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Comparison of grafting versus a molecular genetic technique in identifying clone mates of the reef coral *Porites rus*

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ABSTRACT: The reef coral *Porites rus* is uncommon on reefs along the coast of south Moloka'i, Hawaii, USA, but where found, it covers extensive areas of the reef flat. This pattern suggests a long history of asexual reproduction through fragmentation and dispersal of colonies during large wave events. Genetic comparisons within and between 2 such large isolated populations were made using the classic technique of tissue grafting compared to a more recently developed molecular technique. Both approaches showed that the 2 populations are genetically distinct from each other. All samples taken from one population consisted of a single genotype, while the second population consisted of 2 genotypes that were distinct from the first population. The molecular technique is ultimately a more powerful tool for population studies, but is technically far more complex, expensive and time consuming. The grafting technique has the advantage of simplicity and very low cost, and is suitable for some types of investigations.

KEY WORDS: Population biology · Reef coral · Fragmentation · Clonal structure · Recruitment · Sexual reproduction · Asexual reproduction

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INTRODUCTION

Extensive coral reef surveys were conducted off south Moloka'i, Hawaii. USA, between 1998 and 2006 (Jokiel et al. 2008). An unusual aspect of the coral distribution is that the reef coral *Porites rus* is quite rare along this coastline, but when encountered, colonies cover large areas. A major population of this species was discovered on the shallow fore reef and reef flat on the west side of the channel leading into Kaunakakai, Moloka'i harbor (Site A, Fig. 1). Another smaller patch was located 7 km to the east on the reef crest at Kawela, Moloka'i (Site B). Such patchy population structure can be interpreted as the result of extensive asexual fragmentation of ancestor colonies in a highly favorable area rather than the result of recruitment of many sexually derived larvae (Cox 1992). Highsmith (1982) pointed out that natural fragmentation of corals creates many new colonies of the same clone and expands the territory occupied by a genotype, which increases its ability to produce gametes. Further, the large number of cloned colonies allows a higher chance of genotype survival.

The south coast of Moloka'i in the area of Kaunakakai falls into the 'wave shadow' of surrounding islands and is generally protected from the North Pacific swell and severe trade wind swell (Storlazzi et al. 2005). However, this area is vulnerable to infrequent storm waves associated with hurricanes or 'Kona' storms from the south. Hurricane Iwa caused extensive damage to the Kaunakakai wharf during late November 1982, and presumably the storm waves fragmented corals on the surrounding fragile reefs (Jokiel et al. 2008). Storm waves have built a berm of coral rubble to the west of the study area

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Fig. 1. Location of Site A on reef flat across the channel from Kaunakakai Wharf, and Site B on a reef flat at Kawela in south Moloka'i, Hawaii, USA

Site A (Fig. 1). The study site is characterized by environmental factors that favor rapid coral growth (high irradiance, low wave impact and good water circulation), but with rare storm wave events that lead to fragmentation and scattering of broken coral skeletons.

But how can we identify clone mates? Generalized self versus not-self recognition has been demonstrated in coelenterates (e.g. Hildemann et al. 1975, 1977, Bigger 1988) using grafting technique. These studies utilized autogeneic (self to self), allogeneic (2 different genotypes of the same species) and isogeneic (clone mate to clone mate) graft pairings. The grafting method provides one method of identifying clonal structure in natural populations (Jokiel et al. 1983, Hunter 1985), assuming that tissues of genetically identical colonies will fuse and tissues of genetically different colonies will reject each other. Validation of these assumptions was attempted over 20 yr ago using electrophoresis techniques (Heyward & Stoddart 1985, Willis & Ayre 1985). These 2 studies showed some inconsistencies in results obtained using the graft versus the electrophoretic method. Improved molecular techniques for describing the genotypes of coral are now available and are likely to identify clonal genotypes with a higher accuracy than the older electrophoretic techniques, but have not been tested against the grafting method.

The objective of this study was to compare the results of the classic grafting technique with a more recent molecular technique (microsatellite analysis) for determining the clonal relationships of corals. A double-blind experimental procedure was used throughout the comparison of the 2 methods. P.L.J. and K.S.R. conducted the grafting phase of the study and provided samples of each colony to S.A.K., who determined genetic relatedness of the corals using microsatellite markers without knowledge of the source of the material and without knowledge of the results of the grafting experiments.

MATERIALS AND METHODS

Coral collection

At Kaunakakai (Fig. 1, Site A) a single colony of sufficient size to produce several individual branches for grafting was collected at each of 15 points located at 5 m intervals along a 70 m transect line running from the seaward edge of the reef flat toward shore. The 15 colonies were labeled with their position on the line as numbers A0, A5, A10, A15…A70. GPS points were taken at each location where each colony was collected. Corals were submerged in large plastic tubs while being transported to an inshore site at Kawela (Fig. 1, Site B), where they were maintained on the reef flat on wire frames at a depth of approximately 1 m. The population of *Porites rus* at Kawela (Site B) was much smaller than that at Site A, so only 3 colonies were collected at 10 m intervals and labeled as B1, B2 and B3. These colonies were maintained on the wire frames separate from the wire frames holding the Kaunakakai corals.

Grafting experiments

The collected corals were brought into the field laboratory for grafting. Branches were cut from the colonies using bone shears, and graft pairings were made following procedures previously described (Hildemann et al. 1977, Jokiel et al. 1983). Colony grafting combinations were chosen haphazardly. The graft pairings were held securely in close tissue contact using monofilament nylon fishing line (50-pound test) that was stretched tightly and held securely in place using fishing leader crimps. Each graft pairing was labeled with a plastic tag embossed with a number, and the pairing combinations recorded for each graft. A subset of 42 graft pairings were made out of the 153 possible combinations since time and spatial

limitations prevented all potential groupings. The pairings were secured to 40 × 80 cm wire frames constructed of vinyl coated wire mesh. The completed experimental frames were moved to the area off Kawela where *Porites rus* occurs and secured with stakes driven into the bottom.

Eight months later (October 2007), the frames were recovered and brought to the laboratory, where each pair was photographed and a detailed examination was conducted at 45× magnification. If a boundary or gap persisted between the tissues of the 2 joined colonies (Fig. 2A), the pair was scored as 'rejection'; if the tissues healed completely and became confluent (Fig. 2B), the pair was scored as 'fusion'. The pairings scored as 'rejection' fell apart from each other when the monofilament ties were removed. In contrast, grafts scored as 'fusion' had calcified heavily into a single colony during the 8 mo growth period. There was no ambiguity in the scoring of skeletal fusion and rejection.

Molecular genetic analysis

Subsamples were taken from each colony and genotyped at 7 microsatellite loci following Baranets et al. (2011). A tailed primer method (Gaither et al. 2009) was used to genotype all samples. In this method, PCR reactions are carried out using 3 primers simultaneously: a low concentration of the forward primer with 1 of 4 unique tail sequences added, the reverse primer, and a dye-labeled primer. The dye-labeled tail primers are 19–24 bp in length with 1 of 4 fluorescent dyes attached at the 5′ end, and are identical to the forward primer tail sequences (see Gaither et al. 2009 for details; Applied Biosystems). PCR reactions were carried out in a 10 µl final volume containing 2–25 ng of template DNA, 0.35 pmol of tailed forward primer, 1.5 pmol of reverse primer, 1.5 pmol dye labeled tail primer, and 5 µl of the PCR solution MangoMix (Bioline). After an initial 10 min denaturation at 95°C, there were 38 cycles consisting of denaturation for 30 s at 94°C, 45 s at annealing temperature (see Baranets et al. 2011), and 45 s extension at 72°C with a final 30 min extension at 72°C. PCR products were pooled into 2 groups based on the colors of the fluorescent dye and the anticipated fragment size. PCR products were size sorted on an ABI 3100 Genetic Analyzer (Applied Biosystems).

The probability that 2 individuals randomly chosen from the population have the same multi-locus genotype by chance alone (i.e. probability of identity) was estimated using the Microsoft Excel (Microsoft) plug-in, GenAlEx (vers. 6.5, Peakall & Smouse 2006, 2012).

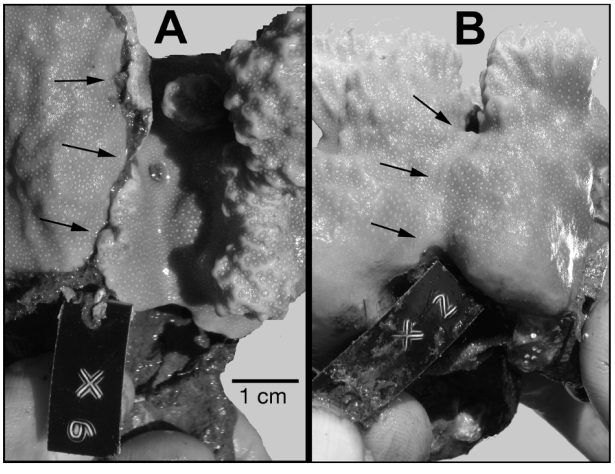

Fig. 2. *Porites rus.* (A) Tissue allograft rejection at points of contact, and (B) isograft tissue fusion

RESULTS

Grafting experiments

Of the 42 pairings, 34 could be scored. The remainder could not be scored due to mortality, breakage or loss. Results of the 34 pairings are shown in Table 1. All 7 autographs fused (Table 1). The 3 colonies (B0, B5, B10) taken at 10 m intervals in the Kawela site all fused with each other (B0 fused with B5 and B5 fused with B10), and thus, are all considered to be isogeneic clone mates. Nine of the Kaunakakai colony pairings also fused, even though the colonies were separated from each other by up to 70 m (Table 1). The 10 remaining Kaunakakai graft pairings showed rejection, indicating the presence of more than one genotype. The 6 pairings between Kawela and Kaunakakai colonies all showed rejection as expected due to the extreme spatial separation of the 2 groups (Fig. 1). Examples of grafting fusions and rejections are shown in Fig. 2. The result of grafting shown in Table 1 is presented visually in Fig. 3. This chart shows the clonal relationships between colonies as determined by grafting and was constructed prior to obtaining results of the molecular analysis. Fusions among the various Site A graft pairings indicate that colonies A25, A30, A35 and A45 are clone mates. Likewise colonies A0, A5, A15, A20, A60 and A70 are members of a second clone. All of the colonies from Site B fused and represent a single clone that is distinct from the clones at Site A.

Table 1. *Porites rus.* Summary of grafting and molecular results. Site A = Kaunakakai, Site B = Kawela. Colonies from Site A were taken at 5 m intervals and named as the distance (in m) along the 70 m transect line, using an 'A' prefix (A0, A5, A10, A15,…A70). The colonies from Site B were taken at 5 m intervals along a transect line and given a 'B' prefix. The Distance column refers to the distance between the 2 source colonies; isografts (from same colony) are shown as a distance of 0.1 m. Four genotypes were detected, but Genotypes I and II differed by only a single allele

Colony no.	Geno-type	Colony no.	Geno-type	Distance (m)	Result	Auto-geneic	Iso-geneic	Allo-geneic
B0	I	B0	I	0.1	Fusion	x		
B5	II	B5	II	0.1	″	x		
B10	II	B10	II	0.1	″	x		
A65	IV	A65	IV	0.1	″	x		
A40	IV	A40	IV	0.1	″	x		
A35	IV	A35	IV	0.1	″	x		
A50	IV	A50	IV	0.1	″	x		
A25	IV	A30	IV	5	″		x	
A0	III	A70	III	70	″		x	
A10	III	A55	III	45	″		x	
A15	III	A70	III	55	″		x	
A5	III	A70	III	65	″		x	
A30	IV	A35	IV	5	″		x	
A45	IV	A25	IV	20	″		x	
A20	III	A60	III	40	″		x	
A0	III	A60	III	60	″		x	
B0	I	B5	II	10	″		x	
B5	II	B10	II	10	″		x	
A0	III	A40	IV	40	Rejection			x
A20	III	A50	IV	30	″			x
A20	III	A40	IV	20	″			x
A5	III	A65	IV	60	″			x
A40	IV	A55	III	15	″			x
A15	III	A50	IV	35	″			x
A65	IV	A15	III	50	″			x
A10	III	A65	IV	55	″			x
A30	IV	A60	III	30	″			x
A10	III	A45	IV	35	″			x
A25	IV	B0	I	7000	″			x
A30	IV	B5	II	7000	″			x
A35	IV	B10	II	7000	″			x
A40	IV	B0	I	7000	″			x
A55	III	B10	II	7000	″			x
A45	IV	B5	II	7000	″			x

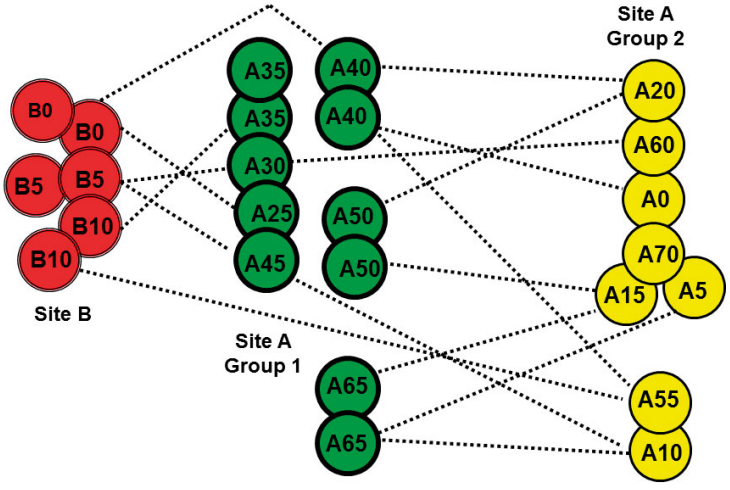

Fig. 3. Kaunakakai (Site A) and Kawela (Site B) grafting data from Table 1 plotted to visualize relationships between colonies as determined by graft pairings, and genotypes as determined in the microsatellite analysis. Overlapping circles = fusion; dotted lines between circles = graft rejection; numbers within circles = source colony name (numbered as distance along sampling line)

Molecular genetic analysis

Most individuals amplified for all loci. The samples from Site B, however, did not amplify at one locus (PRU_5D) even after repeated attempts. Four multi-locus genotypes were identified. Two genotypes from Site B differed by a single allele and corresponded to colony B0 (Genotype I) and B5 and B10 (Genotype II). The 2 genotypes from Site A differed by 11 of the 14 alleles, which is similar to the degree of genetic differentiation seen between the 2 sites (9 to 10 alleles). These genotypes corresponded to Group 1 and Group 2 in Fig. 3. The 2 similar genotypes at Site B fused. All other identical genotypes fused while all non-identical genotypes did not. The probability of identity over all loci was 1.8×10^{-6} when all individuals were used and 3.8×10^{-6} using just the 4 unique genotypes. When locus PRU_5D was excluded, these estimates were 8.5×10^{-6} and 1.8×10^{-5}, respectively.

DISCUSSION

Results of the molecular genetics test agree with the grafting results. A notable observation was the fusion of 2 genotypes at Site B that differed by a single allele, suggesting a common clonal ancestor, but perhaps now with a single mutation that differentiates them. Based on the combined grafting and molecular results, we conclude that all of the colonies tested at Site B were derived asexually from a single ancestor and all of the colonies

tested at Site A are derived asexually from 2 genetically distinct ancestors. The hypothesis governing the original experimental design was that all the colonies at Site A would be clone mates and all the colonies at Site B would be clone mates of a second genotype. The unanticipated fact that 2 genotypes make up the colonies at Site A revealed a shortcoming of this design. The grafting results alone (Fig. 3) do not rule out the presence of up to 6 genetic groups because of missing graft combinations, although the molecular results demonstrate that only 2 clones were present. Over all loci, our power to detect unique genotypes was high (i.e. the changes of 2 colonies being identical in genotype but not by descent is about one in a million), and it is unlikely that we have underestimated the number of genotypes in our samples. In hindsight, a second round of only 4 additional grafts using material from the first round of grafts would have resolved this question. Tagged colonies from the first round should have been retained to resolve any missing connections between clusters of clone mates. Only 4 additional grafts would have been required to establish the genetic relationship of the outliers. Grafting material from colonies A40, A50 and A65 to any members of the main cluster in Group 1 (A25, A30, A35, A45) would have verified the relationship. Also, grafting either A55 or A10 to any member of the major cluster in Group 2 would resolve the genetic identity of these outliers. Future experimental design will benefit from this finding.

The life span of large coral heads is measured in centuries (e.g. Bessat & Buigues 2001). Therefore, it is possible that the cloned fragments documented in this study have been undergoing asexual reproduction through repeated fragmentation for hundreds of years. If that is the case, the genetic difference of the 2 genotypes of a single allele at Site B could be the result of genetic somatic mutation that might have occurred in one of the ancestral sibling colonies.

Inconsistent results between grafting versus allozyme electrophoresis technique in identification of clones have been reported previously for the reef coral *Pavona cactus* (Willis & Ayre 1985). The outcome of grafting tests matched predictions based on electrophoretic results for 60 of 61 pairings, but with fusion in one pairing between electrophorically distinct colonies. Likewise, Heyward & Stoddart (1985) reported in a similar outcome in a study using allozymes. A single locus was used in their investigation, so these results must be viewed with caution. They found that dissimilar genotypes fused 40% of the time. Certainly there has been a need for comparisons between grafting techniques and DNA based techniques such as conducted during the present investigation.

The grafting approach has the advantage of low cost and low technical difficulty, and can quickly yield results. For example, Hunter (1985) mapped the clonal structure of the reef coral *Porites compressa* across a patch reef using this method. Graft fusion or rejection can be detected in days or weeks (Jokiel et al. 1983). Molecular techniques potentially provide a great deal more information on population structure, but require extensive training and elaborate equipment and resources that may not be readily available in some regions. For example, the molecular technique developed in this study for the reef coral *P. rus* required a number of attempts using expensive equipment, technician time and supplies intermittently over a period of several years to produce a method suitable for studying this species. Results of our study suggest that grafting technique can quickly yield the same results as advanced molecular technique. Rapid advances are being made in the further development of methods that directly use variation in DNA to detect differences among individuals and populations. However, Ridgway (2005) argued that the limited number of DNA markers that had been identified for scleractinian coral genetic studies at that time did not necessarily offer greater precision than that offered by the more traditional allozyme approach. We argue the grafting technique cannot be discounted as a legitimate tool for studies of population structure of asexually derived coral colonies. Graft acceptance or rejection provides an important dimension to studies of coral clonal structure based on molecular techniques. Choice of technique will depend largely on resource availability and the question being studied.

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