Phylogenetic Relationships between the Tideland Snails Batillaria flectosiphonata in the Ryukyu Islands and B. multiformis in the Japanese Islands

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Phylogenetic Relationships between the Tideland Snails *Batillaria flectosiphonata* in the Ryukyu Islands and *B. multiformis* in the Japanese Islands

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ABSTRACT—Phylogenetic relationships between two sibling species of Japanese tideland snails, namely, *Batillaria multiformis* from the Japanese Islands and *B. flectosiphonata* from the Ryukyu Islands, were analyzed on the basis of the nucleotide sequences of the mitochondrial gene for cytochrome oxidase I. Populations of *B. multiformis* were genetically distinct from those of *B. flectosiphonata* with the exception of a population from Amami-Oshima Island, which corresponded to the boundary between the distributions of these two species. Individuals with the mitochondrial gene of *B. multiformis* and those with the mitochondrial gene of *B. flectosiphonata* were collected from the same tidal flat on Amami-Oshima Island. All the snails with the mitochondrial gene of *B. multiformis* could be divided into two genetically distinct groups but there was no geographical structure to the distribution of these two groups. Individual populations of *B. flectosiphonata* in the Amami, Okinawa, Miyako and Yaeyama insular groups each consisted exclusively of a unique set of haplotypes, with the exception of a population at a northern site on Okinawajima Island, which included a few individuals with sequences related to those of individuals in the Amami insular group. All individuals from South Ryukyu formed a well-supported monophyletic group, while the monophyly of individuals from Central Ryukyu was not supported. The monophyly of *B. multiformis* was clearly demonstrated but there was no evidence to support that of *B. flectosiphonata*. *Batillaria multiformis* might have been derived from immigrants from the Ryukyu Islands, which became isolated and diverged genetically on the Japanese Islands.

Key words: Japanese Islands, Ryukyu Islands, *Batillaria*, mitochondrial DNA, phylogeography

INTRODUCTION

The Ryukyu Islands and the Japanese Islands are the island arcs of the Asian Continent. The Ryukyu Islands form an arc of about 1,200 km in length, located between Kyushu, which is the southernmost island of the Japanese Islands, and Taiwan. Considerable geological, paleontological and biogeographical evidence suggests that the Ryukyu Islands were connected with the Asian Continent by a landbridge sometime during the Pliocene and the Pleistocene (Kimura, 2000). Many endemic species, some of which are thought to be relics of immigrants from the Asian Continent, have been reported from the Ryukyu Islands. Thus, organisms from the Ryukyu Islands provide very interesting materials for both biogeographic and phylogeographic studies.

The Ryukyu Islands are divided into three parts, namely, North Ryukyu (the Osumi insular group and the northern part of the Tokara insular group), located between Kyushu Island and the Tokara Gap; Central Ryukyu (the southern part of the Tokara insular group and the Amami and Okinawa insular groups), located between the Tokara and Kerama Gaps; and South Ryukyu (the Miyako and Yaeyama insular groups), located between the Kerama Gap and Taiwan Island. Along the Tokara Gap, the Watase Line,

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which is the zoogeographical boundary between two zoogeographic regions, namely, the Palearctic region and the Oriental region, was drawn on the basis of the natural distribution of amphibians, reptiles and mammals (Okada, 1927). Another boundary along the Kerama Gap, designated the Hachisuka Line, was proposed on the basis of avian fauna (Tokuda, 1969). The results of cluster analysis based on amphibian and reptilian fauna suggest that these two gaps might have played very important roles in the establishment of the biogeographical characteristics of the Ryukyu Islands during the Cenozoic (Ota, 1998, 2000).

Snails in the genus *Batillaria* dominate the muddy tidelands in the Japanese Islands. Three species of this genus, namely, *B. multiformis*, *B. cumingi* and *B. zonalis,* are distributed in the Japanese Islands. In addition to *B. zonalis*, individuals with *B. cumingi*-like shells have been found in the Ryukyu Islands. Although it was suggested that these individuals might be a subspecies of *B. cumingi* (Wada *et al*., 1996), Ozawa (1996) described them as the fourth Japanese species of *Batillaria*, namely, *B. flectosiphonata*. The type locality of this species is Iriomotejima Island in South Ryukyu. Molecular phylogenetic analysis, based on nucleotide sequences of the mitochondrial genes for ribosomal RNA (Ozawa, 1996) and cytochrome oxidase I (COI) (Kojima *et al*., 2001), demonstrated that this species is rather closely related to *B. multiformis*. Ozawa (1996) stated that *B. flectosiphonata* inhabits not only the Ryukyu Islands but also the western coast of Kyushu. However, our previous analysis based on the gene for COI, showed that snails with *B. flectosiphonata*-like shells from western Kyushu were not *B. flectosiphonata* but were either *B. cumingi* or *B. multiformis* (Kojima *et al*., 2001). In our previous study, we used only three specimens of *B. flectosiphonata*, which had been collected on Ishigakijima Island, in South Ryukyu, as representatives of this species. In addition to their presence on Iriomotejima Island and Ishigakijima Island, Ozawa (1996) reported finding this species on Kuroshima Island in South Ryukyu and on Okinawajima Island in Central Ryukyu. This species also inhabits some other islands of Central Ryukyu and South Ryukyu (Iijima, unpublished data). Masuda and Hayase (2000) also found *B. flectosiphonata*-like snails on Amami-Oshima Island but they proposed that *B. flectosiphonata* should be treated as a subspecies of *B. multiformis*. In the present study, we analyzed genetic population structures of *B. flectosiphonata* and *B. multiformis*, as well as the phylogenetic relationships among them, on the basis of the nucleotide sequence of the mitochondrial gene for COI.

MATERIALS AND METHODS

Sites at which *Batillaria* snails were sampled are summarized in Table 1 and Fig. 1. DNA was extracted from the head-foot region of each individual by grinding, digestion with sodium dodecyl sulfate (SDS), and extraction with phenol and chloroform. Then part (about 700 bp) of the mitochondrial gene for COI was amplified by the polymerase chain reaction (PCR) using total DNA as template and primers Gastro-2 (Kojima *et al*., 2001) and COI-6 (Shimayama *et al*., 1990). The conditions for PCR were as follows: incubation at 94°C for 60 sec; and then 30 to 40 cycles of incubation at 92°C for 40 sec, 50°C for 60 sec, and 72°C for 90 sec. Genereleaser™ (Bio-Venture Inc., Murfreesboro, TN, USA) was used to sequester products of cell lysis that might have inhibited the polymerase. For spec-

Table 1. Sites at which specimens of *Batillaria* were collected.

No.	Sampling site	Species	*N*
1	Mangokuura Lagoon, Miyagi	*Batillaria multiformis*	20
2	Minaduki Bay, Noto Peninsula	*B. multiformis*	20
3	Lake Hamana, Shizuoka	*B. multiformis*	20
4	Shiokawa Tideland, Mikawa Bay	*B. multiformis*	3*
			17
5	Kushida River, Mie	*B. multiformis*	20
6	Wajiro Tideland, Hakata Bay	*B. multiformis*	20
7	Ariake Bay	*B. multiformis*	17*
8	Kiire Coast, Kagoshima	*B. multiformis*	21
9	Ura River, Amami-Oshima Island	*B. flectosiphonata*-like snail	20
10	Oura River, Okinawajima Island	*B. flectosiphonata*	20
11	Yone Coast, Okinawajima Island	*B. flectosiphonata*	20
12	Okutakejima Island	*B. flectosiphonata*	20
13	Yoneha Bay, Miyakojima Island	*B. flectosiphonata*	20
14	Miyara Bay, Ishigakijima Island	*B. flectosiphonata*	3*
			17
15	Nakama River, Iriomotejima Island	*B. flectosiphonata*	20
16	Kuroshima Island	*B. flectosiphonata*	20

* From Kojima *et al*. (2001)

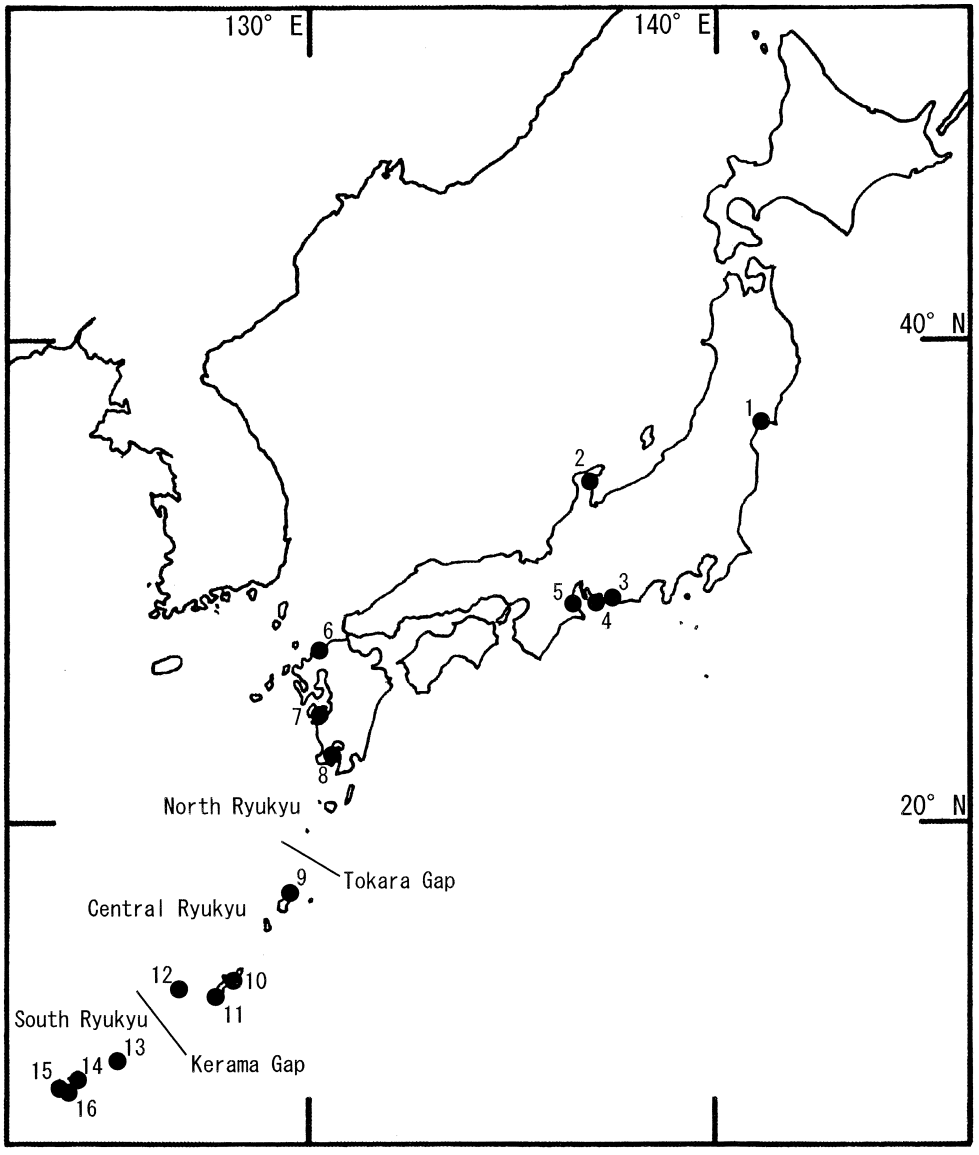

Fig. 1. Sampling sites at which *Batilallia multiformis* and *B. flectosiphonata* were collected. Sites refer to numbers in Table 1. The positions of the Tokara and Kerama Gaps are shown.

imens of *B. multiformis* from the Japanese Islands, both strands of each amplified fragment were sequenced with an automated sequencer (DSQ-2000L; Shimazu Corp., Kyoto, Japan) using internal primers UN-1 and Gastro-4 (Kojima *et al.*, 2001). Nucleotide sequences of DNAs from snails from the Ryukyu Islands were determined with an automated sequencer (ABI3100; Applied Biosystems Inc., California, USA) using primers Gastro-2 and/or COI-6.

For each resultant haplotype, we chose one specimen for amplification of a longer fragment of the gene for COI (about 1,300 bp), using primers LCO1490 (Folmer *et al.*, 1994) and COI-6. The

Table 2. Nucleotide sequences of primers used in the present study. Y, R, S, W and N denote T or C; A or G; G or C; A or T; and G, A, T or C, respectively. Positions refer to the corresponding amino acid residues encoded by the gene for mitochondrial cytochrome oxidase I from *Drosophila yakuba*.

Name	Sequence	Position	Direction
LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	5–14	Forward
Gastro-2	5'-GCGTTCTTTGACCCAGCTGGNGGNGGNGAYCC -3'	216–226	Forward
UN-1	5'-TTRATTTTACCRGGATTYGG-3'	244–250	Forward
Bat-1	5'-AATGTGAGAAATTATTCCRAATCCYGG-3'	247–255	Reverse
Gastro-4	5'-ATAATAAARAARTGNTTNGTYCA-3'	407–415	Reverse
COI-6	5'-GGRTARTCNSWRTANCGNCGNGGYAT-3'	434-442	Reverse

Table 3. Distribution of haplotypes of *Batillaria multiformis* and *B. flectosiphonata*. Sampling sites refer to numbers in Table 1. Abbreviations for haplotypes are the same as in Fig. 3.

	Sampling site															
Type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	6	5	4	11	10	6	5	6	1							
2							1									
3			1													
4	1		1													
5						1										
6									1							
7	1															
8								1								
9				1		1										
10	4	5	2	2		4	2	3	1							
11		1			1											
12	1															
13	2		1	1												
14						1										
15					2		1									
16		1														
17							1									
18	1															
19			1													
20						1										
21					1											
22							1									
23			1													
24				3	2											
25								1								
26			1													
27		1	2													
28								1	2							
29			1			1										
30						1										
31									1							
32				1												
33	4	4	1	1	3	1	2	4								
34								1								
35							3									
36						1		2								
37						1										
38			2		1											
39						1		1								
40		2														
41		1	1				1	1								
42			1													
43									1							
44									12							
45									1							
46										2						
47										18	19	20				
48													20			
49											1					
50														17	17	20
51															1	
52														1		
53															2	
54														1		
55														1		

same regions were amplified from the mitochondrial DNA of single specimens of *B. cumingi* from the Shiokawa Tideland in Mikawa Bay and from Wakamatsujima Island in the Goto Islands and from a specimen of *B. zonalis* from the Yone Coast of Okinawajima Island. The two specimens of *B. cumingi* were representatives of the two genetically distinct groups of this species (Kojima, 2002). In the case of each of these three individuals, the total DNA had been extracted and the nucleotide sequence of the downstream region of the gene for COI had been determined in a previous study (Kojima *et al*., 2001). The nucleotide sequences of the upstream regions of

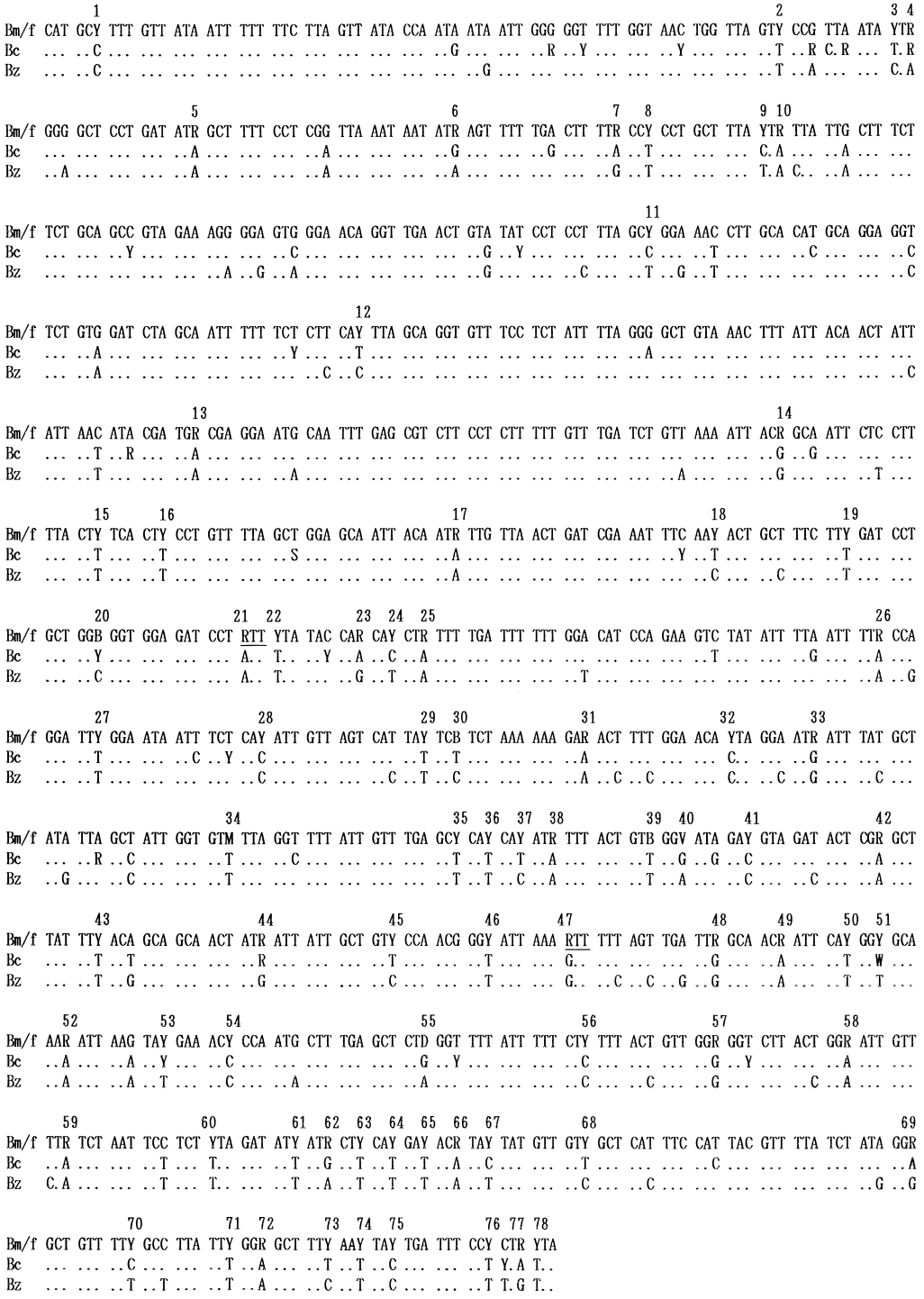

Fig. 2. Nucleotide sequences of mitochondrial genes for cytochrome oxidase I from *Batillaria multiformis* and *B. flectosiphonata* (Bm/f) and from an outgroup, namely, *B. cumingi* (Bc) and *B. zonalis* (Bz). Dots indicate nucleotides that are identical to those in Bm/f. Underlining indicates codons at which amino acid substitutions were detected. Numbers indicate variable positions in Bm/f. R, Y, M, S, W, B, V and D denote A or G; C or T; A or C; G or C; A or T; G, T or C; G, A or C; and G, A or T, respectively. The nucleotide sequences reported in the present study will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under accession numbers AB054364 (Bm/f), AB054365 (Bc), and AB054367 (Bz).

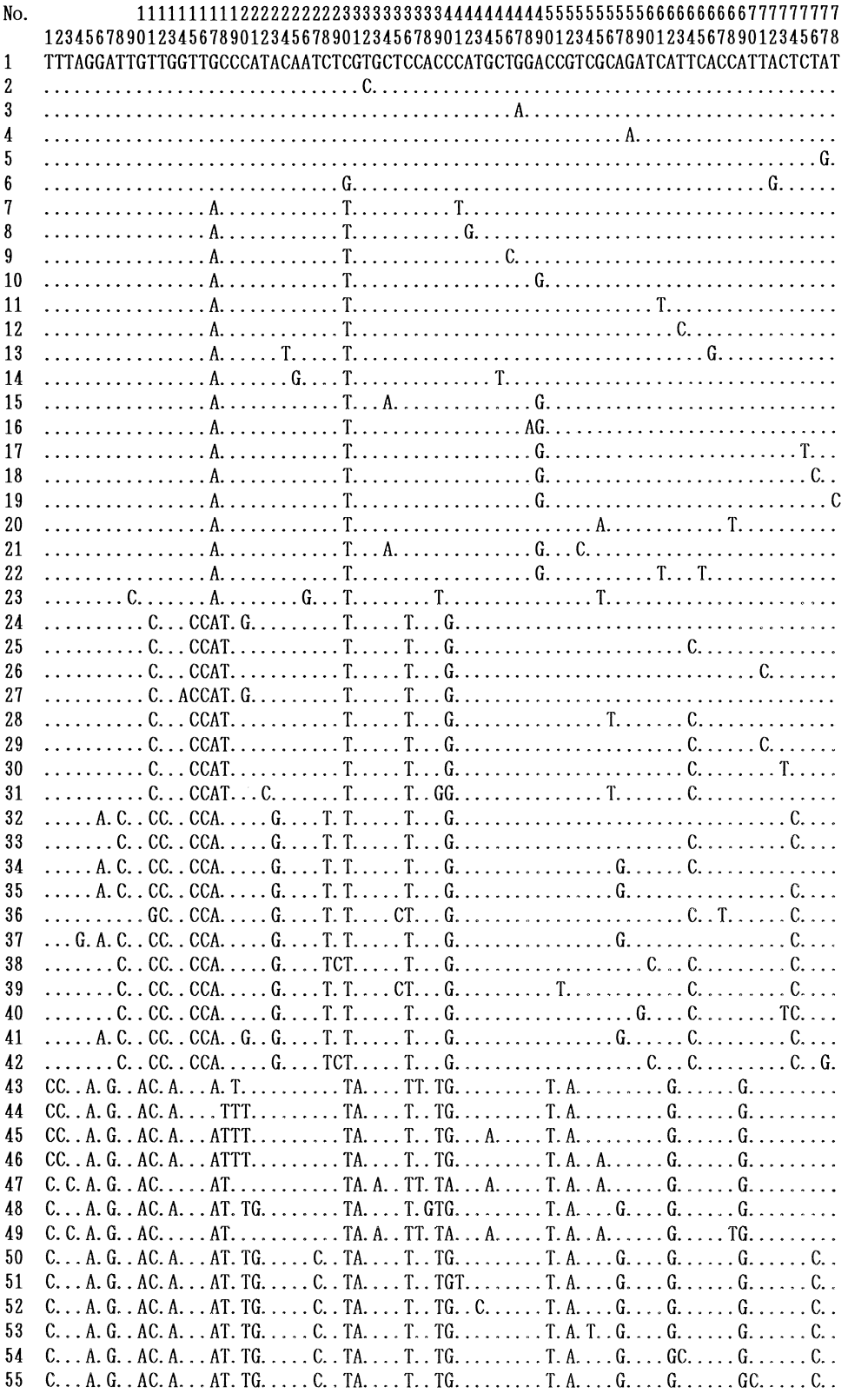

Fig. 3. Nucleotides at variable positions in the sequences of the mitochondrial genes for cytochrome oxidase I from *Batilallia multiformis* and *B. flectosiphonata*. Numbers refer to the positions shown in Fig. 2. Dots indicate nucleotides that are identical to those in the uppermost sequence.

amplified fragments were determined with an automated sequencer (ABI3100) using primer LCO1490. When it was appropriate, the nucleotide sequences of the opposite strands of amplified fragments were determined using primer Bat-1, which was based on the sequences determined with primer LCO1490. The nucleotide sequences of all the primers used in the present study are shown in Table 2.

Amino acid sequences of COI were deduced by reference to the modified genetic code of molluscan mitochondrial DNA (Shimayama *et al*., 1990; Hoffmann *et al*., 1992). The genetic distances, between sequences were calculated by Kimura's two-parameter method (Kimura, 1980). A phylogenetic tree was constructed by the neighbor-joining (NJ) method (Saitoh and Nei, 1987) and the maximum-parsimony (MP) method with the program from MEGA package, Version 2.1 (Kumar *et al*., 2001).

RESULTS

The nucleotide sequence (393 bp) of the mitochondrial gene for COI revealed a total of 55 haplotypes among the 295 individuals analyzed in the present study, and the three and 23 individuals from the Shiokawa Tideland in Mikawa Bay and from Ariake Bay, respectively, whose sequences had been determined in a previous study (Kojima *et al*., 2001; Table 3). We then took one individual of each haplotype and determined the nucleotide sequence (627 bp) of the upstream region of each gene for COI. The nucleotide sequence of each haplotype and those of two species chosen as an outgroup (*Batillaria cumingi* and *B. zonalis*) are summarized in Figs. 2 and 3. Differences among encoded

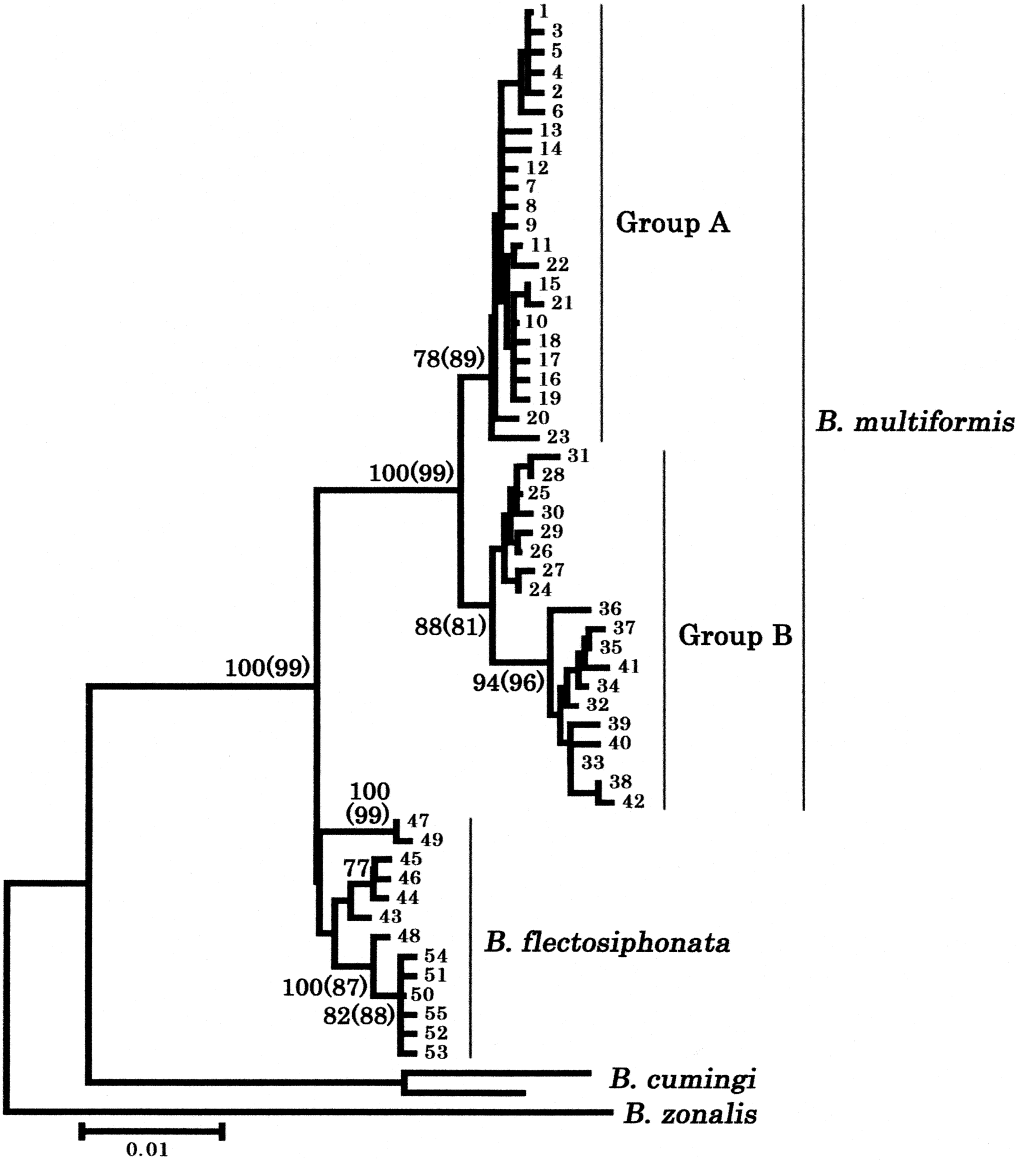

Fig. 4. Phylogenetic relationships among haplotypes of *Batillaria multiformis* and *B. flectosiphonata*. The phylogenetic tree was constructed by the neighbor-joining method using *B. cumingi* and *B. zonalis* as an outgroup. Bootstrap values are shown above branches of clades that are supported by bootstrap values of higher than 70%. Bootstrap probabilities for the maximum-parsimony tree are shown in parentheses when values are higher than 70%.

amino acids were detected at two sites when we compared individuals of *B. multiformis* and *B. flectosiphonata* (Fig. 2).

The phylogenetic relationships among the haplotypes are shown in Fig. 4. All specimens collected from the Japanese Islands and six of 20 individuals from Amami-Oshima Island formed a monophyletic cluster, which was supported by high bootstrap probabilities (100% by the NJ method and 99% by MP method). The remaining individuals also formed a cluster on the NJ tree but the monophyly of this cluster was supported by a rather low bootstrap probability (41%). On the MP tree, the remaining individuals were shown to be paraphyletic but their paraphyly was also supported by only a low bootstrap probability (54%). In this report, individuals belonging to the first and second clusters will be referred to hereafter as *B. multiformis* and *B. flectosiphonata*, respectively, as shown in Fig. 4.

The cluster of *B. multiformis* consisted of two distinct groups, which were supported by relatively high bootstrap probabilities (more than 78%; Fig. 4). All of the analyzed populations of *B. multiformis* contained individuals from both groups (Fig. 5) and no genetic differentiation was detected among sampling sites (Exact test of sample distribution; $p > 0.05$).

Populations of *B. flectosiphonata* from the Amami, Okinawa, Miyako and Yaeyama insular groups, respectively, consisted exclusively of a unique set of haplotypes, with the exception of a population in the estuary of the Oura River on the northern side of Okinawajima Island (Table 3). The population from the Oura River included a few individuals with sequences that were rather closely related to those of individuals in the Amami insular group (haplotype 46). Haplotypes from the Yaeyama insular group were closely related to the sole haplotype identified among individuals of the Miyako insular group. The monophyly of individuals from South Ryukyu, namely, the Miyako and Yaeyama insular groups, was supported by high bootstrap probabilities (100% by the NJ method and 87% by the MP method). Such monophyly was also suggested by the sharing of a single

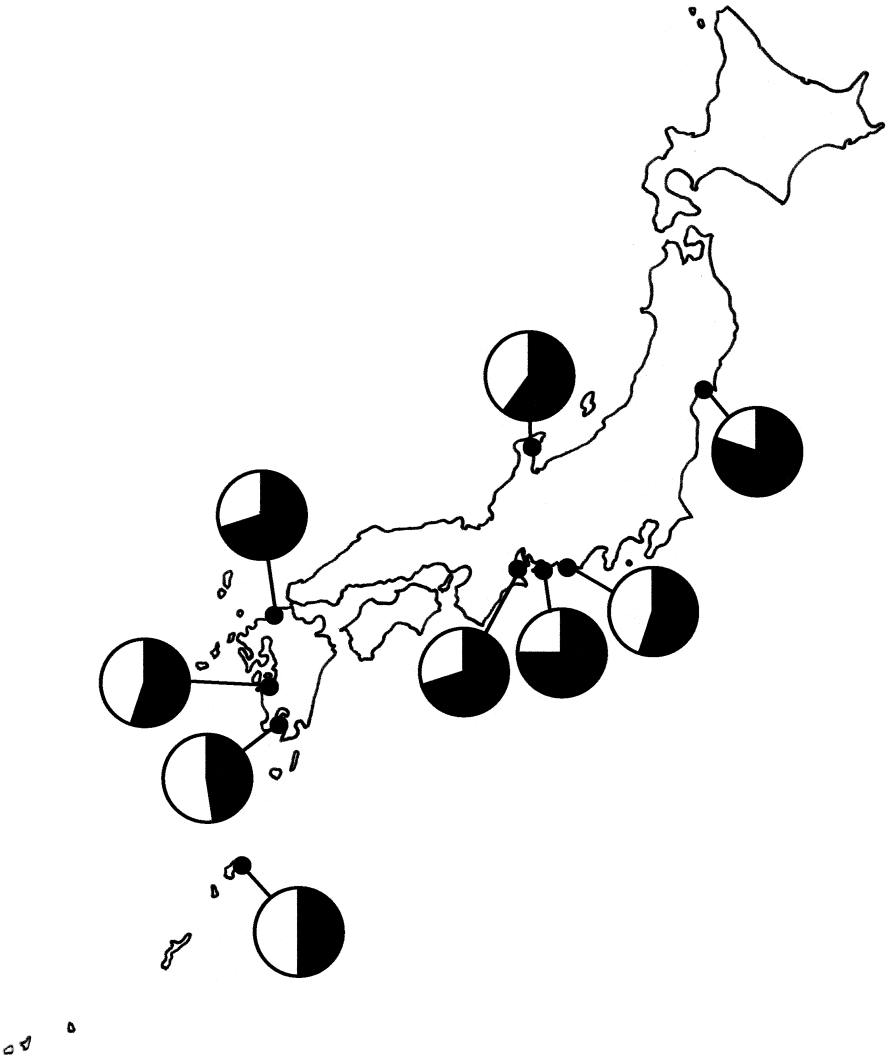

Fig. 5. Geographical distribution of the two groups of haplotypes of *Batilallia multiformis*. Black and white sectors indicate the relative frequencies of group A and group B, respectively, and these groups are defined in Fig. 4.

amino acid substitution (Figs. 2 and 3). The monophyly of haplotypes from the Okinawa insular group, with the exception of one haplotype from the northern site on Okinawajima Island (haplotype 46), was supported by high bootstrap probabilities (100% by the NJ method and 99% by MP method). Haplotype 46 and haplotypes from the Amami insular group also formed a cluster, although bootstrap probabilities supporting this cluster were low (less than 54%). Haplotypes from Central Ryukyu did not form a monophyletic cluster on either the NJ or the MP tree.

DISCUSSION

Our molecular phylogenetic analysis of nucleotide sequences of mitochondrial DNA showed clearly that *Batillaria multiformis* from the Japanese Islands is genetically distinct from *B. flectosiphonata* from the Ryukyu Islands, with the exception that individuals with the mitochondrial sequence of *B. multiformis* and also those with the sequence of *B. flectosiphonata* were collected from Amami-Oshima Island. *Batillaria multiformis* from the Japanese Islands formed a well-supported monophyletic group together with six individuals from Amani-Ohshima Island, while the monophyly of the remaining individuals was supported only by low bootstrap probabilities (Fig. 4). Since *Batillaria* gastropods originated in the tropics, the Japanese Islands during the glacial periods would have been unsuitable as a habitat for them. The bottleneck effect on populations on the Japanese Islands might also have been responsible for their clear monophyly.

Although we found two genetically distinct groups of *B. multiformis* (Fig. 4), there was no difference in terms of geographical distribution between these two groups (Fig. 5). In the case of the congeneric species *B. cumingi* (Kojima, 2002) and the Japanese turban shell *Turbo* (*Batillus*) *cornutus* (Kojima *et al*., 1997, 2000), we also found two genetically distinct groups, but they had a geographical structure. The distribution of the two groups of these two species corresponds to the routes of the two warm currents along the Japanese Islands, namely, the Kuroshio Current and the Tsushima Current, respectively. Although the more detailed genetic structures were shown within each of two groups of *B. cumingi*, no such structures were detected within the groups of *T. cornutus*. While the development of *B. multiformis* includes a planktonic stage (Furota *et al*., 2002), *B. cumingi* is a direct-developer (Adachi and Wada, 1999). Larvae of *T. cornutus* are lecithotrophic, with a short planktonic period of 3 to 5 days (Toyama, 1980). Correspondence between the degree of genetic structuring and the length of planktonic stage strongly suggests that the difference in geographical structure among three gastropod species is attributable to the difference in dispersal ability.

North Ryukyu and Central Ryukyu were isolated from the Japanese Islands upon formation of the Tokara Gap, which was estimated to have been occurred in the Pliocene (Ota, 1998) or Pleistocene (Kimura, 2000). It is likely that *B. multiformis* was derived from a population that was geographically isolated and genetically diverged on the Japanese Islands. Individuals with the mitochondrial sequence of *B. multiformis*, which are found on Amami-Oshima Island at a relatively low frequency, might be attributable to secondary colonization from the Japanese Islands. The existence of individuals with mitochondrial DNA that is closely related to the mitochondrial DNA of individuals on Amami-Oshima Island, on the northern part of Okinawajima Island, might also be a result of southward dispersion. Zhu *et al*. (2003) reported the southwestward current along the southeastern coast of Okinawajima Island when the cold eddy prevailed. Such southwestward currents might have caused southward dispersion of *Batilallia* gastropods.

Individual populations of *B. flectosiphonata* from the Amami, Okinawa, Miyako and Yaeyama insular groups, respectively each consisted exclusively of a unique set of haplotypes, with the exception of a population from a northern site on Okinawajima Island, as mentioned above. The population of each insular group has been isolated from all the others and has evolved independently. Although the mode of development of *B. flectosiphonata* has not yet been reported, the dispersal ability of this species might not be very high. Similarly to many terrestrial species on the Ryukyu Islands (Ota, 1998, 2000), *B. flectosiphonata* exhibits genetic differentiation between Central Ryukyu and South Ryukyu. Haplotypes obtained exclusively from the Yaeyama insular group form a clear monophyletic group with the unique haplotype of the Miyako insular group (Fig. 4). The monophyly of this group is also supported by the single amino acid substitution that is shared. In contrast to the clear monophyly of *B. flectosiphonata* from South Ryukyu, the phylogenetic status of haplotypes of *B. flectosiphonata* from Central Ryukyu was not well supported. The islands of South Ryukyu are smaller than Amami-Ohshima Island and Okinawajima Island, and their small size might have caused a more severe bottleneck of populations in South Ryukyu than in Central Ryukyu during the period when the sea level rose. Such effects should have been especially severe on Miyakojima Island without a high mountain, as shown in the extremely low genetic diversity of a population of *B. flectosiphonata*. By contrast, the large size and elongated shape of Okinawajima Island allow the coexistence of different lineages on the island, as demonstrated for the gecko *Goniurosaurus kuroiwae* (Ota *et al*., 1999).

On Amami-Oshima Island, in the Amami insular group, which corresponds to the distributional boundary between *B. multiformis* and *B. flectosiphonata*, individuals with mitochondrial sequences of the former and the latter were found on a single tidal flat and there was no clear morphological difference between them (Kurozumi, personal communication). The diagnostic characteristics of *B. flectosiphonata* are a distorted and deeply concave columella and a siphonal canal that is reflected to left (Ozawa, 1996). These characteristics are somewhat gradational and not all specimens can been identified on the basis of these characteristics. For

example, individuals with such diagnostic features are rather rare among individuals in the Yaeyama insular group, in South Ryukyu (Kurozumi, personal communication). Masuda and Hayase (2000) questioned the validity of this species. The present results also suggest that it may be necessity to reexamine the taxonomic status of these species. The possibility exists that the mitochondrial sequence of *B. multiformis* in a population on Amami-Oshima Island might be a result of past introgression between *B. multiformis* and *B. flectosiphonata*. Therefore, it is now necessary to assess using genetic markers in the nuclear DNA whether individuals with the mitochondrial sequence of *B. multiformis* on Amami-Oshima Island are *B. multiformis* or *B. flectosiphonata*.

The Ryukyu Islands have been attracting considerable biogeographical interest and many studies of the biogeography, as well as the phylogeography, of terrestrial species have been reported. However, few studies of marine organisms have been performed, in part because it seems that isolation between islands is not as complete for marine organisms as it is for terrestrial ones. For example, the crown-of-thorns starfish *Acanthaster planci* showed very high genetic homogeneity among populations around the Ryukyu Islands (Nishida and Lucas, 1988). In the present paper, we described the robust genetic structure of the dominant gastropod species in intertidal areas of the Ryukyu Islands. Nishikawa *et al*. (2003) also showed the genetic divergence between Central Ryukyu and South Ryukyu, not only for the coral with the restricted larval dispersal ability *Stylophora pistillata* but also for that with the high ability *Acropora tenuis*. These results show that at least some marine species can serve as interesting materials for studies of evolution on these islands. *Batillaria multiformis* and *B. cumingi* have been found on the western coast of the Korean Peninsula (An and Koh, 1992; Sato, 2002). In addition, another congeneric species, *B. sordida*, is found on Taiwan and China. Genetic information on populations of *B. multiformis* and related species in neighboring areas will help us to understand evolutionary processes on the Ryukyu Islands and on the Japanese Islands.

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