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Genetic Diversity and Genetic Heterogeneity of Bigfin Reef Squid "*Sepioteuthis lessoniana*" Species Complex in Northwestern Pacific Ocean

Hideyuki Imai and Misuzu Aoki
University of the Ryukyus
Nara Women's University
Japan

1. Introduction

The bigfin reef squid *Sepioteuthis lessoniana* Férussac, 1831 in Lesson (1830–1831) is widely distributed in the Indo-Pacific, where it is a very valuable fishery resource (Dunning, 1998). Thus, a lot of ecological research of this species were reported (e.g. Ikeda, 1933; Choe & Ohshima, 1961; Segawa, 1987; Ueta, 2003; Ikeda *et al*., 2009). Segawa *et al*. (1993a; 1993b) showed that within *Sepioteuthis lessoninana* have diferrences of egg chracteristics and reproductive trait in Ishigakijima Island. Izuka *et al*. (1994) reported an allozyme analysis found so-called *S*. *lessoniana* around Ishigakijima in Okinawa Prefecture, Japan, includes at least three biological species (Figure 1 & 2). Local fishers call the three species "*aka-ika*," which has a red body, "*shiro-ika*" or "*aori-ika*," which has a white body, and "*kua-ika*," which is smaller than the other two. Of these, the range of "*shiro-ika*" extends to the coast of the main Japanese islands. This is the extent of its taxonomic classification thus far. This is due in part to the limited number of distinguishing morphological characters but also because the type specimens is no longer available and type locality has not been disignated (Lu *et al*., 1995; Jereb & Roper, 2006). This makes it difficult to determain whether genetically recognized species are undescraibed species or one of 13 known synonymies (Young, 2002). In this study, we treated "*aka-ika*" as *Sepioteuthis* sp. 1, "*shiro-ika*" as *Sepioteuthis* sp. 2, and "*kua-ika*" as *Sepioteuthis* sp. 3.

A previous population genetics study found significant differences in the genetic heterogeneity of *Sepioteuthis* sp. 2 between Pacific Ocean and Japan Sea populations using allozyme analysis (Yokogawa & Ueta, 2000). Yokogawa and Ueta (2000) did not include the Okinawan *Sepioteuthis* sp. 2 population in their study. In addition, Pratoomchat *et al*. (2001) found no significant genetic heterogeneity between Japanese and Thai *Sepioteuthis* sp. 2 populations, while our present study tried significant differences in the genetic heterogeneity of the Japanese and Vietnumese *Sepioteuthis* sp. 2 populations. Recently, Aoki *et al*. (2008a) reported significant genetic heterogeneity between Japanese and Vietnumese populations of *Sepioteuthis* sp. 2 using DNA sequencing analysis of the mitochondrial noncoding region.

Therefore, this study examined the genetic diversity (*i.e*., the average heterogeneity) and gene flow among *Sepioteuthis* sp. 2 populations using allozyme analysis and among

populations of *Sepioteuthis* sp. 1 and *Sepioteuthis* sp. 3 using mitochondrial DNA noncoding region sequencing of populations from Japanese, Taiwanese, and Vietnamese waters.

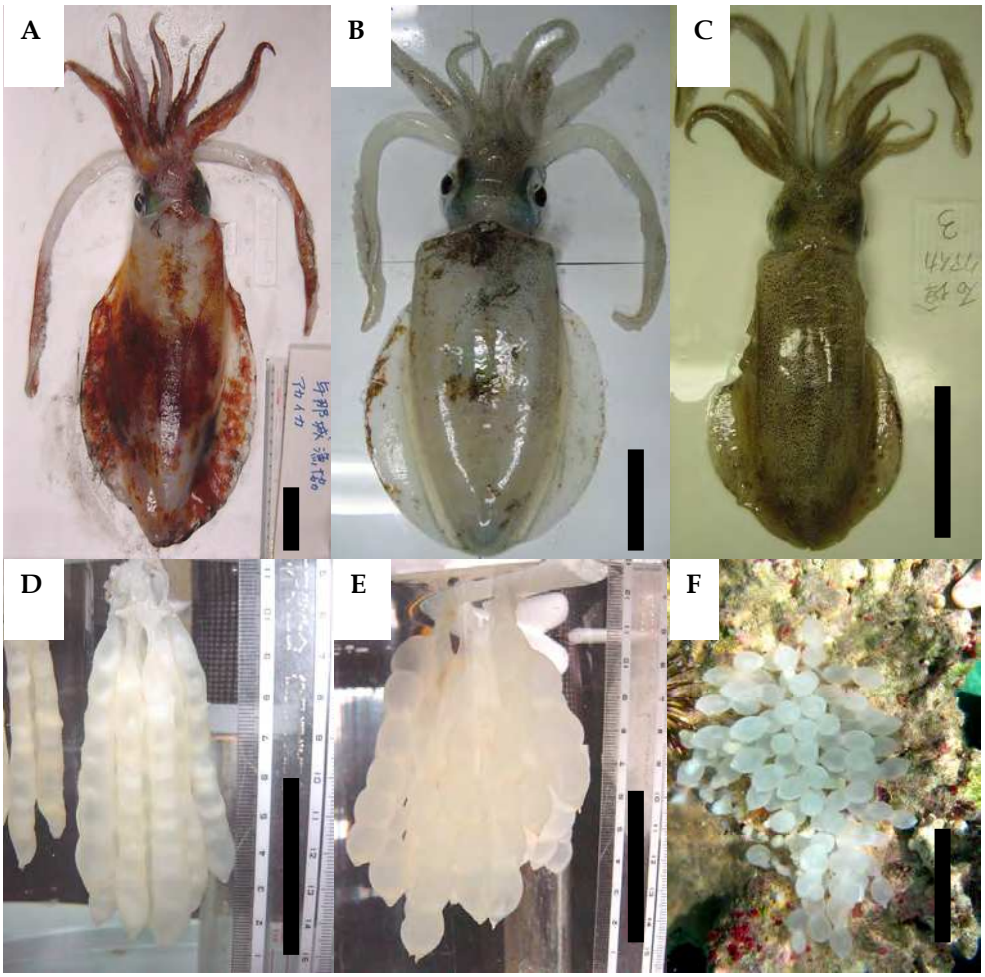

Fig. 1. A: *Sepioteuthis* sp. 1, B: *Sepioteuthis* sp. 2, C: *Sepioteuhis* sp. 3, D: *Sepioteuthis* sp. 1 egg capsules with 5-13 (mean = 9) per capsule, E: *Sepioteuthis* sp. 2 egg capsules with 3-8 eggs (mode = 6) per capsule and F: *Sepioteuthis* sp. 3 egg capsules with consistently two eggs per capsule laid under dead table coral in shallow waters. Black bar indicated 50mm in length.

2. Materials & methods

2.1 Allozyme analysis of *Sepioteuthis* sp. 2

We collected 327 adults between September 1998 and June 2006 from Noto, Ishikawa, Japan (83 individuals), Mugi, Tokushima, Japan (51), the Goto Islands, Nagasaki, Japan (58), Nakagusuku, Okinawa (52), Keelung, Taiwan (23), and the Gulf of Tonkin, Vietnam (60). All

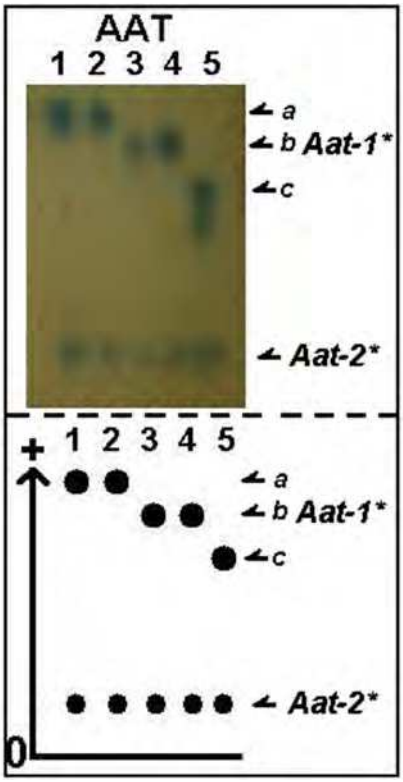

Fig. 2. Electrophoretic patterns of asparate aminotransferase (AAT) of *Sepioteuthis lessoniana* complex. Lane 1-2: *Sepiteuthis* sp. 2, Lane 3-4: *Sepioteuthis* sp. 3 and Lane 5: *Sepioteuthis* sp. 1. These three species are clearly identified by the *Aat-1** marker (Izuka *et al*. 1994).

specimens were fresh and immediately sent to a refrigerator in the laboratory. The buccal bulb muscle was removed and kept frozen at –40°C until the allozyme analysis. Small pieces of liver and skeletal muscle were dissected from selected specimens and minced individually in an equal volume of distilled water on ice. Electrophoresis was conducted in a glass box with ice on top of it. The box was in a refrigerator at a constant voltage (250 V) until the Amido Black 10B marker moved seven cm from the origin. The allozymes were tested using 12.5% horizontal starch–gel electrophoresis and the two buffer systems described by Clayton and Tretiak (1972) and modified by Numachi (1989): citric acid N-(3-aminopropyl) diethanolamine (CAEA, pH 7) and citric acid N-(3-aminopropyl) morpholine (CAPM, pH 6). Each gel was sliced into six 1-mm-thick sheets with a wire gel cutter (Numachi, 1981) and stained for the enzymes aspartate aminotransferase (AAT), isocitrate dehydrogenase (IDHP), lactate dehydrogenase (LDH), phosphoglucomutase

(PGM), and phosphogluconate dehydrogenase (6PGD) according to Shaw and Prasad (1970), Numachi (1970a, b), and Taniguchi and Numachi (1978). The locus and gene nomenclature followed Shaklee *et al*. (1990). Polymorphisms involving several alleles with frequencies of more than 5% were tested at a significance level of 0.05 to determine whether they were consistent with Hardy–Weinberg equilibrium. The average heterozygosity H (Nei, 1978) was calculated as a measure of genetic diversity. The χ^2 homogeneity test of allele frequency among samples was also performed.

2.2 Mitochondrial non-coding region of *Sepioteuthis* sp. 1 and sp. 3

In total, 116 *Sepioteuthis* sp. 1 were collected between April 2005 and September 2006 at three localities: Itoman, Okinawajima (49 individuals), Ishigakijima (38), and Keeling, Taiwan (29). An arm or part of the mantle muscles was kept in 90% ethanol and DNA was extracted with TNES 8M-Urea buffer. For *Sepioteuthis* sp. 3, 60 samples were collected between October 2005 and July 2006 from Nago, Okinawajima (30), and Ishigakijima (30). Crude DNA was extracted by TNES 8M Urea buffer and proteinase K digestion followed by a phenol-chloroform isoamyl method described Imai *et al*. (2004).

We analyzed the noncoding region 2 (NC2) between the Ala and Trp transfer RNAs (tRNAs). The original primers SL-Ala (5'-GGTAACCCTTTCTGTATGATTGC-3') and SL-Trp (5'-AAAGACCTTGAAAGTCTTCAG-3'), which target a portion of tRNA-Ala and tRNA-Trp, respectively, were used with the polymerase chain reaction (PCR) to amplify NC2 (Aoki *et al*., 2008a). The PCR reactions were performed using BIOTAQ (Bioline, UK). A GeneAmp 9700 (Applied Biosystems, USA) thermal cycler was used with the following setting: 94°C for 120 s, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. The PCR products were purified using a PCR Product Pre-sequencing Kit (USB, USA). The nucleotide sequences were determined using ABI 3700 (Applied Biosystems, USA) genetic analyzers. All sequences were initially aligned using ClustalX ver. 1.83.1 (Thompson *et al*., 1997) and then edited manually using MacClade4 ver. 4.08 (Maddison and Maddison, 2005).

The haplotype diversity h (Nei, 1987) and nucleotide diversity π (Tajima, 1983) within populations were calculated using Arlequin ver. 2.000 (Schneider *et al*., 2000). An analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) was used to test the population structure within species for *Sepioteuthis* sp. 1 using Arlequin.

For *Sepioteuthis* sp. 3, AMOVA could not be performed because there were fewer than three localities. Therefore, homogeneity was tested using the chi-square randomization method (Monte Carlo simulation) with 100,000 randomizations of the data (Roff and Benzen, 1989). Significance thresholds were Bonferroni-corrected for multiple pairwise comparisons. Relationships of haplotypes were assessed using a minimum spanning tree created via the Minspanet algorithm in Arlequin and drawn by hand.

3. Results and discussion

3.1 Allozyme analysis of *Sepioteuthis* sp. 2

Regarding the eight loci for the six enzymes analyzed, the five loci *Aat*-1*, *Idhp*-1*, *Ldh*-1*, *Mdh*-1*, and *Mdh*-3* showed no differences among and within localities, and no genetic

polymorphism was recognized. Two *Mdh*-2* heterozygotes were found in Vietnam, although the frequency was 0.017; therefore, it was not considered a polymorphic allozyme locus (Table 1). Genetic polymorphism was detected within a locality for *Pgm** and *6pgd**. The polymorphic allozyme loci were in Hardy–Weinberg equilibrium at the localities. Most of the alleles linked to a locus were monomorphic. A marked excess of homogeneity was found. The average observed heterozygosity H=0.005–0.052 was similar to the values of H=0.037 reported by Izuka *et al*. (1996) and 0.052–0.070 by Yokogawa and Ueta (2000). Other loliginid species have similar heterozygosity values: *Loligo pealeii*, H=0.006; *Lolliguncula brevis*, H=0; *L. plei*, H=0 (Garthwaite *et al*., 1989); *Ommastrephes bartrami*, H=0.004; *Sthenoteuthis oualaniensis*, H=0.011; *Todarodes pacificus*, H=0.043; *Loliolus japonica*, H=0.030 (Fujio & Kawada, 1989); *L. vulgaris reynaudii*, H=0.030; *L. gahi*, H=0.059 (Carvalho & Loney, 1989); *L. bleekeri*, H=0.003 (Suzuki *et al*., 1993); and *L. chinensis*, H=0.006–0.009 (Yeatman and Benzie, 1993). The family Loliginidae appears to be characterized by low genetic diversity.

Locus	allele	Ishikawa	Tokushima	Nagasaki	Okinawajima	Taiwan	Vietnam
Aat-1	**100 A**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
Idh-1	**100 A**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
Ldh-1	**100 A**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
Mdh-1	**100 A**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
Mdh-2	**110 A**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.017**
	100 B	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**0.983**
Mdh-3	**100 A**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
Pgm	**- 60 A**	**0.006**	**0.020**	**0.009**	**0.019**	**0.000**	**0.000**
	-100 B	**0.946**	**0.853**	**0.931**	**0.952**	**0.978**	**1.000**
	-140 C	**0.048**	**0.128**	**0.060**	**0.029**	**0.022**	**0.000**
6Pgd	**150 A**	**0.078**	**0.029**	**0.043**	**0.039**	**0.000**	**0.033**
	100 B	**0.892**	**0.941**	**0.888**	**0.933**	**1.000**	**0.908**
	60 C	**0.030**	**0.029**	**0.069**	**0.029**	**0.000**	**0.058**
Observed	**Ho**	**0.041**	**0.052**	**0.045**	**0.026**	**0.005**	**0.027**
Expected	**He**	**0.038**	**0.046**	**0.042**	**0.028**	**0.005**	**0.025**
	Ho / He	**1.080**	**1.120**	**1.084**	**0.957**	**1.000**	**1.067**

Table 1. Allele frequencies at eight loci and indices of genetic heterozygosities within six localities of *Sepioteuthis* sp. 2.

No allele frequency gap was observed among different localities for the polymorphic allozyme loci *6pgd** allele frequency. In contrast, a significant difference was detected between *Pgm** in the Japanese and Vietnamese localities (Table 2). This result differed greatly from that of Pratoomchat *et al*. (2001), who found the same gene pool in Thailand and Nagasaki, while our result supported the result of Aoki *et al*. (2008). Pratoomchat *et al*. (2001) used *Pgm** and *6pgd**, but the results might have been influenced by differences in the electrophoresis buffer. No difference in allele frequency was observed among localities in Japan. Yokogawa and Ueta (2000) showed replacement of *Ldh*-4* between the main island Japan Sea and Pacific sides, although the allozyme band pattern shown in that paper may have been manipulated, and we find the results suspect. Therefore, we examined *Ldh*-4* with a fresh sample following the advice of Dr. Yokogawa, and we did not find it. Pratoomchat *et al*. (2001) cited Yokogawa and Ueta (2000), but did not detect *Ldh*-4*. When *Ldh*-4* was eliminated, no difference existed between the Japan Sea and Pacific sides. Aoki *et*

al. (2008a) could not show a difference between the Japan Sea and the main island Pacific side, even on analyzing the mitochondrial noncoding region sequence. If *Ldh*-4* of Yokogawa and Ueta (2000) is repeatable, the difference in a highly polymorphic marker among populations would not always be detected. For example, regarding *Theragra chalcogramma*, a restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA and microsatellite DNA could not identify the difference among populations seen in the allele frequency of the superoxide dismutase (SOD) allozyme marker (Iwata, 1975; Mulligan *et al*., 1992; Bailey *et al*., 1999; Chow, 2001). Our finding of gene flow between Taiwanese and Japanese localities detected in the allozyme analysis of *Sepioteuthis* sp. 2 was not consistent with the sequencing analysis of the mitochondrial noncoding region by Aoki *et al*. (2008a), perhaps because of the low level of polymorphic loci for the allozyme analysis.

In addition, noncoding regions such as the mitochondrial control region accumulate more variation than allozyme markers. The relative smallness of the effective population size (female) with nuclear DNA made it easier to detection interpopulation genetic differentiation by genetic drift (Williams *et al*., 2002). Therefore, Aoki *et al*. (2008a) used the mitochondrial noncoding region and found low genetic diversity in Japanese waters. Furthermore, Aoki *et al*. (2008a) revealed the independence of gene flow within the populations in Japanese waters from others.

	Ishikawa	Tokushima	Nagasaki	Okinawajima	Taiwan	Vietnam
Ishikawa	****	**0.026**	**0.591**	**0.296**	**0.784**	**0.009**
Tokushima	**0.0011**	****	**0.131**	**0.241**	**0.113**	**0.000***
Nagasaki	**0.0000**	**0.0008**	****	**0.666**	**0.543**	**0.003***
Okinawajima	**0.0001**	**0.0010**	**0.0001**	****	**0.368**	**0.005***
Taiwan	**0.0012**	**0.0017**	**0.0012**	**0.0003**	****	**0.021**
Vietnam	**0.0004**	**0.0024**	**0.0004**	**0.0002**	**0.0008**	****

Table 2. *P*-value in allele frequencies (*Pgm**; above diagonal) and Nei's genetic distance (below diagonal) among six localities of *Sepioteuthis* sp2. Bonferroni correction $P<0.05$.

3.2 Mitochondrial non-coding region of *Sepioteuthis* sp. 1

We sequenced 552 base pairs (bp) of the NC2 sequence for 116 *Sepioteuthis* sp. 2 specimens from three localities. From a total of 35 haplotypes, 23 variable sites were identified (Table 3). One haplotype was shared among three localities, and the remaining 31 haplotypes were each specific to a single locality. Among the populations, 23.3% of the samples belonged to haplotype no. 1, which was the major haplotype in all Japanese localities. In contrast, haplotype no. 3 was the major haplotype in Taiwan (Figure 3).

The haplotype diversity (h) ranged from 0.7994 for Ishigakijima to 0.8665 for Okinawajima, and the nucleotide diversity (π) varied from 0.0035 for Ishigakijima to 0.0052 for Okinawajima (Table 4). Among the three *Sepioteuthis* sp. 1 localities, the level of genetic diversity did not differ much. The genetic diversity of *Sepioteuthis* sp. 1 was similar to that of *Sepioteuthis* sp. 2: h=0.8972, π=0.0124 in Taiwan and h=0.6828, π=0.0077 in Vietnam. The Japanese values of h=0.2583, π=0.0024 indicate that Japan has three times more genetic diversity than haplotype diversity.

Haplotype	14	19	21	137	199	213	216	228	231	235	236	237	240	242	276	293	294	301	320	330	393	508	536	OK	IG	TA
1	G	A	C	A	T	T	G	C	A	C	A	G	C	A	C	T	C	T	G	T	G	T	A	13	14	0
2	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	12	7	0
3	A	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	·	·	·	·	·	0	0	11
4	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	A	·	·	·	·	0	8	0
5	A	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	A	·	·	·	·	0	0	7
6	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	3	0	1
7	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	2	1	0
8	·	·	·	·	·	·	·	T	·	·	G	·	T	·	·	·	·	·	A	C	·	·	·	4	0	0
9	A	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	A	C	·	·	·	2	0	0
10	·	·	·	·	·	·	·	·	G	·	G	·	·	·	T	·	·	·	·	·	·	·	·	0	2	0
11	A	·	·	G	C	·	·	·	T	·	·	·	·	·	·	·	·	·	A	·	·	·	·	0	0	2
12	A	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	C	A	·	·	·	·	0	0	2
13	·	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	A	C	·	·	G	1	0	0
14	·	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	A	·	·	·	·	2	0	0
15	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1	0	0
16	·	·	·	G	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	1	0	0
17	·	·	·	·	·	·	·	·	·	·	G	·	T	G	·	·	·	·	·	·	·	·	·	1	0	0
18	A	·	·	·	·	·	·	-	T	T	G	·	·	·	·	·	·	·	A	·	·	·	·	1	0	0
19	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	0	1	0
20	A	·	·	G	·	·	·	·	T	·	G	·	·	·	·	·	·	·	A	·	·	·	·	0	1	0
21	A	·	·	·	·	·	A	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0	1	0
22	A	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0	1	0
23	A	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	0	1	0
24	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0	1	0
25	A	·	T	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	·	·	·	·	·	0	0	1
26	A	·	·	G	C	·	·	·	T	·	·	·	·	·	·	·	·	·	A	·	T	·	·	0	0	1
27	A	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	0	0	1
28	A	·	·	·	·	·	·	·	T	·	G	·	·	·	T	·	·	C	A	·	·	·	·	0	0	1
29	A	·	·	·	·	·	·	·	·	T	G	A	·	·	·	·	T	·	·	·	·	·	·	0	0	1
30	A	·	·	·	·	·	·	·	·	·	G	·	·	·	·	C	T	·	·	·	·	·	·	0	0	1
31	A	·	·	·	C	·	·	·	T	·	·	·	·	·	·	·	·	·	A	·	·	·	·	1	0	0
32	A	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	2	0	0
33	G	·	·	·	·	C	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	1	0	0
34	G	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	A	C	·	·	·	1	0	0
35	·	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	A	C	·	C	·	1	0	0
Total																								49	38	29

Table 3. Haplotype distribution and variable sites of mitochondrial NC2 region of *Sepioteuthis* sp. 1 among three localities.

	Okinawajima	Ishigakijima	Taiwan
Haplotype diversity (h)	0.8665	0.7994	0.8079
Nucleotide diversity (π)	0.0052	0.0035	0.0043
Number of individuals (n)	49	38	29
Number of haplotypes	17	11	11

Table 4. Haplotype diversity and nucleotide diversity of *Sepioteuthis* sp. 1 among three localities.

The AMOVA indicated that the genetic variation over all of the Japanese localities was 34.87%, whereas the within-locality variation was 65.13% (p<0.01 [Table 5]). The estimated pairwise *F*st values for the three pairs of three localities ranged from 0.0538 to 0.5329. All combinations of locality samples had significant pairwise *F*st values (p<0.05 [Table 6]). Therefore, each locality had an independent population with restricted gene flow, concurring with Aoki *et al*. (2008a). *Sepioteuthis* sp. 2 had gene flow within the territorial waters of Japan and showed genetic homogeneity. The relationships among the haplotypes

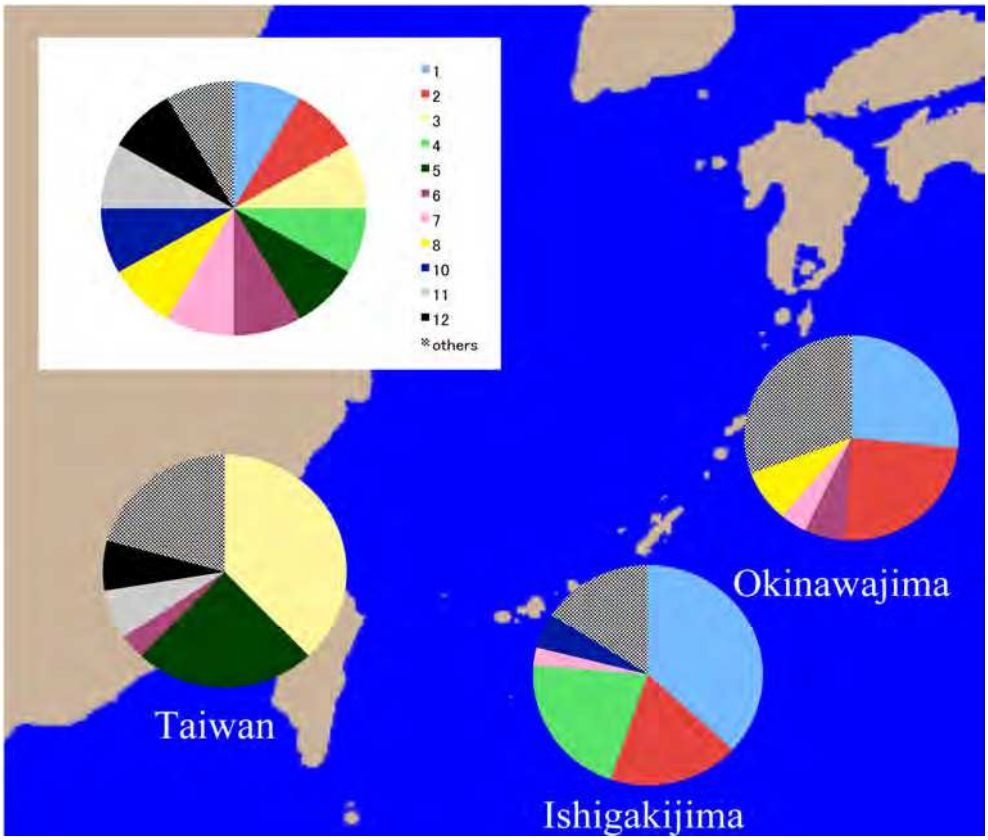

Fig. 3. Pie chart representation of the haplotype frequencies of *Sepioteuthis* sp. 1 of the three localities.

	d.f.	% variation	*F*-statistics	*P*
Among populations within groups	**2**	**34.87**	F_{ST} **= 0.3487**	**< 0.01**
Within populations	**113**	**65.13**		

Table 5. Analysis of Molecular Variance on pairwaise differences and *P*-value of *Sepioteuthis* sp. 1 among three localities is the probability of a more extreme variance component.

	Okinawajima	Ishigakijima	Taiwan
Okinawajima	****	**0.0090***	**0.0000***
Ishigakijima	**0.0538**	****	**0.0000***
Taiwan	**0.4237**	**0.5329**	****

Table 6. Pairwise *F*st and associated probability (*P*) of *Sepioteuthis* sp. 1 among three localities. *F*st values are below the diagonal and corresponding *P* values are above the diagonal. Bonfferroni correction $P<0.05$.

were represented on a minimum spanning tree, and the shape indicated that the population had long-term stability (Figure 4).

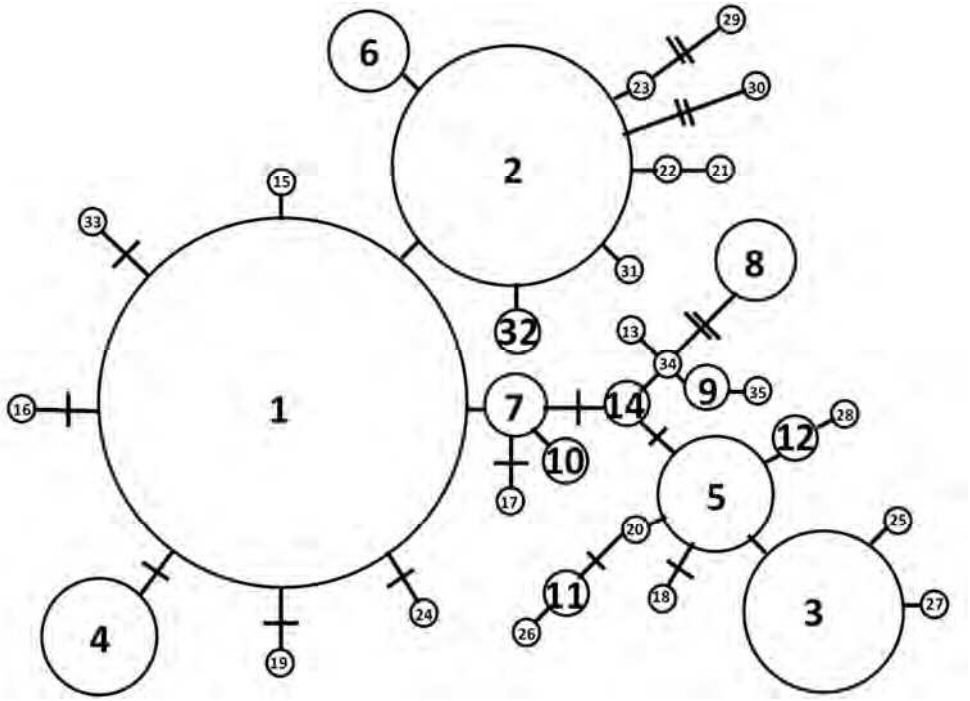

Fig. 4. Minimum spanning tree among 35 hapolotypes of *Sepioteuthis* sp. 1.

3.3 Mitochondrial non-coding region of *Sepioteuthis* sp. 3

We sequenced 557 bp NC2 sequences for 60 *Sepioteuthis* sp. 3 specimens from two localities. From a total of 15 haplotypes, 13 variable sites were identified (Table 7). Seven haplotypes were shared between the two localities, and the remaining eight were specific to a single locality. Overall, 31.6% of the samples belonged to haplotype no. 1, which was not the major haplotype in both localities. Haplotype no. 2 was the major haplotype in Okinawajima (Figure 5). The haplotype diversity (h) ranged from 0.7103 for Ishigakijima to 0.8828 for Okinawajima, and the nucleotide diversity (π) varied from 0.0037 for Ishigakijima to 0.0044 for Okinawajima (Table 8). The level of genetic diversity was similar to that of *Sepioteuthis* sp. 1.

Significant heterogeneity was observed between the Okinawajima and Ishigakijima populations (χ^2=23.89, p<0.01). Therefore, these two populations could be distinguished by the haplotype frequency. Izuka *et al*. (1996) reported that each population was genetically independent based on the allozyme analysis for Ishigakijima and the Ogasawara Islands. These results suggest that *Sepioteuthis* sp. 3 does not experience larval dispersal, but completes its life history within coral reefs. The relationships among the haplotypes were represented on a minimum spanning tree, and the shape indicated that haplotypes could not be divided into clusters (Figure 6).

Haplotype	91	155	209	235	236	244	254	270	300	321	348	510	542	OK	IG
1	A	A	C	A	C	A	C	T	T	C	T	T	G	3	16
2	·	·	·	·	·	·	·	·	·	·	·	·	A	9	3
3	·	C	·	·	·	·	·	·	·	·	·	·	A	4	2
4	·	C	·	·	·	·	·	·	·	·	A	·	·	3	1
5	·	C	·	·	·	·	·	·	·	·	·	·	·	2	2
6	·	·	T	·	·	G	·	C	·	·	·	C	·	1	2
7	·	·	T	·	T	·	·	C	·	·	·	·	·	2	0
8	·	C	·	·	·	·	·	·	·	·	A	·	A	2	0
9	·	·	T	·	T	·	·	C	·	·	·	·	A	1	1
10	·	·	T	·	·	·	·	C	·	·	·	·	A	1	0
11	·	·	T	·	·	·	·	C	·	T	·	·	A	1	0
12	·	·	T	·	·	·	T	C	·	·	·	·	A	1	0
13	C	·	T	·	·	G	·	C	·	·	·	C	·	0	1
14	·	·	·	G	·	·	·	·	·	·	·	·	A	0	1
15	·	·	T	·	·	G	·	C	C	·	·	C	·	0	1
Total														30	30

Table 7. Haplotype distribution and variable sites of mitochondrial NC2 region of *Sepioteuthis* sp. 3 between Okinawajima and Ishigakijima Island.

	Okinawajima	Ishigakijima
Haplotype diversity (h)	0.8828	0.7103
Nucleotide diversity (π)	0.0044	0.0037
Number of individuals (n)	30	30
Number of haplotypes	12	10

Table 8. Haplotype diversity and nucleotide diversity of *Sepioteuthis* sp. 3 between Okinawajima and Ishigakijima Island.

4. General discussion

In this research showed that genetic differentiation between Okinawajima and Ishigakijima population was identified for *Sepioteuthis* sp. 1 and *Sepioteuthis* sp. 3. The result showed that *Sepioteuthis* sp. 1 and sp. 3 prefer coast lines as habitat that limit periodic dispersal of larva and adult among islands. The result showed that there is no gene flow of *Sepioteuthis* sp1 between Ishigakijima and Taiwan, as Aoki *et al*. (2008a) showed for *Sepioteuthis* sp. 2. Geographical distance of these two areas is 300 km, which has no difference of the geographical distance between Okinawajima and Ishigakijima Island. However, genetic structure differentiation between Ishigakijima and Taiwan is bigger than that of Okinawajima and Ishigakijima. Thus, gene flow between Ishigakijima and Taiwan was disturbed for long period. Kuroshio Current possible is possibly disturbing the gene flow Kuroshio Current is warm current with the surface speed of 2m per second, strong flow that moves more than 50 million tons of water. The current axis starts from north equatorial countercurrent, go up towards north between Taiwan and Yonagunijima, through Tokara strait and flows into southern coast of main island Japan (Figure 7). The width between Taiwan and Yonagunijima is small. Kuroshio Current go up to the north along with

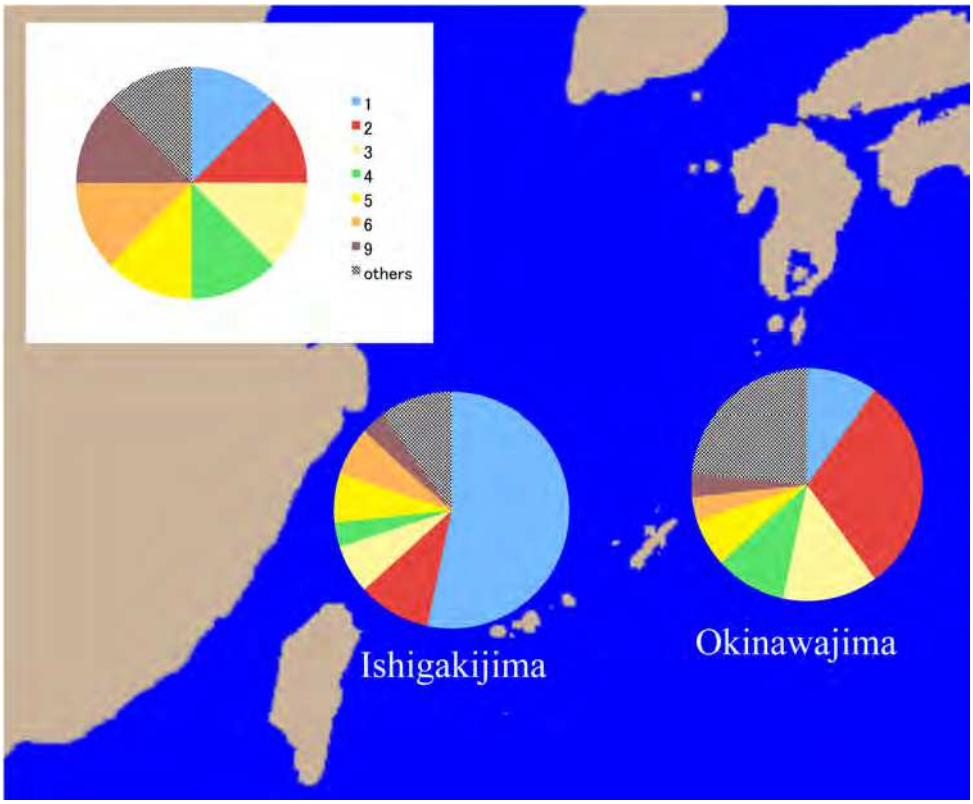

Fig. 5. Pie chart representation of the haplotype frequencies of *Sepioteuthis* sp. 3 of the two localities.

continental shelf. The current split around Kyushu to Tsushima Current along with Japan Sea and main current that goes along with Pacific coast of main island, Japan. The current disperse when it goes to north, the flow of Kuroshio Currnet may take them to very northern part, that has lower temperature of ocean water. Geographical cal distribution of *Panulirus longipes* is another example in which Kuroshio Current is a barrier current between Taiwan and Ryukyu islands (Sekiguchi & Inoue, 2010). Accordingly, several marine organisms, *Uca arcuata* and *Siganus guttatus* shows different genetic structure between Ryukyu Archipelago and Taiwanese population showing no gene flow (Aoki *et al*., 2008b; Iwamoto *et al*., 2012). These genetic sturucture pattern may suggest the influence of Kuroshio Current. Especially squids has short longevity, some of the species has only one spawning season in a life. Drastic environmental change and some other accidental events may destroy population. In order to raise the fitness of squids, water temperature and appropriate environment of growth phase are essential (O'Dor & Coelho, 1993). Kuroshio Current may supply appropriate temperature and abundant feed resources for *Sepioteuthis* spp habitat. The comparative study of *Sepioteuthis* sp. 2 reported by Aoki *et al*. (2008a) and *Sepioteuthis* sp. 1 MST shape shows that these two species can belong to each clade of Japan and Taiwan.

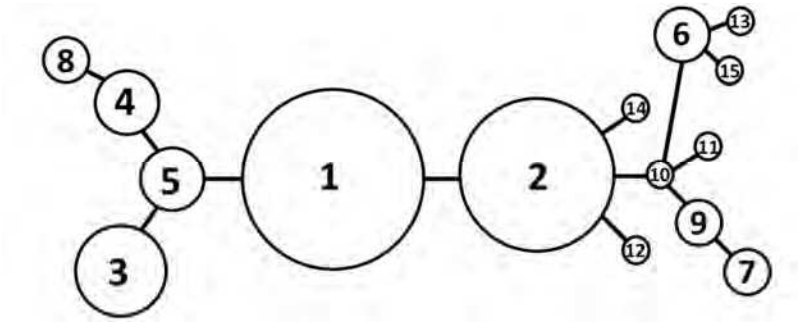

Fig. 6. Minimum spanning tree among 15 hapolotypes of *Sepioteuthis* sp. 3.

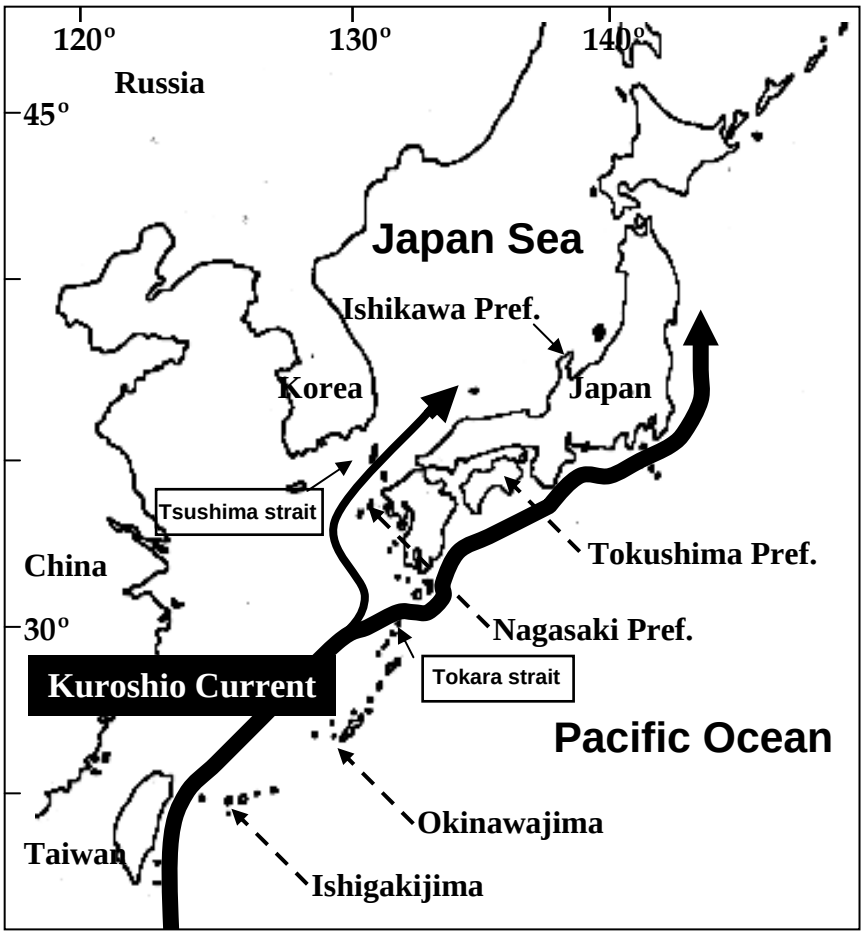

Fig. 7. The location of the main pathway of Kuroshio Current and sampling sites.

5. Conclusion

We analyzed the genetic heterogeneity of populations of *Sepioteuthis* sp. 1 and *Sepioteuthis* sp. 3 in Okinawan waters using DNA analysis. The genetic diversity of *Sepioteuthis* sp. 1 and *Sepioteuthis* sp. 3 was higher than that of *Sepioteuthis* sp. 2 from Japanese waters. Moreover, the genetic heterogeneity of the populations differed significantly. The difference in genetic heterogeneity means that *Sepioteuthis* sp. 1 and *Sepioteuthis* sp. 3 do not have a large gene pool. We postulate that the reason for the genetic differentiation is that these two species prefer coastal habitats. Our results indicate that the Japanese populations of *Sepioteuthis* sp. 2 have very low genetic diversity compared to those of Taiwan and Vietnam. The minimum spanning tree showed that the Japanese populations were of the radiation type, implying that the Japanese populations had experienced founder effects.

The genetic heterogeneity in the Japanese populations was slightly different using AMOVA. This suggests that the mitochondrial noncoding region of the Japanese population lacks sufficient genetic diversity to assess the genetic heterogeneity in the Japanese populations. Moreover, our results suggest that only limited gene flow has occurred between the Ishigakijima and Taiwan populations of *Sepioteuthis* sp. 1 and *Sepioteuthis* sp. 2, implying the presence of barriers to gene flow. Kuroshio Current, a prominent current in this area, which moves at a rate of nearly 50 million m^3/s, may prevent dispersal from Taiwan to Ishigakijima.

Lastly, it should be noted that Prof. Segawa's contribute to ecological research for *Sepioteuthis lessoniana* complex. Further study should focus on resolute species complex as soon as possible to develop ecological research of *Sepioteuthis* spp. It is necessary to identify species identification marker, however, frequently used allozyme marker needs fresh samples. Finding DNA marker that can be used for ethanol sample will be useful. DNA marker development should use allozyme marker of Izuka *et al*. (1994) and Triantafillos and Adams (2005) as the standard specimens. Allozyme analysis is the method to detect nuclear DNA polymorphism by the detection of enzyme molecule polymorphism. It cannot show nucleotide base-substitution mutation when it does not have amino-acid sequence variation. The different condition of electrophoresis buffer may produce different results, even though it is worth noting that it is a reliable tool to find cryptic species (Imai, 2006). The first author of this paper, Imai, H is currently working on development of species identification using DNA marker with some other researchers.

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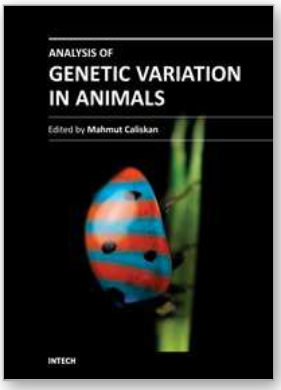

Analysis of Genetic Variation in Animals
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Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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