Supplementary Information for

Assisted gene flow using cryopreserved sperm in critically endangered coral

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Table S1. **Summary of pooled sperm samples used to fertilize *A. palmata* eggs in Curaçao.** Sperm samples were collected and cryopreserved in three locations across the Caribbean. Sperm samples were pooled on the night of collection and/or after thawing to produce pools with as much genetic diversity as possible. Freshly-collected sperm was also used for *in vitro* fertilization experiments. For the CUR (fresh) pool, material was mixed from either 4 or 5 sires depending on the night of spawning and used on the same night it was collected. For the CUR (frozen) pool, material was mixed from two different sets of samples that were collected and frozen on different nights at different reefs (Sea Aquarium; 1 Sire, Spanish Water; 5 Sires). For the FL (frozen) pool, material was mixed from two different sample sets collected from different genets (i.e., genetic clones) at Elbow Reef ("Green" and "Orange" genets). For the PR (frozen) pool, material was mixed from five sires on a single night prior to freezing. Abbreviations: FL: Florida; PR: Puerto Rico; CUR: Curaçao.

Sperm Pool ID for *in vitro* Fertilization	Sperm Collection Location COUNTRY: Site	Sperm Collection Date(s)	Number of Genets Pooled on Collection Date	Total Number of Genets Pooled on Fertilization Date	Date(s) Sperm Pool Used in Experiments
CUR (fresh)	CUR: Spanish Water	7 Sept 2018	4	4	7 Sept 2018
CUR (fresh)	CUR: Spanish Water	8 Sept 2018	5	5	8 Sept 2018
CUR (frozen)	CUR: Sea Aquarium	4 Aug 2018	1	6	7 & 8 Sept 2018
	CUR: Spanish Water	2 Sep 2018	5		
FL (frozen)	FL: Elbow Reef	16 Aug 2016	1: "Orange Genotype"	2	7 & 8 Sept 2018
	FL: Elbow Reef	16 Aug 2016	1: "Green Genotype"		
PR (frozen)	PR: Rincón	7 Aug 2008	5	5	7 & 8 Sept 2018

Table S2. **Summary of gamete handling steps and setup of *in vitro* fertilization crosses using sperm from genetically-isolated populations of *A. palmata*.** Crosses were performed in Curaçao on 7 and 8 September 2018. Low: low-sperm treatments; 475 µL added on both nights. High: high-sperm treatments, 1425 µL added on 7 Sept, 950 µL on 8 Sept. For each pool of sperm, progressive motility was quantified upon thawing.

Spawning Date	Sperm Pool ID	Number of Sires	Total Motility (%)	Stock Sperm Conc. (cells mL^{-1})	Final Sperm Concentration		Number of Eggs Used (Range)
					Low Sperm (cells/mL^{-1})	High Sperm (cells/mL^{-1})	
7 Sept 2018	CUR Fresh	4	<25 to 50%	5.0E+08	1.58E+06	N/A	1,000–2,000
7 Sept 2018	CUR Frozen	6	25%	6.0E+08	1.90E+06	5.70E+06	1,000–2,000
7 Sept 2018	FL Frozen	2	<25%	3.7E+08	1.17E+06	3.52E+06	1,000–2,000
7 Sept 2018	PR Frozen	5	<25%	8.0E+08	2.53E+06	7.60E+06	1,000–2,000
8 Sept 2018	CUR Fresh	5	25 to 50%	6.0E+08	1.90E+06	N/A	3,000
8 Sept 2018	CUR Frozen	6	25%	6.0E+08	1.90E+06	N/A	3,000
8 Sept 2018	FL Frozen	2	<25%	3.7E+08	1.17E+06	N/A	3,000
8 Sept 2018	PR Frozen	5	<25%	8.0E+08	2.53E+06	5.07E+06	3,000

Spawning Date	Dam Colony ID	Spawn Volume	Eggs Allocated Per Bin	Water Per Bin	Number of Bins (Low Sperm + High Sperm)				Total Number of Bins	Total Number of Eggs Used	Colony Self-Fertilization
					CUR Fresh	CUR Frozen	FL Frozen	PR Frozen			
7 Sept 2018	F1	6 mL	2,000	150 mL	1 + 0	1 + 1	1 + 1	1 + 1	7	14,000	NO
7 Sept 2018	F2	2.2 mL	1,500	150 mL	1 + 0	1 + 1	1 + 1	1 + 1	7	10,500	NO
7 Sept 2018	Self1	1.8 mL	1,000	150 mL	1 + 0	1 + 1	1 + 1	1 + 1	7	7,000	YES
7 Sept 2018	Self2	2.0 mL	1,000	150 mL	1 + 0	1 + 1	1 + 1	1 + 1	7	7,000	YES
8 Sept 2018	F3	18 mL	3,000	150 mL	1 + 0	2 + 0	2 + 0	6 + 3	14	42,000	NO
8 Sept 2018	F4	9 mL	3,000	150 mL	1 + 0	2 + 0	2 + 0	6 + 2	13	39,000	NO
8 Sept 2018	F5	6 mL	3,000	150 mL	1 + 0	2 + 0	2 + 0	4 + 0	9	27,000	NO
8 Sept 2018	Self3	7 mL	3,000	150 mL	1 + 0	2 + 0	2 + 0	4 + 0	9	27,000	YES

Table S3. **Fertilization success for *A. palmata* eggs crossed with sperm from genetically-isolated populations.** Crosses were performed in Curaçao on 7 and 8 September 2018. Data represent the percentage of eggs/zygotes that were actively developing (i.e., undergoing embryogenesis) approximately 6 hours after sperm addition. No Sperm: Eggs were kept in FSW after cleaning and no sperm was added from any source. Low: low-sperm treatments; 475 µL added on both nights. High: high-sperm treatments, 1425 µL added on 7 September, 950 µL on 8 September. N/D: No Data: Negative controls for Dam F3 were not replicated due to a pipetting oversight in the early morning hours. A fertilization score was made from the egg stock at 7 AM instead. N/A: Not Applicable: High-sperm treatments were not performed for all sperm pools on all nights. Data are show here by sperm treatment. In Fig. 1, data are shown by sperm source only; using the average across both low and high sperm treatments for each egg donor colony.

		Sperm Pool									
Dam Colony ID	Dam Spawning Date	No Sperm 1	No Sperm 2	No Sperm 3	CUR Fresh Low	CUR Frozen Low	CUR Frozen High	FL Frozen Low	FL Frozen High	PR Frozen Low	PR Frozen High
F1	7 Sept 2018	0.0%	0.7%	0.5%	99%	82%	82%	10%	18%	2%	2%
F2	7 Sept 2018	0%	0%	0%	94%	82%	80%	18%	19%	24%	8%
F3	8 Sept 2018	0.1%	N/D	N/D	91%	58%	N/A	18%	N/A	1%	2%
F4	8 Sept 2018	0%	0%	0%	93%	37%	N/A	3%	N/A	0%	0%
F5	8 Sept 2018	0%	0%	0%	95%	60%	N/A	10%	N/A	0%	0%

Table S4. **Summary of larval settlement and survival rates for *A. palmata* juveniles produced through *in vitro* fertilization with sperm from genetically-isolated populations.** Larvae were reared in Curaçao and shipped to two facilities in Florida for settlement and long-term grow-out. Approximately equal numbers of larvae were shipped to each facility, which employed custom, in-house methods to foster larval settlement and post-settlement growth. AGF larval cohorts were handled using similar methods, but increased care was directed toward AGF larvae, i.e., larvae that resulted from crosses between genetically-distinct populations of the Caribbean (CUR×FL and CUR×PR cohorts). The large decline in survivorship from settlement to month 1 was primarily caused by an outbreak of disease. Larval survival was calculated by the number of survivors at the time point divided by the initial number of settlers.

Category of gamete cross: EGG × SPERM (Sperm Pool)	Number of Larvae	Initial Number of Settlers	Settlement Rate	Number of Settlers at 1 month	Survival Rate at 1 Month	Number of Juveniles at 6 months	Survival Rate at 6 Months
The Florida Aquarium							
CUR × CUR (Fresh Sperm)	3450	1847	54%	964	52%	235	13%
CUR × CUR (Frozen Sperm)	4000	2111	53%	292	14%	29	1%
CUR × FL (Frozen Sperm; AGF)	1107	663	60%	367	55%	80	12%
CUR × PR (Frozen Sperm; AGF)	270	100	37%	63	63%	6	6%
Location Totals	8827	4721	53%	1686	36%	350	7%
Mote Marine Lab							
CUR × CUR (Fresh Sperm)	3400	1258	37%	1205	96%	653	52%
CUR × CUR (Frozen Sperm)	4000	1466	37%	1293	88%	874	60%
CUR × FL (Frozen Sperm; AGF)	1100	584	53%	636	109%	442	76%
CUR × PR (Frozen Sperm; AGF)	270	133	49%	127	95%	98	74%
Location Totals	8770	3441	39%	3261	95%	2067	60%
Both Institutions							
CUR × CUR (Fresh Sperm)	6850	3105	46%	2169	70%	888	29%
CUR × CUR (Frozen Sperm)	8000	3577	45%	1585	44%	903	25%
CUR × FL (Frozen Sperm; AGF)	2207	1247	57%	1003	80%	522	42%
CUR × PR (Frozen Sperm; AGF)	540	233	42%	190	82%	104	45%
Total: All crosses with AGF Sperm	2747	1480	54%	1193	81%	626	42%
Total: All crosses	17597	8162	46%	4947	61%	2417	30%

Table S5. **Summary of genotyping results.** Listed are the sample accession number, the sample date (month/day/year), the sample population, the sample accession number of putative parent 1 and 2, and indication whether the sample was an adult or a juvenile, the DNA quantity (ng/µl), the genetic dissimilarity (GD) for putative parent-offspring triads, and the ancestry proportion in each cluster at K = 2, as estimated by Admixture.

Sample Accession Number	Sample Date	Population	Putative Parent 1	Putative Parent 2	Adult?	DNA quantity (ng/µl)	GD	Ancestry Proportion Cluster 1	Ancestry Proportion Cluster 2
a550962-4381376-121220-857_O03.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	10.560	N/A	0.0000	1.0000
a550962-4381376-121220-857_A05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	5.4500	N/A	0.0000	1.0000
a550962-4381376-121220-857_C05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	9.6600	N/A	0.1212	0.8788
a550962-4381376-121220-857_E05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	6.0700	N/A	0.0000	1.0000
a550962-4381376-121220-857_G05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	5.8500	N/A	0.0000	1.0000
a550962-4381376-121220-857_I05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	1.3300	N/A	0.0000	1.0000
a550962-4381376-121220-857_K05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	2.8400	N/A	0.0000	1.0000
a550962-4381376-121220-857_M05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	6.3600	N/A	0.0000	1.0000
a550962-4381376-121220-857_O05.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	3.7000	N/A	0.0000	1.0000
a550962-4381376-121220-857_A07.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	6.7600	N/A	0.0000	1.0000
a550962-4381376-121220-857_C07.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	12.0900	N/A	0.0000	1.0000
a550962-4381376-121220-857_E07.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	4.2200	N/A	0.0000	1.0000
a550962-4381376-121220-857_G07.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	13.0900	N/A	0.1177	0.8823
a550962-4381376-121220-857_I07.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	9.1800	N/A	0.0000	1.0000
a550962-4381376-121220-857_K07.CEL	11/13/19	CURxCUR (frozen)	N/A	N/A	N	8.6800	N/A	0.0000	1.0000
a550962-4381376-121220-857_G03.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	5.1400	0.0022	0.4714	0.5286
a550962-4381376-121220-857_O01.CEL	11/13/19	CURxFL (frozen)	N/A	N/A	N	5.1300	N/A	0.4813	0.5187
a550962-4381376-121220-857_A03.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	3.2300	0.0026	0.4504	0.5496
a550962-4381376-121220-857_C03.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a100000-4368120-060520-256_C03.CEL	N	5.8800	0.0089	0.5353	0.4647
a550962-4381376-121220-857_M01.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	3.8000	0.0036	0.5136	0.4864
a550962-4381376-121220-857_K01.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a100000-4368120-060520-256_C03.CEL	N	5.4800	0.0087	0.4828	0.5172
a550962-4381376-121220-857_I01.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a100000-4368120-060520-256_C03.CEL	N	8.4100	0.0094	0.4644	0.5356
a550962-4381376-121220-857_G01.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	3.6900	0.0027	0.4759	0.5241
a550962-4381376-121220-857_E01.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	4.6200	0.0027	0.5013	0.4987
a550962-4381376-121220-857_C01.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	1.2700	0.0027	0.4820	0.5180
a550962-4381376-121220-857_A01.CEL	11/13/19	CURxFL (frozen)	N/A	N/A	N	4.9300	N/A	0.1801	0.8199
a550962-4381376-121220-857_K03.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	5.7300	0.0033	0.5084	0.4916
a550962-4381376-121220-857_M03.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	4.5100	0.0045	0.4504	0.5496
a550962-4381376-121220-857_I03.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a550962-4381376-121220-857_E19.CEL	N	6.1500	0.0022	0.4838	0.5162
a550962-4381376-121220-857_E03.CEL	11/13/19	CURxFL (frozen)	a550962-4381376-121220-857_A09.CEL	a100000-4368120-060520-256_C03.CEL	N	4.2700	0.0092	0.5455	0.4545
a550962-4381376-121220-857_M07.CEL	11/4/19	CUR	N/A	N/A	Y	4.5300	N/A	0.0000	1.0000
a550962-4381376-121220-857_O07.CEL	11/4/19	CUR	N/A	N/A	Y	1.8700	N/A	0.0000	1.0000
a550962-4381376-121220-857_A09.CEL	11/4/19	CUR	N/A	N/A	Y	6.1700	N/A	0.0000	1.0000
a550962-4381376-121220-857_C09.CEL	11/4/19	CUR	N/A	N/A	Y	8.1500	N/A	0.0000	1.0000
a550962-4381376-121220-857_E09.CEL	11/4/19	CUR	N/A	N/A	Y	15.2500	N/A	0.0000	1.0000
a550962-4381376-121220-857_E19.CEL	11/15/19	FL	N/A	N/A	Y	9.9000	N/A	1.0000	0.0000
a100000-4368120-060520-256_C03.CEL	10/4/19	FL	N/A	N/A	Y	17.0000	N/A	1.0000	0.0000

Supplementary Information: Extended Materials and Methods

Study Sites and Gamete Collection

Two locations in Curaçao were chosen for spawning observations and gamete collection: Spanish Water (locally known as Spaanse Water; 12°4'13.11"N, 68°52'18.22"W) and the Curaçao Sea Aquarium (12°4'59.94"N, 68°53'42.47"W). Both reefs have large stands of *Acropora palmata*. The reef at Sea Aquarium is known to have high overall genetic diversity (1) and Curaçao in general is known to have higher overall genetic diversity in *A. palmata* relative to other parts of the Caribbean (2). In coordination with the full moons in late July and late August in 2018, divers surveyed between 25 and 100 *A. palmata* colonies per night. Observations were made from 2 days before the full moon to 11 days after the full moon in late July, and from 2 days before the full moon to 13 days after the full moon in late August, for a total of 30 nights of monitoring (see ***Table S1***)

On each dive night, between 4 and 16 divers monitored colonies for at least 60 minutes, spanning the known spawning window of this species in Curaçao, beginning approximately 1 hour and 45 minutes after sunset. Divers examined colonies continuously for signs of setting (i.e., polyps holding egg-sperm gamete bundles in their mouths just prior to release). When setting was observed, the colonies were tented with weighted nylon mesh tents fixed to inverted plastic funnels in order to collect egg-sperm bundles in 50-mL conical centrifuge tubes (polypropylene, BD Falcon) affixed to the neck of each funnel, following methods previously developed by our team (3-8).

On shore, gamete collections from each colony were assessed for their suitability as either sperm donors (sires) or egg donors (dams) based on the volume of material produced; for each egg donor colony, we aimed to collect at least 2 mL of spawn so that replicate fertilization bins could be prepared containing at least 1,000 eggs per bin. The gamete samples that were chosen as egg donors were maintained in their original, closed collection tubes during transport back to the lab. Samples chosen for sperm pooling and cryopreservation were concentrated immediately upon arrival on shore by removing the majority of the seawater from the tubes using a new, sterile plastic transfer pipette for each tube, so that the remaining gamete bundles had approximately a 1:1 ratio of gamete volume to seawater volume in the tube. This ensured that after gamete bundles broke apart, the resultant sperm solution would be concentrated enough for successful cryopreservation. Highly-concentrated sperm

samples can be difficult to collect, but high concentrations help to compensate for losses in viability due to freezing stress, and this allows for a smaller overall volume of the sperm solution and cryoprotectant being added during *in vitro* fertilization. For each sire, we aimed to collect 5 mL of gamete bundles, but in some cases as little as 1 or 2 mL of spawn was used in order to increase the overall number of donor genotypes. All gamete samples were transported approximately 40 minutes by car to the CARMABI Research Station for *in vitro* fertilization experiments.

Sperm samples were collected and preserved from the central and western populations of *A. palmata* in Puerto Rico and Florida, respectively. For the central Caribbean samples, sperm was collected from Tres Palmas Marine Reserve in Rincón, Puerto Rico, in 2008. Sperm was pooled from five donor colonies and cryopreserved in sterile seawater (SSW; 0.2-µm impact filter, 47 mm, Millepore) with a final concentration of 5% dimethyl sulfoxide (DMSO, >99.5% purity, Sigma) as a cryoprotectant. For the western Caribbean samples, sperm was collected from Elbow Reef in Key Largo, FL, in 2016. For these samples, sperm were cryopreserved in SSW with a final concentration of 10% DMSO. Elbow Reef is known to harbor only a few genets (clones) of *A. palmata* (9); therefore, tissue samples were collected from each donor colony and genotyped to confirm that sperm samples originated from two distinct genets. All samples were held in storage under liquid nitrogen in the intervening years, then sent to Curaçao from the USDA National Animal Germplasm Program in Fort Collins, CO, via air using a liquid nitrogen dry shipper. Upon arrival in Curaçao, the temperature of the dry shipper was measured to be below −175°C, and it was immediately re-filled with liquid nitrogen. All samples were then held in storage under liquid nitrogen until immediately before they were thawed for *in vitro* fertilization in the laboratory.

Sperm Motility

We collected egg-sperm bundles from *A. palmata* in September 2018. For efficiency in sperm assessment, each thawed sample was assessed by visual inspection and total motility was estimated by quartile. In fresh sperm samples collected in Curaçao, total mean sperm motility ranged from 25 to 50% (Table ***S2***). Sperm remained motile for at least 6 hours. These observed motility values and durations were within the range observed in *A. palmata* from other locations, including the Florida and Puerto Rico samples collected in 2016 and 2008, respectively. The post-thaw motility for the frozen samples from Curaçao was approximately 25%. Post-thaw motility for the frozen samples from Florida and Puerto Rico was between 0 and 25% (Table ***S2***).

Egg Preparation and Screening

Egg-sperm bundles were allowed to break up with gentle or no agitation and eggs were then rinsed at least five times using filtered seawater (FSW; 47-mm-diameter GF/F filter, Whatman) in polycarbonate fat separators until the surrounding water was clear, indicating that residual sperm, plankton, and detritus had been removed (see Table ***S2*** for details of gamete production and handling by colony). To avoid transferring any sperm from one parent colony to another, egg batches from each colony were kept in individual fat separators during the entire rinsing process, and separate beakers were used to pour water into each fat separator. Egg batches were then screened for the occurrence of self-fertilization. If eggs from a specific donor colony were observed to undergo primary cell cleavage, this indicated that fertilization had occurred within the collection tubes, and this material was not used for *in vitro* fertilization. Egg batches were observed for up to 4 hours after bundle breakup to avoid using eggs that were already fertilized. Trial experiments in Curaçao showed that the eggs remained viable for at least seven hours and *Acropora* eggs from the Pacific remain viable for at least this long (10, 11).

Sperm Preparation and Assessment

The concentrated samples chosen for sperm collection were gently agitated to break-up the gamete bundles. The free sperm solution was removed from the bottom of the tube using a plastic transfer pipette and then filtered through a clean cell strainer (70-µm nylon mesh, BD Falcon) to remove plankton, detritus, and any coral eggs carried over. Sperm samples were first kept separate by parent colony while they were assessed for motility using a phase microscope (Olympus BH2) and video system following method developed previously (4). To verify these data, additional motility and concentration data were collected by visual examination using a Leitz Orthoplan microscope with a phase contrast condenser and phase contrast objectives. Motility was scored by visual examination of 10-µL aliquots of the sperm solution (diluted 1:10 in clean FSW) that were spotted onto clean glass microscope slides and observed at 125×. Sperm concentrations were measured by direct observation using a cell counting chamber (sperm and bacteria counting chamber, Petroff-Hausser) at a total magnification of either 125× or 500×.

Sperm Freezing and Thawing

Sperm samples were cryopreserved as described previously (4, 12). Briefly, samples were kept as concentrated as possible, with the goal of achieving a sperm concentration above 1×10^9 cells mL^{-1}. Samples in which total motility was at least 50% were pooled to create a mixed population of sperm from as many donor colonies as possible, with the goal of pooling material from at least 5 donor colonies per night to ensure pooled sperm contained substantial genetic diversity. The sperm concentration of each pooled sample was measured, then known volumes of the pooled sperm were diluted 1:1 (vol:vol) with freshly-prepared 20% DMSO in sterile seawater (SSW; 0.22-µm Sterviex HA syringe filter, Millepore). The 20% DMSO solution was added very gradually to the concentrated sperm with constant swirling to offset exothermic heating, which can potentially damage the sperm. The sperm were allowed to equilibrate in the 10% DMSO for 10 min. During that time, 1-mL aliquots were placed into cryovials (2.0 mL, externally threaded, Corning), which were capped, loaded into a custom-built cryofreezer, and frozen at 20 ± 2°C $minute^{-1}$ (calculated as the slope of the line from −10 to −80°C) until they reached −80°C, at which time they were submerged directly in liquid nitrogen.

During nights when only a small number of colonies spawned in Curaçao, sperm was assessed, frozen, loaded into cryocanes, and maintained in liquid nitrogen for several days, then used on nights when sufficient eggs could be collected to perform the *in vitro* fertilization experiments. For the *in vitro* fertilization experiments conducted on 7 and 8 September 2018, the cryopreserved Curaçao sperm samples were frozen on 4 August 2018 (1 donor colony from Sea Aquarium) and 2 September 2018 (5 donor colonies from Spanish Water). These samples were pooled upon thawing, yielding a sperm pool for the CUR×CUR (frozen) crosses with eggs from six donor colonies. The inclusion of the sample from Sea Aquarium helped to ensure that the pooled sperm contained diverse genotypes. Tubes from these same batches of frozen Curaçao sperm were used for the *in vitro* fertilization trials on both 7 and 8 September 2018. Weather conditions at Sea Aquarium prevented monitoring and collection during the last four nights of the project, therefore fresh sperm from Sea Aquarium was not available for the CUR×CUR (fresh) crosses. For the crosses described here, fresh eggs from Spanish Water and frozen sperm from the Sea Aquarium and Spanish Water were used (Tables ***S1*** and ***S2***).

To thaw cryopreserved sperm samples for *in vitro* fertilization, the cryovial was removed from liquid nitrogen and swirled gently in warm FSW (approximately 30°C) keeping the cryovial constantly moving (but

without shaking) for about 2 minutes until the contents were completely thawed. The vial was then gently inverted to mix its contents, aliquots of sperm were quickly assessed for motility and concentration, and sperm was added to each fertilization container very gently using a micropipette to avoid placing heavy shear stress on the cells.

In Vitro Fertilization Experiments

On 7 and 8 September 2018 (nights 12 and 13 after the late August full moon), massive spawns were observed at Spanish Water, with approximately 75% of all colonies spawning during this window. On these nights, a series of large-scale *in vitro* treatments was performed using freshly-collected eggs from the Spanish Water colonies with four different pools of sperm. The four categories of crosses were: CUR×CUR (fresh sperm), CUR×CUR (frozen sperm), CUR×FL (frozen sperm), and CUR×PR (frozen sperm).

For all four categories of crosses, sperm was added to clear polystyrene containers (21.0 × 21.0 × 7.6 cm, ClearSeal clear hinged lid containers, Dart, Catalog #C90PST1) containing a starting water volume of 150 mL of FSW. Our goal was to aliquot 3,000 eggs per container and hold the number of eggs per container consistent between containers for each egg donor colony. In cases where donor colonies did not produce enough eggs to reach this target number, eggs were distributed evenly between containers for a total of 1,000 to 3,000 eggs per container.

In vitro crosses were designed to balance both experimental and conservation goals. First and foremost, we aimed to test whether AGF is possible in *A. palmata* to any degree, and if so, to produce as many AGF juveniles as possible with limited amounts of irreplaceable cryopreserved material. In a small-scale trial leading up to the mass spawning nights, we observed low overall fertilization in both the FL and PR sperm pools. Therefore, we added sperm to the large-scale *in vitro* crosses at two different concentrations; the low-sperm treatments were conducted to keep the final concentration of DMSO well below levels that can be toxic to sperm, while the high-sperm treatments boosted the overall sperm concentration to increase the number of encounters between sperm and egg, while potentially edging closer toward toxic levels of DMSO. For low-sperm treatments, we added 475 µL of the stock solution per container on both nights. For high-sperm treatments on 7 September, we added three times this amount (1425 µL per container). The only high-sperm treatment performed on 8

September was conducted in the CUR×PR (frozen) crosses using 950 µL of the stock solution (see Table ***S2*** and ***S3*** for details).

Due to the natural variation in spawning volume on various nights when sperm samples were cryopreserved, there was also slight variation in the starting concentration of the cryopreserved sperm samples. For CUR (fresh sperm), starting sperm concentration was 5×10^8 cells mL^{-1} and 6×10^8 cells mL^{-1}, respectively, on 7 and 8 September 2018 (Table ***S2***). For the frozen sperm samples, identical samples were used on both nights. Starting sperm concentrations were 6×10^8 cells mL^{-1} for CUR (frozen sperm), 3.7×10^8 cells mL^{-1} for FL (frozen sperm), and 8×10^8 cells mL^{-1} for PR (frozen sperm).

Across both nights, final sperm concentration was between 1.17×10^6 and 2.53×10^6 cells mL^{-1} for the low-sperm treatments and between 3.52×10^6 and 7.60×10^6 cells mL^{-1} for the high-sperm treatments (Table ***S2***). Overall, the final sperm-to-egg ratio in the *in vitro* fertilization experiments were chosen to span the ratio considered optimal for corals (100,000:1) (13). We endeavored to maintain the final sperm:egg ratios in all treatments at or above 25,000:1.

To continue monitoring egg batches for self-fertilization, replicated aliquots of eggs were taken from each donor colony and kept separate as no-sperm controls. Approximately 200 eggs were placed in 40 mL of FSW in 100-mm polystyrene Petri dishes, replicated three times per egg donor. These dishes were examined at multiple time points during the night to determine whether any cell division had taken place. These observations were not performed at the same large scale as the *in vitro* fertilization crosses because we prioritized the goal of producing as many juveniles of this threatened coral species as possible.

For each *in vitro* fertilization replicate, 1,000 to 3,000 thoroughly-rinsed eggs were placed in 150 mL of FSW in a polystyrene container, then either fresh or cryopreserved sperm was added by very slow pipetting and the solution was swirled gently every 1 to 2 minutes for approximately 10 minutes. Although fertilization may occur within minutes of adding sperm, cryopreserved sperm may have lower motility than fresh sperm, and the cryoprotectant in these samples can reduce sperm motility. Therefore, sperm-egg mixtures were left at this density for one hour with occasional swirling before any rinsing steps were started. After one hour, the volume in the containers was raised to 500 mL to dilute the cryoprotectant. During the next two hours, eggs and zygotes in every bin were rinsed several times with FSW to remove residual sperm, bacteria, and cryoprotectant, using a

clean fat separator for each cross, then eggs and zygotes were transferred to clean containers containing new FSW as they began cell cleavage and embryogenesis.

Between 6 and 8 hours after sperm was added, fertilization success in each category of cross was assessed by sub-sampling between 40 and 150 embryos from each container. Fertilization was quantified during this window of time because the visual difference between unfertilized eggs and developing *A. palmata* embryos is most striking during gastrulation: unfertilized eggs remain round and intact while embryos undergoing gastrulation have the appearance of a "prawn chip" or "cornflake" (14). Furthermore, when coral eggs are fertilized with cryopreserved sperm, their time to first cleavage can be delayed by one or more hours (15). Therefore, we assessed fertilization and development 6 to 8 hours after fertilization, rather than immediately after the onset of cleavage, as is done for other coral species (16), to leave ample time for slower-developing embryos to proceed through cell division. The total number of unfertilized eggs and developing embryos were counted under a stereomicroscope (Nikon SMZ800) at between 10× and 63× magnification and the number of developing embryos was recorded. Fertilization percentages were then determined for each cross.

Larval Propagation and Transport

After fertilization was quantified, all unfertilized eggs were removed from the containers by pipetting. The developing embryos were transferred into new FSW and new containers by pipetting, then embryos were distributed across additional containers as needed to maintain a density below 1 embryo mL^{-1}. This low density has been found to improve larval survival (3). Larvae were maintained in a dimly-lit, air-conditioned laboratory at 27°C with indirect natural light and a 12h:12h light:dark cycle with overhead fluorescent lights. Containers were held static with gentle agitation 4 to 5 times per day. Container changes and 95% water changes were performed every 24 to 48 hours by consolidating and rinsing larvae in a fat separator or by pipetting larvae into new containers. The number of swimming larvae in each treatment was assessed on 11 September 2018 (i.e., 3 and 4 days after spawning) in preparation for air transport to Miami, Florida, USA on 13 September 2018.

A variety of ultra-insulated coolers were tested using temperature loggers for their ability to maintain water temperatures between 27 and 28°C for at least 12 hours during air transport. Additionally, a variety of single-use and re-useable drinking water bottles were tested to identify which shape and material would cause the

least shear stress and turbulence to the larvae. A simulated larval transport experiment was performed in which a small batch of *A. palmata* larvae was subjected to packaging, transportation, vibration, handling, and temperature stresses similar to those that would be experienced during air transit. Coolers produced by Ozark Trail (52 Quart High Performance Cooler; interior dimensions 58 × 29 × 28 cm L × W × H) and Pelican (Pelican Elite 30; interior dimensions 37 × 26 × 28 cm L × W × H), and 1.5 L HDPE clear plastic bottles with narrow necks (Lovers Ice Water, Curaçao; 9 × 28 cm W × H) were chosen to transport the larvae. Bottles were kept sealed prior to shipping the larvae, and only opened immediately before use. Fresh water was decanted and bottles were rinsed once with FSW.

Larvae were consolidated in freshly-prepared FSW using clean glass and plastic transfer pipettes. Approximately 150 to 1000 larvae were packaged per bottle, depending on the relative rarity of each larval cohort (i.e., corals from AGF crosses were packed at lower density to maximize survival). For all bottles, larval density was kept below 1 larva mL^{-1} FSW to maintain high dissolved oxygen concentrations and discourage bacterial growth. Bottles were filled so that less than 1 cm of vertical air space remained in the neck, which reduced water sloshing and subsequent shear stress on the larvae. Lids were tightened only in the final 10 minutes before closing the coolers. Each cooler contained 12 bottles. Excess space in the Ozark Trail cooler was filled with foil-lined, ultra-insulating bubble wrap to stabilize the larvae and further buffer against temperature changes.

Larvae were packaged into the bottles beginning at 03:00 Eastern Time (ET) on the day of transport. Air travel began at 07:00, arrival in Miami was completed by 11:00, customs clearance was completed by 12:00, and ground transportation was completed to the destination facilities by 15:00 ET. Thus, larvae spent approximately 12 hours in the bottles. Upon arrival, larvae could be seen actively swimming in the bottles. A total of 60 L of water and over 20,000 larvae were transported using four coolers. At the two settlement facilities, Mote Marine Lab and The Florida Aquarium Center for Conservation, larvae were carefully poured into holding containers (1-L polystyrene clamshell containers) to recover from shipment. The number of larvae from each bottle was estimated and compared to the number of larvae shipped.

Larval Settlement and Grow-out at Mote Marine Lab

Over 20,000 coral larvae were transported by air to Florida, where the larval cohort was divided for settlement and grow-out at Mote Marine Laboratory and The Florida Aquarium. At Mote, larval settlement was carried out in static 19-L glass aquaria containing seawater from a well system. Seawater exiting this well (pH 7.5) was immediately aerated to off-gas carbon dioxide and hydrogen sulfide, filtered through a moving bed biological filter containing SWX media (Sweetwater) followed by a sand filter (Pentair Aquatic Ecosystems), then passed through a pleated filter (100 µm, Pentair Aquatic Ecosystems) to remove sediment before being introduced to settlement aquaria (pH 8.0, Salinity 37 ppt). Aquaria were maintained in an indoor, temperature-controlled wet lab at approximately 27°C. Aquaria were also halfway submerged in flow-through fiberglass raceways measuring 2.5 × 1.0 × 0.3 m to act as a second temperature control failsafe. Swimming larvae were added to the aquaria each containing 12–20 replicate ceramic substrates (Ceramic Coral Frag Plugs, Boston Aqua Farms) measuring 3 cm in diameter.

To estimate larval numbers, larvae were transferred to a vessel with a known volume and the total volume of seawater was raised to 2 L. The water was gently mixed to disperse the larvae uniformly in the container, then a glass pipette (10 mL, Pyrex) was used to draw up a volume of seawater and larvae. The pipette was viewed under a stereomicroscope (American Optical) at 10× magnification and larvae were quantified by counting the number present in 3 separate, 1-mL sections of the pipette. This entire process was then repeated two more times and the resulting values were averaged to arrive at an estimate of larval number mL^{-1}. The larvae were then aliquoted in know numbers to settlement tanks.

Settlement was fostered by sprinkling ground pieces of live crustose coralline algae (CCA) onto the tops of each substrate. The CCA used was a mixture of unidentified species cultured in Mote's land-based coral facility in the same location. Importantly, the addition of this mixture of diverse CCA encouraged most of the swimming larvae to settle onto the tops of each substrate rather than onto the undersides of the substrates or onto glass surfaces in the tanks. Given the high conservation value of the two larval cohorts produced from AGF sperm (i.e., larvae from the CUR×FL and CUR×PR crosses), and to maximize the number of settlers obtained from these two groups, extra effort was taken to foster their settlement by keeping them at a lower density compared to the other larval cohorts. For these AGF cohorts, approximately 250 larvae were added per settlement tank. Similarly, larvae from the CUR×CUR (frozen) cross were given higher priority than larvae from the CUR×CUR (fresh)

cross because larvae were more abundant in the latter cross, and the former has conservation value as the largest coral cohort ever produced through cryopreservation. Thus, approximately 750 larvae were allocated per settlement tank for the CUR×CUR (frozen) cross and approximately 1700 larvae were allocated per settlement tank for the CUR×CUR (fresh) cross. After larvae had settled, the substrates were moved to a dimly-lit, indoor, flow-through raceway (same dimensions as above) for post-settlement care and grow-out. The raceway was fed by flow-through seawater at 1.0 L minute^{-1} and 6–7.5-cm air stones were used to provide water circulation within the raceway.

When settled juvenile corals had established dense populations of *Symbiodiniaceae* spp. at approximately 1.5 months post-settlement, all juveniles were moved to a clean, outdoor grow-out raceway with the same dimensions and number of air stones as above, fed by filtered seawater at a rate of 4.0 L minute^{-1} at 23–27°C and pH 8.0. The settlement substrates were marked according to each cross on the bottom of each substrate with permanent marker covered by extra thick cyanoacrylate super glue gel (Bulk Reef Supply) and placed on modular plastic egg crate racks elevated 3 cm above the raceway floor using small PVC segments.

Algal fouling was controlled by the addition of the snails *Lithopoma americanum* and *Batillaria minima* (approximately 50 and 700 snails/raceway respectively). Each day, the bottom of the raceway was siphoned to remove detritus and grazers were collected and redistributed evenly across the raceway. When egg crate racks became inundated by nuisance algae, they were lowered to the raceway floor for 24 hours to increase localized grazing pressure on the substrates, and the racks themselves were cleaned and replaced as needed. Similarly, individual substrates were monitored proactively for the recruitment of undesirable fouling organisms, such as *Cyanobacteria* spp. suspected to hinder growout. Once found, these organisms were smothered with super glue to prevent proliferation on the substrates. Finally, when cyanobacteria and filamentous algae became conspicuous in the raceway, coral juveniles were temporarily removed to a clean vessel and the fouled raceway was cleaned and replaced. Air stones and egg crate racks were then replaced with clean backups. Juveniles were reintroduced 24 hours later, after draining and refilling the raceway a second time.

Diligent mitigation of algal growth contributed to the competitive advantage and relative dominance of CCA in the grow-out environment. Although CCA can at times overgrow coral settlers, this group of encrusting organisms is easier to monitor and manage compared to other coral competitors such as turf algae, fleshy algae,

and cyanobacteria. In addition to the steps taken above, all substrates containing AGF crosses were periodically cleaned; encroaching CCA was removed around each recruit using a blade (X-Acto knife, Elmer's Inc.) to prevent smothering of the coral polyps. Finally, at 3 months post-settlement, each AGF juvenile was removed from its original substrate using a scalpel and glued to a clean substrate.

Larval Settlement and Grow-out at Florida Aquarium

Upon arrival at the grow-out facility, larvae were poured gently into polystyrene clamshell containers and water from the settlement aquarium was mixed to a final ratio of 50% shipping water to 50% settlement aquarium water. Temperature and salinity were matched within 0.5°C and 1 ppt between the shipping water and the settlement aquarium. Larvae were then transferred by gentle pouring into the settlement containers. Settlement containers were constructed out of 17-L polyethylene dish pans with four 7.6 cm holes drilled into the sides. Holes were covered with 150-µm nylon mesh attached to the bin with food-grade silicone sealant. Settlement containers were placed on a PVC rack inside of a well-established, 2270-L recirculating aquarium system with live rock, a deep sand bed, and protein skimming. A pump and valve manifold supplied a slow trickle of water through each settlement bin from the main aquarium.

Larvae from the AGF crosses (i.e., CUR×FL and CUR×PR) were placed at a lower density in the settlement bins, ranging from approximately 250 to 550 per bin. Larvae from the Curaçao fresh and frozen sperm crosses (i.e., CUR×CUR (fresh) and CUR×CUR (frozen)) were placed at a higher density of larvae per bin, at 1000 and 1500 larvae per bin, respectively. Settlement tiles were smooth ceramic squares (Ceramic Reef Squares, Boston Aqua Farms) that were conditioned for three months in recirculating coral aquaria with small colonies of *Acropora cervicornis* and *A. palmata.* In addition, fragments of CCA (tentatively identified as *Hydrolithon boergesenii*) were ground using a mortar and pestle and sprinkled into the settlement aquaria. In order to provide a source of free-living coral symbionts and to provide additional settlement cues for the larvae, a small amount of sediment from well-established aquaria was also added to the settlement aquaria.

Within one week of settlement, settled polyps were counted (Table ***S4***) and transferred into two separate recirculating aquarium systems located in a climate-controlled greenhouse. One system consisted of a single shallow raceway (2.4 m × 0.9 m × 0.3 m) and a sump (1700 L total) and the second system consisted of two

shallow raceways (1.5 m × 0.6 m × 0.4 m) and a sump (1325 L total). Each system contained live rock, a deep sand bed (10–12 cm deep), and several fragments of *A. cervicornis* to serve as sources of additional symbionts. Each system contained a protein skimmer, titanium immersion heaters, titanium chilling coils, and a media reactor with activated carbon. Temperature was set at 25 ± 0.5 ºC. Aquaria were covered with shade cloth so that they received 50–75 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) at solar noon. Algae that grew around coral settlers was manually removed as needed using aluminum clay needle tools and curved, stainless steel tweezers. To reduce algal fouling, both aquaria were stocked with small herbivorous snails (*Batillaria minima*, *Cerithium lutosum*, and juvenile *Lithopoma americanum*).

At approximately two weeks post-settlement, a visible tissue loss syndrome was observed beginning in the CUR×CUR (frozen) cohort, which rapidly spread to other groups of settlers. To halt further losses, all settlers at the Florida Aquarium Center for Conservation were given a 10-day bath in ampicillin dosed at 100 μg mL^{-1} based on previous studies (17, 18). Recruits were moved into a single system and isolated from filtration, and a 100% water change was conducted daily with a re-dose of antibiotics each day. This treatment stopped the spread of the tissue loss syndrome, after which recruits were moved into a new aquarium system with cycled live rock and returned to circulation with filtration. After one month, PAR levels were increased to 150 μmol m^{-2} s^{-1}.

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