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RESEARCH ARTICLE

Overcoming barriers to reef restoration: field-based method for approximate genotyping of *Acropora cervicornis*

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Reef restoration efforts aim to enhance resilience by safeguarding the genetic diversity of coral populations. This could be facilitated by genotyping methods that are relatively inexpensive, and field-based. A potential method for assessing coral genotypic diversity arises from self-recognition phenomena. Past studies have shown that contact between tissues from the same genet (isogeneic) will fuse whereas tissue from different genets (allogeneic) will result in rejection. However, the accuracy of this method has been questioned. Here, we revisit the grafting method as a tool to estimate genotypic diversity in a Caribbean coral restoration target, *Acropora cervicornis*. Ten ramets of unknown genetic relation were arranged in 82 grafting tests consisting of 5-fragment bundles that replicated all possible combinations between ramets. After 10 weeks, we found that outcomes of acceptance and rejection were highly consistent (96.7% across all combinations and replicates). The proposed existence of 4 genets across the 10 ramets based on response outcomes was confirmed by two SNP-based genotyping methods. Both genet pairing (isogeneic or allogeneic pairs) and genetic distance significantly affected the odds of acceptance or rejection responses. Moreover, a significant correlation was found between the degree of fusion between fragments and their genetic distance, supporting that the most related ramets are accepted more strongly compared to those that are more unrelated. This field-based contact method can be a powerful tool to estimate genotypic diversity in coral nurseries, facilitating the management of genetic diversity within the nursery and genotype-level tracking of key traits like disease and bleaching resistance.

Key words: 2b-RAD, Caribbean, coral histocompatibility, genetic distance, genotypic diversity, grafting method, SNP-chip

Implications for Practice

- The grafting method allows estimating genotypic diversity while reducing methodological and economic bottlenecks that advanced genotyping methods bring to some restoration programs.
- The information obtained can be applied to increase the genetic diversity of restoration programs, as well as to facilitate monitoring specific genotypes that stand out in important traits such as resistance to bleaching, diseases, or phenotypic plasticity, implying progress toward reef resilience in the face of climate change.
- Although this study only focused on *A. cervicornis*, the method could likely be applicable to other species, broadening its utility globally.

Introduction

Anthropogenic stressors continue to increase the threat to the world's coral reefs (Hughes et al. 2003; Hoegh-Guldberg et al. 2007; Hughes et al. 2017), which keep on degrading at alarming rates that rates of natural recovery processes cannot meet (Donner et al. 2005; Young et al. 2012; IPCC 2018). This is of particular concern for Caribbean reefs, where phase shifts from coral to macroalgae have been pronounced since its documentation began in the 1970s (Hughes 1994; Mumby et al. 2007; Cramer et al. 2021). The increasing frequency and intensity of bleaching events, as well as the outbreak of the devastating Stony Coral Tissue Loss Disease (SCTLD), have been

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among the major focus of concern in the last years for this region as remaining coral communities are being heavily impacted (McWilliams et al. 2005; Precht et al. 2016). Curbing these factors by reducing greenhouse gas emissions and overexploitation of natural resources is imperative to decelerate the degradation of reef systems and allow their recovery. Along with these efforts, active reef restoration through interventions such as coral gardening, larval enhancement, or assisted evolution can help repair ecosystem functions (Rinkevich 2015; Hein et al. 2021; Van Oppen & Aranda-Lastra 2022).

Despite successful biomass increases of nursery-grown corals (Lohr et al. 2015), restoring reefs and their functions at scale in the face of climate change is a challenge that still faces many barriers (Boström-Einarsson et al. 2020). For instance, outplanting efforts that do not consider genetic diversity and/or thermotolerance traits can remain vulnerable to increasingly frequent bleaching events (Bowden-Kerby & Carne 2012). There is a need to improve restoration practices to promote more resilient reefs (Wilkinson & Souter 2008; Van Oppen et al. 2015; Blanco-Pimentel et al. 2022). To do so, efforts should include enhancing genetic diversity (allele diversity) (Shearer et al. 2009; Baums et al. 2019), as well as genotypic diversity (the number of genets on a reef, Baums 2008). The greater the variation of alleles in the population, the more likely it would be for some individuals carrying certain alleles to survive the new conditions of their environment, and hence, the more chances the population will have to adapt (Reed & Frankham 2003; Bay et al. 2017; Drury et al. 2017). While populations poor in genetic diversity may lead to problems such as inbreeding depression or fertilization failure, sexual reproduction of genetically diverse populations will contribute to species recovery (Baums 2008; Shearer et al. 2009). Moreover, with only a few genets, the chances of the whole population surviving when exposed to sudden high temperatures or disease outbreaks, among other stressors, are smaller than in a genetically and genotypically diverse population.

It was only relatively recently that genetic and genotypic diversity was considered in reef restoration (Baums 2008; Shearer et al. 2009; Young et al. 2012). While genetic diversity in coral nurseries can be enhanced by sourcing individuals from distinct environments and phenotypes, and by boosting sexual reproduction (Rinkevich 2015; Calle-Triviño et al. 2018; Baums et al. 2019), the actual genotypic diversity would still be uncertain unless genotyping is conducted. Genotyping techniques used in Caribbean coral species include microsatellites, single nucleotide polymorphisms (SNPs), allozymes, and amplification fragment length polymorphisms (AFLP) (Shearer et al. 2009; Lundgren et al. 2013; Baums et al. 2019). However, current tools to assess genotypic diversity are not affordable for most restoration programs (Puckett 2017). Not only the costs for the genetic analyses must be considered, but also the time and effort of sample exportation, the computational infrastructure, as well as the time investment and availability of bioinformatically-trained personnel required for the processing and analysis of the results. For restoration practitioners with limited or no lab and computational resources, microarray- and microfluidics-based SNP approaches are recommended as the best option by Baums et al. (2019) (Kitchen et al. 2020). Although some efforts have been made in the Dominican Republic to include genotyping of *A. cervicornis* coral nurseries (Lirman et al. 2014; Drury et al. 2017; Calle-Triviño et al. 2020), the high genotyping costs can limit genetic considerations in restoration programs. A major gap thus remains between access to genetic studies and their application in vulnerable reefs.

The use of a rapid and inexpensive field-based method that allows us to approximate the existing genotypic diversity in nurseries and restoration programs could go a long way to fill this gap. This method was previously referred to as grafting (Neigel & Avise 1983; Jokiel et al. 2013) and it is based on self/non-self-recognition phenomena by which the living tissue and skeleton of two isogeneic fragments (i.e. of the same genotype) will fuse when grown in contact with each other whereas allogeneic fragments (i.e. of different genotype) will reject each other (Hildemann et al. 1977; Hughes & Jackson 1980; Neigel & Avise 1983). Acceptance/rejection responses based on self/non-self-recognition processes are the base of cnidarian immunology. Underlying mechanisms are surprisingly complex and diverse, with both analogies and homologies to the vertebrate immune system (Palmer et al. 2011; Palmer & Traylor-Knowles 2012; Parisi et al. 2020).

Self-recognition phenomena of the genus *Acropora* were first described by Hildemann et al. (Hildemann 1974; Hildemann et al. 1975). The rejection response was described by Hildemann et al. (1975) as a narrow zone of calcareous cementation devoid of connecting soft tissue, and by Neigel and Avise (1983) as a suture line at the skeletal interface separating the tissues of the two fragments. Allospecific interactions also tend to result in the overgrowth of one of the fragments over the other on a linear hierarchy pattern (Chadwick-Furman & Rinkevich 1994; Frank & Rinkevich 1994). Bleaching, anomalous growth, soft tissue death, and incomplete development of the polyps have been also observed in the rejection response (Hildemann et al. 1977; Rinkevich 2004). Validation of the grafting method for genotyping has been attempted in the past by electrophoresis for species of the genera *Porites*, *Seriatopora*, and *Montipora* (Heyward & Stoddart 1985; Resing & Ayre 1985; Willis & Ayre 1985). However, more recent molecular techniques such as microsatellite analysis have shown a positive validation of the method for the species *Porites rus* (Jokiel et al. 2013). To date, this method has not been validated using the most recent and precise genotyping techniques available for *Acropora* spp., which is the focal genus for restoration in the Caribbean (Bayraktarovn et al. 2020).

This study aimed to demonstrate the viability of the grafting method as a potential tool to approximate the genotypic diversity of *Acropora cervicornis*. For this purpose, grafting tests were performed using fragments from 10 nursery-grown ramets of *A. cervicornis*. The number of fusion or fission instances across replicates of the same pairwise combinations was evaluated to assess response consistency. The feasibility of the method to distinguish ramets from the same genet (isogeneic) versus those from different genets (allogeneic) was evaluated by comparing grafting test results with those of SNP-chip and

2b-RAD genotyping of the ten ramets assessed. We hypothesized that replicates of the same pairwise combination of *A. cervicornis* ramets would consistently show the same outcome, whether it is acceptance or rejection. Also, we hypothesized that fusion of the fragments (acceptance) would only happen between isogeneic ramets, and rejection responses would only happen between allogeneic ramets when grown in contact, thus adequately predicting genotypic diversity. The verification of these hypotheses is a first step toward validating the injection method as a low-cost tool that allows differentiating genetic variants in a population.

Methods

Study Site

The grafting method study was conducted in the in-water nursery of Iberostar hotel located in the southern region of the Dominican Republic (18° 20′ 20.94" N; 68° 49′ 35.075" W) in September 2020. The nursery is 13 m deep and contains approximately 1,200 ramets of *Acropora cervicornis* distributed in 44 structures (including ropes, reinforcing steel in the shape of A-frames and domes, and PVC trees). Whereas ropes, frames, and domes contained ramets of mixed origin, each PVC tree contained fragments that originated from a specific colony of wild-collected *A. cervicornis*. However, both the genet identity and the geographical origin of the wild collection were unknown at the outset of the study. A subset of fragments of *A. cervicornis* from 10 coral trees of unknown genetic nature was collected for genetic analysis, and another subset was assembled into test bundles for tissue contact method evaluation (Fig. 1A). Tests were hung from a 3 × 3 m three-level polyethylene-rope structure anchored with galvanized anchors (Duckbill 68 II) and sustained by air-filled bottles (Fig. 1B).

Grafting Method Operational Framework

To replicate the grafting method, it is necessary to evaluate certain specific factors of the restoration program in question, such as the logistical and economic resources available, the number of genotypes to be tested, and for what objective. Some guidance around these considerations, as well as a detailed guide to the step-by-step process, is included in the protocol by Blanco-Pimentel et al. (2023). The details on the preparation of the tests are collected in the section below.

Grafting Test Arrangement

A more detailed explanation of the test arrangement is presented in the protocol by Blanco-Pimentel et al. (2023). All possible pairwise combinations of the 10 ramets were arranged and replicated across different test bundles. Isogeneic tests were also carried out as a control to corroborate fusion between ramets from the same nursery tree. Each test consisted of a five-fragment bundle: one larger fragment (10 ± 2 cm) and four smaller fragments (5 ± 1 cm) zip-tied to both the apical and basal parts of the larger piece (Fig. 1A). The five-fragment design was intended to optimize the number of tests needed

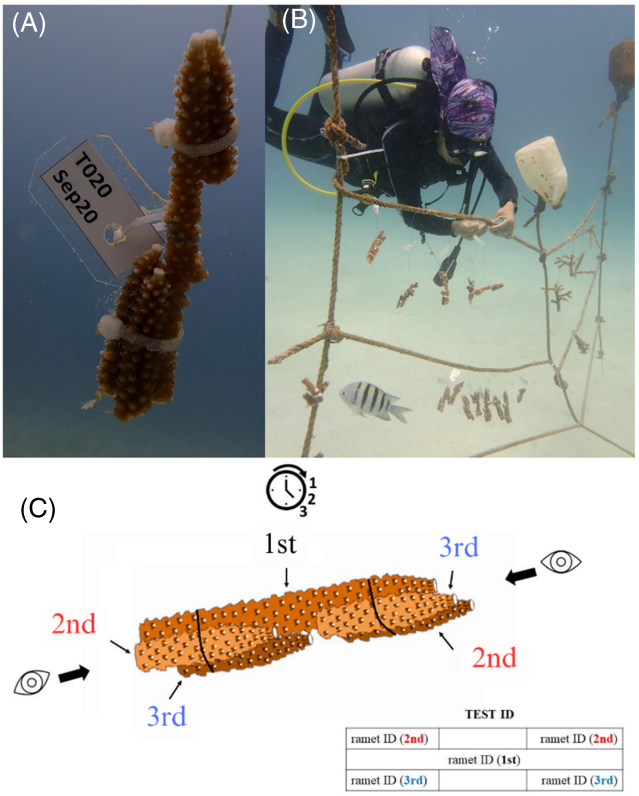

Figure 1. Experimental grafts of *Acropora cervicornis* fragments. (A) Test bundle consisting of five fragments of *Acropora cervicornis* (four smaller ones zip-tied to both the anterior and posterior parts of a larger fragment). (B) Rope structure with graft tests in the underwater coral nursery. Picture credits: Macarena Blanco-Pimentel (September 2020). (C) Test bundle arrangement and table representation. Clockwise order is followed by looking toward the apices in the case of anterior fragments of the bundle and toward the bases in the case of posterior fragments. In both cases clockwise order starts from the larger fragment. Note that all apices are oriented in the same direction which corresponds to left to right direction in the table. The second and third fragments in the bundle correspond, respectively, to the top red and bottom blue ramet numbers in the reference table. Note that numbers 1 to 3 in the figure indicate the order of ramets, not the ramet ID number itself.

for all contacts without compromising the ability to distinguish fragments once tissue grew. For some of the isogeneic bundles, only two medium-sized fragments (8 ± 2 cm) comprised the test. Combinations were arranged in a way that each pairwise combination (1–1, 1–2…,9–10,10–10) was replicated at least five times. Combinations did not follow a specific numerical pattern (i.e. both the larger and smaller fragments could be any one from 1 to 10) but each of the fragments had to be in contact with the rest at least five times across the tests. Some redundant replicates were inevitably obtained. During the test arrangement, the coral tree containing ramet number 7 was missing, so a second round of tests was performed when tissue was retrieved from a backup nursery. A total of 82 test bundles were arranged, representing 55 different pairwise combinations and 347 pair contacts to evaluate (see supplement S1). However, for 10 ramets in

one round of tests, an average of 43 tests and 239 contacts would be needed for 5 times replicated pairwise combinations (see supplement S2).

Combinations of each test were planned before the underwater arrangement. They were represented in tables that included test numbers and ramet numbers to be used as well as their position in the test (Fig. 1C). Both anterior and posterior fragments in the test were arranged with all apices pointing toward the same direction and in a clockwise fashion to keep track of ramets used in each test. Clockwise order always started from the larger fragment and was continued by looking toward the apex in the case of anterior fragments and toward the bases in the case of posterior fragments of the bundle. Following indications of the reference tables, the second and third fragments of the clockwise order (either anterior or posterior fragments) corresponded to the top red and bottom blue ramet numbers, respectively.

Tests were left to grow for 10 weeks. This time was based on literature demonstrating tissue regeneration in acroporids (García-Urueña et al. 1995) and field observations. Test bundles were checked every two weeks to assess growth, evidence of responses, and good conditions of the animals and structure.

Data Collection. Tests were retrieved and brought to the Iberostar's coral laboratory over two consecutive days using 83 L buckets. Pairwise combinations were promptly analyzed under a stereoscopic microscope (SMZ-143 N2LED, MOTIC). Responses were broadly categorized as acceptance or rejection, where acceptance involved the fusion of both skeletal components and soft tissues and rejection involved a lack of fusion of the tissue in its different forms of avoidance (skeletal barrier, overgrowth, bleaching, etc.). However, due to the observation of two consistent types of rejection responses, rejection was subclassified into strong and weak rejection for some of the analyses. Pictures were taken for all combinations and replicates. Consistency of responses was calculated based on the predominant outcome across replicates in a specific pairwise combination (i.e., 40% fusion - 60% rejection would imply a 0.6 constancy in that pair).

Acceptance (fusion) percentages across replicates of each pairwise combination were arranged in a matrix to perform a correlation plot in RStudio (R Core Team 2022, version 2022.07.1) using the corrplot package and hclust function with ward.D2 as the method, to visualize the possible ramets with a greater or lesser genetic relationship.

Genotyping

SNP-Chip Analysis. Around 2 cm samples of the 10 ramets of *A. cervicornis* object of the study were collected with clippers and placed in 5 mL sample tubes filled with ethanol 95%. Samples were sent to Eurofins BioDiagnostics laboratory (WI, U.S.A.) for DNA extraction and SNP-chip analysis. Genetic information was analyzed through the Galaxy web-based portal described by Kitchen et al. (2020). In brief, multi-locus genotypes were assigned for each fragment based on allele calls from 19,694 SNPs. These multi-locus genotypes were then compared against each other and a database of acroporid reference genotypes to identify genets. Relatedness among samples was estimated using identity-by-state (IBS) analysis in the R package SNPRelate (Zheng et al. 2012), and Prevosti's absolute genetic distance was calculated using poppr (Prevosti et al. 1975; Kamvar et al. 2014).

2b-RAD Analysis. An additional, replicated set of tissue samples, preserved as described above, was sent to the University of Southern California for 2b-RAD genotyping analysis (Wang et al. 2012). DNA was extracted using Wayne's method (Wilson et al. 2002). 2b-RAD libraries were prepared following the modifications for Illumina sequencing protocol using Bcg I and a 1/16 reduction scheme and resulting reads were trimmed and filtered following a standard protocol (https://github.com/z0on/2bRAD_denovo). Briefly, PCR duplicates were removed, reads exhibiting matches, and only reads exhibiting base quality scores greater than 20 over 90% of the read were retained. High-quality reads were mapped using bowtie2 (Langmead & Salzberg 2012) to the *A. cervicornis* reference genome (Kitchen et al. 2019) concatenated to representative symbiont genomes for the four dominant Symbiodiniaceae genera which associate with reef-building coral: *Symbiodinium microadriaticum* (formerly clade A, (Aranda et al. 2016), *Breviolum minutum* (formerly clade B, Shoguchi et al. 2013), *Cladocopium* spp. (formerly clade C1, [Shoguchi et al. 2018]), and *Durusdinium trenchii* (formerly clade D, [Dougan et al. 2022]). Reads exhibiting best matches to the host reference were extracted, then sorted and indexed using SAMtools (Danecek et al. 2021). Genotyping to infer clonal groups was accomplished using ANGSD v0.933 (Korneliussen et al. 2014). Briefly, loci were required to be present in at least 80% of individuals, with a minimum minor allele frequency of 0.05, a minimum mapping quality score of 20, a minimum base quality score of 30, an SNP $p < 0.00001$, a strand bias $p > 0.00001$, heterozygosity bias > 0.00001, removing all triallelic sites and reads having multiple best hits. Hierarchical clustering of samples based on pairwise IBS distances was used to infer clones following Manzello et al. (2019) using technical replicates to identify the appropriate height cutoff.

Statistical Analyses

Consistency of responses across replicates of the same pairwise combinations was represented as a percentage to evaluate the reliability of responses (i.e., number of replicates of a specific ramet combination presenting the same outcome by the total number of replicates for that combination). After evaluating the consistency of responses, each pairwise combination was assigned a score based on the type of response/s shown across its replicates. Scores were used to evaluate the correlation between the degree of fusion and the genetic distance obtained from the SNP-chip analysis. The score was between 1 (fusion across 100% of the replicates for a specific combination) and 0 (strong fission across 100% of the replicates). Weak fission

was scored as 0.5. For pairwise combinations with two different outcomes across replicates, the score was calculated as indicated in Equation (1).

$$\text{Total pair score} = \text{Frequency}_{\text{outcome1}} \times \text{score}_{\text{outcome1}} + \text{Frequency}_{\text{outcome2}} \times \text{score}_{\text{outcome2}} \quad (1)$$

Spearman's rank correlation between pairwise combination scores and genetic distance was then conducted. For the rest of the analyses, responses were categorized into acceptance or rejection (including both strong and weak fission). To validate the association between grafting method and genotyping results and account for nesting into pairwise combinations, we used two generalized linear mixed effects models (GLMM) with Laplace approximation method (Raudenbush et al. 2000; Bolker et al. 2009). These models included contact outcome as a categorical response variable (acceptance = 1; rejection = 0) with pairwise ID as a random factor. The first model also included genet pairing (isogeneic pairs =1; allogeneic pairs = 0) as a fixed categorical factor, whereas the second model included genetic distance as a fixed continuous factor. Both models were compared against a null model using the Akaike Information Criterion (AIC). All analyses were performed in RStudio (R Core Team 2022, version 2022.07.1) using the glmer function in the lme4 package (Bates 2005) with binomial family for GLMM.

Results

Tissue Contacts Outcomes

During the evaluation of the contacts under the microscope, the acceptance responses obtained were characterized by the complete fusion of tissue between both fragments of the test. These responses occurred in 100% of the isogeneic control tests (10 replicated combinations of ramets 1 to 10 between themselves) and 33% of the combinations of unknown genetic relationships (45 replicated pairwise combinations between ramets 1 to 10). Clear rejection responses (in this study referred to as strong fission) were characterized by a clear suture or bridge between both fragments, sometimes with tissue overgrowth from one of the fragments above the other. There were also cases of death of one of the fragments, for instance, in four replicates of the combination of ramet 1 with 3 in different test bundles, fragment 3 died. A second type of rejection response, as an intermediate response between the previous ones, was categorized as weak fission. In this response, what seemed like a certain fusion was observed, without reaching a sealed and smooth fusion as in the case of total acceptance. In addition, in most of the replicates, a white area was observed between both fragments, probably indicating a lack of colonization by the symbionts (Hildemann et al. 1977). For both types of rejection, a lack of soft tissue growth across small gaps between the fragments was also observed. Examples of these responses are shown in Figure 2. More pictures for each category can be found in the protocol by Blanco-Pimentel et al. (2023). The consistency of outcomes across replicated combinations was 96.7%, indicating high reliability of contact responses (Fig. 3).

The percentages of fusion between combinations across all replicates were arranged in a correlation matrix (Fig. 4). Correlation scores revealed a strong relatedness between ramets 3 and 9 which were 100% fused across replicates, and both showed no apparent relatedness to the rest of ramets, since there was 100% strong rejection between the rest of combinations and replicates. Ramets 2,4,6,7,8 and 10 also fused in the majority of instances, except for the combinations: 2–6, 2–8, and 4–7, where there was a 40% fusion / 60% weak fission; 50% fusion / 50% weak fission and 80% fusion / 20% weak fission, respectively. Ramets 1 and 5 showed signs of both strong and weak fission in all cases with the rest of the ramets, likely indicating a different genetic nature. In no pairwise combination was both fusion and strong fission across replicates observed.

Molecular Genotyping

Genotypic diversity obtained from SNP-chip analysis revealed four genets across the 10 ramets of the study. Genet "A" corresponded to ramets 2,4,6,7,8 and 10; Genet "B" corresponded to ramets 3 and 9, Genet "C" corresponded to ramet 1, and Genet "D" corresponded to ramet 5 (Fig. 5A). 2b-RAD genotyping revealed a similar major genet grouping (Fig. 5B).

Grafting Method Validation

The proposed genotypic relatedness of the ten ramets based on acceptance/rejection results (Fig. 4) was in accordance with genotypic diversity obtained both from SNP-chip and 2b-RAD analyses (Fig. 5A & 5B). Apart from the isogeneic tests that showed 100% acceptance, fusion occurred in 91.3% of replicated contacts between ramets of the same genotype. The remaining 8.7% was recorded as weak rejection. In the case of identified unique genotypes, strong fission occurred in 65.5% of replicated contacts, followed by 33.1% weak rejection and 1.4% fusion.

The predominant outcome of pairwise combinations presenting both fusion and weak rejection was used to calculate the constancy of responses (96.2%) before obtaining genotyping results. Once the genotypes were unveiled, the accuracy of responses was calculated using the expected outcome (i.e., constancy of fusion for the same genotypes and constancy of rejection for different genotypes). This accuracy represented 96.2% since only in one pairwise combination (2–6) there was 60% weak rejection and 40% fusion being the same genotype. For the rest of the pairwise combinations, the predominant outcome corresponded to the expected one.

When scoring outcomes as 1 (fusion), 0 (strong fission) and 0.5 (weak fission) for each replicate in the pairwise combination, total scores of the pairs showed that the degree of fusion between them was significantly correlated with their genetic distance (Spearman's rank; rho = −0.89; $p < 0.001$) (Fig. 6). That is, responses of ramets with low or near zero pairwise genetic diversity, indicative of them being clone mates, were more likely to undergo fusion upon contact.

Genet pairing (isogeneic pairs = 1; allogeneic pairs = 0) significantly affected the odds of acceptance or rejection responses

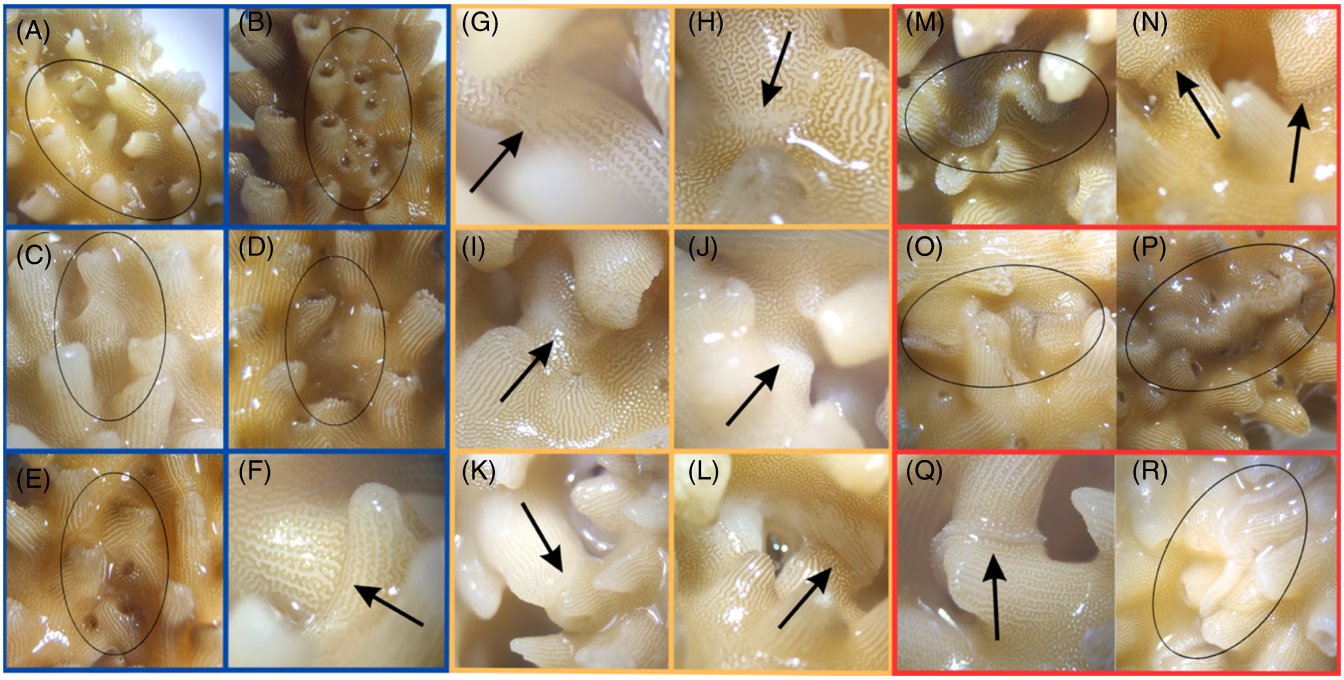

Figure 2. Example pictures of contact between pairs of fragments under the stereoscopic microscope. The first two columns in blue to the left (A–F) show acceptance outcomes where tissue between fragments is fully fused. The third and fourth columns in yellow (G–L) show weak rejection where neither fusion nor strong fission is observed, and in some cases, there is a white line between both fragments indicating rejection. The two columns in red to the right (M–R) exemplify strong fission with both a suture line and/or overgrowth of one of the fragments over the other. Contacts between the two fragments are indicated by a circle or arrow.

(1 = fusion; 0 = rejection) (Generalized Linear Mixed Model (GLMM) 1; Pseudo-$R^2 = 0.98$; $p < 0.001$) (Fig. 7A). Responses were also significantly associated with genetic distance (GLMM 2; Pseudo-$R^2 = 0.98$; $p < 0.001$) (Fig. 7B). The Akaike Information Criterion (AIC) value is reduced when fixed factors are added to the model ($AIC_{null} = 150.730$; $AIC_{model\ 1} = 69.6$; $AIC_{model\ 2} = 70.2$), hence both models better represent patterns of variation in the response outcomes in comparison to the null model. Moreover, according to AIC, the lower value of the first model indicated a slightly better fit, implying that genet pairing is a strong predictor of acceptance or rejection responses.

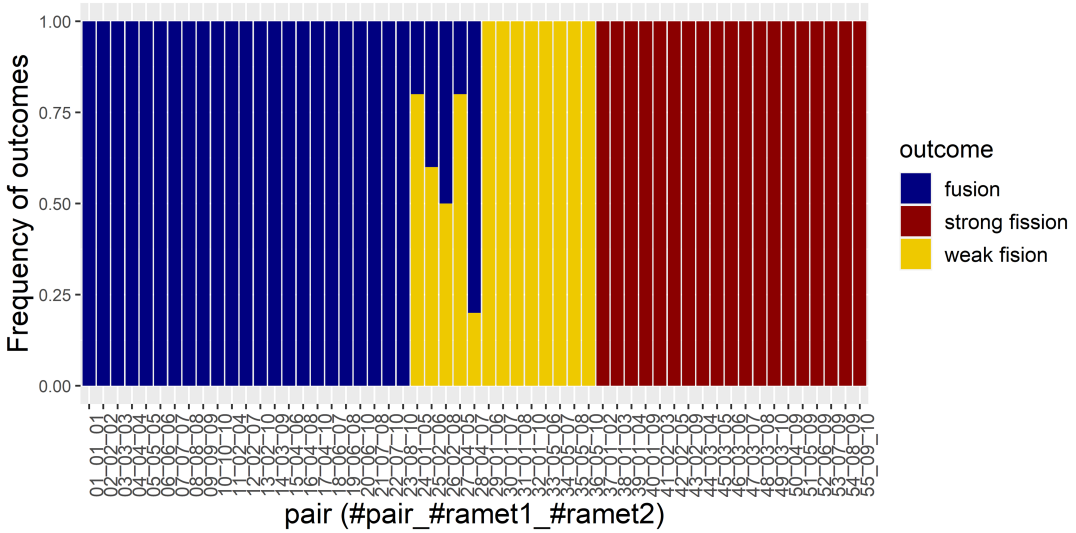

Figure 3. Frequency of contact tissue outcomes for each pairwise combination of ramets 1 to 10 across replicates. Fusion is represented in blue, weak fission in yellow, and strong fission in red. Consistency of outcomes across replicated combinations was 97.3% when including isogeneic control tests, and 96.7% when excluding isogeneic controls. Combinations with incongruity corresponded to combinations 1–5, 2–6, 2–8, 4–5, and 4–7. Cases of incongruity occurred only between fusion and weak fission outcomes. Pairs were ordered by outcome using a pair number (#pair) only for aesthetic purposes.

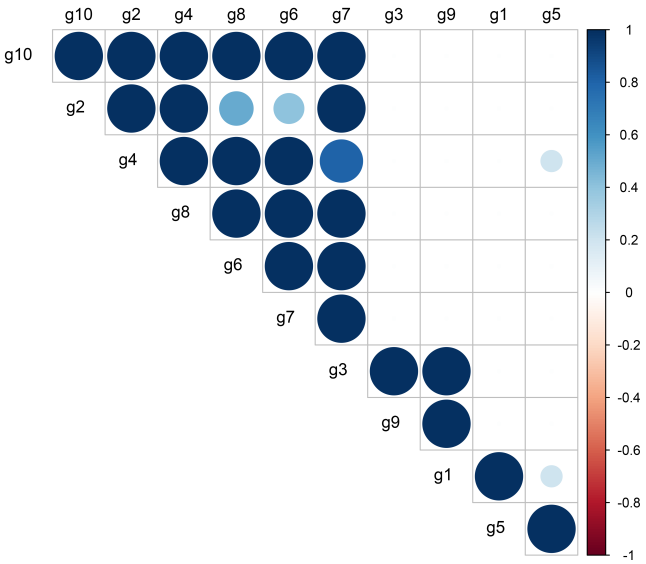

Figure 4. Correlation matrix of fusion percentages between ramets (r) 1 to 10 of *Acropora cervicornis* in pairwise combinations across replicated tests. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. The legend color shows the correlation coefficients and the corresponding colors. White cells imply no correlation between the corresponding ramets in the matrix. Ramet order was arranged by correlation scores for aesthetic purposes.

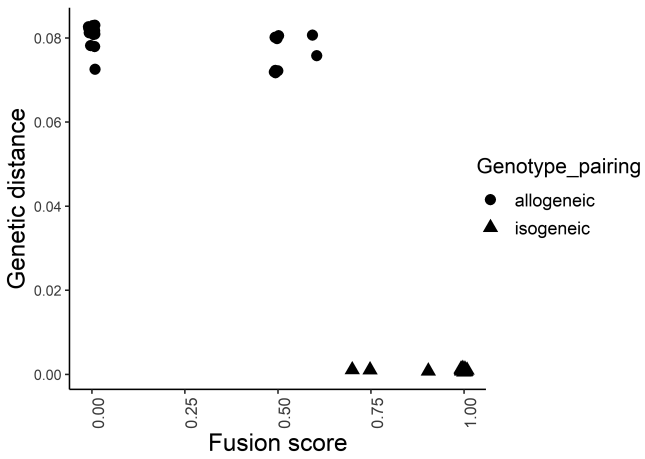

Figure 6. Correlation between degree of fusion (1 = 100% fusion; 0 = 100% strong fission across replicates) and Prevosti's absolute genetic distance of pairwise combinations (Spearman's rank; rho = −0.89; $p < 0.00$).

Discussion

Tissue Contacts Outcomes

Acceptance/rejection outcomes were highly consistent across replicates of pairwise combinations of *A. cervicornis* ramets. Tissue fusion occurred in both known isografts (100%) and those that were later proven to belong to the same genet (91.3%). The incongruity of responses across replicated pairwise combinations (3.8%) was in all cases observed as both fusion and weak fission but never involved both fusion and strong fission outcomes. This supports the idea that acceptance and rejection responses are not random but determined by underlying genotypic differences. Consistent fusion of tissue and skeletal elements within genets coincides with that observed by Hildemann et al. (1975) in *Acropora formosa* and *Porites andrewsii*, and later corroborated in *Montipora verrucosa* (Hildemann et al. 1977), *A. cervicornis* (Neigel & Avise 1983), *M. dilatata* (Heyward & Stoddart 1985), *M. ramosa*

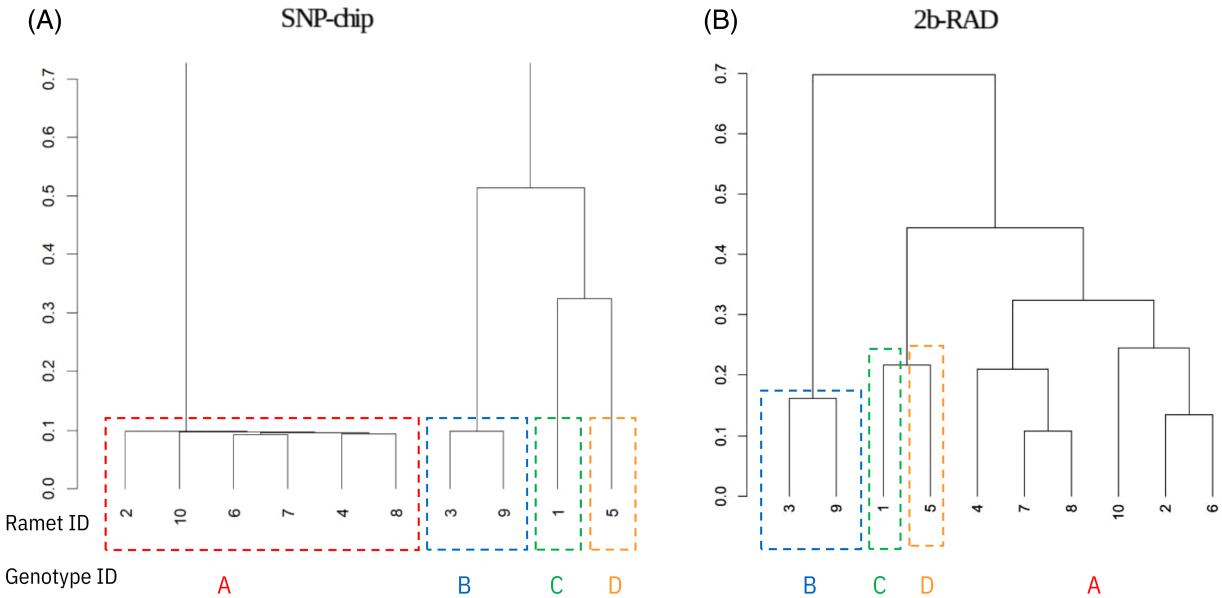

Figure 5. Hierarchical clustering dendrogram of *Acropora cervicornis* ramets obtained by SNP-chip (A) and 2b-RAD (B) genotyping. Dendrograms included ramets 1 to 10 used in the study which corresponded to six unique genotypes: genotype "A" corresponded to ramets 2, 4, 6, 7, 8, and 10; genotype "B" corresponded to ramets 3 and 9, genotype "C" corresponded to ramet 1, and genotype "D" corresponded to ramet 5.

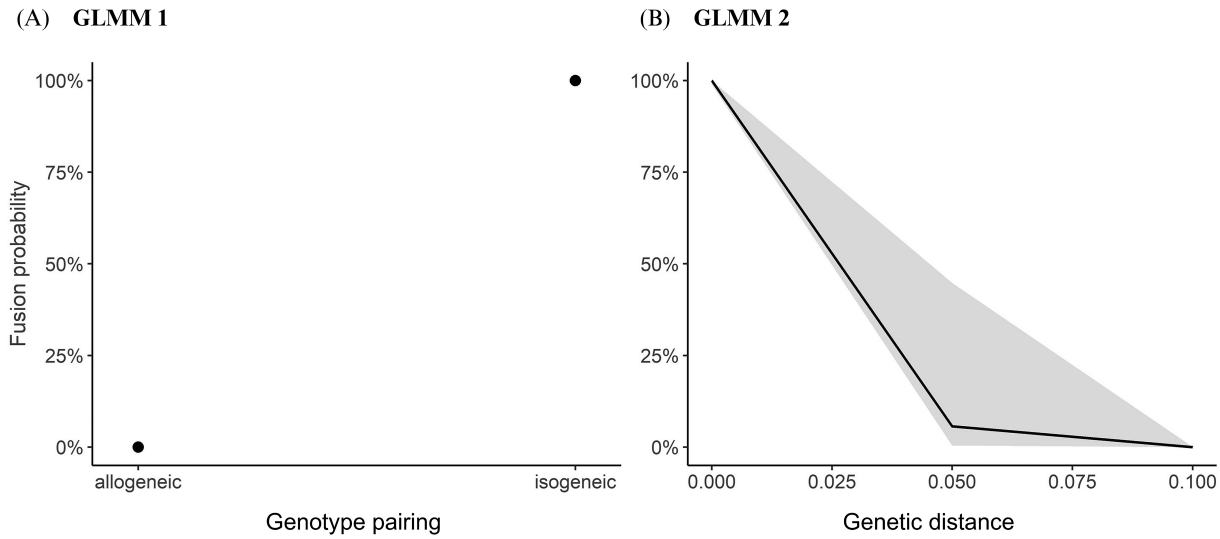

Figure 7. Fusion probability predictions based on generalized linear mixed models (GLMM) for the association between tissue acceptance/rejection and (A) genotype pairing (same = isogeneic combination; different = allogeneic combination) and (B) Prevosti's absolute genetic distance. Model 1 (A): Pseudo-$R^2 = 0.98$; $p < 0.001$; Model 2 (B): Pseudo-$R^2 = 0.98$; $p < 0.001$. $AIC_{model1} = 69.6$; $AIC_{model2} = 70.2$.

(Heyward & Collins 1985), *Pavona cactus* (Willis & Ayre 1985), *A. hemprichi* (Rinkevich et al. 1994) and *Stylophora pistillata* (Chadwick-Furman & Rinkevich 1994). These and other studies also demonstrated consistent rejection responses between supposedly allogeneic colonies reported as the formation of a morphologically distinct bridge or undulated suture line at the skeletal interface separating the tissues (Neigel & Avise 1983; Chadwick-Furman & Rinkevich 1994), as well as an opalescent cementation area or naked skeleton lacking soft tissue (Hildemann et al. 1975; Johnston et al. 1981), and include cases of growth failure between gaps (Hildemann et al. 1975) and overgrowth of one fragment over the other (Willis & Ayre 1985; Rinkevich et al. 1994), all of which was observed in the present study. Outcomes in the present study did not reflect a clear hierarchy of overgrowth or aggressive competition between allogeneic grafts as observed in other studies (Chadwick-Furman & Rinkevich 1994; Frank & Rinkevich 2001), except for the specific case of ramets 1 and 3, with 3 exhibiting greater mortality.

Even though growth and sample survival were monitored every 2 weeks, acceptance/rejection responses were evaluated at a specific point in time corresponding to 10 weeks after growth. This time was enough to be able to distinguish fusion and fission responses between the fragments. However, the ideal response evaluation time may vary for each study depending on several factors such as species, genotypes, temperature, and life stage, among others. For instance, whereas in the study of Chadwick-Furman and Rinkevich (1994) with *Stylophora pistillata* clear rejection was observed after 6 to 23 months, Hildemann et al. (1977) reported a mean time to definitive rejection between allografts of 20 days for *Montipora verrucosa*. Required growth time could also vary depending on the specific genets (i.e., different growth rates) and graft combinations (Johnston et al. 1981). For instance, among genetically closer allografts rejection time may be longer compared to grafts composed of more distant genotypes. This could explain the weak rejection response reported here, since this may have developed into a clear rejection response after a longer observation period. This is supported by results obtained by Chadwick-Furman and Rinkevich (1994), who observed fusion-like responses between *S. pistillata* allogeneic combinations after the first months (i.e., weak fission) that later developed into suture barriers and overgrowth (i.e., strong fission). This implies that in some cases, too short a time could produce unclear responses or be insufficient to reveal hierarchies of overgrowth and competition, and therefore insufficient time may be the cause of reports of fusion between allografts in previous studies (Heyward & Stoddart 1985; Resing & Ayre 1985; Willis & Ayre 1985). On the other hand, early observation of these responses can provide important information about the underlying immunological and physical processes of self/non-self-recognition. This would provide a better understanding of the stages of the fusion and fission processes and hence a standardization of results between studies and species. Therefore, growth time is a key factor that must be considered when designing or comparing graft experiments. Acceptance and rejection responses may also be influenced by other factors such as temperature (Johnston et al. 1981) and life stage (i.e., the immune system maturation). For example, in juvenile corals whose immune systems are immature, fusion between allogeneic corals can occur, known as chimerism (Barki et al. 2002; Puill-Stephan et al. 2012). Maturation of the allorecognition system can vary between species, being slower in spawning corals such as *A. cervicornis* (Puill-Stephan et al. 2012). Also, a more similar immune system between more genetically related but distinct individuals could explain the appearance of a weak rather than a strong fission response.

Grafting Method Validation

The genetic relationship among the 10 ramets coincided with the proposed genetic relationship based on the acceptance and rejection responses before obtaining the SNP-based genotyping results. This highlights the potential of the grafting method to distinguish clonal ramets from those of different genets. The grafting method had never been compared to current genotyping techniques before, which represents a novel advance. Despite major genet groupings agreed between SNP-chip and 2b-RAD genotyping methods, pairwise relationships seem to differ slightly, likely because the genetic distance method differs, and different loci were used. Cases of different genotypes showing both fusion and weak rejection (pairs 1–5 and 4–5), as well as cases of different genotypes showing 100% weak fission (for instance both 1 and 5 with 6, 7, 8, 10), could be explained by a greater relatedness between the genotypes, which could have influenced the type of rejection, or the time required to exhibit a definite strong rejection. To prove this, longer-term experiments would be needed. The low percentage of weak rejections observed between pairs of ramets from the same genotype (2–6, 2–8, and 4–7) could not be explained in terms of genetic distance as all ramets appear similarly related in both SNP-chip and 2b-RAD results with no specific trend of dissimilarity between those combinations. However, it is interesting to consider that even though ramets 2, 4, 6, 7, 8, and 10 are considered to belong to the same genet according to SNP-chip results, these ramets have been growing separately in different nursery structures for more than 5 years, which could have promoted the differentiation of certain traits at the phenotypic or even epigenetic and genetic level and thus explain slightly different recognition responses. A similar case to this is the observation by Jokiel et al. (2013) of the fusion of two fragments that only differed by a single allele, suggesting that they are descendants from the same genet, but a somatic mutation had occurred in one of the colonies.

Grafting Method Strengths

The grafting method represents an inexpensive tool to estimate genotypic diversity even for low-income, resource-limited small islands. The main financial and logistical needs associated with this method are those of any underwater restoration operations (i.e., scuba tanks and gas costs, trained personnel, boat logistics, dive gear, and underwater security, among others) so the cost of the method itself is not high when there already is a restoration program running that includes regular dive operations. If that is the case, a minimum investment in materials such as rope, nylon, labels, zip ties, and clippers would be needed, and the biggest cost associated with this method would then be the stereoscopic microscope. When the number of genotypes to be tested is high (for instance in large restoration programs), the number of tests and thus underwater time and diving costs increase. A solution to this would be to arrange the tests out of the water, which would imply a tradeoff between costs, time, and coral stress. Although simple training of the personnel is necessary to assemble the fragments of the test in the correct order as well as to evaluate the results under the microscope, the technical level required is minimal. It is also easily applicable to various objectives and scalable to any location.

Grafting Method Versus Molecular Genotyping

Molecular genotyping methods used in this study can provide more accurate results that are not influenced by growth time and other aforementioned factors as in the grafting method. Also, these can provide additional information beyond the identification of management units, such as host-symbiont population genetics and symbiont communities. In addition, genotyping techniques can provide valuable information on the current allelic diversity of the population, as well as provide a better understanding of gene flow among target and neighboring populations. The grafting method is thus an additional tool to genotyping that, while more approachable for some researchers and practitioners, still requires investment of resources into trained personnel and water accessibility. Furthermore, the accurate estimation of genotypic diversity is subject to the researcher's experience in visually differentiating fusion and fission responses. Using the grafting method implies rerunning all possible pairwise combinations whenever a new wild collection is added to the nursery as the data is purely comparative, which could be time-consuming. Therefore, the selection of this method over molecular genotyping methods will depend on the human, financial, and logistical resources available and the objectives of the study. Despite the knowledge gaps remaining on the genetic mechanisms of coral self/non-self-recognition (Wijayanti & Hidaka 2018), outcomes can be a very useful component of the genetic toolbox for applying genetic information in restoration operations.

Application to Reef Restoration

Including genetic considerations to boost genetic diversity and increase reproductive success in reef restoration programs to avoid long-term genetic constraints in restored populations is increasingly recognized to promote ecosystem resilience and adaptation to future environmental conditions (Baums 2008; Baums et al. 2019). Knowing the genotypic diversity in coral nurseries is crucial to tracking genotype-specific features such as growth rate, disease resistance, bleaching tolerance, and recovery, among other key traits. This could improve decision-making in restoration practices (i.e., which individuals are selected for out-planting) without compromising genetic diversity (Morikawa & Palumbi 2019; Caruso et al. 2021; Cunning et al. 2021). The grafting method could then help to overcome some of the barriers of genetic-based restoration and to distribute more evenly the development of genetic studies across active restoration programs on degraded reefs in resource-limited areas where time and access to the water are not a limitation. Moreover, previously mentioned studies conducted with other species show the possibility that the method may be applicable to other species, although a different test arrangement would be necessary for non-arborescent growth forms.

Future Research

Many knowledge gaps remain in the field of immunology that could explain the underpinning mechanisms of acceptance and rejection, as well as the influences of growth time and life stages for different species (Hildemann et al. 1977; Palmer & Traylor-Knowles 2012; Parisi et al. 2020). Greater knowledge in these areas would allow a more precise interpretation of the results of the grafting method. In addition, knowing if there are different rejection responses between grafts of the same species and grafts of cryptic species would help to address some taxonomic bottlenecks, especially in the Indo-Pacific (Sheets et al. 2018). Also, validation of the method with different species will be beneficial to account for species diversity in reef restoration.

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Supporting Information

The following information may be found in the online version of this article:

Supplement S1. Tests and combinations used for the 10 ramets in five-fragment tests.

Supplement S2. Example of tests and combinations for 10 ramets in five-fragment tests.

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