Genetic Variation in Populations of the Diamond-shaped Squid
*Thysanoteuthis rhombus* as Examined by Mitochondrial DNA Sequence Analysis

Jun Kitaura,*1 Gunji Yamamoto,*2 and Mutsumi Nishida*2,†

*1Department of Biological Science, Faculty of Science, Nara Women's University, Kitauoya-nishimachi, Nara 630-8263, Japan
*2Department of Marine Bioscience, Fukui Prefectural University, Obama, Fukui 917-0003, Japan

(Received January 5, 1998)

The genetic variation in populations of the diamond-shaped squid *Thysanoteuthis rhombus* in the Pacific was examined through mitochondrial DNA analysis. Part of the cytochrome oxidase subunit I gene was amplified with the polymerase chain reaction (PCR) and sequenced for 23 individuals, five from each of three samples collected off Kyoto/Fukui, Okinawa Island, and the Ogasawara Islands in the western Pacific, and also for eight individuals from a sample caught off Galapagos Islands in the eastern Pacific. Resultant sequence data showed that there were seven variable nucleotide positions in the 399 base-pair segment of the gene and that all 23 sequences were grouped into six haplotypes. No marked genetic difference was observed between populations on both sides of the Pacific. The PCR-mediated SSCP (single-strand conformation polymorphism) analysis applied to a larger number of individuals from more than 200 from adjacent waters around Japan (off Kyoto/Fukui and Okinawa Island) revealed no divergence in haplotype frequency between these two regional samples.

**Key words:** diamond-shaped squid, *Thysanoteuthis rhombus*, the Pacific Ocean, genetic population structure, mitochondrial DNA, cytochrome oxidase I gene, DNA sequencing, PCR-SSCP

The diamond-shaped squid *Thysanoteuthis rhombus* is distributed in the tropical-subtropical waters on a global scale.1-3) Around Japan, this species occurs both in Kuroshio and Tsushima Current regions,4) and is one of the important fishery resources in the western coast of Japan along the Sea of Japan. Increasing fishing pressure on this species in the waters around Japan has prompted us to examine its population structure for providing useful information for resource management. In the present study, we investigated genetic constitution of some selected samples of the diamond-shaped squid from the waters around Japan and a sample from the eastern Pacific through examining nucleotide sequence of part of the cytochrome oxidase subunit I (COI) gene in the mitochondrial DNA (mtDNA). This DNA segment of the COI gene was examined by polymerase chain reaction (PCR)-mediated direct sequencing for some individuals in each sample and also by the PCR-SSCP (single-strand conformation polymorphism) analysis for a larger number of remaining individuals. Intraspecific sequence variation within mtDNA has proven a powerful tool for examining population structure in marine organisms.5)

**Materials and Methods**

**Samples**
Diamond-shaped squid *Thysanoteuthis rhombus* samples were collected from three locations in the waters around Japan; one in the Sea of Japan (off Kyoto and Fukui) and two in the Pacific Ocean (Off Okinawa Island and the Ogasawara Islands). In addition, one population sample collected off the Galapagos Islands in the eastern Pacific Ocean was used for comparison. Fifteen individuals from Kyoto/Fukui, Okinawa, and Ogasawara (five individuals from each location), and eight individuals from the Galapagos were sequenced prior to SSCP analysis. For SSCP analysis, 100 individuals from the Sea of Japan (Kyoto/Fukui) and 101 from the Pacific (Okinawa) were used (Table 1).

**DNA Extraction, Amplification and Sequencing**
Total genomic DNA was extracted from arm musculature of each squid. Removed musculatures were minced, and digested by protease K/SDS solution and purified

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sequencing</td>
</tr>
<tr>
<td><strong>North western Pacific</strong></td>
<td></td>
</tr>
<tr>
<td>The Sea of Japan</td>
<td>5</td>
</tr>
<tr>
<td>Off Kyoto/Fukui</td>
<td></td>
</tr>
<tr>
<td>The Pacific</td>
<td>5</td>
</tr>
<tr>
<td>Off Okinawa Is.</td>
<td>5</td>
</tr>
<tr>
<td>Off Ogasawara Iss.</td>
<td>5</td>
</tr>
<tr>
<td><strong>Eastern Pacific</strong></td>
<td></td>
</tr>
<tr>
<td>Off Galapagos Iss.</td>
<td>8</td>
</tr>
</tbody>
</table>

† To whom correspondence should be addressed.
Genetic Variation in Diamond-shaped Squid Populations

359

by standard phenol/chloroform extraction. DNA was concentrated with Microcon-100 (Amicon) and dissolved in Tris-EDTA (pH 7.5) buffer. The target DNA segments were amplified by PCR. The primers used for the amplification of partial COI gene were HCO2198 (5'-TAAAC-TTGAGGGTACCAAAAAAT-3') and LCO1491 (5'-GGTCACCAACATATAGGATATTG-3') as described by Folmer et al.6) Double-stranded PCR products were obtained in a total volume of 25 μl reactions containing 2.5 μl of 10 × PCR buffer II (Perkin Elmer), 0.2 mM each dNTP, 0.5 μM of each primer, 3.5 mM MgCl2, 0.5 units of Taq polymerase (Perkin Elmer), and 1 μl template. The temperature regime for 30 cycles was 40 sec at 94°C, 1 min at 50°C, and 1 min at 72°C.

Amplification products were checked for size by loading 5 μl on a 2% NuSieve agarose gel with 0.5 μg/ml ethidium bromide. The remaining product was filtered twice with 500 μl of distilled H2O in Microcon-100 (Amicon). These filtrated products were sequenced directly using the dye-terminator cycle sequencing reaction (Perkin Elmer) with ABI DNA sequencer 373A. Sequencing reactions were performed by following the protocol suggested by manufacturer’s instructions. Primers used for the sequencing were the same as those for PCR. All final sequences were obtained from both strands for verification.

Phylogenetic and Sequence Difference Analyses

The extent of sequence difference between individuals was calculated by averaging pair-wise comparisons of sequence difference across all individuals. For examining phylogenetic relationships among haplotypes, unrooted maximum parsimony network was constructed by PAUP version 3.1.17 using exhaustive search algorithm.

PCR-SSCP Analysis

PCR-SSCP method is a simple and effective technique for detection of single base mutations in PCR products.8) This method relies on the fact that the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is not only determined by its size but also by its nucleotide sequence. Even a single nucleotide substitution may alter the three-dimensional structure of the molecule, which affects its migration mobility.

DNA was extracted with a simplified method using Chelex-100: Removed musculatures were incubated at 95°C in 5% Chelex-100, and vortexed for 15 seconds.9) This solution was directly used as template for the PCR amplification. A segment of approximately 250 base-pair (bp) segment of the COI gene was sequenced for 23 individuals from three locations (Kyoto/Fukui, Okinawa, and Ogasawara) on the west side of the Pacific and one location (Galapagos) on the east side. Among these individuals, a total of seven nucleotide positions (positions 86, 89, 194, 218, 221, 308, and 332) were found to be variable (Fig. 1). Only variation at one nucleotide position of 218 was shared with more than one individual, while each of the other variations was observed in a single individual only (Table 2). As expected, all these variable sites were at third position of codon triplets (Fig. 1), and all but one changes were transitions (Table 2). All the base substitutions were silent (synonymous) changes that do not result in amino acid changes, so that all the variants can be considered as selectively neutral.

Analysis of Relationships and Occurrence Frequency of Haplotypes

Nucleotide sequence difference between 23 individuals from both sides of the Pacific ranged from 0.00 to 1.25% with an average (=nucleotide diversity) of 0.25%. Among these individuals, six unique haplotypes (A-F) were found in consequence (Table 2). The haplotype A was most commonly observed on both sides of the Pacific, and the haplotype C followed (Table 2, Fig. 2). The other haplotypes were found respectively in only one individual; the haplotypes B and D in the western Pacific, and the haplotype F in the eastern Pacific. The maximum parsimony network

Statistical Testing

Tests for differences in haplotype frequencies among samples were performed with a Monte-Carlo method according to Roff and Bentzen,10) in which the significance is assessed by comparing to chi-square values generated by repeated randomizations. The test was carried out using the MONTE program from REAP package12) and each test was based on 10000 randomizations.

Results

Sequence Variation of the COI Gene

Three hundred and ninety-nine base-pair (bp) segment of the COI gene was sequenced for 23 individuals from three locations (Kyoto/Fukui, Okinawa, and Ogasawara) on the west side of the Pacific and one location (Galapagos) on the east side. Among these individuals, a total of seven nucleotide positions (positions 86, 89, 194, 218, 221, 308, and 332) were found to be variable (Fig. 1). Only variation at one nucleotide position of 218 was shared with more than one individual, while each of the other variations was observed in a single individual only (Table 2). As expected, all these variable sites were at third position of codon triplets (Fig. 1), and all but one changes were transitions (Table 2). All the base substitutions were silent (synonymous) changes that do not result in amino acid changes, so that all the variants can be considered as selectively neutral.

Analysis of Relationships and Occurrence Frequency of Haplotypes

Nucleotide sequence difference between 23 individuals from both sides of the Pacific ranged from 0.00 to 1.25% with an average (=nucleotide diversity) of 0.25%. Among these individuals, six unique haplotypes (A-F) were found in consequence (Table 2). The haplotype A was most commonly observed on both sides of the Pacific, and the haplotype C followed (Table 2, Fig. 2). The other haplotypes were found respectively in only one individual; the haplotypes B and D in the western Pacific, and the haplotype F in the eastern Pacific. The maximum parsimony network

Statistical Testing

Tests for differences in haplotype frequencies among samples were performed with a Monte-Carlo method according to Roff and Bentzen,10) in which the significance is assessed by comparing to chi-square values generated by repeated randomizations. The test was carried out using the MONTE program from REAP package12) and each test was based on 10000 randomizations.

Results

Sequence Variation of the COI Gene

Three hundred and ninety-nine base-pair (bp) segment of the COI gene was sequenced for 23 individuals from three locations (Kyoto/Fukui, Okinawa, and Ogasawara) on the west side of the Pacific and one location (Galapagos) on the east side. Among these individuals, a total of seven nucleotide positions (positions 86, 89, 194, 218, 221, 308, and 332) were found to be variable (Fig. 1). Only variation at one nucleotide position of 218 was shared with more than one individual, while each of the other variations was observed in a single individual only (Table 2). As expected, all these variable sites were at third position of codon triplets (Fig. 1), and all but one changes were transitions (Table 2). All the base substitutions were silent (synonymous) changes that do not result in amino acid changes, so that all the variants can be considered as selectively neutral.

Analysis of Relationships and Occurrence Frequency of Haplotypes

Nucleotide sequence difference between 23 individuals from both sides of the Pacific ranged from 0.00 to 1.25% with an average (=nucleotide diversity) of 0.25%. Among these individuals, six unique haplotypes (A-F) were found in consequence (Table 2). The haplotype A was most commonly observed on both sides of the Pacific, and the haplotype C followed (Table 2, Fig. 2). The other haplotypes were found respectively in only one individual; the haplotypes B and D in the western Pacific, and the haplotype F in the eastern Pacific. The maximum parsimony network

---

Fig. 1. The nucleotide and deduced amino acid sequence of partial COI gene in the diamond-shaped squid Thysanoteuthis rhombus.

Asterisks above the sequence indicate the variable nucleotide positions (see also Table 2).
Table 2. Variable nucleotide positions in part of the COI gene region of six haplotypes, and number of individuals of each haplotype found in each locality

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Site 86</th>
<th>Site 89</th>
<th>Site 194</th>
<th>Site 218</th>
<th>Site 221</th>
<th>Site 308</th>
<th>Site 332</th>
<th>KYO</th>
<th>OKI</th>
<th>OGA</th>
<th>GAL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>G</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Position number of the variable sites follows Fig. 1. Nucleotide identity to reference sequence of Okinawa-#1 (haplotype A) is indicated with a dot.
* KYO = Kyoto/Fukui, OKI = Okinawa, OGA = Ogasawara, GAL = Galapagos.

Figure 2. Maximum parsimony network detailing evolutionary relationships among haplotypes observed in the diamond-shaped squid.

The letter in the circle refers to the haplotype (see Table 2), and the area of each circle is roughly proportional to the number of individuals possessing each haplotype. The short bar crossing each radial arm denotes a nucleotide substitution. Tree length was seven steps with the consistency index (CI) = 1.00.

Figure 3. Example of result of the PCR-SSCP analysis.

Sample haplotype determined by nucleotide sequencing: lane 1, haplotype B (Kyoto-#5 individual); lane 2, haplotype D (Okinawa-#2); lane 3, haplotype A (Kyoto-#1); lane 4, haplotype A (Kyoto-#2); lane 5, haplotype C (Ogasawara-#1); lane 6, haplotype C (Ogasawara-#2). ds, double-strand DNA; ss, single-strand DNA; M, HincII digest of φX174DNA for a size marker.

Table 3. Number of individuals with each haplotype in two Japanese samples from the Sea of Japan and the Pacific as examined by the PCR-SSCP analysis

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>The Sea of Japan (Kyoto/Fukui) (N=100)</th>
<th>The Pacific (Okinawa) (N=101)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>83</td>
<td>87</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>C+D</td>
<td>13</td>
<td>14</td>
</tr>
</tbody>
</table>

The SSCP method was suitable to the mass screening of haplotype composition when the haplotype D was pooled into the haplotype C.

One hundred individuals from the Sea of Japan (Kyoto/Fukui) and 101 from the Pacific (Okinawa) were subject to the SSCP analysis. The results are shown in Table 3. The haplotype A was the most common haplotype in these locations, and the haplotype C+D was the next common. The haplotype B was found only in low frequency in the Sea of Japan. Banding patterns which should be assigned into new haplotypes other than known ones were not observed throughout. The frequency of occurrence of these haplotypes (A, B, C+D) was not significantly different between the two locations, Okinawa and Kyoto/Fukui (P=0.173).

PCR-SSCP Analysis

Then we investigated whether there was subtle genetic population structuring within the north western Pacific by using the PCR-SSCP method for a larger number of individuals. Prior to the mass screening, ability of nucleotide substitution detection of SSCP analysis was checked with sequence determined samples. The haplotypes A-C could be distinguished from each other by the differential mobility of single-strand bands (Fig. 3). The haplotype D was not distinguished from the haplotype C (Fig. 3), but this haplotype D was one of the most minor one so that pooling this into the haplotype C seemed hardly to affect the result of mass screening. This result thus showed that geographical proximity has no relevance to genetic-phylogenetic relationships among these haplotypes: although most divergent haplotypes (B and F) were found on the different sides of the Pacific, respectively, next divergent pairs (B and D, and E and F) were observed within the same side. The frequency of occurrence of the six haplotypes were not different significantly between populations on both sides of the Pacific (P=0.538).
Discussion

**DNA Extraction and Amplification from Squid**

The difficulty of DNA extraction from mollusks has been frequently observed primarily because of the mucus produced by the animals. However, in the present diamond-shaped squid, DNA was easily extracted by both standard phenol/chloroform and simple Chelex extractions, with which PCR and PCR-mediated direct sequencing could be made successfully. Though it is not clear at present whether or not this observation can be applicable to all cephalopods, this indicates that DNA extraction, amplification and sequencing from, at least, some of cephalopods could be done easily.

**Utility of PCR-SSCP Analysis for Mass Screening of DNA Sequence Variation**

There are various DNA methods for examining of nucleotide sequence variation. Of these, DNA sequencing provides the most precise information of course, but requires considerable time and cost. For assaying a large number of individuals for examination of genetic population structuring, PCR-RFLP (restriction fragment length polymorphism) analysis may be one of the best choice among the most time and cost effective methods. However, sometimes suitable restriction enzymes are unavailable to detect known nucleotide sequence variations, as was the case in the present study. In the PCR-SSCP analysis, appropriate experimental condition can be surveyed for best resolution of known sequence variations. In this study, three of the four haplotypes observed in the northwestern Pacific samples could be discriminated on a relatively small (90 mm × 80 mm) gel with usual ethidium bromide staining as shown in Fig. 3. Screening of more than 200 individuals could be made through spending a reasonable amount of time and money. If much larger gels are used, the forth haplotype (haplotype D) might be discriminated. When an unidentified banding pattern is detected, one can examine the haplotype designation of the sample through sequencing it. The present result shows the utility of the PCR-SSCP analysis for assaying nucleotide sequence variation in a large number of individuals.

**Genetic Relationships of Populations in the Waters around Japan**

The present mtDNA analysis suggests there was no evidence for genetic differentiation in the diamond-shaped squid in the waters around Japan. Spawning site of the diamond-shaped squid around Japan is estimated in the Kuroshio Current region from Kii Peninsula to Taiwan and the squid in the Sea of Japan are considered to be transported from the south by the Tsushima Warm Current, and not to reproduce in the Sea of Japan. The present result is compatible with these prevailing views on population structure of this species around Japan.

**Genetic Relationships of Populations on the Both Sides of the Pacific**

In the present study, no marked genetic difference was observed even between populations on both sides of the Pacific, though the statistical power to test this was limited due to lesser number of individuals examined. Pelagic organisms in the open ocean are generally regarded as having low levels of population differentiation, resulting from probable ample opportunities of dispersal in various life history stages and the lack of physical barriers in the environment. In recent years the population genetic structure of large pelagic fishes in the open oceans, such as tunas and marlins, has been studied, and indeed these organisms have been observed to show none or only very low levels of population structuring within the oceans. Furthermore, some species have revealed virtually no genetic differentiation even between populations in the Pacific and Atlantic Oceans. The result in this study suggests that the present diamond-shaped squid, as a large pelagic animal with considerable mobility, also possess homogeneous population structure similar to that observed in those large mobile fishes.

It is premature, nonetheless, from these observations to conclude that most pelagic organisms have such homogeneous structure of populations, because unexpectedly high level of genetic structuring of populations has been discovered in an ubiquitous mesopelagic fish. The validity of the present suggestion that the diamond-shaped squid is genetically uniform throughout the Pacific needs to be further examined through obtaining much extensive data from more geographical samples and also from more DNA markers.

**Acknowledgments**

This study was conducted as part of the project of the Basic Research on the Development of Offshore Fishery Ground organized by the Japan Marine Fisheries Resource Development Center. We are most grateful to the staff of Japan Marine Fisheries Development Center, Kyoto Institute of Oceanic and Fishery Science, Fukui Prefectural Fishery Experiment Station, and National Research Institute of Far Seas Fisheries of sample collection. We thank T. Ohkawa for his help in performing randomization test.

**References**


