2009; Mao, Gao, Yanagimoto, & Xiao, 2011). A mitochondrial gene, cytochrome *b* (*Cyt b*), has been widely used to study marine invertebrate species, such as *Paracentrotus lividus* (Maltagliati, Di, Barbieri, Castelli, & Dini, 2010), *Octopus variabilis*(Li, Lv, Liu, Wu, & Zhang, 2013), and *Perinereis aibuhitensis* (Deng, Song, Liu, & Gao, 2014). Previously, complete mitochondrial genome information of *P. esculenta* has been revealed, which was expected to apply to conservation biology (Shen, Ma, Ren, & Zhao, 2009). In this study, considering the lack of genomic information of *P. esculenta*, we used *Cyt b* gene sequence as a molecular marker to investigate the genetic diversity and genetic structure of *P. esculenta* population in the coastal zone of south-eastern China, preliminarily. Based on the results, possible effects of life history, paleoclimate, and aquaculture on the population genetic differentiation of *P. esculenta* were discussed.

Materials and Methods

Sampling

From April to July 2014, a total of 200 wild individuals of *P. esculenta* were collected from the intertidal zones at four sites in China (Figure 1). Species identification was conducted by its morphology described by Li (1989). Twenty organisms sampled per site were used for genetic analyses. Pieces of muscle tissue were preserved in 95% ethanol until genomic DNA extraction.

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DNA extraction, Amplification, and Sequencing

Genomic DNA was extracted from the muscle tissue of each *P. esculenta* individual by proteinase K digestion followed by a phenol-chloroform method. Subsequently, the *Cyt b* gene in *P. esculenta* was amplified and sequenced. Based on the *P. esculenta* mitochondrial genome（GenBank Number: EF583817.1), the following primers were designed for polymerase chain reaction (PCR) amplification: *Cytb*-F (5′-GCAACCCTTCCAAACCATCACTT-3′) and *Cytb*-R (5′-GGCCAGGTGT ATAGAAGAGTCG-3′). The PCR amplification of *Cyt b* molecular markers was carried out in a 50 μL reaction volume, containing 4 μL genomic DNA (50ng/ μL), 25 μL 2X Taq PCR MasterMix (Tiangen, China), 2 μL of each primer (10 μM), and 17 μL ddH_2O. PCR was conducted as follows: initial denaturation at 94°C for 5 min, followed by 35 amplification cycles (comprising denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min) and a final extension at 72°C for 10 min. Ultimately, the reaction products were checked by electrophoresis on 1.0% agarose gel, stained with Gelview (BioTeke, China), and visualized under UV light. Purification and bi-directional sequencing were completed by Invitrogen (Shanghai, China).

Data Analysis

Sequences were aligned by BioEdit 7.1.3.0 (Alzohairy, 2011). The important indices for estimating molecular diversity, including haplotype diversity (*h*), nucleotide diversity (*Pi*), and the average number of nucleotide differences (K), were calculated using the DNAspV5 program (Librado & Rozas, 2009). Based on the *Cyt b* gene sequences, the mean genetic distances within and between *P. esculenta* populations were computed using the MEGA 5.10 program (Tamura et al., 2011). Based on the results, a neighbor-joining (NJ) tree was constructed, in which the bootstrap values were estimated using 1000 replicates with Kimura's two-parameter model. Population genetic differentiation were estimated using analysis of molecular variance (AMOVA). The median-joining network was consulted using NetWork 5.0 (Polzin & Daneshmand, 2003). Tajima's D and Fu's FS test of neutrality were actualized by the Arlequin 3.01 software (Excoffier, Laval, & Schneider, 2005). Population expansion time (T) was investigated by using the equation $T=\tau g/4\mu k$ (Song et al. 2012), where τ is the peak value of nucleotide mismatch distribution, g is the generation time, μ is the rate of molecular evolution, and k is the number of nucleotides.

Results

Information of Base Composition and Variable Sites

The 1136-bp *Cyt b* gene sequences from 80 individuals of *P. esculenta* collected from 4 regions were aligned. The contents of A, T, C, and G base content of the sequences were 29.8, 31.0, 22.6, and 16.6%, respectively, indicating A-T rich regions in the *Cyt b* gene as reported for most invertebrate mtDNA sequences (Liu et al., 2012a; Deng et al., 2014; Yang et al., 2014) and coinciding with the feature of *P. esculenta* mitochondrial genome (Shen et al., 2009). A total of 59 variable sites (5.2% of the entire sequence), including 27 singleton variable sites and 32 parsimony informative sites, were detected (Table

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1). These variable sites consist of 45 transition mutations, 8 transversion mutations, and 6 sites in which transition and transversion mutations coexist, while there are no insertion or deletion mutations (Table 1). In addition, 49 haplotypes (GenBank Number: KY401184-KY401232) were detected that occurred in 61.25% of the total samples (Table 1). Total haplotype diversity (*h*), nucleotide diversity (*Pi*), and the average number of nucleotide differences (K) were 0.976±0.007, 0.00439±0.00027, and 4.98165, respectively (Table 2). Furthermore, the haplotypes Hap1, Hap26, Hap28, Hap4, Hap6, Hap7, Hap8, Hap11, Hap12, Hap19, and Hap24 were shared by at least two populations, whereas the other haplotypes were singletons (Table 3).

Population Genetic Structure

Genetic distance within and between *P. esculenta* populations based on *Cyt b* sequences were 0.00410–0.00471 and 0.00397–0.00457 (Table 4), respectively, revealing no significant genetic differentiation. The *Fst* value was −0.01069–0.09358 between populations (Table 4), which suggested that the inter-population differences were relatively low except between WL and ND and WL and ZJ (Dong et al., 2013). AMOVA showed that the genetic variance resulted mainly from the difference between individuals within populations (Table 5). Besides, haplotype neighbor-joining tree of *P. esculenta* suggested that the haplotypes were randomly distributed and their clustering was not correlated with their geographical distributions (Figure 2). Therefore, no clear geographic genetic was observed, which was also revealed by median-joining network (Figure 3).

Estimate of Population Expansion

Tajima's D and Fu's Fs test of neutrality of the four groups (Table 2), based on 5,000 simulated samplings, showed a negative value. While all groups were analyzed as one population, Tajima's D and Fu's Fs values were negative and 1% significant, strongly indicating population expansion. Tajima's D values reflect the ancient events, while the Fu's Fs values reflect recent events of a population (Fu, 1997; Su, Fu, Wang, Jin, & Chakraborty, 2001). In our study, the absolute values of Fu's Fs were far greater than the Tajima's D values; therefore, the *P. esculenta* populations accumulated more mutations recently.

The mismatch analysis results showed that the observed nucleotide mismatch distribution had a single peak shape, which did not significantly deviate from the expected distribution of population expansion model (Figure 4). Supporting the results of the neutrality test that the population expansion occurred in *P. esculenta*, the low SSD value (0.00212), raggedness value (0.00895), and no statistically significant difference ($P>0.05$) of the goodness-of-fit tests revealed a higher matching degree between the observed value and model. Further, we estimated the expansion time using τ (4.37891), the peak value of nucleotide mismatch distribution, and 2%/Ma (million years) as the rate of molecular evolution (Gillooly, Allen, West, & Brown, 2005). The *P. esculenta* population expansion time, approximately 0.046 million years ago, was calculated by $T=\tau g/4\mu k$.

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Discussion

Genetic Diversity

Genetic diversity, also called gene diversity, is the basis and core of biodiversity, which guarantees species evolution. High level of genetic diversity suggests the strong adaptability and survival ability of populations (Barrett & Schluter, 2008). Conservation of germplasm resources and rational development and utilization of marine organisms can be actualized according to the study of genetic diversity. The *Cyt b* gene, an effective molecular marker, has been applied to analyze the genetic information of many species (Maltagliati et al. 2010; Li et al. 2013; Deng et al. 2014). In our study, the *Cyt b* gene was used to check the genetic diversity of *P. esculenta* in the coastal zone of south-eastern China.

The haplotype diversity index (*h*) and nucleotide diversity index (*Pi*) are important parameters of genetic diversity and high *h* and *Pi* values indicate high polymorphism and genetic diversity. In our investigation, 59 polymorphic sites were detected and 49 haplotypes (61.25% of the total samples) were identified in all the sequences. The total haplotype diversity (*h*) and nucleotide diversity (*Pi*) were 0.976 and 0.00439 (Table 2), respectively. Therefore, with the rapid quantitative depression of wild *P. esculenta* population, its genetic diversity and germplasm resource have not been seriously damaged and irreparable, a good news for the conservation and utilization of *P. esculenta* populations.

Genetic Differentiation

Genetic fixation index (*Fst*) between different geographic populations is the main measure to estimate genetic differences, but also can reflect the genetic relationship between populations. The *Fst* value between *P. esculenta* populations were −0.01069-0.09358 (Table 4). Genetic differences between *P. esculenta* populations were relatively low, except between WL and ND and WL and ZJ (Dong er al. 2013). As the 4 groups as one population, the *Fst* value was 0.0345, suggesting no clear genetic differentiation. Besides, no clear significant difference was observed in the genetic distance within and between *P. esculenta* populations. AMOVA showed that the genetic variance resulted mainly from the difference between individuals within populations, and there was no genetic differentiation among populations. NJ phylogenetic tree and median-joining network suggested that the haplotype clustering based on *Cyt b* markers was not evident in geographical regions. In other words, our results suggest that the *P. esculenta* populations in the coastal zone of south-eastern China showed low genetic differentiation, which was consistent with the *Cyt b* sequence analysis of the *P. aibuhitensis* populations in Qingdao coastal waters and *Ommastrephes bartramii* populations in the North Pacific Ocean (Deng et al. 2014; Liu, Xu, & Chen, 2012b).

In the ocean, the lack of a geographical barrier influencing population diffusion will benefit gene exchange among geographical populations, and subsequently, result in genetic homogeneity in many species (Zhao, Zhuang, Zhang, & Shi, 2011). The *P. esculenta* adults live in the intertidal zone and have weak movement ability, but their free-swimming larvae last for approximately 15 days (Jin et al. 2011). Most importantly, in July and August, its breeding fastigium, the summer monsoons are frequent. Therefore, with the transport of coastal current in the coastal zone of south-eastern China, the free-swimming larvae can spread in a long

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distance (Deng et al. 2014). Furthermore, *P. esculenta*, a eurythermal and euryhaline organism, shows strong resistance to environmental stress (Zeng, Hong, Ding, & Zhu, 2006). Thus, the invasive *P. esculenta* larvae can acclimatize themselves, allowing dispersion and gene exchange between *P. esculenta* populations. In addition, with the development of artificial breeding, the endemic wild seedlings are continually imported, thereby increasing the chances of gene exchange.

Historical Demography and Its Influences

Periodic climatic oscillations over the Pleistocene are thought to have greatly influenced the range contractions and expansions in many species. Much evidence, including the information on population genetic variation, has been proposed (Hewitt, 2000). Therefore, the *P. esculenta* populations might also undergo range contractions and expansions. During the Pleistocene glacial maximum, the 120–140 meters declines in sea levels have been noted (Imbrie et al., 1992), which might result in the extinction of *P. esculenta*. However, with interglacial climate warming, the melting ice caused a rise in sea level, which might have caused expansions in the populations of *P. esculenta*. In our study, Tajima's D and Fu's Fs test of neutrality were negative and 1% significant as 80 samples as one population, which supported our speculation. In addition, the mismatch analysis showed that the observed nucleotide mismatch distribution coincided with the expected expansion model, confirming population expansion in a certain period. Interestingly, as we speculated, the expansion time was roughly estimated to be approximately 0.046 MY ago (during late Pleistocene). However, due to the lack of the mutation rate of *P. esculenta Cyt b* gene, the rate of 2% /Ma was applied to our study (Gillooly et al. 2005), making it difficult to associate the expansion time with a particular Pleistocene glacial event. Nevertheless, the periodic climatic oscillations influenced the *P. esculenta* population. Similar events were found in other marine invertebrates and fishes. For instance, *P. aibuhitensis* near the coasts of Shandong Peninsula (in China) and *Mytilus coruscus* along the coastal waters of Zhejiang and Fujian (in China) were also found to have undergone population expansion during Pleistocene (Liu et al. 2012a; Yang et al. 2014). Besides, *Larimichthys polyactis*, *Miichthys miiuy*, and *Pampus argenteus* distributed in the northwest Pacific had also undergone population expansion in the same period (Peng, Zhang, Zhao, & Yue, 2010; Xia, Zhang, Gao, Chen, & Li, 2013; Zhao et al., 2011).

In addition, our results support a hypothesis that a high *h* and low *Pi* is attributed to the population expansion after a period of low effective population size (Grant & Bowen, 1998). In our study, the high *h* and low *Pi* values were detected (Table 2). Coincidentally, the population expansion of *P. esculenta* was revealed by the neutrality test and mismatch-distribution analysis. Many marine invertebrate species, such as *S. nudus* (Du, Chen, Deng, & Wang, 2009), *P. aibuhitensis* (Liu et al. 2012a), and *M. coruscus* (Li et al. 2013), were also found to be in accordance with this hypothesis. Interestingly, these species exhibit expansion-promoting characteristics such as strong reproduction and diffusion ability. Rapid population growth and short mutation time could result in high *h* and low *Pi*. Therefore, we can infer the population history of a species from its genetic diversity (Grant & Bowen, 1998). In other words, the expansion of *P. esculenta* populations influenced its genetic diversity and resulted in the phenomenon of high *h* and low *Pi*. Besides, the short evolution time of each population after expansion might be a reason for weak genetic differentiation among *P. esculenta* populations. Much evidence has been proposed that paleoclimate-

derived population expansion could influence the phylogeographic structure of invertebrate (Yang, Ye, Xin, Zou, & Xia, 2016) and vertebrate species (Wang, Jiang, Xie, & Li, 2013).

In this study, we investigate the genetic diversity of wild *P. esculenta* populations in the south-eastern coast of China, which suggested that genetic diversity and germplasm resource of *P.esculenta* have not been seriously damaged and irreparable. Furthermore, no genetic differentiation influenced by the life history, paleoclimate, and recent human disturbances was observed among *P. esculenta* populations. Although three years have passed since our sampling, during which time the exploitation of wild *P. esculenta* resource was limited in small scale, and destructive utilization was not happen. In addition, enhancement and releasing has not been carried out because of the condition of immature artificial breeding. Therefore, under the condition of lack of remarkable human disturbance on wild populations, the status of wild *P.esculenta* resource today is similar with that three years ago. We believe that our results can still represent the current wild population genetics information of *P.esculenta*, which will certainly contribute to the protection and reasonable exploitation of *P. esculenta* resource.

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Table 1. Variable sites of *Cyt b* gene in *P. esculenta*

Haplotypes	Variable sites
	1 1 1 1 1 1 1
	1 1 1 1 1 2 3 3 3 3 3 3 3 3 4 4 4 4 5 5 5 5 5 5 5 5 6 6 6 7 7 7 7 7 7 7 8 8 8 8 8 9 9 9 9 0 0 0 0 0 1 1
	2 5 6 6 1 1 6 6 9 4 0 0 1 2 4 5 6 9 1 2 7 8 0 2 3 4 5 7 7 8 9 4 8 9 2 3 3 5 5 9 9 0 2 2 4 5 7 0 4 5 7 8 2 5 7 8 9 0 1
	7 0 6 9 4 8 5 8 8 7 6 9 6 7 6 1 6 3 7 9 7 0 4 8 0 0 5 0 4 3 1 0 1 6 9 5 6 4 6 2 8 7 2 5 9 5 0 6 6 4 2 4 6 9 2 3 4 4 6
Hap1	C G T C A C T C G A A C G A C G A C A T C C C C A T C C G G C G C A C A C A C G A T C T A T C C C C T C T G A T T C A
Hap2	. A . . . . . A . . . . C . A . . . . . T . . . . T . . . . . C . . . . . A . . . . . G . T . . . . .
Hap3	. . . . . T A . . . . C . . . . . T T . . . . T C . T . . . . . C . . T . . . . . G
Hap4	. . . . . A . . . . C . . . . . T . . . . . C . . . . T . . . . . T . T . . . . .
Hap5	. . . . . A . . . . C . . . . . A . . . . T . . . A . C . . . . . T . . . T . T . . . . .
Hap6	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . T . T . . . . .
Hap7	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . G . T . . . . .
Hap8	T . . . . . A . . . . C . . . . . T . . . . . C . . . . T . . . . . T . T . . . . .
Hap9	. . . . . A . G . . C . . . . . T . . . . T C . . . . . T . T . . . C . . . . .
Hap10	. . . . . T . . A . . . . C . . . . . T . . . . . C . . . . . C . . T . . . . .
Hap11	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . T . T . . . . A . . . . .
Hap12	. . . . . A . . . . C . . . . G . . . T . . . . T . . . . . C . . . . . T . T . . . . .
Hap13	. . C . . . . . A . . . . C . . . . . C . . T . . . . T . . . . . C . . T . . . . . T . . . . .
Hap14	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . G A T . . . . .
Hap15	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . T . . . . .
Hap16	. . . . . A . . . . C . . . . . T . . . . . C T . . . T . . . . . T . T . . . . .
Hap17	. . . . . A . . A . C . . . . . T . . . . T . . . . . C T . . . . . T . T . . . . .
Hap18	. . . . . T . . . . .
Hap19	. . . . . A . . . . C . . . . . T . . . T T . . . . . C . . . . . T . T . . . . A . . . . .
Hap20	. . . . . A . . . . C . . . . . G . . T . . . . . T . . . . . A . . . . . C . . T . . . . .
Hap21	. . . . . A . G . . C . . . . . T . . . . . T . . . . T C . . . . . T . T . . . . .
Hap22	. . . . . A . . . . C . . . . . T . . . . T C . . . . . T . T . . . . .
Hap23	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . T . T . . . . . C . .
Hap24	. . . . . A . . . . C . A . . . . . T . . . . T . . . . . C . . . . . T . T . . . . . T . . . .
Hap25	. . . . . A . . . . C . . . . . . C . T . . . . . C . . . . T . . . . . T . T . . . . .
Hap26	. . . . . A . . . . C . . . . . T . . . . . C . . . . T . . . . . T . T T . . . . . C . . .
Hap27	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . G . T . C . . . . .
Hap28	. . . . . A . . . . C . . . . . T . . . . . C . . . . T . . . . . G . T . T . . . . .
Hap29	. . . . . A . . . . C . . . . . T . . . . . C . . . . . C . . . . . T . T . . . . .
Hap30	. . . . . A . . . . C . . . A . . . . T . . . . T . . . . . C . . . . . T . . . . A . . . . .
Hap31	. . . . . A . . . . C . . . . . T . . . . T . A T . . C . . . . . T . . . . .
Hap32	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . A . . . . . T . T . . . . .
Hap33	. . . A . . . . A . . . . C . . . . . T . . . . . C . . . . . C . . T . . . . .
Hap34	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . C . . A . T . . . . .
Hap35	. . . . . A . . . . C . . . . . T . . . . T C . . . . . G . . . G . T . T . . T . . . . .
Hap36	. . . T . . . . A . . . . C . . . . . A . . . . . T . . . . . C . . . G . . . . . T . . . . .

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Hap37	. . . . . A . . . . C . A . . . . . T . . . . T . . . . . C . . . . . G . T . . . . .
Hap38	. . . . . A . . . . C . . . . . C . . T . . . . T . . . . . C . . T . . . . . T . . . . .
Hap39	. . . . . T . T A . . . . C . . . . . T . . . . T . . . . . C . . . . . T . T . C . . . . .
Hap40	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . A . T . . . . .
Hap41	. . . . . A . G . . C T . G . . . . . T . . . . T C . . . . . T . T . . . . .
Hap42	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . T . T . . . . . T .
Hap43	. . . . . A . G . . C . . . . . T T . . . . . T . . . . T C . . . . . T . T . . . . .
Hap44	. . . . . A . . . A C . . . . . T . . . . T C . . . . . T . T . . . . .
Hap45	. . . . . A T . . . C . . . . . T . . . . T A . . . . C . . . . . G . T . . . . .
Hap46	. . . . . A . . . . C . . . . . T . . . . T . . . . . C . . . . . C . . . . G . T . . . . .
Hap47	. . . . G . C . A . . . . C . . . . . T . . . . T . . . . . T . . . . . A . . . . . T . T . . . . .
Hap48	. . . . . A . . . . C . . . . . T . . . T . . . . T C . . . . . T . T . . . . .
Hap49	. . . . . A T . . . C . . . . . T . . . . T . . . . . C . . . . . G . T . . . . .

Table 2. Statistics of genetic variation parameters and Neutral test in *P. esculenta* populations based on *Cyt b* sequences

Genetic variation parameters	XS	WL	ND	ZJ	Total
Sample size	20	20	20	20	80
NO. of haplotypes	13	15	18	15	49
H	0.953±0.028	0.942±0.043	0.989±0.019	0.968±0.021	0.976±0.007
π	0.00449±0.00065	0.00455±0.00037	0.00408±0.00033	0.00395±0.00045	0.00439±0.00027
K	5.10526	5.16842	4.63684	4.48947	4.98165
Tajima's D test	-1.37789	-0.63806	-1.52073	-1.59610	-1.91360
P-value	0.06900	0.29500	0.04500	0.04400	0.00300
Fu's Fs test	-19.17688	-19.03653	-20.29491	-20.67743	-25.53801
P-value	0.00000	0.00000	0.00000	0.00000	0.00000

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Table 3. The haplotype distribution of *Cyt b* gene in four *P. esculenta* populations

Haplotypes	XS	WL	ND	ZJ	Total	Haplotypes	XS	WL	ND	ZJ	Total
Hap1	3	5			8	Hap26			2	1	3
Hap2	1				1	Hap27			1		1
Hap3	1				1	Hap28			1	1	2
Hap4	1		2		3	Hap29			1		1
Hap5	1				1	Hap30			1		1
Hap6	3	1		3	7	Hap31			1		1
Hap7	2	2			4	Hap32			1		1
Hap8	1	1			2	Hap33			1		1
Hap9	1				1	Hap34			1		1
Hap10	1				1	Hap35			1		1
Hap11	2		1		3	Hap36			1		1
Hap12	2	1			3	Hap37			1		1
Hap13	1				1	Hap38			1		1
Hap14		1			1	Hap39				1	1
Hap15		1			1	Hap40				1	1
Hap16		1			1	Hap41				1	1
Hap17		1			1	Hap42				1	1
Hap18		1			1	Hap43				1	1
Hap19		1	1		2	Hap44				1	1
Hap20		1			1	Hap45				1	1
Hap21		1			1	Hap46				2	2
Hap22		1			1	Hap47				2	2
Hap23		1			1	Hap48				1	1
Hap24			1	2	3	Hap49				1	1
Hap25			1		1						

Table 4. Genetic fixation index (*Fst*) (below diagonal) and Genetic distance within (diagonal) and between (above diagonal) *P. esculenta* populations based on *Cyt b* sequences

population	XS	WL	ND	ZJ
XS	0.00452	0.00450	0.00434	0.00433
WL	-0.01069	0.00457	0.00468	0.00471
ND	0.00793	0.07279	0.00410	0.00410
ZJ	0.02095	0.09358	0.01497	0.00397

Table 5. AMOVA analysis of *P. esculenta* populations based on *Cyt b* sequences

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	3	12.475	0.08667 Va	3.45
Within populations	76	184.300	2.42500 Vb	96.55
Total	79	196.775	2.51167	100

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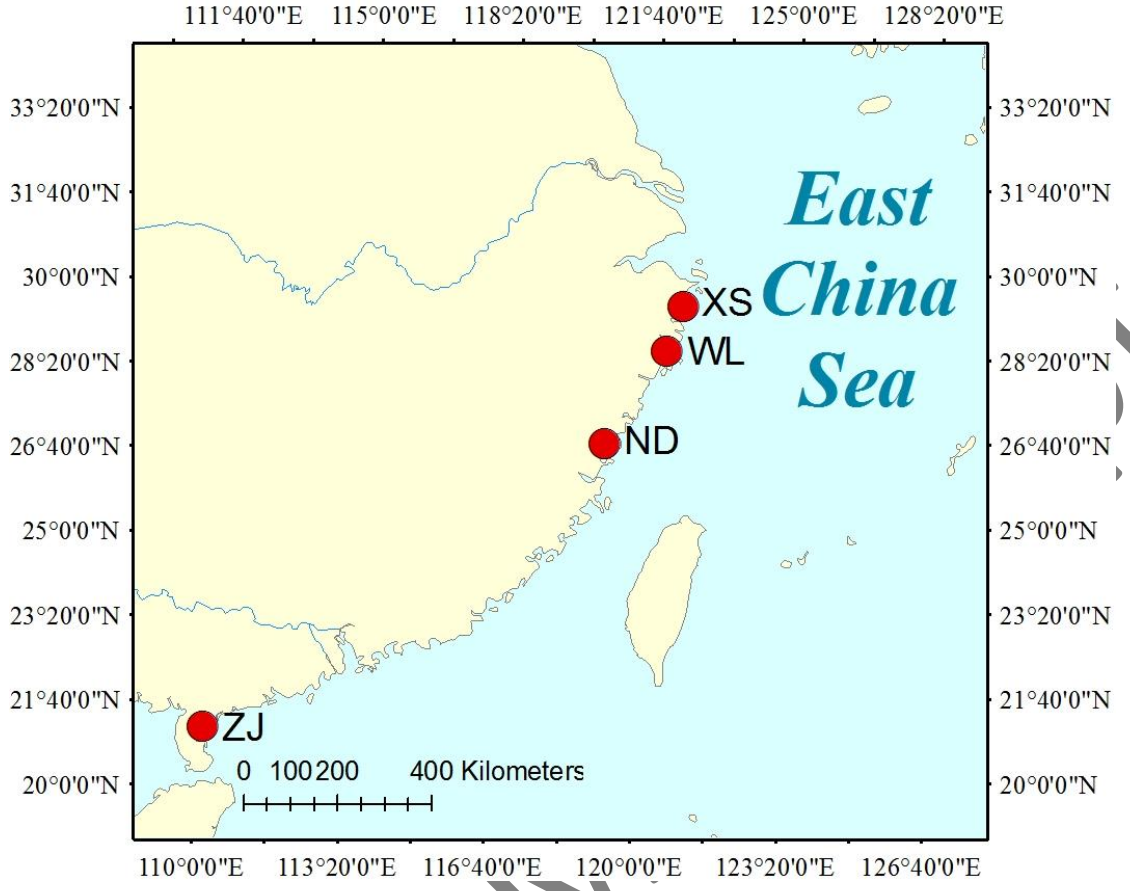

Figure 1. Sample sites of four *P. esculenta* populations. Xiangshan (XS); Wenling (WL); Ningde (ND); Zhanjiang (ZJ).

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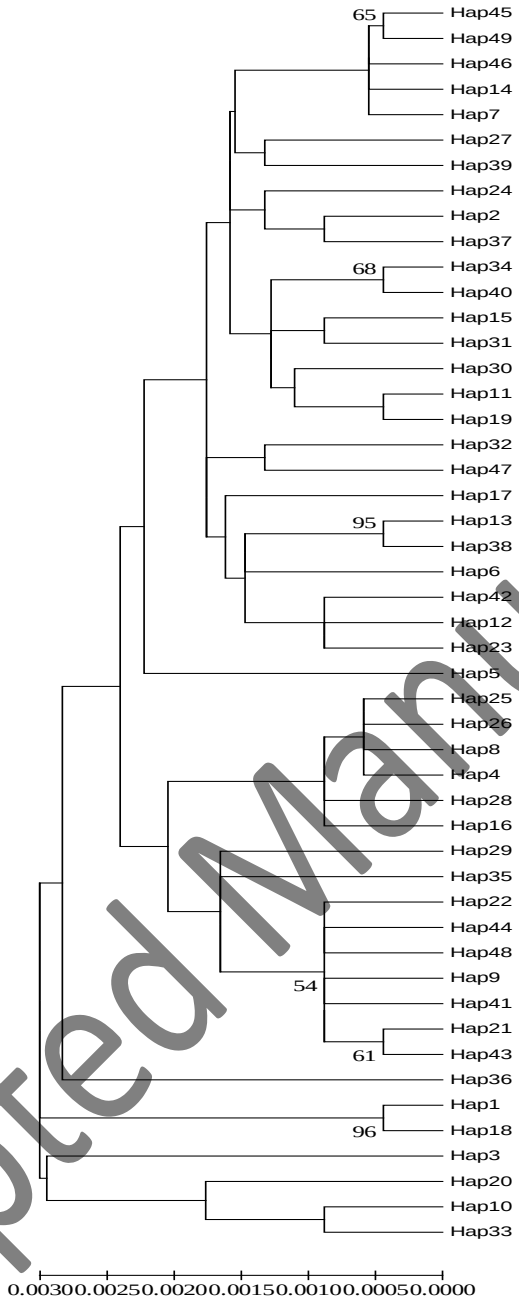

Figure 2. Neighbor-joining tree of *P. esculenta* resulted from mtDNA *Cyt b* gene.

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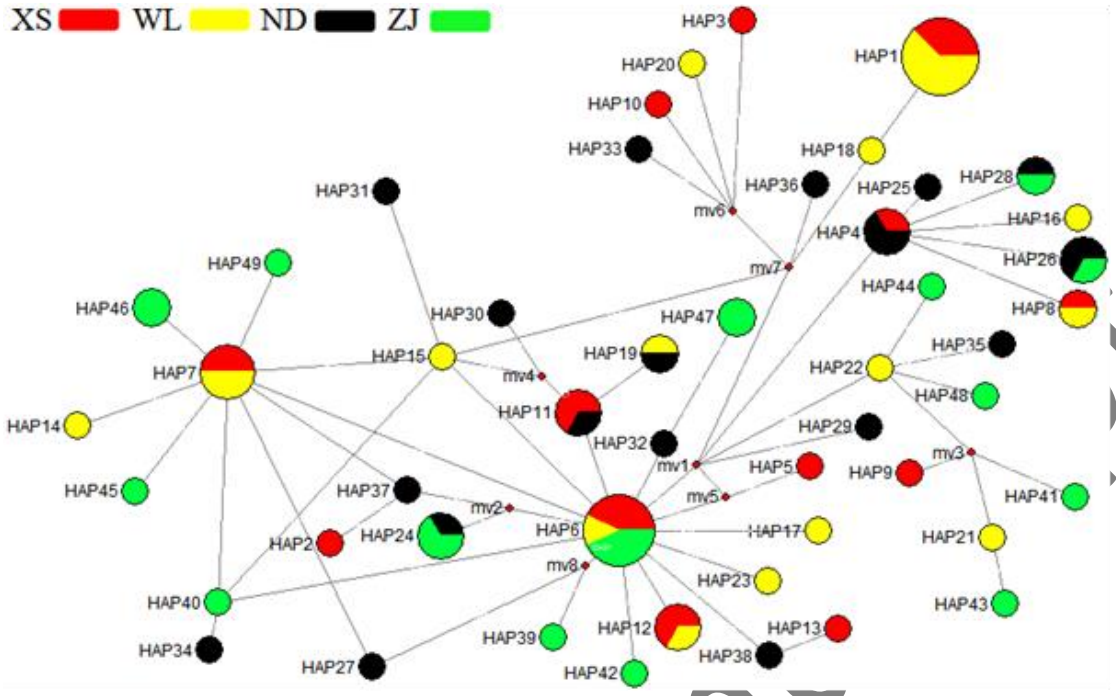

Figure 3. The median-joining network based on 49 haplotypes of *P. esculenta* found by sequencing *Cyt b* gene of 80 individuals from 4 populations in south-eastern China. The colors represent the various populations, the size of a circle is proportional to the number of haplotypes represented. Xiangshan (XS); Wenling (WL); Ningde (ND); Zhanjiang (ZJ).

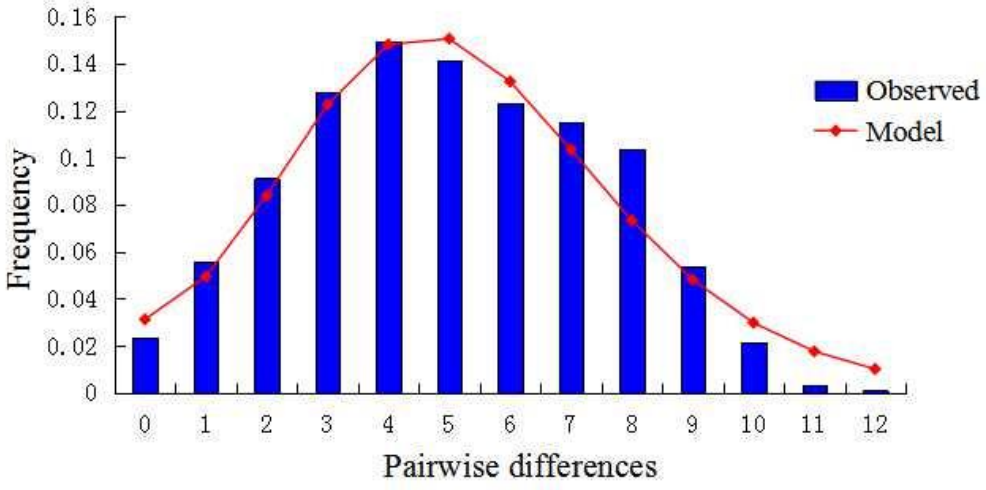

Figure 4. Nucleotide mismatch distribution for Cytb gene in *P. esculenta*.