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Population Genetic Structure of *Nuchequula mannusella* (Perciformes: Leiognathidae) Population in the Southern Coast of China Inferred from Complete Sequence of mtDNA *Cytb* Gene

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Authors' Contribution
BBG conceived and designed the study, executed the experimental work, analyzed the data, and wrote the article. NS helped in conceiving and designing the study. ZLL helped in sampling of specimens. TXG and LQL helped in preparation of manuscript.

Key words
***Nuchequula mannusella*, Cyt*b* gene, Complete sequence, Genetic structure, Population expansion.**

ABSTRACT

Population genetics of marine fishes is vitally important in fishery management and conservation. The ponyfish species *Nuchequula mannusella* is a dominant species along southern coast of China with important economic and ecological value, and little of its population genetic structure has been known. We use mitochondrial DNA markers to investigate the genetic structure of the species along southern coast of China. The complete sequences of Cytochrome *b* (*Cytb*) gene of 163 individuals collected from 6 locations were obtained and used for genetic analysis. No insertion or deletion were found among 26 mutation loci and 27 haplotypes were detected. All six populations were characterized by low haplotype diversity (0.39–0.59) and low nucleotide diversity (0.00038–0.00073). The result of AMOVA and pairwise F_{st} values indicated weak or no genetic differentiation among different populations. Haplotype network and NJ phylogenetic trees revealed that there was no distinctly geographical distribution pattern among the haplotypes. Furthermore, the mismatch distribution of pairwise nucleotide and the negatively selective neutrality test suggested that *N. mannusella* might have experienced a recent population expansion about 29, 000 years ago. The population expansion and coastal currents may be responsible for the homogeneity among the six populations.

INTRODUCTION

Nuchequula mannusella is a warm-water inhabitant of coastal waters of China (Chen and Zhang, 2016). It is a small rhomboid-shaped ponyfish, characterized by a distinct saddle-shaped nuchal marking, the ventrally protracting mouth and a big yellow spot on the upper part of the spinous region of the dorsal fin (Chakrabarty and Sparks, 2007; Chakrabarty *et al*., 2010). *N. mannusella* is also a dominant species in the southern coastal waters of China (Huang *et al*., 2010, 2015).

Population genetic analysis have been proved to be the best tool for evaluating genetic divergence and for obtaining information about the conservation genetics for species (Crandall *et al*., 1999). In recent years, more and more molecular genetic markers are used as common approach to manage sustainable yields and to monitor populations for conservation purposes (Liu and Cordes, 2004). Due to characteristics of simple structure, maternal inheritance, a faster evolutionary rate relative to that of nuclear DNA and absence from intermolecular genetic recombination, the mtDNA is widely used in population structure, phylogeography, and phylogenetic studies (Xiao and Zhang, 2000; Han *et al*., 2008; Wu *et al*., 2014; Xiao *et al*., 2016).). Cytochrome *b* (Cytb) is a component of the mitochondrial oxidative phosphorylation system III protein complex, encoded by the *Cytb* gene of the mitochondrial genome. In view of the moderate rate of evolution, the *Cytb* gene is not only a mtDNA fragment easy to amplify and sequence using universal primers, but also the only mtDNA protein coding gene whose structure and function is better understood (Irwin *et al*., 1991; Ardoya and Meyer, 1996). The *Cytb* gene has been documented to be sensitive in understanding germplasm resources situation and population genetic structure of marine fishes and has become one of the most commonly markers in detecting genetic diversity of fishes in recent years (Xiao and Zhang, 2000).

Researches on *N. mannusella* were mainly concentrated in taxonomy, phylogeny and ecology

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(Chakrabarty and Sparks, 2007; Seah *et al*., 2008; Chakrabarty *et al*., 2010, 2011; Huang *et al*., 2010, 2015). However, population genetic structure of *N. mannusella* has not been studied yet. Here we analyzed the population structure of *N. mannusella* based on the mitochondrial DNA (mtDNA) Cytb complete sequences to provide genetic structure information for the management and conservation of *N. mannusella.*

MATERIALS AND METHODS

Sampling

We collected a total of 163 specimens from 6 localities in southern coastal waters of China during November 2014 to September 2015 (Fig. 1, Table I). All the muscle samples were preserved in 95% ethanol and stored at −20°C until genomic DNA extraction.

DNA extraction, polymerase chain reaction amplification, and sequencing

Total genomic DNA of each individual was extracted from muscle tissue using a standard phenol/chloroform method (Sambrook *et al*., 1989). Three pairs of primer were designed to amplify the *N. mannusella* complete sequence of mtDNA *Cytb* gene (Accession No: KY851077–KY851103). The primers, respectively, are:
L14734-F: (5′-AACCACCGTTGTTATTCAACT-3′)
Cytb-R: (5′-CTCAGAATGACATTTGTCCTCA-3′)
14810-L:(5′-TACCTCTACAAAGCAACCTGAAACG-3′)
14810-H: (5′-GAGCTACTAATGCAATTTCATCCGA-3′)
R-L: (5′-TTCTCACCTGAATCGGAGGAATGCC-3′)
R-H: (5′-TAGCTTTGGGGGTTAGCGATGAAGG-3′)
A complete Cytb sequence of *Nuchequula nuchalis* (Accession No: AB355911) as outgroup was downloaded in GenBank.

PCR was performed on a Veriti Thermal Cycler (Applied Biosystems, USA) in a total volume of 25 µl containing 0.4 µM of each primer, 0.2 mM of each dNTP, 1×PCR buffer, 2 mM of MgCl2, 1 unit Taq polymerase (TaKaRa, Japan) and 10–100 ng DNA. The amplification profile included an initial denaturing at 94°C for 5 min, 35 cycles of 45 s at 94°C, at the locus-specific annealing temperature for 45 s, and 72°C for 45 s, and final period at 72°C for 10 min. The PCR products were visualized on 1.5% agarose gels and the most intense products were selected for sequencing. PCR products were purified by Gel Extraction Kit (Shanghai Watson biological science and Technology Co., Ltd.) and sequenced using an ABI Prism 3730 automated DNA sequencer.

Data analysis

All sequences were edited and aligned using Clustal (Thompson *et al*., 1994). Genetic diversity indices, including number of haplotypes, polymorphic sites, transitions, transversions and indels, were calculated using DnaSP 5.0 (Librado and Rozas, 2009). The haplotype diversity, nucleotide diversity and the mean number of pair-wise differences were calculated using Arlequin 3.0 (Excoffier *et al*., 2005).

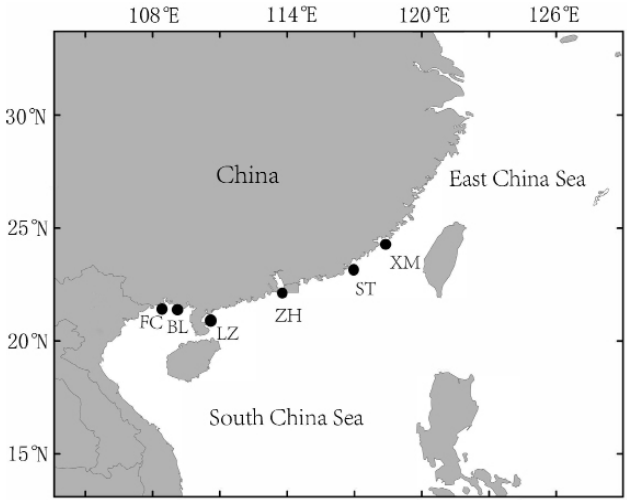

Fig. 1. Sampling sites of *N. mannusella.*

Table I.- Sample information and genetic diversity parameters in different populations of *N. mannusella.*

Population	Code	Date	Number of samples	Number of haplotypes	Mean pairwise difference	Haplotype diversity	Nucleotide diversity
Xiamen	XM	August 2015	32	7	0.43±0.40	0.39±0.11	0.00038±0.00039
Shantou	ST	November 2014	30	8	0.47±0.42	0.42±0.11	0.00041±0.00041
Zhuhai	ZH	September 2015	30	8	0.59±0.49	0.51±0.11	0.00052±0.00048
Leizhou Bay of Zhanjiang	LZ	November 2014	12	5	0.80±0.62	0.58±0.16	0.00071±0.00061
Lianzhou Bay of Beihai	BL	March 2015	30	9	0.84±0.61	0.59±0.10	0.00073±0.00060
Fangchenggang	FC	November 2014	29	8	0.69±0.54	0.43±0.12	0.00060±0.00052
Total			163	27	0.62±0.49	0.47±0.05	0.00054±0.00048

Table II.- Variable sites among 27 mitochondrial haplotypes of *N. mannusella*.

Haplotype	Variation sites																									
	49	**57**	**156**	**240**	**303**	**348**	**408**	**498**	**546**	**555**	**565**	**573**	**576**	**592**	**597**	**675**	**696**	**702**	**741**	**762**	**921**	**927**	**1083**	**1086**	**1116**	**1139**
Hap1	G	C	T	G	G	C	A	G	C	G	A	T	C	T	C	T	C	C	T	T	T	T	C	C	G	A
Hap2																			C							
Hap3											G															
Hap4															T											
Hap5					A																					
Hap6															T					C						
Hap7																						C				
Hap8								A																	A	
Hap9				A																						
Hap10									T						T											
Hap11																					C					
Hap12										A																
Hap13															T											C
Hap14																			C				T			
Hap15																C										
Hap16		T																								
Hap17			C																							
Hap18		T												C												
Hap19								A																		
Hap20												C														
Hap21																								T		
Hap22																		A								
Hap23							G																			
Hap24																	T									
Hap25													T													
Hap26	A																									
Hap27						T																				

All haplotypes are compared with Hap1.

The neighbour-joining (NJ) tree of the haplotypes was constructed using MEGA 4.0 (Tamura *et al*., 2007) by the optimal substitution model obtained in Modeltest 3.7 (Posada and Crandall, 1998). Genetic differentiation between pairs of population was evaluated with the pairwise fixation index F_{st} (Excoffier *et al*., 1992). The significance of the F_{st} was tested by 10,000 permutations for each pairwise comparison in Arlequin 3.0. To further examine hierarchical population structure, analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was used.

Historical demography of *N. mannusella* was examined by neutrality tests and mismatch distribution analysis (Tajima, 1989; Fu, 1997). The *D* test of Tajima (1989) and F_s test of Fu (1997) were used to test for neutrality. Significant negative *D* and F_s statistics can be interpreted as signatures of population expansion. Historical demographic expansions were also investigated by the examination of frequency distributions of pairwise differences between sequences (mismatch distribution), while τ (time since expansion, expressed in units of mutational time) was estimated (Rogers and Harpending, 1992). The distribution is usually unimodal in samples drawn from populations at demographic equilibrium following a recent population demographic expansion or population range expansion (Excoffier *et al*., 1992; Rogers and Harpending, 1992; Ray *et al*., 2003). The concordance of the observed with the expected distribution in the sudden-expansion model was tested by a least-squares approach (Rogers and Harpending, 1992). Expansion parameter (τ) was estimated by the general non-linear least-squares approach. The values of τ were transformed to estimates of real time since expansion with the equation $\tau = 2 \times \mu t$ where μ is the mutation rate for the whole sequence under study and t is the time since expansion. Both mismatch analysis and neutrality tests were performed in Arlequin 3.0.

RESULTS

A 1141 base pairs sequence was obtained from all 163 individuals. The average base composition of the *Cytb* gene was shown as following: C = 31.55%, T = 28.84%, A = 25.51%, G = 14.10%, indicating a strand compositional bias characterized by a slight excess of C relative to G. The content of A+T (54.35%) was slightly higher than that of C+G (45.65%).

Twenty-six polymorphic sites were detected from the whole sequence of all 163 individuals, including 9 parsimony informative sites and 17 singleton variable sites (Table II). They were defined as 26 nucleotide substitutions (24 transitions and 2 transversions). Insertions or deletions were not found. Twenty-seven haplotypes were identified from 163 individuals and have been deposited in GenBank with the following accession number: KY851077–KY851103. Among the haplotypes, 8 haplotypes were shared haplotypes, while the others were population specific haplotypes (Table III). Obviously, Hap1 was the most dominant haplotype, accounting for 72.4 % of all *N. mannusella* specimens. The Hap1 and Hap5 were shared in all six populations (Table III).

The haplotype diversity index ranged from 0.39 to 0.59; nucleotide diversity ranged between 0.00038 and 0.00073 (Table I). Among the specimens, the mean haplotype diversity and nucleotide diversity were 0.47 ± 0.05 and 0.00054 ± 0.00048, respectively (Table I). The BL population had the highest values of haplotype diversity and nucleotide diversity, the XM population had the lowest (Table I).

Table III.- Distributions of haplotypes among six populations.

Haplotype	XM	ZH	ST	LZ	BL	FC
Hap1	25	21	23	8	19	22
Hap2					1	
Hap3					1	
Hap4	1	2	1		1	
Hap5	2	1	1	1	4	1
Hap6					1	
Hap7					1	
Hap8					1	
Hap9					1	
Hap10						1
Hap11						1
Hap12			1			1
Hap13						1
Hap14						1
Hap15						1
Hap16		1		1		
Hap17	1		1	1		
Hap18				1		
Hap19			1			
Hap20			1			
Hap21			1			
Hap22	1					
Hap23	1	1				
Hap24	1					
Hap25		2				
Hap26		1				
Hap27		1				

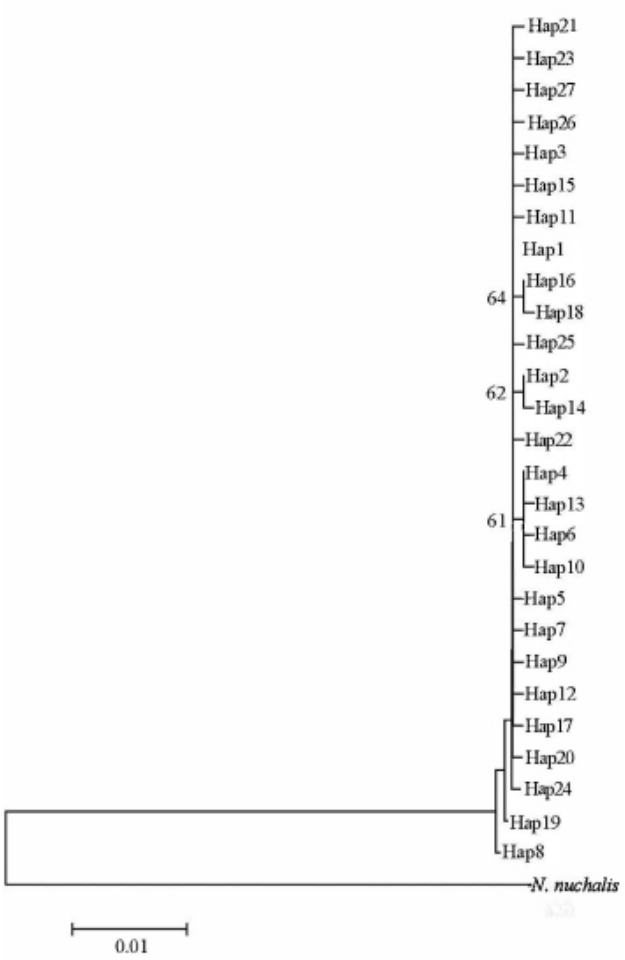

Fig. 2. NJ phylogenetic trees based on complete sequence of *Cytb* gene of *N. mannusella* using *N. nuchalis* as outgroup.

Table IV.- Analysis of molecular variance (AMOVA) of *N. mannusella* based on complete sequence of *Cytb* gene.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	F index (P value)
Among populations	5	1.510	−0.00024 Va	−0.08	F_{ST}= −0.00077 (P = 0.48974)
Within populations	157	48.415	0.30838 Vb	100.08	
Total	162	49.926	0.30814		

The pairwise F_{st} values among the six populations were between −0.03194 and 0.00617 (Table V), but none of them had significant difference statistically (P>0.05). Consistent with the pairwise F_{st} values, the AMOVA test revealed that 100.08% of the genetic variation occurred within populations, whereas −0.08% of the genetic variation occurred among populations (Table IV). The fixation index (F_{ST}) evaluating the level of differentiation among populations was −0.00077 (P>0.05) (Table IV), suggesting a lack of genetic differentiation in *N. mannusella* populations.

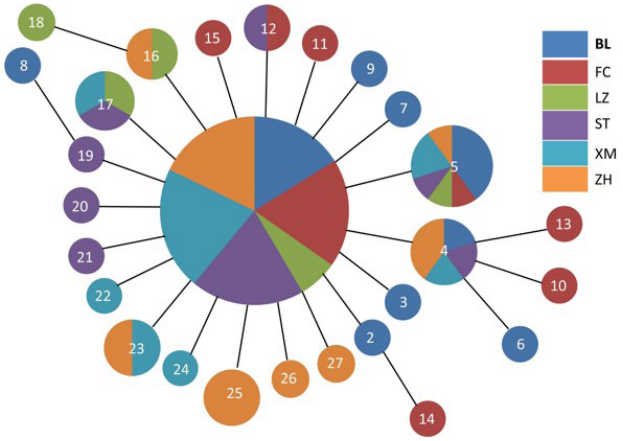

Fig. 3. Median-networks showing genetic relationship among haplotypes of *N. mannusella* based on parsimony method.

The neighbor-joining (NJ) tree among 27 haplotypes and an outgroup (*Nuchequula nuchalis*) was constructed based on the Kimura-2-Parameter distance model (Fig. 2). No significant genealogical branches or clusters were observed. To further depict the phylogenetic and geographical relationships among the identified sequences, haplotype networks were constructed based on the simplified intermediate network method (Fig. 3) and revealed a star-like structure. Hap1 was the most dominant haplotype and lied at the center of the network, connecting the other haplotypes by one or several step mutations.

Tajima's D and Fu's F_s tests were performed to test whether the complete Cyt*b* sequence of *N. mannusella* evolved under neutrality or not. Tests for neutral evolution resulted in the negative Tajima's D and Fu's F_s values, and statistical tests were up to a significant level (P<0.05). This statistical finding was consistent with the population expansion from six populations. Mismatch distributions were unimodal (Fig. 4), and matched the expected distributions under the sudden expansion model, revealing *N. mannusella* had experienced population expansion event. Using the test of goodness to fit the consistency between the observed values of the nucleotide mismatch and the

Table V.- Pairwise F_{st} (below diagonal) and non-differentiation exact *P* values (above diagonal) among *N. mannusella* populations.

Population	XM	ZH	ST	LZ	BL	FC
XM		0.60938	0.99902	0.68262	0.29102	0.99902
ZH	−0.01088		0.75781	0.90820	0.53711	0.58398
ST	−0.02236	−0.01199		0.78516	0.25781	0.99902
LZ	−0.01901	−0.02874	−0.02148		0.91504	0.73340
BL	0.00593	−0.00522	0.00617	−0.03194		0.28320
FC	−0.01696	−0.00869	−0.02071	−0.01811	0.00538	

expected value of the population expansion model based on minimum variance, the result showed that the *SSD* value and raggedness index were both small, and statistical tests were not significant (P>0.05), indicating a significant fit between the observed and expected distributions. Based on a mtDNA Cyt*b* gene mutation rate of 2 % per million years (Bermingham *et al*., 1997; Bowen *et al*., 2001), we used the τ = 0.654 (95% CI: 0.350–1.256) to derive that population expansion for *N. mannusella* may have occurred 29,000 years ago (95% CI: 15,000–55,000 year).

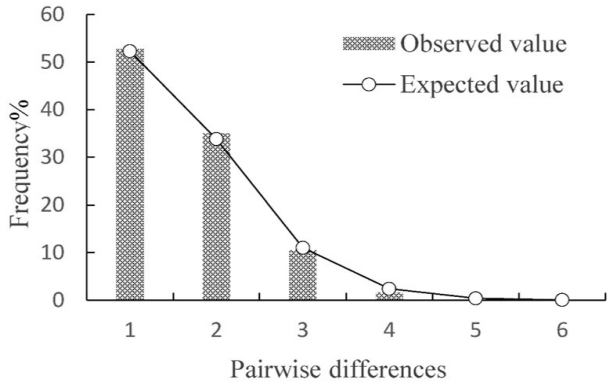

Fig. 4. The observed frequency distribution and the expected frequency distribution for the number of pair wise differences of *N. mannusella* individuals.

DISCUSSION

Genetic diversity and phylogenetic pattern

The present study employed sequences of the complete Cyt*b* gene to investigate the genetic diversity and population structure of *N. mannusella* among six populations from the southern coast of China. Low haplotype diversity and low nucleotide diversity were detected in all *N. mannusella* populations. Among the specimens, the mean haplotype diversity and nucleotide diversity were 0.47 and 0.00054, respectively. Based on the values of haplotype and nucleotide diversities, Grant and Bowen (1998) interpret four basic categories of population history of marine fishes. The present result fitted in with the first category, which was low haplotype diversity and low nucleotide diversity of marine fishes (Grant and Bowen, 1998). According to their models, populations of *N. mannusella* had a regional extinction during Pleistocene glaciation, followed by a postglacial range expansion. During the late Pleistocene period (the last 1 million years), especially over the last 800,000 years, the sea level may decline for 120–140 m (Lambeck *et al*., 2002). With the flooding of the most shelves across Chinese coastal, most species went extinct over large parts of their ranges and some migrated to the new environment or refugiums for survival (Hewitt, 2000). When the glaciers receded, these surviving populations may have expanded north with the rise in temperature. The results of mismatch distribution and Tajima's *D* and Fu's F_s statistics supported population expansion for *N. mannusella*. The recent expansion of *N. mannusella* populations was 29,000 years ago, in the late Pleistocene period. Such range changes can be expected to have genetic consequences (Hewitt, 2000; Tamaki and Honza, 1991). In present study, the NJ tree and haplotype network proved the existence of no significant genealogical branches or clusters for *N. mannusella*, which may originate from refugium of South China Sea. So the founder event following a single mtDNA lineage is probably an important factor for how to explain the genetic pattern of low haplotype diversity and low nucleotide diversity.

Population genetic structure

Compared with freshwater and anadromous fishes, marine fish usually have low levels of genetic differentiation among geographic regions. This is largely due to a high dispersal potential of different life-history stages coupled with an absence of physical barriers to movement between ocean basins or adjacent continental margins (Ward *et al*., 1994; Grant and Bowen, 1998; Hewitt,

2000). In present study, the result of AMOVA showed that the genetic variations of *N. mannusella* occurred mainly within populations. Moreover, all pairwise F_{st} values of 6 comparisons were very low and not statistically significant between the localities. So *N. mannusella* conforms to this pattern without significant genetic structure or population sub-division.

N. mannusella is a warm-water inhabitant and generally inhabits upper layer of coastal waters. Its spawn occurs estuarine and nearshore waters between the months of June to July in the southern coast of China (Huang, 1985; Smith, 2001). Behaviors of *N. mannusella* cannot be prevented because Chinese southern coastal waters are continuous areas. The ocean current circulation between the western and eastern sea areas of Leizhou Peninsula is exchanged by currents in the Qiongzhou Strait (Ke, 1983). The summer coastal current at northern South China Sea flows from southwest toward northeast and then passes through the Taiwan Strait (Xue *et al*., 2001). In addition, the oceanic circulation in the Beibu Bay is year-round (Liu and Yu, 1980). When the glaciers receded, these individuals survived in the refugium of South China Sea may have expanded north with the rise in temperature. The above ecological characteristics and three kinds of currents facilitate the dispersal of individuals among distant populations, and are sufficient to compensate for the possible genetic bias among six populations.

The data obtained from the present study revealed the presence of genetic homogeneity in the *N. mannusella* populations of the southern waters of China. Moreover, the low genetic diversity found in the current study raised our concerns. This result may be caused by the genetic marker based on the Cyt*b* complete sequence. We examined only a portion of the entire genome in this study, the mtDNA control region sequence is used in analyzing population structure for understanding further genetic diversity of *N. mannusella.* It is necessary to conduct a full research on population structure and genetic diversity of *N. mannusella* using other molecular markers (such as SSR, AFLP, SNP *etc*.), promoting its resource protection and sustainable development in the future.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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