PROTOCOL – GRAFTING METHOD FOR ESTIMATING GENOTYPIC DIVERSITY IN *ACROPORA CERVICORNIS*

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ABSTRACT

The grafting method has been proposed as a potential tool to differentiate corals that share the same genotype from those with different genotypes (Neigel & Avise, 1983; Jokiel et al. 2013). This is based on the principle of histocompatibility whereby corals of the same genotype will fuse their tissues upon contact, while those of different genotypes will produce rejection responses such as fission and overgrowth (Hildemann et al. 1977; Hughes & Jackson, 1980; Neigel & Avise, 1983). It is a simple method, based on the field, with low cost and wide scalability. This method does not replace advanced genotyping methods, but it can serve as an additional tool to include genetic information in restoration programs with economic or logistical constraints for genotypic analysis in the laboratory. The application of this method has great implications for reef restoration, as the genetic information obtained can help restoration decision-making that seeks to enhance genetic diversity, crucial for reef resilience. Estimating genotypic diversity allows, in turn, to monitor genotypes that stand out in important resilience traits such as resistance to bleaching, diseases or phenotypic plasticity. Here we present a protocol for the implementation of the method in underwater nurseries with the species *Acropora cervicornis*.

Keywords

Acropora cervicornis; Caribbean; fission; fusion; genotypic diversity; genotyping; grafting method; reef restoration.

STEPS

1. DEFINE OBJECTIVES OF THE STUDY

The overarching goal of this method is to establish who are the unique genets and who the clonal ones among a set of ramets of unknown genetic nature. That is to say, to unveil genotypic diversity. This in turn will ideally have the objective of ensuring or increasing the genetic diversity of restored reefs, thus promoting reef resilience. However, prior to designing the plan, it is important to establish what are the specific objectives of the study, or in other words, what will be the specific application of the genetic information obtained, since that will give a better idea of how appropriate it is to use this method compared to other more advanced methods of genotyping.

Some examples of its utilization are:

- To organize coral ramets in a nursery by genotype-specific structures or labels, for later evaluation of important traits such as bleaching tolerance, growth rates, disease resistance, etc.
- To organize coral ramets in a nursery by genotype-specific structures or labels, for later genetically organized outplant.
- To ensure genotypic uniqueness of new wild-collected ramets introduced in a nursery.

2. EVALUATE RESOURCES AVAILABLE

Available resources will determine the viability of performing this method. These include human, economic, and logistical resources for both in-water and on land operations. Primarily, you must have access to a diving center for the provision of tanks, boat, and dive gear; and have a team of restoration technicians or trained divers who can carry out the tests, as well as monitor and retrieve them. In addition, it is imperative to have the necessary government permits to handle coral and for the establishment of underwater structures if necessary. In already up and running restoration programs, this would incur no additional cost to ongoing restoration operations. However, if it is done occasionally without any established restoration program, all expenses associated with this activity must be considered (tanks, dive gear, boat, payment of personnel and training-associated costs, permit fees, etc.). Also, some tools and materials would be needed:

- Clippers
- Nylon
- Labels (for instance laminated waterproof papel, or plastic numbered labels)
- Zip ties
- Manpower and nursery structures materials in the case of setting up a new structure where to hang the tests from. For instance:
    - Rope structure: rope, anchors, and buoys.

- Table, dome, or A-frame: rebar.
- PVC tree: PVC pipes, driller, nylon, anchor, buoy.

For the analysis of the tests once the tissue growth period is concluded, a stereoscopic microscope is required (for instance: SMZ-143 N2LED, MOTIC).

3. DETERMINE SUBJECTS OF STUDY

Depending on the objective, it will be necessary to decide how many and which corals are going to be studied. The more corals you want to evaluate, the greater the number of combinations necessary between them, and therefore the necessary amount of tissue and the time required will increase. Regarding tissue availability is important to take into account that the fragments to be collected of each ramet will ideally correspond to the apical parts of the ramet, so the more branched the coral, the easier it will be to collect fragments and the more tests can be performed.

4. DESIGN GRAFTING COMBINATIONS

Combinations between tissue of different ramets will be done in pairs (i.e., individual 1 with individual 2) but will be arranged in a five-fragment bundle test to optimize number of tests needed for all contacts without compromising the ability to distinguish fragments once tissue grows. The five-fragment bundle is comprised of a 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. 1). In this way, for each test there will be 6 contacts in pairs between the fragments (the two short ones from the apical part with the large one, the two short ones from the basal part with the long one, and the small ones from both parts between them).

Figure 1. Grafting test comprised of five fragments of *Acropora cervicornis*.

Ramets in the study will be assigned a number from 1 to as many ramets there are, in order to facilitate combinations planning and arrangement. Letters can also be used instead but we will be using numbers in the present example. Each five-fragment test will also be identified by a number or a letter. Everyone will be combined with the rest of individuals (i.e., 1-2, 1-3, 1-4, and so on). Combinations between oneself could also be

performed as acceptance controls. These combinations should be replicated across the tests in order to provide rigor to the outcomes. It is recommended to arrange a minimum of 4 or 5 replicates, depending on the tissue, time and logistics available. There is no specific order in which the combinations should be done, but they must be designed in such a way that the number of tests with the desired combinations is optimized, ideally without obtaining many redundant combinations. An example of combinations for 10 ramets is shown in the figure 2 below. Table representations will be important to follow a specific placement of the shorter fragments when arranging the test. More details in this regard are explained in section 7.

TEST #001		**TEST #002**		**TEST #003**		**TEST #004**	
2	4	4	5	4	5	6	9
1		2		3		5	
3	5	6	7	8	6	8	10

TEST #005		**TEST #006**		**TEST #007**		**TEST #008**	
1	7	1	3	2	10	4	3
6		7		8		9	
9	10	8	9	9	4	7	5

TEST #009		**TEST #010**		**TEST #011**		**TEST #012**	
1	3	8	4	4	7	6	9
10		1		2		5	
2	4	3	5	6	5	8	10

TEST #013		**TEST #014**		**TEST #015**		**TEST #016**	
1	7	4	3	2	10	4	1
6		7		8		9	
10	9	8	6	9	7	10	7

TEST #017		**TEST #018**		**TEST #019**		**TEST #020**	
6	2	2	4	4	5	4	6
10		1		2		3	
x	3	3	5	6	7	8	8

TEST #021		**TEST #022**		**TEST #023**		**TEST #024**	
1	9	1	x	1	3	2	10
5		6		7		8	
8	10	9	10	10	x	9	6

Figure 2. Example of combinations for ten ramets in five-fragment tests. The middle number of each table represents the larger fragment, whereas the numbers of the left column of each table represent the basal fragments and the ones to the right column represent the apical fragments. Note that for each test there are six possible pairwise combinations (between shorter fragments and between short ones and the larger one) except for those with missing fragments, which were not included to avoid redundancies, making a total of 239 pairwise instances. In this example there are 5 replicates for each pairwise combination across the tests, with some redundancies.

5. CALCULATE NECESSARY TISSUE

Depending on the number of corals for study and the planned combinations and replicates, the number of fragments needed should be calculated in advance to assess whether there is enough tissue available to collect for each individual without compromising the health of the colony. For the previous example of test for 10 individuals (Fig. 2), the required tissue is presented in table 1 below.

Table 1. Number of fragments needed to arrange combinations between the ten ramets (Fig. 2).

	Number of fragments needed	
Ramet ID	**Large**	**Short**
1	5	14
2	5	13
3	4	17
4	1	23
5	5	14
6	5	16
7	5	16
8	5	14
9	4	18
10	4	17

6. DESIGN ASSEMBLY, MONITOR AND RETRIEVAL PLAN

Firstly, the structure where tests will be hanged from needs to be determined. It can be any kind of nursery structure (ropes, frames, coral trees...) although it is recommended that the tests hang with nylon off the structure rather than being fixed to it, as this way tissue will grow faster. An example of a rope structure with grafting tests is shown in figure 3. If there is no such structure already in the nursery, then the setup of this prior to test arrangement must be taken into account.

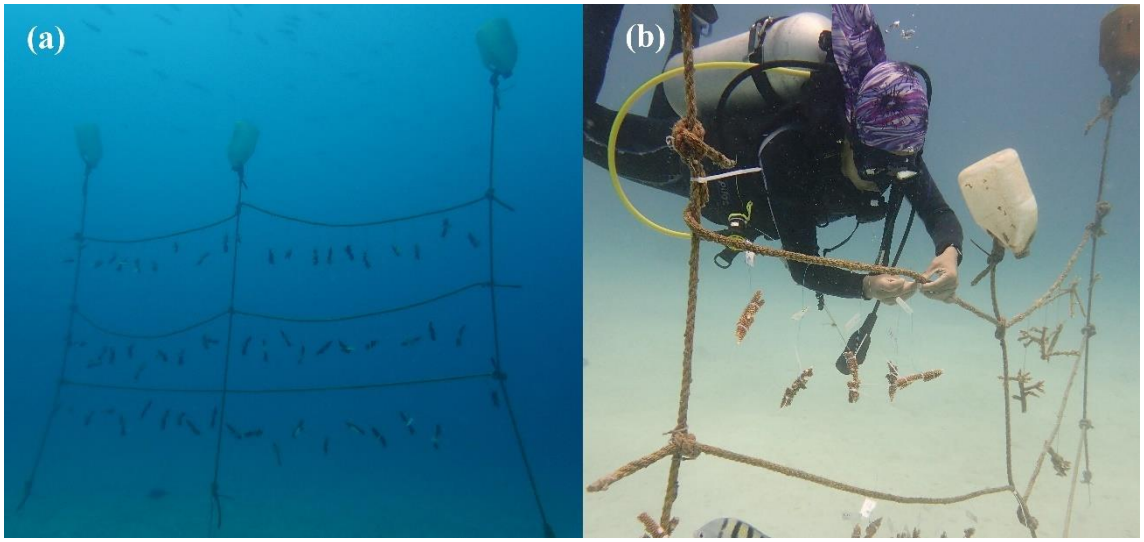


Figure 3. Rope structure in underwater nursery with grafting test (a) and process of hanging them by a SCUBA diver (b).

It is also important to think about who is going to set up the structure, assemble the tests, who is going to monitor their growth and clean them of macroalgae if necessary, who is going to collect and analyze them, etc; and when will all these activities take place. In addition, it is necessary to decide how long they are going to let them grow. In our experience and based in previous literature (García-Urueña et al. 1995), 10 weeks is enough to get a clear outcome between the fragments. Other logistical aspects associated with the previous step need to be considered (i.e., dive logistics for structures setup, transport of fragments to the lab or space where they will be analyzed once retrieved, etc.)

7. GRAFTING TESTS ASSEMBLY

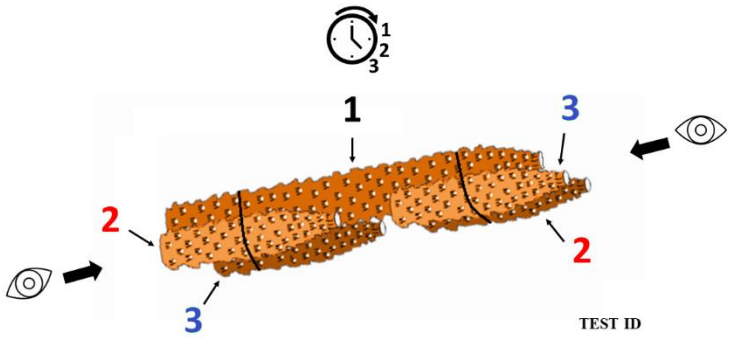
7.1. Fragment placement and orientation

Combinations of each test must be planned before underwater arrangement and should be represented in tables that include test number and ramet numbers to be used (see section 4). It is recommended that this information is printed in a waterproof paper or written down on a diving slate for when tests are performed (Fig. 4).

Figure 4. SCUBA Diver arranging the test using a diving slate with guidance of fragment combinations and number of fragments to collect from coral trees.

To arrange the test bundles, these instructions should be followed:

1) The five fragments should all have their apex pointing towards the same direction (to the right in the example of this protocol).
2) This way, the apical fragments would be represented by the third (right) column of the table; and the basal ones would correspond to the first (left) column of the table).
3) Both apical and basal fragments will be zip tied to the larger fragment following a clockwise order. To do this, the person arranging the test should be looking towards the apex (for apical ones) and then towards the bases (for the basal ones) (Fig. 5).
4) The clockwise order will always start with the larger fragment (number 1 on the clock) and will be followed by the fragment represented on the first row of the table. The third fragment to be placed following the clockwise order will then be the one on the third row of the table.
5) The same principle stated in the previous step will be followed for both apical and basal fragments.



TEST ID

ramet ID (2nd)		ramet ID (2nd)
ramet ID (1st)		
ramet ID (3rd)		ramet ID (3rd)

Figure 5. Five-fragment test bundle arrangement and table representation. The clockwise order is followed by looking towards the apices in the case of anterior fragments of the bundle and towards 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 order of ramets, not the ramet ID number itself.

7.2. General procedure

1) To optimize the time needed to carry out the tests, it is advisable to first fragment all necessary tissue (thus the importance of having the number of fragments needed as in table 1 and figure 4) and keep it separated by ramet ID. This can be done by using previously numbered plastic nests or bags (Fig. 6).
2) Fragments will then be joined together using thin zip ties and following the afore mentioned steps (section 7.1). Additionally, a small label indicating the test number and desired additional information such as the date will be added to each bundle. Labels can be laminated pieces of waterproof paper, or any kind of manufactured label that can resist being underwater for a long period. This label can be tied to the test with nylon (preferably tied to one of the zip ties). Also, some nylon from the label could be left longer and used later on to hang it from the structure.
3) The previous step can be either done underwater or at the surface. This decision would be a trade-off between time availability and reducing coral stress.
4) Once arranged, the labeled tests can be tied with nylon to the structure, making sure they do not crash into each other to avoid stress.

Figure 6. Plastic containers labeled by ramet ID (letters A to I) to facilitate fragment collection prior to test arrangement.

8. GROWTH MONITORING AND RETIREVAL

The monitoring frequency can vary depending on the interest in evaluating the evolution of the acceptance or rejection response. If there is interest in this aspect, retrieving the tests to be examined under the microscope several times before the final retrieval could be a possibility. However, this will increase the stress in the corals and could affect their health and competition performance. It is recommended that tests are checked at least every two weeks, to ensure that they do not get covered in macroalgae, get entangled or other possible events that would have detrimental effects on the tests.

9. MICROSCOPE EVALUATION

Before evaluating the contacts under the microscope, it is recommended to prepare a spreadsheet or table that contains a row for each combination and replicate, as well as the corresponding test number. Each test will be evaluated contact by contact (sometimes the flange can be removed to see if the contact between fragments keeps them together). It is also recommended to take photographs of each contact and write down whether acceptance or rejection is observed, as well as any additional information of interest.

Here we will present three types of responses that we have previously identified for *Acropora cervicornis*, one of them corresponding to an acceptance or tissue fusion response, and the other two corresponding to rejection or fission responses, one in a stronger way than the other.

9.1. Acceptance or fusion response

Acceptance responses involve the fusion of both skeletal components and soft tissues. When this happens, it is no longer possible to establish the start and end of both fragments, as they are smoothly sealed. Some examples under the microscope are shown in figure 7.

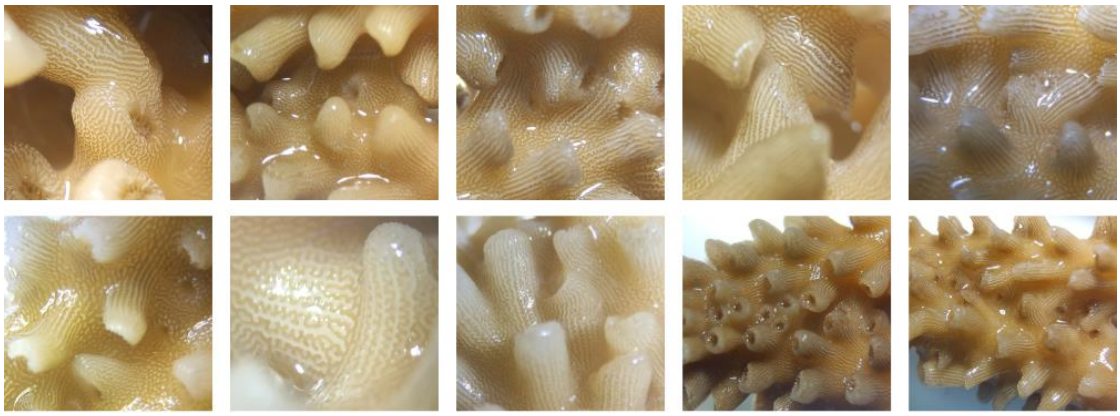


Figure 7. Microscope pictures of fusion between fragments of *Acropora cervicornis.*

9.2. Rejection or fission response

9.2.1. Strong fission

Here we call strong fission what has previously been described as rejection in order to distinguish it from a weaker, less clear rejection described in the next section. The rejection response normally involves the formation of a suture line at the skeletal interface separating the tissues of the two fragments Neigel & Avise (1983). This area is devoid of connecting soft tissue. Rejection responses can also be characterized by the overgrowth of one of the fragments over the other, sometimes 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). Some examples under the microscope are shown in figure 8.

Figure 8. Microscope pictures of strong fission between fragments of *Acropora cervicornis.*

9.2.2. Weak fission

In this type of response, what seemed like a certain fusion of the skeletal components was observed, not as clear and smooth as what is described as acceptance response. Also, in most of cases a white area was observed between both fragments, probably indicating lack of colonization by the symbionts (Hildemann et al. 1977). Some examples under the microscope are shown in figure 9.

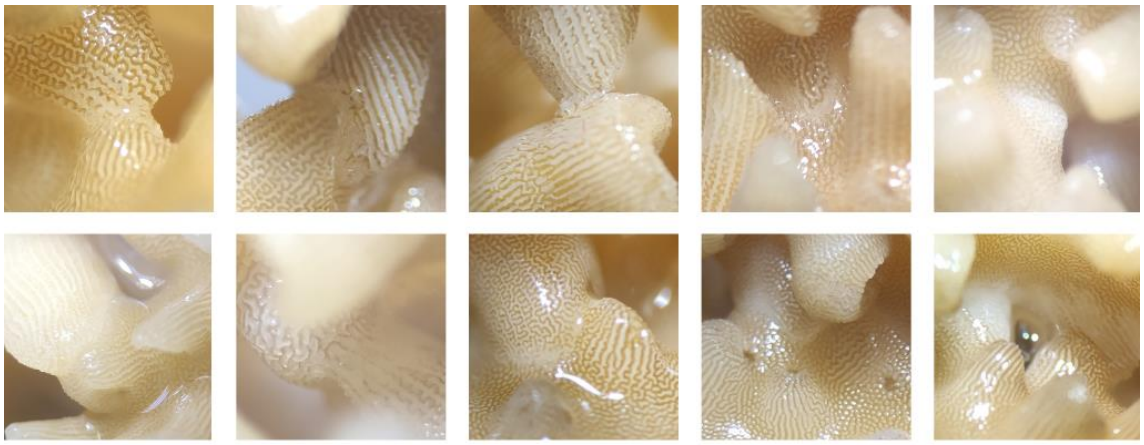


Figure 9. Microscope pictures of weak fission between fragments of *Acropora cervicornis.*

10. GENOTYPIC DIVERSITY ESTIMATION

Once outcomes data is collected, it is important to assess their consistency for each combination across replicates in case there is any inconsistency. That is to say, that the same combination (for example 4-5) sometimes fuses and sometimes fissions. If this happens, it is recommended to redo some more tests of the same combination. This may be due to human error during the test arrangement, using the wrong ramet. With this information, an estimation of the genetic relationships between the individuals in the study can now be established: those that are always fused will be taken as belonging to the same genotype, and those that are always rejected will belong to different genotypes. It is possible that those that reject each other weaker are more genetically related despite not being the same genotype.

ADDITIONAL NOTES

The grafting method could 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.

Other genotyping methods can provide additional information beyond identification of management units, such as host-symbiont population genetics and symbiont communities, as well as valuable information on the current allelic diversity of the population, and a better understanding of gene flow among target and neighboring populations. The grafting method, 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 laboratory genotyping

methods will depend on the human, financial and logistical resources available and the objectives of the study.

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. Also, validation of the method with different species will be beneficial to also account for species diversity in reef restoration.

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