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REPORT

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Genotyping the clonal structure of a gorgonian coral, *Junceella juncea* (Anthozoa: Octocorallia), using microsatellite loci

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Abstract The identification of different clones is fundamental to the study of population structure among organisms with mixed reproductive modes such as cnidarians. However, due to the low genetic variation of coral mtDNA and contamination by zooxanthellate DNA, very few molecular markers are available for studying the clonal structure of cnidarians. Herein we used four polymorphic loci of microsatellite DNA isolated from a zooxanthellae-free octocoral, *Junceella juncea*, to study its clonal structure in seven populations collected from three localities in Taiwan. In total, 40 multilocus genotypes were found among 152 colonies, and the number of genotypes (clones) identified in the seven populations ranged from 2 to 16. Each of the 40 multilocus genotypes was restricted to a single population, even where adjacent populations were only 100 m distant. The ratio of observed to expected genotypic diversity (G_o:G_e) ranged from 0.217 to 0.650, and G_o showed a significant departure from G_e ($p < 0.05$) at each site indicating that asexual fragmentation may play a major role in the maintenance of established populations. Mean relatedness (R) values showed that genotypes within reefs were more closely related than those between regions. The results indicate that microsatellites are useful for discerning the clonal structures among and within populations at different spatial scales.

Introduction

Clonal propagation is a common feature of marine benthic organisms. It facilitates the rapid recolonization of disturbed areas and allows established genotypes to dominate benthic habitats (Jackson 1985). Anthozoans are typical benthic organisms that possess a mixed mode of reproduction, utilizing sexually produced propagules for long-distance dispersal, and vegetative propagules for rapid expansion (Hughes 1987; Hughes et al. 1992). Such mixed reproductive modes have profound effects on population structures. Clonal growth can increase the reproductive output of a genetic individual, while allowing an individual to expand and monopolize resources. It may also affect the tempo and mode of evolution by increasing generation times and altering both genetic diversity and effective population size (Hughes et al. 1992).

The levels of sexual and asexual reproduction may vary with biotic and abiotic factors including environmental variation and disturbance (Coffroth and Lasker 1998). It has been suggested that asexual reproduction will be favored in either geographically isolated or ecologically stable habitats (Hughes et al. 1992; Hunter 1993). However, recent studies have shown that populations of *Pocillopora damicornis* on high latitude reefs as well as on the tropical Great Barrier Reef are maintained mainly through sexual reproduction, and that asexual reproduction contributes little to overall population structure (Miller and Ayre 2004). Given this controversy, studies on clonal structure take on added importance for discerning the contribution of sexual and

Electronic supplement: Unique multilocus genotypes (clones) revealed by 4 polymorphic loci for *Junceella juncea* colonies collected from Xiashuijui (Reefs A, B, C, Transplant, Transect), Nanwan and Shicheng

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asexual reproduction, and for testing hypotheses concerning the evolution of colonial organisms (Hughes et al. 1992). However, it is difficult to discriminate asexually derived ramets from sexually produced genets by morphological characteristics and field experiments (Coffroth et al. 1992).

Several molecular markers including allozymes, mitochondrial DNA, and a few nuclear genes have been applied to study evolutionary and population genetics in anthozoans. Population genetic studies in corals have traditionally relied on analyses of allozymes (Adjeroud and Tsuchiya 1999; Yu et al. 1999; Ayre and Hughes 2000; Dai et al. 2000; Ng and Morton 2003; Miller and Ayre 2004). However, allozyme data may overestimate levels of clonality due to the lower level of allelic diversity at loci (Stoddart et al. 1985; Willis and Ayre 1985). Minisatellites have also been applied to study the clonal structure of the Caribbean gorgonian, *Plexaura kuna* (Coffroth et al. 1992; Coffroth and Lasker 1998). Although the resolution of minisatellite data is sufficient to identify genotypes within a population, there is potential confusion caused by the fingerprint of zooxanthellae, and it should be excluded before genotype analyses (Coffroth et al. 1992).

Microsatellites, tandem simple sequence repeats of 1–6 bp, are distributed throughout the genomes of eukaryotes (Ashley and Dow 1994). Because of their high variability in repeat size, microsatellites have become the molecular marker of choice to distinguish among closely related individuals and/or taxa (Marquez et al. 2003; Li et al. 2002). In cnidarians, Chen et al. (2002) isolated a microsatellite locus from the 5′-terminus of the IGS (intergenic spacer) of the ribosomal RNA gene and used it to assess the clonal structure of *Junceella fragilis* in Taiwan. Results indicated that the local population of *J. fragilis* was mainly composed of asexual recruits from only a few sexual founders. It seems that the high polymorphic level of microsatellites may provide a useful tool to solve the complexity of clonal structure in anthozoans.

Junceella juncea is an azooxanthellate whip-like octocoral widely distributed on Indo-Pacific reefs (Chen and Chang 1991). In some localities, it forms dense patches, but at other localities it is sparsely scattered. It is gonochoric with an annual cycle of gametogenesis (TY Fan, unpublished data). *J. juncea* conducts active fragmentation by weakening its axial skeleton and tissue matrix. Asexual propagules are formed when the axial skeleton fails and a fragment from the colony tip detaches, which is often facilitated by strong wave forces (Fan TY, unpublished data). Since spontaneous fragmentation frequently occurs among colonies of *J. juncea* (TY Fan, unpublished data), it is very difficult to distinguish genets from ramets in an established local population. We used four microsatellite loci isolated from *J. juncea* to reveal the clonal structure of seven populations collected from three localities, and to assess the relative contributions of sexual and asexual reproductive modes.

Materials and methods

Sampling

Samples of *J. juncea* were collected from three localities, Xiashuijui and Nanwan in southern Taiwan, and Shicheng in northern Taiwan (Fig. 1). The sampling method at different sites was mainly constrained by the number of coral colonies we could find. At Xiashuijui, where *J. juncea* colonies were abundant, three types of sampling schemes were applied. First, 36 colonies were collected at a regular distance (1 m) along a 50-m long transect to avoid collecting clonemates. Second, three patch reefs (A–C), each separated by more than 100 m, were sampled, and all colonies in an area of about 16 m^2 were mapped by drawing their relative positions on waterproof paper in situ. Sample sizes (numbers of colonies) for reefs A–C was 11, 13, and 8, respectively. Third, samples were collected from an area of about 100 m^2 at 10 m depth, where 50 colonies of *J. juncea* had been transplanted from 17 m depth and had been monitored since May 2000. The original colonies were longer than 25 cm, but in March 2002 there were 21 colonies >25 cm and 36 colonies <25 cm, the latter generated by partial mortality or fragmentation of original transplants (Fan TY, unpublished data). Samples from all 57 colonies that were found were collected. At

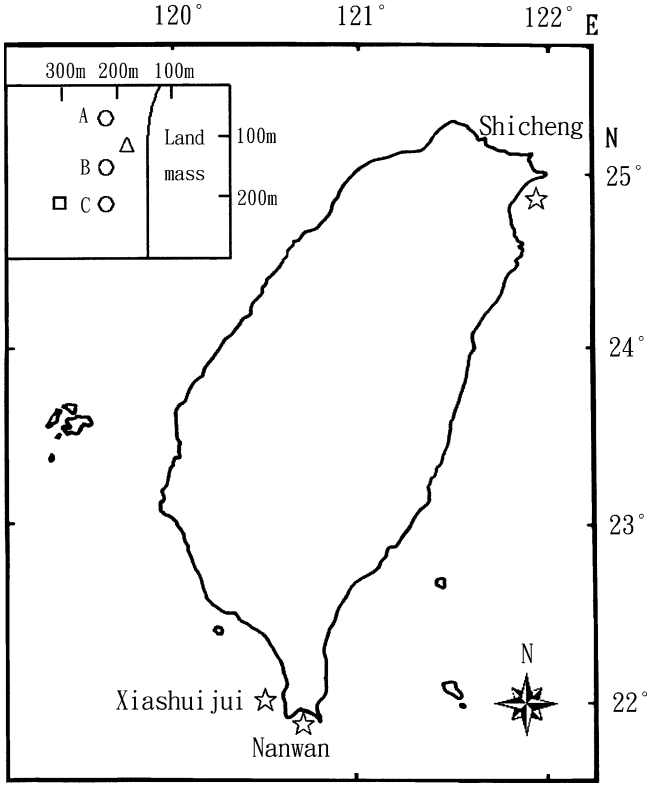

Fig. 1 Map showing the collection sites (?). *Inset* on the upper corner indicating the positions of three patch reefs (A–C) in *hollow circles*, the transplant population in triangle, and the transect in *square*

Nanwan and Shicheng, where colonies of *J. juncea* were sparse, only 21 and 6 samples were collected in an area about 10,000 m^2, respectively.

A small fragment was broken off from the tip of each colony and preserved in 95% EtOH for DNA extraction. Genotypes of these colonies were then analyzed to infer the clonal structure of *J. juncea* on the three reefs.

Isolation of microsatellite loci, PCR optimization, and genotyping

The DNA extraction protocol followed the description in Liu et al. (2005). Four polymorphic loci were applied to the analysis of clonal structure. The *J*4 locus was isolated from 235 potential clones that were generated using the enrichment method (Hamilton et al. 1998). The *J*201, *J*349, and *J*639 loci were isolated from 3856 partial library clones generated using procedures modified from Yu et al. (2002). Grading PCR conditions were optimized as follows: 5 min at 95°C; 30 cycles of 45 s at 94°, 40 s at the annealing temperature (45–60°), and 45 s at 72°; followed by 72° for 10 min. Standard reaction conditions after optimization were 25 ng of each primer, 1.5–3.0 mM $MgCl_2$, 10 times PCR buffer, 2.5 nM dNTPs, 1 unit of Taq DNA polymerase, and 2–5 ng DNA in a 25-μl reaction volume. Primers were labeled with fluorescent dye (TAMRA, FAM, and HEX), and PCR products were visualized and sized by automated detection with gel electrophoresis using a MegaBACE 500 automated sequencer.

Data analysis

The genetic diversity of loci was calculated using the MSA analysis program (Dieringer and Schlotterer 2003). The expected heterozygosity (H_e) and observed heterozygosity (H_o) of each locus and geographic regions were calculated. Hardy–Weinberg expectations for each locus and region were tested by the Markov-chain method with the program GENEPOP Version 3.3 (Raymond and Rousset 1995; available at http://wbiomed.curtin.edu.au/genepop), which implements Fisher's exact tests for multiple alleles (Guo and Thompson 1992).

Indices of genotypic diversity were calculated from the number of genotypes identified on the seven reefs. First, the observed genotypic diversity, G_o, was calculated as

$$\mathrm{Go} = 1 \Big/ \sum_{l}^{k} g_i^2,$$

where g_i is the relative frequency of ith genotype and k is the number of genotypes (Stoddart 1983). G_o varies from a minimum of 1, when all individuals belong to a same genotype, to a maximum of k, when numbers of individuals of each genotype are evenly distributed. The ratio of observed genotypic diversity (G_o) to the expected genotypic diversity (G_e) under conditions of random sexual reproduction can be used to assess the relative importance of asexual fragmentation. Departures of G_o:G_e were assessed by *t*-tests (Stoddart and Taylor 1988). The proportion of unique multilocus genotypes, N_c/N, where N_c is the number of unique genotypes and N is the sample size, was used to represent the genotypic diversity and the extent of clonal propagation (Ellstrand and Roose 1987). Values of N_c/N and G_o/G_e range from 0 to 1, with higher values indicative of higher genotypic diversities. In addition, we calculated the Shannon–Weaver index of diversity (H') as an index of clonal diversity (Magurran 1988). The evenness index, $E = H'/\ln N_c/N$, was also calculated. E approaches 0 when a single clone dominates the population, and its maximum value is 1, when all individuals are distributed evenly among clones.

Genetic variation

Relatedness (R) was calculated to represent the genetic relationship between multilocus genotypes (Queller and Goodnight 1989):

$$R = \sum_{x}\sum_{k}\sum_{l}(Py - P*) \Big/ \sum_{x}\sum_{k}\sum_{l}(Px - P*)$$

where x indicates individuals in the data set, k is the locus, l is the allelic position (i.e., $l = 1$ or 2 for a diploid or 1 for a haploid individual), and P_x is the frequency within the current x individuals of the allele at x's locus k and allelic position l. The P_x value in a diploid must be either 0.5 or 1.0. P_y is the frequency of an allele in the set of "partners" of x individuals for measuring x's relatedness. $P*$ is the frequency of the allele in the population at large, with all putative relatives of x excluded. Since the relationship between colonies is unknown, we assume that $P* = 0$ when calculating R.

Results

Levels of polymorphism varied among the four loci (*J*201, *J*349, *J*639, and *J*4). The observed heterozygosity (H_o) and expected heterozygosity (H_e) within regional populations ranged from 0.17 to 1.00 and 0.22 to 0.93, respectively (Table 1). Significant deviation (heterozygote deficiency, $p < 0.01$) occurred in 4 of 12 possible tests, among two regional populations (Xiashuijui and Nanwan) and three loci (*J*201, *J*349, and *J*639) except *J4* (Table 1).

In total, 152 samples of *J. juncea* were analyzed using the four polymorphic loci. Among these samples, we identified 40 unique multilocus genotypes. In the transplanted population of 57 colonies, only three genotypes were identified with one dominant genotype containing 55 colonies and two minor genotypes each represented

Table 1 Hardy–Weinberg test of heterozygosity deficiency within regional populations and individual locus

Locus	Population	H_e	H_o	Mean H_o
*J*639	Xiashuijui	0.58	0.48*	0.37*
	Nanwan	0.69	0.62	
	Shicheng	0.73	1.00	
*J*201	Xiashuijui	0.90	0.75*	0.75*
	Nanwan	0.77	0.75	
	Shicheng	0.93	0.66	
*J*349	Xiashuijui	0.69	0.24*	0.19*
	Nanwan	0.65	0.37*	
	Shicheng	0.61	0.33	
*J*4	Xiashuijui	0.22	0.17	0.03
	Nanwan	0.00	0.00	
	Shicheng	0.00	0.00	

H_e expected heterozygosity, H_o observed heterozygosity, *Mean* H_o mean heterozygosity of locus, an *asterisk* (*) indicates H_o significantly differs from H_e ($p < 0.01$)

by a single colony. Sixteen genotypes were recognized in 36 colonies collected along the transect at Xiashuijui. The low values of N_c/N and G_o:G_e at reefs B and C, and the transplanted population suggest that a high degree of asexual propagation occurs in these populations (Table 2). Evenness varied markedly among reefs, ranging from 0.046 for the transplanted population to 0.665 for reef A where six genotypes were identified among 11 colonies. G_o differed significantly from G_e among all populations (Table 2). Populations from reef A and the transect had higher evenness values, while those at Nanwan and Shicheng showed moderate evenness values. The distribution of genotypes on the three patch reefs at Xiashuijui (Fig. 2) indicated that reefs B and C were mainly dominated by single clones.

Results of the Hardy–Weinberg test showed significant departures from expectations in most of the polymorphic loci either in whole samples or unique multilocus genotypes. The deviation of loci and the bias due to different sample sizes suggest that *F*-statistics may not be suitable to calculate genetic difference among reefs. We then used unique multilocus genotypes to analyze the relatedness within and between populations. The *R* values within and between sites ranged from 0.416 to 0.970 and 0.290 to 0.587, respectively. Similar *R* values were found on both local and regional spatial scales, e.g., among five populations separated by 100 m at Xiashuijui (0.416–0.580) and among populations separated by more than 300 km at Xiashuijui, Shicheng and Nanwan (0.420–0.490). Moreover, some of the *R* values between the five populations at Xiashuijui were lower than those between Xiashuijui and Nanwan which were separated by a distance of 6 km (Table 3). This suggests that segregated populations on different spatial scales were likely formed by sexually reproducing founders that readily dispersed over the spatial scales that we sampled.

Discussion

The four polymorphic microsatellite loci provided sufficient resolution to discern the clonal structures of *Junceella juncea* populations since the genotypes among localities did not overlap even within closely located patch reefs at Xiashuijui. Nonmetric multidimensional scaling analysis, which was used to visually describe the relationship of multilocus genotypes, also supports the conclusion that the four polymorphic loci can discern coral individuals and clones (Liu et al. 2005).

The significant deviations from Hardy–Weinberg expectations were primarily due to heterozygote deficits, which can be generated by clonal propagation, inbreeding or the stochastic error of small sample size (Hoffmann 1986; Stoddart 1984; Ayre and Hughes 2000). The low ratios of observed and expected genotypic diversity and the fact that most colonies at a site belong to one genotype suggest that the heterozygote deficits were mainly caused by the predominance of clonal propagation of *J. juncea*.

The genotypic diversity of *J. juncea* populations, indicated by both N_c/N and G_o/G_e, was lower than expected for sexually reproducing populations. Low genotypic diversity has been reported in other marine clonal species (Stoddart 1984; Hoffmann 1986; Burnett et al. 1995; Coffroth and Lasker 1998; Chen et al. 2002), suggesting that these species rely heavily on clonal population growth. This was especially evident for the transplanted population. The dominance of a single clone in the transplanted population indicates the ge-

Table 2 Genotypic diversity measures of *Junceella juncea* collected from Xiashuijui (Reefs A–C, Transplant, Transect), Nanwan and Shicheng

	N	N_c	N_c/N	G_o	G_e	G_o:G_e	*E*	*p*
Xiashuijui								
Reef A	11	6	0.550	4.187	7.631	0.549	0.665	< 0.05
Reef B	13	2	0.150	1.166	3.046	0.383	0.105	< 0.05
Reef C	8	2	0.250	1.278	3.259	0.392	0.184	< 0.05
Transplant	57	3	0.053	1.054	3.895	0.271	0.046	< 0.05
Transect	36	16	0.440	5.394	23.014	0.234	0.644	< 0.05
Nanwan	21	8	0.380	2.512	11.573	0.217	0.454	< 0.05
Shicheng	6	3	0.500	2.574	3.962	0.650	0.564	< 0.05

N sample size, N_c number of unique multilocus genotypes, N_c/N proportion of distinguishable genotypes, G_o observed genotypic diversity, G_e expected genotypic diversity, *E* evenness index (*p*), *p* values of *t*-test for G_o:G_e

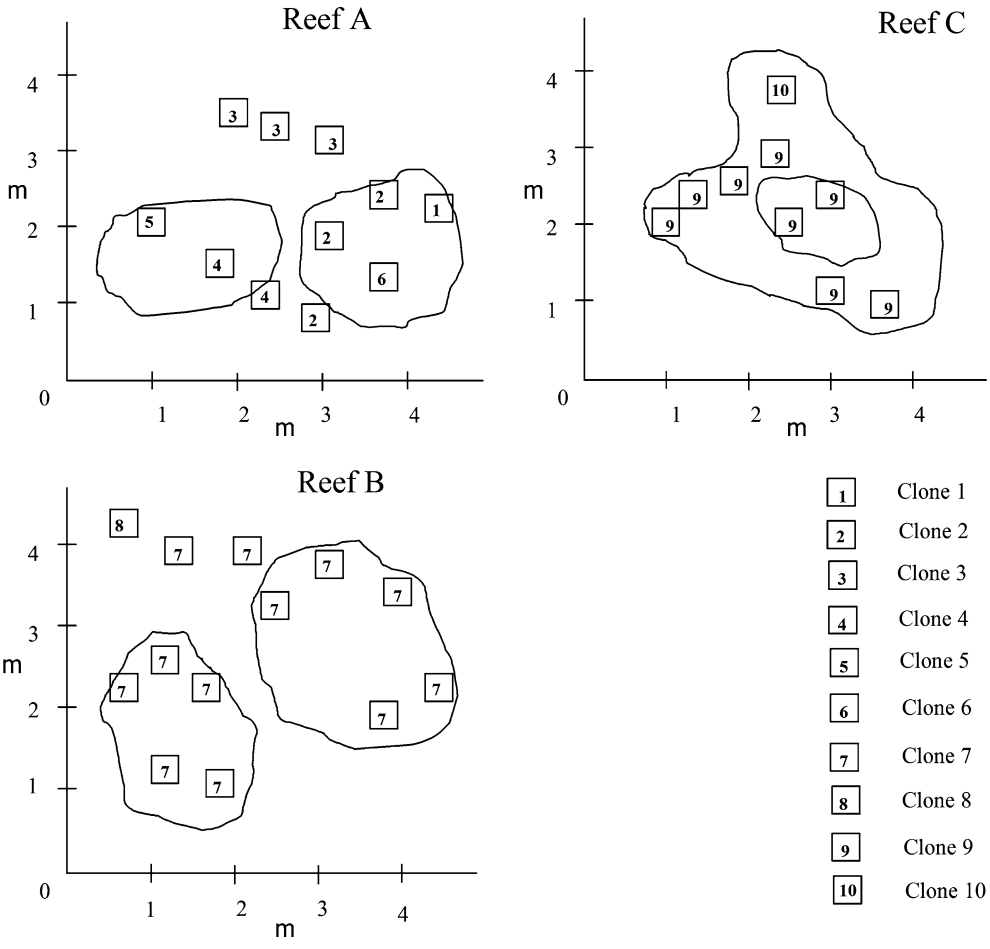

Fig. 2 Maps showing the distribution of *Junceella juncea* clones at three patch reefs at Xiashuijui. *Numbers in squares* indicated the clone number (see the electronic supplement). *Solid lines* on each patch reef indicate the contour of blocks on reef substrate

netic variation of the original population was very low. Furthermore, all the new recruits were generated through asexual fragmentation.

Low genotypic diversity has been observed in other gorgonians, e.g., *Plexaura kuna* in the Caribbean (Coffroth and Lasker 1998) and *J. fragilis* at Lanyu (Orchid Island), Taiwan (Chen et al. 2002). Deviation of N_c/N from 1 has also been observed among several cnidarian taxa, indicating that asexual reproduction commonly occurs in cnidarians (Ayre 1984; Willis and Ayre 1985; Benzie et al. 1995). The low genotypic diversity of *J. juncea* suggests that asexual recruits are the major source for maintaining local populations, while sexual recruits of genotypically diverse larvae are the primary source of recruits on isolated reefs.

Clonal competition in sessile organisms may lead to the exclusion of subordinate genets and to the dominance of a few genets in an area (Hughes 1987; Hunter 1993). Disturbances including physical and biological factors may affect the clonal structure by increasing fragmentation rates and inducing differential mortality of genets (Coffroth and Lasker 1998). The frequent ty-

Table 3 Measurements of relatedness (R) between populations of *Junceella juncea* collected from Xiashuijui (Patch Reefs A–C, Transplant and Transect), Nanwan, and Shicheng

	Reef A	Reef B	Reef C	Transplant	Transect	Nanwan	Shicheng
Xiashuijui							
Reef A	0.416						
Reef B	0.416	0.667					
Reef C	0.575	0.550	0.700				
Transplant	0.587	0.580	0.523	0.970			
Transect	0.422	0.437	0.504	0.465	0.534		
Nanwan	0.472	0.290	0.461 $Mean_1 = 0.447$	0.501	0.511	0.432	
Shicheng	0.440	0.461	0.541 $Mean_2 = 0.490$	0.508	0.501	0.420	0.580

$Mean_1$ = average relatedness between the Nanwan and Xiashuijui populations
$Mean_2$ = average relatedness between the Shicheng and Xiashuijui populations

phoons around Taiwan are the possible disturbance factor that acts as a major source of mortality and controls the genotypic diversity of *J. juncea*.

Clonal species with a sex-ratio bias will decrease their effective population size and thus increases the degree of genetic drift. The sex-ratio bias in *J. juncea* (approximately one male in four to five colonies; Fan, unpublished data) suggests that the influences of genetic drift on isolated populations may be high and thus the low genotypic diversity. Moreover, genotyping revealed that some genets within a reef were closely related to one another. The degree to which a population receives recruits from local resources versus other populations has important ecological and management ramifications.

The high polymorphic levels of the four microsatellite loci applied in this study were useful in discerning coral genets and ramets. Despite the difficulties in isolating coral microsatellites from cnidarians (Marquez et al. 2003), microsatellite markers may provide a useful tool in estimating local retention and population connectivity in the future.

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