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Genetic Diversity and Population Structure of the Peanut Worm (*Sipunculus nudus*) in Southern China as Inferred from Mitochondrial 16S rRNA Sequences

Du Xiaodong*, Chen Zian, Deng Yuewen, Wang Qingheng and Huang Ronglian

Fishery College, Guangdong Ocean University, 524025 Zhanjiang, China

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Abstract

Genetic diversity and population structure of the peanut worm (*Sipunculus nudus*) were investigated by using 536 base-pair fragments of the mitochondrial 16S ribosomal gene. Populations were collected from three locations along the southern coast of China - Beihai, Sanya, and Xiamen. Amplified polymerase chain reaction products were sequenced in both directions and data were analyzed using ClustalX, Arlequin, and MEGA. A total of 69 polymorphic sites and 21 distinct haplotypes were revealed. The mean haplotype and nucleotide diversity of the three populations were 0.814% and 0.37%, respectively. The Beihai population had the greatest haplotype and nucleotide diversity, followed by the Xiamen and Sanya populations. Analysis of molecular variance (AMOVA) showed significant genetic differentiation among the three populations (Fst = 0.0619, $p<0.05$) and distinct population structures among the three sites.

Introduction

Genetic diversity, the fundamental hierarchy of species and ecosystem diversities, is the primary subject of biodiversity research (Avise and Himrick, 1996). Documentation of genetic diversity is needed to utilize and manage genetic resources. Genetic diversity can be assessed by using morphological, cytological, physiological, biochemical, and molecular markers. The most frequently used molecular markers include nuclear and mitochondrial markers. Mitochondrial DNA (mtDNA) has a high mutation rate relative to single-copy nuclear DNA (Brown et al., 1979) and, under equilibrium conditions, maternal inheritance contributes one quarter of nuclear DNA (Birky et al., 1983). Thus population differentiation is expected to evolve more rapidly within mtDNA than in allozymes and other coding regions of

* Corresponding author. E-mail: duxd@gdou.edu.cn

nuclear DNA, making mtDNA a more sensitive indicator of population structure. Mitochondrial DNA markers are frequently used to evaluate genetic diversity in marine invertebrates (e.g., Boulding et al., 1993; Su et al., 2005; Kumar et al., 2007).

In China, the peanut worm, *Sipunculus nudus*, is mainly distributed along the southern coast. The species is widely cultured in southern regions of China, especially in Beihai, Guangxi. *Sipunculus nudus* culture relies entirely on seed collected from the wild, which may result in over-exploitation of wild populations. Therefore, it is urgent to obtain information on the genetic diversity of wild populations so as to manage commercial species.

Data on population genetics of *S*. *nudus* have been reported on the basis of random amplified polymorphic DNA (Wang et al., 2006). In the present study, genetic diversity and the population structures of *S*. *nudus* collected from three locations along the coast of southern China were investigated using 16S rRNA gene sequence analysis. The objective was to provide useful information for resource conservation and fishery management of the species.

Materials and Methods

Sample collection. Wild samples of *S*. *nudus* were collected from Beihai in Guangxi, Sanya in Hainan, and Xiamen in Fujian, all in the South China Sea. Fresh tissues from the muscle and whole individuals were preserved in 70% ethanol.

Total DNA extraction. Total DNA was extracted from the ethanol-preserved muscle tissues. The tissues were incubated overnight in lysis buffer with proteinase K at 37°C. DNA was purified using phenol-chloroform according to Wang et al. (2006). Isolated DNA was dissolved in 50-100 µl distilled water. This solution was further diluted in distilled water to 100 ng/µl for polymerase chain reaction (PCR).

Amplification and sequencing. A 536-bp segment of 16S rRNA was amplified by PCR. The primers were: 16SAR (forward) 5'-CGCCTGTTTATCAAAAACAT3' and 16SBR (reverse) 5'CCGGTTTGAACTCAGATCATG-3' (Zhang and Ryder, 1993). Amplification was performed in a 50 µl reaction volume containing 1 µl DNA dilution, 25 pmol primers, 5 µl 10x reaction buffer, 25 mM $MgCl_2$, 200 µM dNTP, and 2 U Taq polymerase (Sangon, Shanghai). The cycling conditions were 2 min denaturation at 94°C, 35 cycles of 45 s denaturation at 94°C, 1 min annealing at 50°C, 1 min extension at 72°C, and a final extension at 72°C for 5 min. PCR products were separated on 1.5% agarose gels; bands were stained with rhodium bromide and viewed under a UV light source.

A total of 5 µl of each PCR product was used for 1.5% agarose gel electrophoresis to confirm the amplified fragment length with a marker. Here, bands were made visible using ultraviolet light after an ethidium bromide bath. The remainder of each PCR product was used as a template for automated sequencing reactions performed with a T3 Thermocycler and run on an ABI 310 DNA sequencer. The primers used for sequencing reactions were the same as those for PCR amplification. Sequence data are available from GeneBank (accession numbers EU260100-EU260120).

Data analysis. A total of 536 bp sequences from 30 individuals were aligned by using ClustalX (Thompson et al., 1997). Data were analyzed using Arlequin vers. 3.0 (Excoffier et al., 2005). The numbers of transition, transversion, and haplotype, as well as haplotype diversity (*h*), and nucleotide diversity (*pi*) values (Nei, 1987), were calculated for separate and combined populations. The total number of nucleotide differences, mean sequence divergence values, and Jukes-Cantor genetic distances (Jukes and Cantor, 1969) were calculated for each pair using molecular evolutionary genetics analysis (MEGA vers. 2.1; Kumar et al., 2004).

Analysis of molecular variance (AMOVA) was used to partition the total genetic variation into variance components and produce fixation indices (Fst). Fst values were calculated for the three populations separately and together for all pairs of populations on the basis of information regarding haplotypes and their frequencies. The statistical significance

of the Fst values was tested by permutation tests (10,000 replicates). AMOVA was conducted by using Arlequin vers. 3.0 (Excoffier et al., 2005).

Results

Mitochondrial DNA 16S rRNA polymorphism. The 536 bp sequences of the 16S rRNA gene from 30 individuals produced 69 polymorphic sites that defined 21 haplotypes (Table 1). One haplotype (F) was shared by the Beihai and Sanya populations while the remaining twenty (95.2%) were unique to one of the three populations. Forty (58.0%) of the nucleotide substitutions in the 16S rRNA gene were synonymous and 29 were non-synonymous. Variant substitutions of parsimony informative sites appeared in the 21 haplotypes at position 482 (A↔G, A↔T, T↔G, three variants).

Haplotype, nucleotide diversity, and other population-specific diversity indices are showed in Table 2. For all indices, the Beihai population had the greatest values, followed by the Xiamen and Sanya populations.

Population structure. AMOVA from 16S rRNA gene sequence analysis indicated significant genetic structure and population differentiation. It showed that a significant percentage of the variation was attributable to among-population differences (6.19%) and population subdivision (Fst = 0.06193, $p<0.01$) among all populations (Table 3). Pairwise relationships among the population pairs are given in Table 4.

Discussion

The present study reports on 16S rRNA gene sequence analysis of *S*. *nudus* sampled from three locations along the southern coast of China. The data reveal high variation in the 16S rRNA gene sequence of *S*. *nudus*, although this region of the mitochondrial genome usually shows low variation. Our results (21 haplotypes in 30 individuals at 69 polymorphic sites) were more variable than in other marine invertebrates. For example, in 592-bp16S rRNA gene sequences of the scallop, *Chlamys farreri*, there were 23 haplotypes in 47 specimens at 31 nucleotide positions (Kong et al., 2003) and in 500-bp 16S rRNA gene sequences of the oyster, *Crassostrea rivularis*, there were 12 haplotypes in 105 samples at 23 polymorphic sites (Su et al., 2005). Possible explanations for the high level of variation in *S. nudus* are higher mutation in the 16S rRNA gene sequence and large effective population sizes of the species.

The mean haplotype and nucleotide diversity of the three populations were 0.814 and 0.37%, respectively. These results are comparable with those on other marine invertebrates, for example, 0.57 and 0.14% in *Crassostrea virginica* from the Atlantic Ocean (Reeb and Avise, 1990) and 0.07-0.55 and 0.08%-0.6% for *Penaeus monodon* from the Indian Ocean (Kumar et al., 2007). The high level of genetic diversity in the present study indicates that the 16S rRNA gene sequence might be useful as a genetic marker for aquaculture purposes such as maintaining stock diversity and distinguishing hatchery stocks from wild populations.

The assessment of genetic diversity and population structure of *S*. *nudus* is important for appropriate conservation and management of the species. Commercial culture of *S*. *nudus* is expanding in southern China (Lan et al., 2007). With increased culture, a common concern is the loss of genetic diversity in wild populations. Genetic monitoring and evaluation can help identify negative effects of aquaculture on genetic diversity. A high level of genetic diversity and significant differentiation between population structures can help buffer *S*. *nudus* from detrimental genetic effects of diseases and population decline and facilitate improvement of stocks of commercially important species through breeding programs.

In the current study, the significant Fst value among all the populations shows that *S*. *nudus* populations are genetically structured. This could be a result of insufficient gene flow among the locations. Gene flow is the exchange of genetic material between populations caused by movement of individuals or their successfully fertilizing gametes (Klinbunga et al., 1998). A long planktonic larvae stage influences the gene flow over large geographic areas in marine invertebrates

Table 1. Distribution of 536 bp sequences of 16S rRNA gene from 30 peanut worms (*Sipunculus nudus*) from three locations produced 69 polymorphic sites that defined 21 haplotypes.

	Haplotype (n = 21)																			
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*
Polymorphic site (n = 69)																					
30 etc.*	T	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	C	C	C	C	C
32	A	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	T	T	T
33 etc.*	C	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	T	T	T
47	G	.	.	.	.	.	T	T	T	T	.	.	.	.	.	.	.	.	.	.	.
50 etc.*	C	.	.	.	.	.	.	.	.	.	.	.	.	A	A	A	A	A	A	A	A
59	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.
66 etc.*	A	.	.	.	.	.	.	.	.	.	.	.	.	G	G	G	G	G	G	G	G
85	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.
86	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
104	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
107	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
147	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
163 etc.*	G	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	C	C	C	C	C
174 etc.*	G	.	.	.	.	.	.	.	.	.	.	.	.	A	A	A	A	A	A	A	A
215 etc.*	T	.	.	.	.	.	.	.	.	.	.	.	.	A	A	A	A	A	A	A	A
216	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
258	T	.	.	.	C	.	.	.	.	.	.	.	.	C	C	C	C	C	C	C	C
282 etc.*	C	.	.	.	.	.	.	.	.	.	.	.	.	G	G	G	G	G	G	G	G
317	T	.	.	.	.	.	.	.	.	.	.	.	.	G	G	G	G	G	G	G	G
335 etc.*	A	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	C	C	C	C	C
373	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
412	G	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
462	G	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	T	T	T
474	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
482	A	.	.	.	.	.	.	.	.	.	.	.	T	G	T	T	T	T	T	T	T
522	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
523	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	G
525	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
526	T	.	C	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C
534	A	G	.	G	.	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Location																					
Beihai						1	1	2	1	1	1	2	1								
Sanya	1	2	1	3	1	2															
Xiamen														1	1	1	2	1	1	1	2

* Other occurrences of this haplotype are: for 30 at 35, 179, 181, 197, 264, 279, 324, 326, and 344; for 33 at 34, 50, 70, 180, 184, 218, 265, 271, 272, 329, 340, 366, and 461; for 50 at 67, 270, 330, 336, and 481; for 66 at 177, 214, 256, and 261; for 163 at 343; for 174 at 281 and 463; for 215 at 276 and 331; for 282 at 358; and for 335 at 341 and 359.

Table 2. 16S rRNA gene polymorphism of *Sipunculus nudus* collected from three locations along the southern Chinese coast.

Population	*No. sequences analyzed*	*Haplotype diversity*	*Nucleotide diversity (%)*	*Avg no. nucleotide differences*
Beihai	10	0.871	0.43	2.29
Sanya	10	0.756	0.32	1.43
Xiamen	10	0.816	0.37	1.96
Average	10	0.814	0.37	1.89

Table 3. Analysis of molecular variance (AMOVA) of 16S rRNA sequences for three populations of *Sipunculus nudus*.

Source of variation	*Degree of freedom*	*Sum of squares*	*Variance components*	*Percentage of variation*
Among populations	2	1.557	0.03095Va	6.19
Within populations	27	12.658	0.46880Vb	93.81
Total	29	14.215	0.49975	-
Fst: 0.06193	-	-	-	-

Table 4. Pair Fst values among the populations based on Jukes-Cantor genetic distances of 16S rRNA.

	Beihai	*Sanya*	*Xiamen*
Beihai	0.00000	-	-
Sanya	0.07007*	0.00000	-
Xiamen	0.03622*	0.07778*	0.00000

* $p<0.05$

such as *Strongylocentrotus purpuratus* and *S. droebachiensis* (Palumbi and Wilson, 1990) and *C. farrei* (Yu et al., 2007). The long pelagic period in *Crassostrea virginica* larvae could result in a moderate gene flow among wild populations (Hare and Avise, 1996). The planktonic larvae stage in *S. nudus* is 7.5-27 days at 21.5-34.0°C (Lan et al., 2007). Yet, the estimated inter-population migration in our study was a mere 5.9-13.4 individuals per generation. The reason why *S. nudus* larvae have not dispersed in the South China Sea, resulting in genetic homogeneity, may thus be explained by the existence of some yet-undiscovered natural barrier between the locations.

In summary, 16S rRNA gene sequences revealed a relatively high level of genetic diversity and significant genetic differentiation among three populations of *S. nudus* collected along the coast of southern China. Our results will provide useful information for genetic resource conservation and fishery management of this species.

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