**Introduction**

Reef-building (or hermatypic) corals build the trophic and structural bases of the coral reef ecosystems in the shallow sea of the tropical and sub-tropical zones. Hermatypic corals are cnidarians that form calcified hard skeleton and harbor dinoflagellate endo-symbionts *Symbiodinium* spp. (Baker 2003, Coffroth & Santos 2005), and their majority are scleractinian corals that form colonies consisting of numerous polyps. Taxonomy of hermatypic corals was traditionally based on skeletal morphology (Veron 2000). In recent years, revised frameworks of the phylogeny of the order Scleractinia have been proposed using molecular tools (e.g. Romano & Palumbi 1996, Fukami et al. 2004). On the other hand, the majority of hermatypic corals, including many common species, are yet to be carefully examined for their species status due to the lack of molecular markers and morphological traits that are useful in distinguishing closely related species or cryptic species.

**Galaxea fascicularis** (Occuliniidae) is a hermatypic coral that forms massive colonies, and is widely distributed in the Indo-Pacific Ocean and Red Sea (Veron 2000). This species is still commonly found throughout the Ryukyu Archipelago in southwestern Japan, even around Okinawa Island where the decline of hermatypic coral populations is marked. In this species, occurrence of variants in nematocyst morphology and color pattern is known (Hidaka & Yamaizato 1985, Kusen 1987, Hidaka 1992, Wewengkang et al. 2007).

Galaxea fascicularis colonies in Okinawa are classified into two morphotypes (the ‘soft’ and ‘hard’ types that will be referred to as type S and H, respectively) based on the morphology of a nematocyst type, microbasic *p*-mastigophore (MpM), in the acrosphere of the external tentacle (Hidaka 1992, Watanabe et al. 2005). Type S MpMs have relatively thick capsules and shafts about a half of the capsule length, whereas in type H MpMs the capsule is relatively slender and the shaft is shorter than one third of the capsule length (Hidaka 1992). Occasionally, individual colonies are found that possess mixtures of the two MpM types in the same tentacle; they are referred to as 'mixed'
A previous study (Watanabe et al. 2005) showed that the MpM type (H or S) was highly correlated with the presence or absence of a 290 bp deletion in a mitochondrial (mt) cyt b-ND2 intergenic region. The haplotypes with or without the deletion will be referred to as mt-S (short) and mt-L (long), respectively. These results have suggested the possibility that G. fascicularis in Okinawa consists of two genetically differentiated groups that can be distinguished based on the MpM morphology and the mt genotype.

The present study was performed to evaluate the extent of inter-breeding between the two groups in G. fascicularis. To address this issue, the genotype at a nuclear microsatellite locus was analyzed in more than 200 colonies, and the frequency of individuals carrying a hybrid genotype was determined. Using those samples, we also aimed at assessing correlation between color pattern variants in G. fascicularis, and MpM types or the genotypes. The diversity of genotypes, color morphs of G. fascicularis, and the correlation with these two factors will be discussed.

**Materials and Methods**

**Coral samples**

*Galaxea fascicularis* samples were collected in the following five locations: a reef-slope near Cape Zampa in Okinawa Island (“Zampa” in Fig. 1), reef-slopes near Sunabe in Okinawa I. (“Sunabe”), reefs around Aka I. (“Aka”), a reef on the northwestern side of Taketomi I. (“Taketomi”), and Nagura Bay of Ishigaki I. (“Nagura”). Two to three polyps were collected per colony, and kept in seawater until their nematocyst morphology was observed. Thereafter, the samples were kept in 95% ethanol to preserve genomic DNA.

**Determination of the nematocyst type and color morph**

The morphological type (S vs. H) of microbasic p-mastigophores (MpMs) was determined as described by Hidaka (1992). In some specimens, mixtures of the two MpM types were found. When the minor type exceeded 10% in counting more than 50 MpMs, the individuals were called type M (mixed). *Galaxea fascicularis* individuals were classified into the following six color morphs by visual inspection *in situ* during sampling: B (brown or pale brown polyps), BG (brown polyps with greenish oral region), Gs (brown polyps with green septal tentacles), Gt (brown polyps with green external tentacles), Wt (brown polyps with pale green (almost white) fluorescent tentacles), and Ft (brown polyps with fluorescent green tentacles). The B, BG, Gs, Gt and Wt morphs are the same as described by Hidaka & Yamazato (1985).

**Genotype analysis**

Determination of the mt haplotype class was performed as described by Watanabe et al. (2005). Briefly, genomic DNA was isolated from 1–2 polyps per colony, and subjected to PCR with primers 188-2 and 188-R2 to amplify a part of the mt intergenic region between the cyt b and ND2 loci. The PCR products were electrophoresed with molecular weight markers (100 bp DNA ladder, TaKaRa) on 1.5% agarose gels, and the haplotype class (mt-L and -S) was determined based on the mobility. The PCR products from

![Fig. 1. Locations (shown with asterisks) of the five sampling sites in Okinawa, Japan.](image-url)
mt-S and mt-L samples are approximately 430 and 720 base pairs (bp), respectively.

Total genomic DNA isolated from the sperm of an mt-S individual (collected near Cape Zampa, Okinawa, Japan) was used to construct an (AG)n/(AC)n-enriched library. A modified hybrid capture method adapted from Carleton et al. (2002) and the Kocher Laboratory protocol (http://hcgs.unh.edu/protocol/msat) was followed, except that the cycle numbers of the pre-hybridization polymerase chain reaction (PCR) and the PCR to amplify captured DNA fragments were set to 15 and 24, respectively. In sequencing clones in the library, a genomic sequence containing a (GACT)n repeat was found (Fig. 3). The PCR to amplify this sequence was performed with primers (MS-F1, 5'-TGAGCTACAGTGCTAATA-3'; MS-R1, 5'-TAGYGGGGAATTGTTTGTC-3') as described by Takeshima et al. (2005), except that the annealing and extension were at 62°C for 30 s and at 72°C for 20 s, respectively. Allele sizes were determined using GENEMAPPER version 3.0 (Applied Biosystems) and 500LIZ size standard (Applied Biosystems). Hardy-Weinberg equilibrium was tested using GENEPOP (Raymond & Rousset 1995).

Table 1. MpM type, color morph and mt haplotype in Galaxea fascicularis colonies at five locations in the Ryukyu Archipelago

| Location   | N of samples | MpM type | Color morph | mt haplotype |
|------------|--------------|----------|-------------|--------------|
|            |              | S | H | M | ND | B | Ft | Wt | Gs | BG | Gt | ND | mt-L | mt-S |
| Zampa      | 35           | 15 | 12 | 5 | 3  | 10 | 8  | 7  | 2  | 0  | 8  | 0  | 5    | 13   |
| Sunabe     | 27           | 11 | 14 | 2 | 0  | 4  | 2  | 13 | 1  | 2  | 0  | 5  | 0    | 12   |
| Aka        | 71           | 42 | 11 | 12 | 6  | 15 | 9  | 17 | 6  | 7  | 2  | 15 | 15   | 16   |
| Taketomi   | 30           | 12 | 17 | 0 | 1  | 14 | 11 | 0  | 0  | 1  | 4  | 14 | 14   | 16   |
| Nagura     | 61           | 18 | 41 | 2 | 0  | 26 | 19 | 1  | 4  | 2  | 5  | 4  | 21   | 40   |
| Total      | 224          | 98 | 95 | 21| 10 | 69 | 49 | 38 | 13 | 11 | 8  | 36 | 127  | 97   |

ND: not determined.

Table 2. Occurrence of seven genotypes at the MS-1 locus at five locations in the Ryukyu Archipelago

| Location       | Number of individuals with given genotypes at the MS-1 locus |
|----------------|-----------------------------------------------------------|
|                | 118/118 | 118/122 | 118/130 | 126/126 | 126/130 | 130/130 | 130/134 | He  | Ho   |
| Zampa (35 colonies) | 20      | 0       | 0       | 1       | 7       | 7       | 0       | 0.5669 | 0.2000*** |
| Sunabe (27)     | 12      | 0       | 0       | 0       | 6       | 8       | 1       | 0.6084 | 0.2593**  |
| Aka (71)        | 53      | 1       | 0       | 1       | 9       | 6       | 0       | 0.3999 | 0.1408*** |
| Taketomi (30)   | 12      | 0       | 2       | 3       | 6       | 7       | 0       | 0.6378 | 0.2667*** |
| Nagura (61)     | 16      | 1       | 0       | 5       | 24      | 15      | 0       | 0.6473 | 0.3770*** |
| Total (224)     | 113     | 2       | 2       | 10      | 52      | 44      | 1       | 0.6047 | 0.2487*** |
| Group 1 total (115) | 113     | 2       | —       | —       | —       | —       | —       | 0.0172 | 0.0174   |
| Group 2 total (107) | —      | —       | —       | 10      | 52      | 44      | 1       | 0.4465 | 0.4766   |
| Hybrid pattern (2) | —      | —       | —       | 2       | —       | —       | —       | —     | —       |

He: expected heterozygosity; Ho: observed heterozygosity.
** and *** indicate statistical significance in the deviation from HWE (p<0.01 and 0.001, respectively).

Coral samples

Fragments were collected from a total of 224 Galaxea fascicularis colonies from five locations of the Ryukyu Archipelago (Fig. 1). These specimens were observed for MpM type and color morph, and analyzed for genotypes at the mt intergenic region and a nuclear microsatellite locus (Tables 1 and 2).

In observing the nematocyst morphology (Table 1), almost equal numbers of colonies possessing type S and H MpMs were found (98 and 95 colonies, respectively). Twenty-one colonies were of type M. The MpM type could not be scored in ten samples due to failure to dissect tentacle acrospheres.

It was possible to determine the color morph in 188 colonies, but not in the other 36 colonies, which exhibited intermediate or rare color patterns. In exploring new sampling sites, a new color morph, Ft (fluorescent tentacle), was found. This color morph was similar to the Wt (white tentacle) morph, but could be distinguished based on higher levels of fluorescent green pigmentation in external tentacles (Fig. 2). The Ft morph occurred at high frequencies in the Yaeyama area (Taketomi and Nagura).
The mt genotypes were scored in all of the 224 individuals.

Genotype at the MS-1 locus

PCR was performed with the genomic DNA from the 224 samples to amplify a genomic region containing (GACT)n repeats (EMBL/GenBank/DDBJ accession number AB272101), and five alleles of 118, 122, 126, 130 and 134 bp were found. These alleles were named allele 118, 122, 126, 130 and 134. Alleles 118 and 130 were cloned and sequenced (Fig. 3). The two sequences contained 3 and 6 copies of the GACT repeat, respectively. This microsatellite locus was named MS-1.

Seven genotypes were observed in the 224 samples (Table 2). Of these, homozygotes for allele 118 (118/118) occurred at the highest frequency, followed by 130/130, 126/126 and 118/118, which were commonly observed at all of the five locations (Table 2). On the other hand, alleles 122 and 134 were found only in 2 and 1 individuals in total, respectively. At all of the locations, the observed heterozygosity (Ho in Table 2) was much lower than the expected heterozygosity (He), and deviation from the Hardy-Weinberg equilibrium (HWE) was significant (Table 2). Of the three possible heterozygous combinations (126/130, 118/130 and 118/126) of the three common alleles, the latter two were rare or absent (found only in 2 and 0 colonies, respectively).

The absence or deficiency of 118/130 and 118/126 is consistent with the possibility that the *G. fascicularis* populations in Okinawa consist of two isolated groups, one carrying alleles 118 and 122 (Group 1 in Table 2) and the other carrying 126, 130 and 134 (Group 2). The two colonies heterozygous for alleles 118 and 130 (118/130) were assumed to be hybrids or their offspring. Within the two groups, Ho was nearly equal to He, and significant deviation from HWE was not observed (Table 2).

The mt genotype, MpM type and color morph in the two groups

Frequencies of the two mt haplotypes (mt-L and mt-S),
three MpM types (S, H and M), and six color morphs (B, Ft, Wt, Gs, BG and Gt) were compared between Group 1 and 2 (Table 3). All of the Group 1 colonies carried the mt-L haplotype, whereas in Group 2 more than 90% of the colonies were mt-S. Thus, the frequencies of the two haplotypes were significantly different between the two groups ($\chi^2$ test, $p<0.01$). Similarly, difference in the frequencies of the MpM types between the two groups was significant ($\chi^2$ test, $p<0.01$) (Table 3). Type S MpMs were observed in 76% of the Group 1 colonies, whereas in Group 2, type H MpM was predominant (observed in 82% of the colonies). In contrast, the two groups did not show significant difference in the frequencies of the six color morphs ($\chi^2$ test, $p>0.05$) (Table 3). The occurrence of the color morphs did not differ significantly among the three groups of colonies with type H, S and M MpMs, or between the groups of mt-S and mt-L colonies (data not shown).

Discussion

The results of the present study support our previous view that Galaxea fascicularis in Okinawa consists of two genetically differentiated groups (Watanabe et al. 2005). The results shown in Table 2 argue that the reproductive barrier between Groups 1 and 2 is nearly impermeable. The analysis shown in Table 3 indicates that the two groups are highly different in the mt genotype and MpM morphology, but not in occurrence of the color morphs. We discuss below (1) the possible mechanisms of reproductive isolation between the two groups, and (2) genetic basis of the MpM morphology and color pattern.

Reproductive barrier between the two lineages of Galaxea fascicularis

In this study, more than 99% (222 out of 224) of the Galaxea fascicularis colonies collected in the Ryukyu Archipelago were classified into two groups based on the allelic difference at the MS-I locus. In Group 1, all of the examined individuals carried the mt-L haplotype, and in their majority the MpM type was S (Table 3). By contrast, the mt-S haplotype and type H MpM were predominant in Group 2. Thus, the grouping in the present study is consistent with the results of our previous report (Watanabe et al. 2005). Only two individuals could be found to carry an allele combination (118/130) expected for hybrids or their offspring, suggesting that the breeding between the two groups takes place very rarely, or the fitness of the hybrid offspring is low. Thus, the two groups may be regarded as cryptic or sibling species (Knowlton 1993). This information will be useful in population analyses of this common hermatypic coral.

The mechanisms underlying the reproductive barrier between the two sympatric groups have not been fully understood. However, the barrier may be in part due to different timing of spawning. Difference in reproductive timing was observed between colonies of the two MpM types (Heyward et al. 1987, Yamazato 1988), suggesting that the breeding within the groups is more likely to occur than inter-breeding. The reproductive isolation may also be explained by relative incompatibility between gametes from different groups. A cross-fertilization experiment was attempted between a Group 1 father (mt-L) and Group 2 mother (mt-S), when their spawning took place the same night (Abe et al. in press). The proportion of eggs that initiated cleavage was about 5%—a fertilization rate much lower than in crosses between colonies of the same MpM types (usually 40% or higher, Abe et al. in press). Thus, both pre-zygotic and post-zygotic factors are likely to contribute to the prevention of breeding between the two groups.

Nematocyst types and color morphs

Currently, the genetic basis for the MpM morphology is unknown. A question that remains unsolved is which of (or whether either of) the S and H MpM phenotype is dominant over the other. The presence of both type S and H MpMs in type M individuals may suggest the possibility that these individuals are heterozygous for hypothetical type S and H alleles with co-dominant phenotypes. However, this possibility was not supported by the results of this study. Both of the two individuals heterozygous for 130 and 118 had type S MpMs. Moreover, none of the 21 type M individuals examined in the present study exhibited heterozygosity that was indicative of hybridization (e.g. 118/130 or 118/126) (Table 3).

In previous studies (Kusen 1987, Hidaka 1992, 1993, Inoue 1998), only two individuals could be found to carry an allele combination (118/130) expected for hybrids or their offspring, suggesting that the breeding between the two groups takes place very rarely, or the fitness of the hybrid offspring is low. Thus, the two groups may be regarded as cryptic or sibling species (Knowlton 1993). This information will be useful in population analyses of this common hermatypic coral.

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Wewenkang et al. 2007), observations were made on the nematocyst morphology and color patterns in *G. fascicularis* collected at three locations around Okinawa I. Correlation between certain color morph and MpM types was suspected (Hidaka 1992), but the results were not conclusive due to small sample sizes. In this study, observing a larger number of colonies in five locations, no correlation was observed between the six color morphs and the *MS-1* genotype (or the mt genotype and MpM type). All of the six color morphs occurred in both groups, and their relative abundance did not differ greatly between the two groups. This result argues that the evolution of the genetic systems that produce various color patterns had taken place before the divergence of *G. fascicularis* into the two lineages.

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