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Evidence of Sexual Reproduction in Out-Planted Coral Colonies

Violeta Martínez-Castillo, Alma Paola Rodríguez-Troncoso * and Amílcar Leví Cupul-Magaña

Laboratorio de Ecología Marina, Centro Universitario de la Costa, Universidad de Guadalajara, Puerto Vallarta 48280, Mexico; viomarcast@gmail.com (V.M.-C.); amilcar.cupul@gmail.com (A.L.C.-M.)
* Correspondence: pao.rodriguezt@gmail.com or alma.rtroncoso@academicos.udg.mx; Tel.: +52-322-134-8009

Abstract: Intervention techniques to restore coral communities have become an important management tool to help recover and rehabilitate damaged reefs. The direct transplantation of healthy coral fragments is the most common method; however, there is controversy in the long-term success, as using coral clones may diminish the genetic diversity of the coral population. Genetic recombination can be achieved when the coral colony produces gametes and eventually reproduces; therefore, it is important to provide evidence that restored colonies produce gametes as their naturally recruited counterparts with similar colony size (age). Natural and restored *Pocillopora* coral colonies of the same size range (between 40 and 50 cm in diameter) were tagged and sampled during the rainy season to determine gamete maturation. Our results show no differences in the reproductive activity among colonies: natural and restored coral colonies matured gametes from June to October, with a peak in sexually active coral colonies in July. Also, gamete malformation was not observed. During the gamete production period, the area's temperature ranged from 27.9 to 30.02 °C. The results' evidence that coral colonies formed through active restoration contribute not only to the increase in live coral cover as seen in previous studies but potentially contribute in the medium term (>5 years after out-planting) to the production of larvae and local and subsidiary recruitment, since they exhibit the same reproductive patterns as their naturally formed counterparts and no differences in the reproductive activity among coral colonies. Therefore, long-term coral restoration projects contribute to maintaining the live coral cover and the genetic diversity in the region, eventually rehabilitating the coral community.

Keywords: coral reefs; coral reef recovery; Scleractinia; *Pocillopora*; reproductive activity; reproductive season

Citation: Martínez-Castillo, V.; Rodríguez-Troncoso, A.P.; Cupul-Magaña, A.L. Evidence of Sexual Reproduction in Out-Planted Coral Colonies. *Oceans* **2023**, *4*, 350–359. https://doi.org/10.3390/oceans4040024

Academic Editors: Michael W. Lomas and José Lucas Pérez-Lloréns

Received: 30 June 2023
Revised: 6 September 2023
Accepted: 18 October 2023
Published: 25 October 2023

1. Introduction

Coral reefs are declining worldwide at alarming rates, due to climate change and human activities [1]. On a global scale, climate change (i.e., ocean warming and acidification) affects coral reefs and their inherent ecological processes while increasing the frequency of thermal disturbances associated with inter-annual El Niño Southern Oscillation events [2,3], resulting in massive bleaching and mortality events, depending on the intensity and duration of thermal anomalies. On a local scale, destructive fishing, changes in land use, increasing pollution, decreasing water quality, and expanding urban developments contribute to rapid reef decline [4,5]. In response, there is an urgent need to implement intervention techniques (e.g., restoration programs) that promote coral recovery and ecosystem rehabilitation in addition to other local actions that help manage and conserve these ecosystems [6].

Active fragment nursing and transplantation is one of the intervention techniques applied as a management tool to promote coral recovery and maintain and preserve coral ecological and genetic diversity [7]. The final goal is not only for the ecosystem to recover its pre-decline live coral cover and maintain its associated biodiversity and services, but also to out-plant hermatypic corals that, due to their acclimatization response, can resist and cope with the critical conditions associated with climate change. This can be achieved

by finding the most resistant coral genotypes or populations by assessing individuals using genetic markers or with evidence from long-term monitoring [8–10]. Besides long-term viability, active coral restoration protocols face a series of challenges, so the assistance with coral restoration, its feasibility, and its success remain controversial within the scientific community [7]. First, active coral restoration must be adapted and designed considering each site's local characteristics (oceanographic and socio-ecological), and there should be a trial of different protocols to determine the most suitable restoration technique [11]. Also, the design of all restoration programs must contemplate the biology and physiology of the targeted species since they respond differently to environmental and anthropogenic stress depending on their life history [9,10,12]. Another concern, and probably one of the most relevant, is that most of the protocols used worldwide rely on asexual propagation; hence, coral clones are being re-attached to the reef, which may reduce or stratify the genetic diversity of the community [8]. While the success of active restoration is often measured by the survival of coral fragments (i.e., out-planted clones), recovery of the ecosystem does not only rely upon the survival rates of the out-planted corals, it also relies on the capacity of such recruits to grow, reproduce, and successfully contribute to the sexual recruitment of the reef [13].

Within the Central Mexican Pacific (CMP), a coral rehabilitation program has been implemented in Islas Marietas National Park (IMNP). This program uses direct transplantation of naturally formed coral fragments (i.e., "corals of opportunity") with a low-cost technology to mitigate the loss of coral seen in the last decade [14,15]. The contribution of coral restoration to the recovery of this ecosystem has been evaluated through survival rates of out-planted coral fragments, coral attachment to the substratum over time, and estimates of coral growth rates following out-planting [14–17]. While these are key features in coral recovery, another important trait has been left aside: coral reproduction. So far, we do not know if coral colonies are able to contribute to sexual recruitment as their naturally formed counterparts, which is important when maintaining the inherent genetic diversity and resilience capacity of coral ecosystems [7,13]. Here, we documented for the first time the sexual reproduction of naturally and assisted recruited *Pocillopora verrucosa* coral colonies as a complementary approach to evaluate restoration effectiveness in the mid-term. We hypothesize that assisted restoration will increase live coral cover and contribute to maintaining the diversity in the community through sexual reproduction a few years after assisted out-planting of coral fragments.

2. Materials and Method

Study area: the Central Mexican Pacific, located in the Northeastern Pacific, is an oceanographic transition zone between tropical and temperate waters where three water masses converge: the California Current with cold water, the Gulf of California water mass with warm and saline water, and the warm Mexican Coastal current running up north along the coast [18]. This transition zone is a dynamic region characterized by the influence of tropical cyclones that typically strike or travel along the Mexican coast between June and November [19]. Within this region lies Islas Marietas National Park, a natural protected area with both insular and marine territory that comprises two continental islands (Isla Larga and Isla Redonda) and several islets located 6 km from the shore [20] (Figure 1).

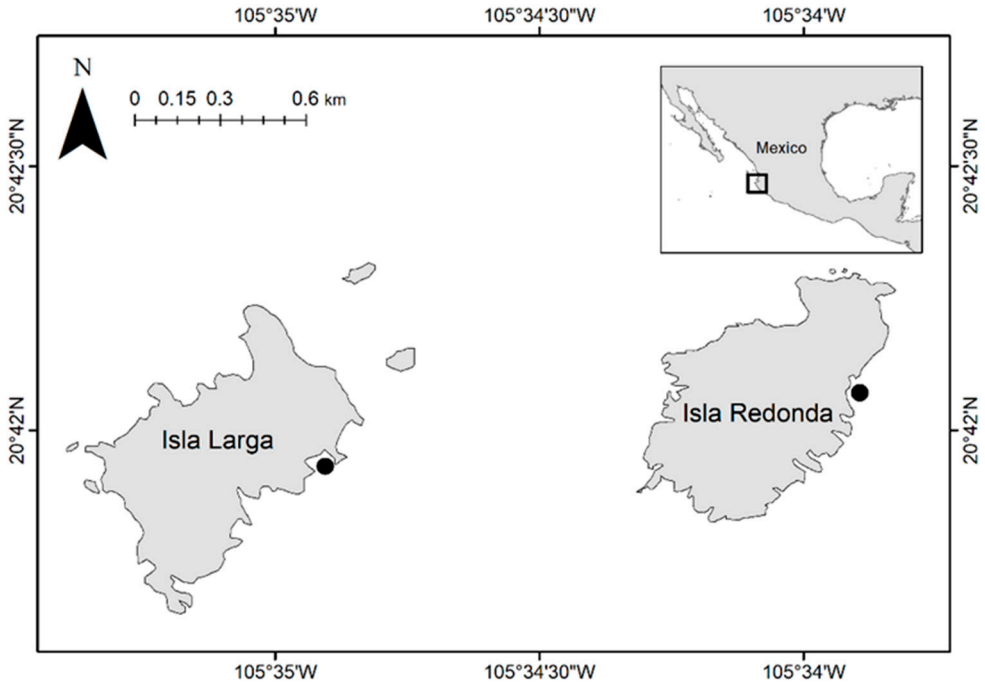

Figure 1. Location of the two restoration sites at Islas Marietas National Park. Black marks signal where coral colonies were sampled.

Isla Larga harbors a coral community along a shallow (1–5 m) and small platform comprised mainly of *Pocillopora* corals that are exposed daily to wide tidal ranges and wave action; in contrast, Isla Redonda has a rocky bottom and a deep slope (30 m) and lacks a platform, with the presence of *Pocillopora* and *Pavona* corals at a depth range from 2 to 18 m [21]. As mentioned before, a coral rehabilitation program in both islands has been implemented since 2013 using branching *Pocillopora* fragments naturally formed (i.e., fragments of "opportunity") out-planted at approximately 5–6 m depth. *Pocillopora* fragments were chosen due to their abundance and prevalence in both sites and because they are currently the region's main reef-building and resistant genera [22]. Fragments re-attached to the natural substratum have been monitored since out-planting [14,15].

Sample collection: the sample collection was performed by trained SCUBA divers with experience in coral identification and sample extraction to ensure that coral colonies were not damaged throughout the study. In both restoration sites, 10 naturally recruited *P. verrucosa* colonies and 10 *P. verrucosa* assisted recruited colonies (N = 40, 20 per site) were tagged using cable ties and plastic tags to ensure the correct monthly identification of each colony in sampling the same individuals throughout the study. To avoid bias, all colonies had a diameter of 40–50 cm, which is a marker that the coral colony has reached its reproductive age [23]. Since the study seeks to demonstrate the presence/maturation of gametes, colonies were sampled monthly from June to October, when reproductive activity has been previously recorded in the region [24,25]. From each coral colony, a 1 cm fragment was removed each month using a chisel and a hammer and fixed in 10% seawater formaldehyde until histological analyses were made. The temperature was recorded at the site every 15 min, using a Hobo® pendant data logger.

Coral reproductive assessment: fixed coral fragments were decalcified in 10% acetic acid, and the coral tissue was preserved in 70% ethanol. Each tissue was dehydrated in a TP1020 Leica® tissue processor and embedded in paraplast X-Tra® [26]. Coral samples were then cut using an RM 2125RT Leica® microtome in 8 µm slides and stained following the hematoxylin and eosin protocol [27]. Each slide was examined using an Axiolab Zeiss® microscope to evidence the gametes' presence and maturation. Female and male gamete maturation was characterized according to the specific description of the Pocilloporidae family [24]. A Mann–Whitney test (alpha level of 0.05) was performed to determine

differences in the proportion of coral colonies exhibiting gametes, using the software SigmaPlot® ver. 11.0 [28].

3. Results

Both natural and restored *P. verrucosa* colonies matured gametes from June to October, and the highest proportion of sexually active colonies was recorded in July (85% of all coral colonies) and the lowest by the end of the reproductive season, in October (10% of all coral colonies, Figure 2). The observed gametes presented all the typical characteristics described for the Pocilloporidae family, and colonies appeared as asynchronous hermaphrodites exhibiting gametes in different developmental stages within the same polyp. There was evidence of immature female gamete reabsorption at the end of the reproductive season, but no evidence of gamete malformation along the reproductive period. All gamete maturation stages were observed in the studied corals.

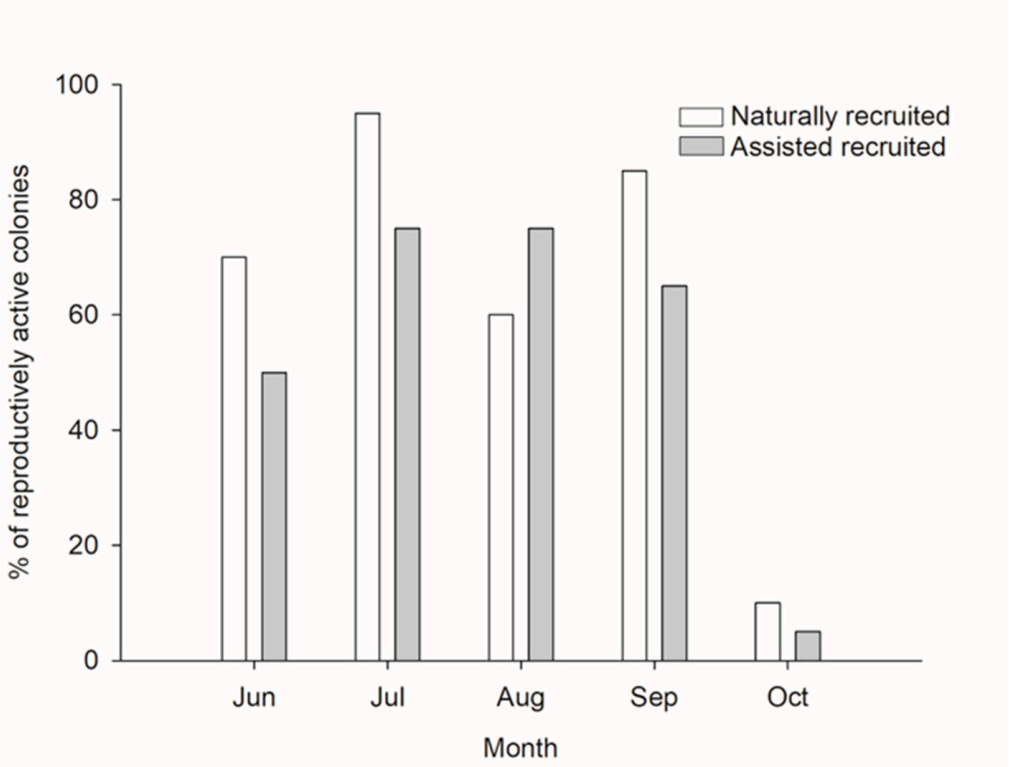

Figure 2. Percentage of *Pocillopora* naturally and assisted recruited colonies maturing gametes throughout the study.

During the gamete production period, the area's temperature ranged from 27.9 to 30.02 °C, with the minimum temperature recorded during June, of 28.04 ± 0.08 °C, and with a maximum of 29.9 ± 0.12 °C in October.

Evaluation of gamete maturation in both naturally and assisted recruited coral colonies showed that early stages of oocytes and spermaries were more abundant in July and August, while mature gametes were observed from July to October (Figure 3). All four maturation stages of oocytes were observed simultaneously from July to September (Figure 3a); in contrast, not all four maturation stages of male gametes were observed simultaneously (Figure 3b).

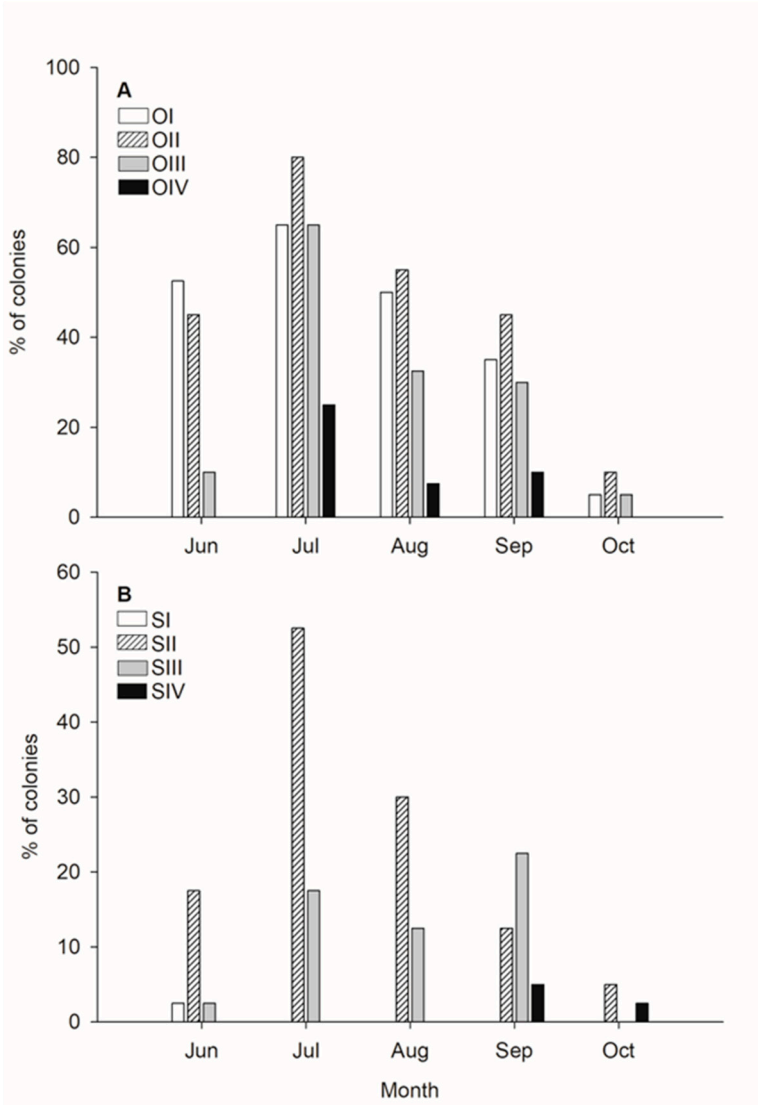

Figure 3. Percentage of total coral colonies exhibiting gametes in each studied month: (**A**) Stage I, II, III, and IV oocytes; (**B**) Stage I, II, III, and IV spermatids. O: oocyte, S: spermary.

When assessing natural vs. assisted recruited individuals, all coral colonies presented gametes; therefore, there was no difference in the reproductive activity among colonies, and no mortality or damage to the colonies sampled was observed. Therefore, there was no significant difference in the number of colonies presenting gametes each month (U = 9; $p = 0.55$).

4. Discussion

Active and passive strategies for coral reef rehabilitation have been developed in recent years to mitigate global coral loss and its associated biodiversity [7,29,30]. Active intervention involves the implementation of different protocols such as the use of "fragments of opportunity" or micro-fragmentation, and, in both cases, coral fragments can be either grown in nurseries before they are re-attached to the bottom or they can be directly out-planted in restoration sites if there are suitable conditions to do so [7]. The design of such restoration strategies is defined by spatial, temporal, and cost scales [11]. An advantage when using fragments of opportunity is that these fragments detach from the coral colony by natural physical disturbances such as wave action, storms, and hurricanes, among others; therefore, this protocol avoids the fragmentation of "donor" colonies, which is the most common technique for obtaining more individuals [7] and no damage or stress

is inflicted on the healthy adult colonies. Moreover, because these fragments are close to sites to be restored, they do not need a nursery or acclimatization period and can be immediately re-attached to the substratum. In addition, each fragment of opportunity before the transplantation is "visually assessed" [14] to obtain those healthy fragments that are survivors of physical disturbances such as constant waving or sedimentation. Therefore, this protocol is considered as giving a "second opportunity" to highly- resistant individuals, and their chances for survival are expected to be high, due to its life history traits. Within this context, corals that have thrived under disturbances are more likely to survive future environmental changes due to their resilience.

Even though the re-attachment of fragments of opportunity is a strategy widely used due to its low cost and simplicity [7,13,14,17,31], its long-term efficiency is still debatable [7] since there are still a lot of questions regarding the techniques used; the follow-up of restoration activities (i.e., monitoring programs) are designed to evaluate the survival of out-planted fragments and are often conducted in short time spans that cannot assess the mid- and long-term effects of restoration. In addition, the survival of out-planted fragments varies across reef sites and coral species and is influenced by the life history of the organisms, making it difficult to predict restoration outcomes [32]. Because of this, the success of active restoration through fragments of opportunity remains questionable. This highlights the necessity for restoration programs to have constant monitoring, using different metrics according to the phase of the project, by including physiological markers, such as growth or self-fixation rate [14] during the first months after implementation, in order to evaluate the success of the technique used and also the survival ability of the individuals. In the medium term, reproduction would become a metric that shows that the colony is in "optimal" conditions, as gamete production is a high-energy physiological process and can be inhibited when the individual becomes stressed, usually because of the environmental conditions [23]. Furthermore, in the long term, other ecological metrics must be included [9,11] to determine if the community can achieve rehabilitation. For this reason, any coral restoration initiative needs a thorough follow-up in the short, medium and long term to ensure that the restored reefs can eventually achieve their recovery and self-maintenance, even in the absence of human efforts, programs, and interventions.

In this context, so far, the success of coral restoration efforts in the Central Mexican Pacific has been evaluated in terms of coral survival and the increase in live coral cover. For example, there has been an increase in >15% of live coral cover in Punta de Mita, despite it suffering one of the region's most severe bleaching and mortality events [33]. This recovery has been promoted by the growth rates of *Pocillopora* corals, with assisted recruited fragments exhibiting linear extension rates of ~4 cm year^{-1}, resulting in a 3-fold increase in their size within the first two years [18] and allowing them to become available microhabitats and resources for associated fauna [31]. Also, the restoration protocol used in the CMP region has shown high attachment (78%) and survival rates ($\geq$80%) [15]. Altogether, studies show that calcium carbonate production has increased by 42% in these communities [14], an important achievement of the restoration protocol. However, the success of these active restoration efforts, regardless of the technique being used, has yet to be measured in the mid- to long term, not only through the survival rates of re-attached coral fragments but also through their capacity to reproduce and successfully contribute to the genetic diversity of the community [13].

Within this context, our study evaluates the next step in the contribution of coral restoration to the rehabilitation of degraded sites beyond the survival of re-attached coral fragments and the increase in live coral cover. With this new approach, we evaluate the contribution of assisted recruited coral colonies to maintain the coral ecosystem and the community's health through a key physiological trait they rely on: sexual reproduction. Active restoration via asexual recruits can rapidly increase coral coverage, as already seen in Eastern Tropical Pacific (ETP) populations [19,34,35], but it does not directly increase the genetic diversity and resilience capacity of coral communities unless opportunity fragments can grow and fully develop into adult colonies that can reproduce sexually. Our results

provide a first insight into out-planted colonies' production and maturing of gametes in the CMP. We observed that assisted recruited colonies were reproducing just as their naturally recruited counterparts were five years after their out-planting. Indeed, we observed the four maturation stages in both female and male gametes throughout the reproductive season. Since sexual reproduction is a physiological process which is sensitive to environmental stress, the fact that assisted recruited colonies have the same gamete maturation as naturally recruited ones indicates that the whole protocol used for coral out-planting is adequate, as already seen in previous studies [14,15,31]. Therefore, we can assume that the design of this protocol has been adequate [11].

Evaluating the success of a restoration program using different markers on each time scale is relevant, as the success of coral restoration initially depends on the technique (protocol) and the local conditions of the site, the life history traits, and the acclimation capacity of out-planted corals [27]. While we do not know the exact moment of the recruitment of naturally formed coral colonies, we can assume that they are of similar ages to their assisted recruited counterparts, based on their size during the study. All coral colonies sampled were ~5 years old and were out-planted in early 2015, coinciding with the most severe El Niño Sothern Oscillation event recorded so far [3]. Moreover, the sampling time coincides with La Niña thermal anomalies, with records of −1.3 °C. The fact that out-planted corals survived the most severe ENSO event to date and that five years later reproduction, a physiological process that is sensitive to thermal anomalies and other disturbances [29], was not hindered by low thermal anomalies, demonstrates the acclimation capacity and the resilient nature of *Pocillopora* corals in the Eastern Tropical Pacific [22,27,29–32]. Notably, during the study, detrimental effects from disturbances such as gamete malformation were not observed; all gametes exhibited the typical characteristics described for Pocilloporidae [15], and all coral colonies matured their gametes. This is important, because this process has been suggested as identifying coral communities and populations that are genetically more tolerant to stressors [6,9,12]. While we do not have information on the genetic composition of these coral communities, we have observed that CMP corals can withstand disturbances from global and local stressors [16,17,22,33,36]. Hence, they represent communities that are suitable for active coral restoration and that, in the long term, can be sites that are a suitable source of fragments, given their capacity for resilience.

It is important to note that this aforementioned capacity for resilience of corals depends not only on the physiological plasticity of the corals' host, but also on that of their symbionts. *Pocillopora* corals in the region harbor microalgae from the genus *Durusdinium* [37], a group of organisms that are extremophiles and are adapted to survive in environments with large temperature and turbidity fluctuations [38]. This also explains why reproduction was not negatively affected by thermal anomalies: as their endosymbionts can survive in wide temperature ranges, their photosynthetic activity was not constrained, and therefore there was enough energy translocated to the coral to produce and mature gametes [39]. This reinforces the idea that *Pocillopora* corals of the Eastern Pacific can withstand global disturbances and recover from them [15–17,22,33,34,36].

One recent major concern surrounding active restoration with asexually formed coral fragments (via micro-fragmentation or with fragments of opportunity) is that, while these techniques are effective in increasing live coral cover [15–17], out-planting coral fragments that are clones may eventually compromise the genetic diversity of the whole community.

As high genetic diversity within a population translates into a better acclimation and resilience capacity [6,9,12], which is necessary to withstand environmental disturbances, out-planting clonal fragments may limit resilience in the long term [7,40]. While the approach used in this study relies on the re-attachment of coral clones, our study evidences that in the mid-term (five years from out-planting), assisted recruited coral colonies can contribute to the genetic pool via recombination during sexual reproduction. In this context, it is important to mention that restoration efforts aim to help the recovery of a degraded or damaged ecosystem and preserve and enhance genetic diversity and resilience [40,41].

The genus *Pocillopora* represents the most abundant and resilient hermatypic coral throughout the ETP region [22], and as a branching coral, fragmentation is the most common reproduction mode as a strategy to self-seed the community [41] and survive local stressors, especially in marginal habitats [42]. Therefore, the use of fragments of opportunity in restoration efforts resembles a natural process for the species, which has been shown to have low genetic diversity throughout the region [43]. However, there is evidence of sexual larvae and recruits in the CMP region [44], confirming that *Pocillopora* can mature viable gametes. Hence, the restoration protocol may contribute to both types of propagation (asexual and asexual), contributing to the natural genetic structure of the community. Although in low proportions, the out-planted colonies can contribute to sexual recruitment in the mid-term, and at the moment, monitoring of the recruitment using semi-permanent transects in the restoration sites is being assessed.

Given the resilient nature of these communities, further restoration efforts may benefit from identifying such resilient genotypes using different physiological and genetic markers, as other communities may benefit from them. Furthermore, our results highlight the importance of assessing coral communities under different scopes. Altogether, our results evidence the effectiveness of this technique when restoring branching *Pocillopora* corals and highlight the resilience capacity of Central Mexican Pacific coral communities. Out-planted coral colonies not only survived manipulation during restoration activities, but they have also survived at least two ENSO events, and, five years later, they are contributing to the sexual recruitment of the community while facing thermal anomalies. The presence of colonies with reproductive capacity in the restored sites is crucial so that those that survive can contribute with new recruits to the recovery of both this area and neighboring sites.

Author Contributions: A.P.R.-T. and A.L.C.-M. conceptualized the study. Fieldwork and data collection were performed by A.P.R.-T., A.L.C.-M. and V.M.-C. Laboratory work and data analyses were performed by V.M.-C. The authors V.M.-C. and A.P.R.-T. developed the initial discussion. The first draft of the manuscript was written by V.M.-C. Funding was obtained by A.P.R.-T. and A.L.C.-M. All authors have read and agreed to the published version of the manuscript.

Funding: The present research was funded by NGS-100354C-23 and NGS-55349R-19 to A.P.R.T and PROCER/CCER/DROPC/09/2016 to A.L.C.M. During fieldwork and the writing of this manuscript, V.M.C. received a scholarship and a postdoctoral fellowship from the Consejo Nacional de Ciencia y Tecnología (CONACyT; I.D. 332939).

Institutional Review Board Statement: The present study was conducted using PPF/DGOPA-085/22 permit for sample collection and fieldwork in a Marine Protected.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank J.J. Adolfo Tortolero-Langarica for his help in field activities during the restoration program and Ángel Moisés Rivera-Quintero for his assistance in sample processing and in the histological laboratory procedures. They also want to thank the Comisión Nacional de Áreas Naturales Protegidas for providing all the necessary permits to conduct the restoration program and the present study.

Conflicts of Interest: The authors declare no conflict of interest.

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