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Mendelian microsatellite loci for the Caribbean coral *Acropora palmata*

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ABSTRACT: The genus *Acropora* constitutes the most species-rich clade of hermatypic corals, and its members are important reef builders throughout their broad tropical range. In the Caribbean, acroporid populations have declined over the last 2 decades due to disease, hurricanes, predation, and bleaching episodes, and some are now subjects of conservation efforts. Genetic estimates of population connectivity and clonal structure should be part of these efforts, but such studies have been hampered by low levels of mitochondrial DNA variation in corals, and an apparent dearth of variable single-copy nuclear markers. Developing microsatellite markers in *Acropora* has proven especially difficult. We used Southern blotting to reveal that, indeed, some microsatellite motifs (AAC, AAG) are rare in the genome of the Caribbean species *Acropora palmata*. However, repeats with the motif AAT are both abundant and variable. We developed 8 polymorphic microsatellite markers for *A. palmata*, and performed crosses to confirm co-dominant inheritance patterns. Five of the 8 markers tested show simple Mendelian inheritance (mean observed heterozygosity = 0.84, mean number of alleles per locus = 8.6). Along with outcrossed sexual larvae, individual egg donors also produced some triploid and selfed larvae that developed normally and survived for 80 h, when the experiment was terminated. The markers reveal variation among 3 Florida populations of *A. palmata* and among clones within 1 of these populations. Seven of the markers amplify DNA from *A. cervicornis* and 8 from the hybrid *A. prolifera*. These markers should prove to be valuable tools for developing conservation strategies for Caribbean acroporid species.

KEY WORDS: *Acropora palmata* · Scleractinia · Caribbean · Microsatellite · Clonal structure · Mendelian inheritance · Triploid larvae · Self-fertilization

INTRODUCTION

Understanding the evolution and population ecology of hard corals has been hampered by a scarcity of suitable population genetic markers (van Oppen et al. 2000b). Anthozoan mitochondrial DNA lacks the high levels of intraspecific variation that have facilitated population and low-level phylogenetic studies in other animals (Shearer et al. 2002, M. E. Hellberg unpubl. data). rDNA ITS-sequences have been used to study reticulate evolution, specifically in the genus *Acropora*; however, the extremely high levels of intra- and inter-individual diversity of these multi-locus markers complicate their interpretation in a population genetic context (Marquez et al. 2003). Sequences from a limited number of introns have been used to address speciation and hybridization questions (mini-collagen: Hatta et al. 1999; Pax c 47/48 intron: van Oppen et al. 2001; mini-collagen, calmodulin and mitochondrial putative control region: Vollmer & Palumbi 2002), but intraspecific variation in these markers is so low that they would prove impractical for most studies of clonal

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structure and gene flow. Allozyme studies have provided data on population structure and the contribution of asexual reproduction in cnidarians (Jokiel et al. 1983, Stoddart 1984, Johnson & Threlfall 1987, Ayre & Willis 1988, Brazeau & Harvell 1994, Hellberg 1994, McFadden 1997, Ayre & Hughes 2000, Ridgway et al. 2001); however, the need for preserving fresh tissue in liquid nitrogen makes its use impractical, considering the often remote settings of coral reefs. Thus, there is a need for single copy, variable, DNA-based, nuclear markers.

Microsatellite markers have proven useful for population genetic questions in numerous organisms due to their high degree of polymorphism and the relatively low cost of genotyping many individuals (reviewed in Jarne & Lagoda 1996, Schlotterer 2000, Sunnucks 2000). Maier et al. (2001) reported the development of microsatellite markers for the coral *Seriatopora hystrix* (Pocilloporidae). Microsatellites have been subsequently developed in 2 Pacific faviids (Miller & Howard 2004) and in the Caribbean faviid *Montastraea annularis* (Severance et al. 2004). Shearer & Coffroth (2004) developed microsatellite markers for *M. cavernosa* and the poritid *Porites porites*. Chen et al. (2002) report the use of a multi-gene microsatellite repeat located in the ribosomal intergenic spacer region (IGS) to investigate clonal population structure in a gorgonian. Multiple attempts (3 different labs, 5 methodologies) to develop microsatellites in acroporids have, however, been unsuccessful (Marquez et al. 2000).

Beyond a lack of variability, population genetic analyses are hindered when multiple paralogous loci are present in the genome (Harris & Crandall 2000). This possibility is raised in *Acropora* because species vary in their ploidy levels (Kenyon 1997). Mendelian inheritance of microsatellites can be confirmed by performing crosses of known parental genotypes, as is routine in the plant and yeast literature (e.g. Jakse et al. 2001, Dobrowolski et al. 2002).

In addition to their application to questions of connectivity among coral populations, microsatellite loci can also be used to assess other aspects of coral reproductive biology. Although fragmentation is the most well-studied and obvious means of asexual reproduction in reef cnidarians (Highsmith 1982, Coffroth & Lasker 1998, Lirman 2000), genetic comparisons of parents and offspring have revealed several cases of selfed and/or parthenogenic larvae (Ayre & Resing 1986, Ayre et al. 1997, Brazeau et al. 1998). These have involved both brooded and broadcasted larvae, but just how common this phenomenon is remains unknown because few species have been analyzed in this manner.

Here, we use a DNA hybridization approach to assess the relative abundance of different microsatellite repeats in the *Acropora palmata* genome. Using this information, we then develop microsatellite markers from an enriched library and confirm their Mendelian inheritance by controlled crosses. The goal of this work is to produce a suite of loci suitable for assessing aspects of demography and population genetic structure in *A. palmata* across its Caribbean range.

MATERIALS AND METHODS

DNA extraction. Because adult *Acropora palmata* Lamarck harbor zooxanthellae (*Symbiodinum* spp.), but their free-spawned gamete bundles do not, we extracted DNA from gametes to build a genomic library. *A. palmata* reproduces sexually once a year (Szmant 1986). Spawn was collected during the August 2001 spawning event at Horseshoe Reef, Key Largo, Florida (N 25° 08.392, W 80° 17.649) and Green Turtle Cay, Abacos, Bahamas (N 26° 42.448, W 77° 09.215). DNA from 3 to 5 gamete bundles was extracted using the Phytopure DNA extraction kit (Amersham Pharmacia), yielding high molecular weight DNA.

Adult DNA for assaying levels of variation in clonal and population structure was extracted from colonies with the QIAmp DNA Mini Kit (Qiagen). Between 3 and 10 polyps were scraped off with a sterile razor and ground with a plastic pestle in a 1.5 ml microcentrifuge tube. Extraction was performed overnight at 65°C following the manufacturer's instructions. DNA was quantified with a spectrophotometer (BioRad Smart Spec 3000).

Larval DNA was extracted by transferring each ethanol-preserved larva into a 0.2 ml strip tube and squashing it with a pipette tip. Any remaining ethanol was evaporated by heating the larvae for 2 min at 100°C. Twenty µl of 5% Chelex and 2 µl Proteinase K (20 mg ml^{-1}) were added to the dried larvae and incubated overnight at 56°C. Proteinase K was inactivated by heating for 15 min at 100°C. After spinning at 3400 × *g* for 2 min, the supernatant containing the DNA was transferred to a fresh tube. This DNA extract (2 µl) was added to the multiplex PCR reactions. Larval DNA concentrations were not quantified.

For a Southern blot to compare repeat abundances, DNA was isolated from additional species (roseate tern, *Sterna dougallii*; giant Asian turtle, *Orlitia borneensis*; bicolor damselfish, *Stegastes partitus*) using a standard proteinase K/SDS digestion (Mullenbach et al. 1989) followed by phenol/chloroform extraction. *Acropora palmata* DNA was extracted using the Phytopure DNA extraction kit (see above).

Southern blotting. DNA (4 µg) was digested with 15 U of *Dpn*II (New England Biolabs). Digests were divided among 4 lanes on a 1.2% agarose gel, which

was then run at 60 V for 16 h; the 500 bp band in the size marker had run 16.2 cm. The gel was stained with ethidium bromide, photographed (Fig. 1, left panel, size standard from New England Biolabs), and subjected to a standard Southern blot. The nylon membranes (Nytran N, Schleicher and Schuell Keene N.H.) were probed with the 5′ ^{32}P-labeled oligonucleotides $(AAT)_{10}$, $(AAC)_{10}$ and $(AAG)_{10}$, and then exposed to X-ray film. Relative signal intensity was compared among species and among probes to determine whether particular repeat types were more frequent than others.

Microsatellite-enriched genomic library development. A genomic library was developed using a repeat-enrichment protocol by Hamilton et al. (1999), following the modifications given for blunt-end cutters. Genomic DNA was cut with a set of restriction enzymes (*Rsa*I, *Snab*, *Hae*III; New England Biolabs) that yielded fragments between 200 bp and 1 kb. These restriction fragments were ligated to an SNX linker (see Hamilton et al. 1999 for sequence). The cut genomic DNA with attached SNX linkers was heat denatured and then hybridized to biotinylated nucleotides ($[AAT]_{10}$ and $[AC]_{10}$ in 2 separate trials) at 45°C overnight. Streptavidin-coated magnetic beads (Dynabeads, Dynal) were added to this mixture, and incubated at 43°C overnight. Fragments that did not bind to the beads were eliminated during a set of 3 washes, each lasting 5 min, with increasing stringency (first wash: 2× at RT with 2× SSC, 0.1% SDS wash buffer; second wash: 2× at 33°C with 1× SSC, 0.1% SDS; third wash: 2× at 43°C with 1× SSC, 0.1% SDS). Fragments bound to the magnetic beads (i.e. those which had hybridized to biotinylated oligos) were retained using a magnet that pulled the beads to the side of the tube during the washes. The AAT- and AC-repeat enriched DNA was made double-stranded using a primer that annealed to the SNX linker, then digested with *Stu*I to produce cloning ends. The repeat-enriched DNA was ligated into *Eco*RV-digested pBluescript SK (Stratagene) vector. Supercompetent cells (Epicurian Coli® XL1-Blue MRF′, Stratagene) were transformed following the manufacturer's instructions and plated on Ampicillin/X-gal/IPTG-containing plates. Colonies were picked, and DNA was prepared (Promega Wizard Plus SV Miniprep Kit, Promega) and sequenced using BigDye 3 chemistry (Applied Biosystems) on an ABI 377 automated sequencer in both directions. Sequences were aligned with the program Sequencher (Gene Codes Corporation).

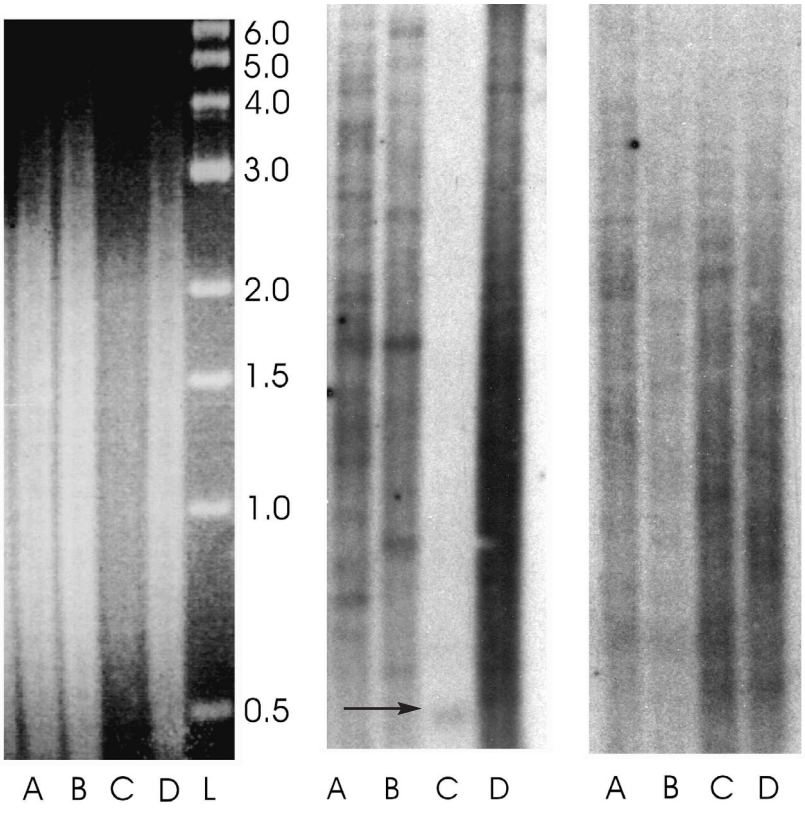

Fig. 1. Abundance of microsatellite motifs in *Acropora palmata* and 3 other species. The left panel shows the gel stained with ethidium bromide and UV-illuminated. It shows the relative amounts of DNA in each lane. This gel was Southern blotted; the membrane was probed and exposed to x-ray film (middle and right panels). The middle panel shows the autoradiograph produced by the $(AAC)_{10}$ probe. The arrow indicates a repetitive element in the coral genome. The right panel shows the autoradiograph produced by the $(AAT)_{10}$ probe. A: roseate tern, *Sterna dougallii*; B: giant asian turtle, *Orlitia borneensis*; C: elkhorn coral, *A. palmata*; D: bicolor damselfish *Stegastes partitus*; L: ladder (kb)

Primer design and testing. Primers were designed for sequences that contained 8 or more AC or AAT repeats with the program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi, Table 1). Some sequences were similar to others in the library; these were excluded from marker development because they are likely to represent multi-copy loci. The non-labeled (reverse) primer of marker 201 has an addition (GTTTCTT) to the 5′ end to ensure a high percentage of adenylation (Brownstein et al. 1996). Three populations of *A. palmata* from the Key Largo area of Florida were screened for polymorphism. Eight primers were polymorphic (most common allele frequency <90%) and amplified consistently in the adults (Table 1). Two sets of multiplex PCRs were developed for the 8 primers so that they could be

Table 1. *Acropora palmata*. Microsatellite primers for the coral. All primers amplify regions with AAT repeats. The size of the amplicon (bp) and the number of AAT repeats in the bacterial clones to which the primers were first designed are given. PET, VIC, NED and 6FAM are fluorescent dye labels (Applied Biosystems)

Primer	Primer sequence	Multiplex	Product length (bp)	No. of AAT repeats	Mendelian
166	TCTACCCGCAATTTTCATCA PET-CGCTCTCCTATGTTCGATTG	I	168	28	Yes
180	VIC-TTTCTCAGTGGGTTCCATCA CCTTTCGTTGCTGCAATTTT	II	134	19	No
181	NED-TTCTCCACATGCAAACAAACA GCCAGGATAGCGGATAATGA	I	152	10	Yes
182	6FAM-TCCCACAACTCACACTCTGC ACGCGGAAATAGTGATGCTC	II	165	18	Yes
187	NED-CGGATCTCACACTGATGCAA CATATAGATATCTGCGCGAATAAG	II	112	11	No
192	6FAM-TTTGAGCATTTAAGGAGCAACA CAGCAGACTCAACAGCAGGA	I	180	28	Yes
201	VIC-CCAAAACTCAGAAACCCCATT GTTTCTTCGCAGAATCCATGTTGATAGC	I	134	12	No
207	ATCCACGCCCAAACAATGTA PET-CTATTCGCTACCCACGCTTC	II	183	20	Yes

efficiently scored on an automated sequencer (ABI 3730). The total product length obtained via genotyping may differ by 1 bp relative to those obtained by direct sequencing (Table 1) due to the reaction conditions and the adenylation of PCR products.

Microsatellite scoring. Two 10 µl multiplex PCR reactions (M-I and M-II, Table 1) were performed per sample. M-I consisted of 0.2 µl each of primer pairs 166-PET (5 µM), 201-VIC (3 µM), 192-6FAM (5 µM) and 181-NED (3 µM), 1 µl 10× PCR Reaction Buffer (Promega), 0.8 µl of MgCl2 (25 mM), 0.2 µl of dNTPs (10 mM), 0.2 µl of *Taq*-Polymerase (5 U μl^{-1}, Storage Buffer B, Promega) and 6 µl H_2O. M-II consisted of 0.2 µl each of primer pairs 207-PET (5 µM), 180-VIC (5 µM), 182-6FAM (5 µM) and 187-NED (4 µM), 1 µl Promega 10× PCR Reaction Buffer, 1.2 µl of $MgCl_2$ (25 mM), 0.2 µl of dNTPs (10 mM), 0.2 µl of *Taq*-Polymerase (5 U μl^{-1}) and 5.6 µl H_2O. Adult DNA (100 to 200 ng, 1 µl) was added to each reaction. Adults and larvae were amplified using the same recipes except that twice the amount of *Taq* was used per reaction for the larvae. In addition, because 2 µl of larval DNA were added to the reactions, the amount of H_2O was adjusted to achieve a 10 µl final volume. Thermal cycling was carried out with MJ Research PT200 or PT100 cyclers with an initial denaturation step at 95°C for 5 min followed by 35 cycles of 95°C for 20 s, 50°C for 20 s, 72°C for 30 s. A final extension of 30 min at 72°C ensured that the majority of amplicons were +A (Brownstein et al. 1996). PCR products were visualized using an ABI 3730. An internal size standard (Gene Scan 500-Liz, Applied Biosystems) was used for accurate sizing. Electropherograms were analyzed with GeneMapper Software 3.0 (Applied Biosystems).

Sampling of *Acropora palmata*. We sampled *A. palmata* stands at Horseshoe Reef (see DNA section above), Little Grecian Reef (N 25° 07.106, W 80° 19.029), and Sand Island (N 25° 01.074, W 80° 22.117), located off of Key Largo, Florida. These sites are separated by distances ranging from 3.3 (Horseshoe–Little Grecian) to 15.4 km (Sand Island–Horseshoe). As no information was available on the clonal structure of *A. palmata* in these populations, we sampled at 3 spatial scales within the Little Grecian and Horseshoe populations to detect both common and rare genets. Random numbers were generated for each of 3 nested circles with radii of 15, 10 and 5 m. The random numbers generated were precise to 5° of arc and 50 cm along strike. At each reef, a stake was placed in the center of the patch. Using a measuring tape attached to the center stake and a compass, the coordinates were located (e.g. 15° and 150 cm). The colony underneath each of the coordinates was sampled; this was repeated 8 times per circle (= 24 samples per set of circles of 15, 10 and 5 m radius). If there was no colony at a particular random coordinate, that coordinate was crossed out and the next random number was sampled. No colony (defined here as a continuous, upright entity of skeleton with a stalk that attaches it to the bottom) was sampled twice. At Sand Island reef, >90% of the colonies were sampled, making a random sampling approach unnecessary. A single 1 cm long tip per identified colony was snipped off using a bolt cutter and placed in a labeled zip lock bag. Coral samples were transferred to 70% ethanol upon returning to shore and stored at –80°C until genotyping. Only samples where amplicons could be scored at all 8 loci were included in the analyses (13 of 107 were excluded).

Crossing experiments. Gametes were collected from 5 colonies (representing 4 genets) at Sand Island Reef (Fig. 2) on 17 August 2003 using nets (ca. 80 µm mesh size). Egg/sperm bundles were transferred into 15 ml centrifuge tubes, where they remained until the bundles broke apart (60 to 90 min). Eggs were carefully siphoned off the surface and washed at least 3 times in filtered sea water until no sperm remained. Crosses were set up by adding eggs and sperm to a culture dish and left to fertilize for 1 h (Table 2). Sperm concentrations (10^6 to 10^8 sperm ml^{-1}, Table 2B) used here are within in the range reported to give good fertilization success in Pacific acroporids (Willis et al. 1997). The fertilized eggs were then transferred to Petri dishes and reared at 25°C until they were 80 h old. Larvae were pipetted to dishes with fresh filtered sea water whenever unfertilized eggs and sperm began to foul the dish. Larvae were observed under a dissecting microscope to check for normal development. Larvae (80 h old) were preserved in 95% ethanol and stored at –80°C until analysis. In some cases (mean = 3 per cross, Table 3), fewer than 4 loci could be scored for a larva, resulting in the exclusion of that sample.

The fertilization solution containing the sperm was preserved with Lugol's solution (10 parts potassium iodide, 5 parts iodine, 85 parts deionized H_2O). Sperm concentration was determined by counting sperm in a hemocytometer in duplicate.

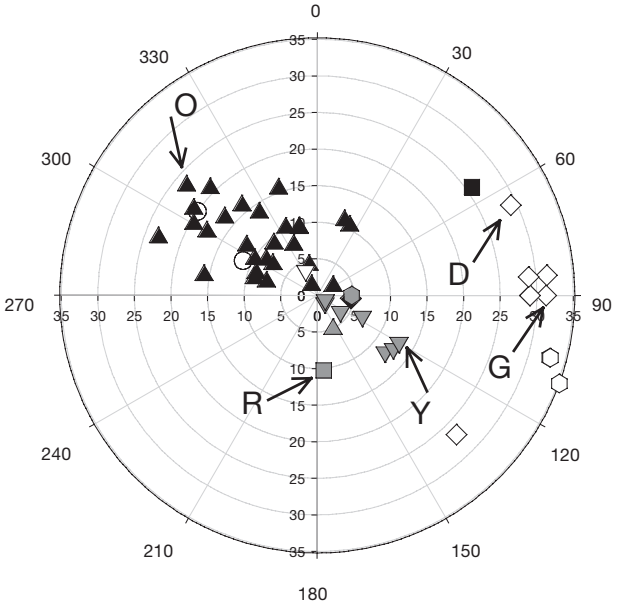

Fig. 2. *Acropora palmata*. Polar plot of population at Sand Island Reef. Each mark represents a colony. Ramets of the same genet are indicated by common shape and shading. Arrows indicate colonies that served as gamete donors during the August 2003 spawning event. Letters correspond to parental designation in Table 2. Radial axis: distance in (m); angular axis: angle in degrees. Number of colonies = 53

Table 2. *Acropora palmata*. Crosses between colonies. Letters identify colonies (n = 6) from which gametes were collected (n = 6). As the supply of gametes was limited, not all crosses were attempted (empty cells). (A) Number of larvae that survived until Day 4. (B) Sperm concentration (no. of sperm × 10^6 ml^{-1}). Bold numbers indicate self-crosses. Some samples were lost (dashes). All parental colonies were located at Sand Island Reef (see Fig. 2) except for colony H which was from Horseshoe Reef. Colonies D and G are clonemates (i.e. share the same alleles at all 8 loci). na = not applicable

(A)

Sperm donor	Egg donor R	O	G	D	Y	H	Total
R	**2**	259		224	868	19	1372
O	6		3	4	30		43
G	386	54		**1**	119		560
D	94			**2**	175		271
Y	96	210		265	**9**		580
H	0						0
Total	584	523	3	496	1201	19	2826

(B)

Sperm donor	Egg donor R	O	G	D	Y	H
R	**177**	182	na	248	129	30.5
O	10.3	na	87.8	–	23.7	na
G	11.6	13.6	na	5.27	1.35	na
D	23.2	na	na	**–**	–	na
Y	162	241	na	–	**–**	na
H	400	na	na	na	na	na

Larval genotypes were classified as being outcrossed sexual, triploid, or maternal. Outcrossed sexual larvae had 1 maternal and 1 paternal allele at all loci, and were used for tests of co-dominant inheritance of the 8 microsatellite loci. We compared expected and observed ratios of offspring classes using chi-square tests. Chi-square tests are sensitive to small sample sizes; the expected number of observations should not be <5 per class. For a cross between 2 heterozygous individuals, 4 classes of gametes are possible and the expected Mendelian ratio is 1:1:1:1. Hence, we only analyzed crosses for which >25 larvae were scored.

Larvae were scored as triploid (for reasons discussed below) if 3 alleles were found at least at 1 locus and the alleles scored at the other loci did not exclude the possibility that the larva could be triploid (i.e. 1 of the parents was homozygous at that locus). The third peak at a locus in a triploid larva was clearly distinguishable from stutter peaks because its height and morphology were comparable to the other 2 peaks. The majority (>96%) of larvae scored as triploid showed 3 peaks at 2 or more of the 5 Mendelian loci (see below). All 3 larval peaks were also present in the parental genotypes. As triploid larvae showed 3 peaks at more than 1 locus and showed only alleles that were also present in parental genotypes, it is improbable that these triploid patterns were the result of PCR error.

Table 3. *Acropora palmata*. Larval genotypes. See 'Materials and methods' for explanation of larval classes. Prop: proportion; n: sample size; SD: standard deviation. Self-crosses are indicated in bold. Note that colonies D and G are clonemates. Means are calculated excluding empty cells

Cross (sperm × egg)	Total larvae	<4 Loci genotyped	≥4 Loci genotyped		Outcrossed sexual		Maternal genotypes only		Triploid pattern	
		n	n	Prop	n	Prop	n	Prop	n	Prop
R × D	31	5	26	0.84	23	0.88			3	0.12
G × R	31	11	20	0.65	16	0.80			4	0.20
D × R	31	4	27	0.87	17	0.63			10	0.36
Y × R	31	0	31	1.00	18	0.58			13	0.42
D × D	**2**	**1**	**1**	**0.50**	**0**	**0.00**				
D × Y	31	4	27	0.87	26	0.96	1	0.04		
G × O	31	4	27	0.87	10	0.37	17	0.63		
G × Y	30	1	29	0.97	29	1.00				
O × D	4	3	1	0.25	1	1.00				
O × G	3	0	3	1.00	2	0.67	1	0.33		
O × R	6	1	5	0.83	5	1.00				
O × Y	8	3	5	0.63	2	0.40	3	0.60		
R × H	1	1	0	0.00	0	0.00				
R × O	31	12	19	0.61	6	0.32	13	0.68		
R × R	**2**	**0**	**2**	**1.00**	**0**	**0.00**	**2**	**1.00**		
R × Y	31	2	29	0.94	29	1.00				
Y × D	31	3	28	0.90	28	1.00				
Y × O	31	4	27	0.87	11	0.41	16	0.59		
Y × Y	**9**	**1**	**8**	**0.89**	**0**	**0.00**	**8**	**1.00**		
D × G	**1**	**0**	**1**	**1.00**	**0**	**0.00**	**1**	**1.00**		
H × R	0	0	0	0.00						
Total	**376**	**60**	**316**	**0.84**	**223**	**0.71**	**62**	**0.20**	**30**	**0.09**
Mean ± SD	**19 ± 14**	**3 ± 3**	**16 ± 12**	**0.74 ± 0.31**	**12 ± 11**	**0.73 ± 0.27**	**7 ± 7**	**0.65 ± 0.33**	**8 ± 5**	**0.28 ± 0.14**

Some larvae only had maternal alleles at all loci. As these larval genotypes were not always identical to the maternal genotype at all heterozygous loci, they were considered to be the products of self-fertilization.

RESULTS

Southern blot

Fig. 1 shows that the sequence motif AAC is nearly absent from the *Acropora palmata* genome, while the sequence AAT is comparatively abundant. Indeed, despite the underloading of *A. palmata* DNA (see left panel), the coral lane is as dark or darker than all the other species when probed with AAT. Note also the appearance of dark bands in the AAC lanes, indicative of some repetitive element that contains an AAC microsatellite. No species showed an abundance of AAG repeats (data not shown).

Microsatellite marker development

Of 40 bacterial clones sequenced from the AAT-enriched library, 15 (38%) contained AAT-repeats. The number of repeat units in these positive clones ranged from 4 to 28 (mean ± SD = 13 ± 7.9). Primers were designed and tested for the 10 sequences that contained AAT repeats with more then 8 repeat units. Of these 10 primer pairs, 8 amplified and were polymorphic in the Florida populations (Table 1, see next section). All 8 loci had perfect AAT repeats. The 20 perfect AAT repeats were followed by ATA, 3 AAT, A, 3 AAT, A, 1 AAT at locus 166. The motif at locus 192 switched to ATT (repeated 21 times) after 6 iterations of TAA. The AAT repeat of locus 181 was preceded by 4 repeats of TCA.

Screening 144 bacterial clones of an earlier library constructed using AC as the enrichment motif produced 25 repeat-containing clones. Of these 25, 9 *Acropora palmata* repeats aligned with a sequence from the histone gene cluster of *A. formosa* (Miller et al. 1990); specifically, the *A. palmata* repeat ($[GT]_3TT\ [GT]_2GA[GT]_4$) aligned with the non-coding region between the H2A and H4 coding regions of the histone box (alignment available from I.B. upon request) of *A. formosa*. The prevalence of the multi-copy histone-box repeat limited the usefulness of the AC-library for Mendelian marker development. Primers were designed for 12 non-histone AC-loci; however, none of these were polymorphic.

Spawning and crosses

Acropora palmata was observed spawning during August 2003 at Sand Island Reef, Key Largo, Florida.

On 17 August 2003, the 6th night after the August full moon, bundles formed between 21:37 and 21:55 Eastern daylight time. Egg/sperm bundles were released from 22:00 until 22:25 h. Of the 60 colonies present at Sand Island reef, 8 colonies spawned. These 8 colonies represent 4 different genets.

A total of 21 crosses were attempted (Table 2A), resulting in 2826 larvae. Some crosses could not be performed due to limited amounts of sperm or eggs (empty cells in Table 2A). Sperm concentration ranged from 10^6 to 10^8 ml sperm ml^{-1} (Table 2B). *Acropora palmata* larvae followed the development pattern described by Hayashibara et al. (1997) and Ball et al. (2002). Larvae had obtained an elongated shape with an indentation around the center indicating gastrulation at ca. 14 h after fertilization. By 31 h, larvae were round again. The first signs of cilia were observed after 41 h, along with a change in shape from round to pear-like. Larvae began swimming at 78 h.

The number of larvae remaining after 80 h varied widely among crosses (Table 2A). In self-fertilization trials, fewer than 10 larvae survived for 80 h (Table 2A). This also holds true for the cross between clonemates G and D (Table 2A). Most eggs in the self-fertilization trials had broken apart 14 h after the fertilization attempt was made.

Inheritance patterns

Larval genotypes were compared to known parental genotypes (Appendix 1). Primers for presumptive loci 187 and 201 commonly exhibited more than 2 peaks per larva; 187 amplified between 2 and 5 peaks per individual, while 201 amplified up to 8 peaks per individual. Thus, these 2 primer pairs likely amplify multiple loci. In addition, primers for presumptive locus 180 commonly failed to amplify one of the parental alleles in larvae, creating apparent homozygote excess; this probably indicates the presence of null alleles. Thus, expectations for offspring classes were impossible to formulate for these 3 primer sets and chi-square tests were not performed.

Tests of co-dominant inheritance were conducted for the 5 remaining presumptive loci (166, 181, 182, 192, 207). In each of the 4 reciprocal crosses that produced over 25 scorable larvae, chi-square tests for departure from expected Mendelian ratios were non-significant for these 5 markers (Appendix 1).

In 4 (19%) of the 21 attempted crosses, at least some larvae were scored as triploids (Table 3). The proportion of triploid larvae ranged from 0.12 to 0.42 in those crosses that showed triploids (Table 3). Selfed larvae occurred in 6 of the 17 (35%) outcrosses (Table 3). In outcrosses that produced selfed larvae, the proportion ranged from 0.04 to 0.68 (Table 3). The proportion of both triploid and selfed larvae varied among egg donors. At least some triploid larvae developed from eggs in 3 out of 4 outcrosses in which colony R was the egg-donor (Table 3). Additionally, triploid larvae were observed in cross D × R (36%) and cross G × Y (7%).

Selfed larvae were found mainly in crosses where colony O was the egg donor. Also noteworthy is the low number of larvae surviving when colony O was the sperm donor (Table 2A), even though sperm concentrations were similar between crosses with O as a sperm donor and other sperm donors fertilizing eggs from the same colony (compare cross O × G and O × Y, Table 2B). Note that colonies G and D are clonemates as previously identified through genotyping. Both G and D were crossed with Y, producing the same classes of larval genotypes (Appendix 1).

Marker testing in adult populations

Samples from 3 *Acropora palmata* populations from Key Largo were genotyped using the 5 scoreable loci. The number of alleles sampled per locus ranged from 5 to 11 (Table 4) in a sample of 93. These 93 colonies represented 14 unique genotypes. Observed heterozygosity per locus was high, ranging from 0.58 to 0.85 (Table 4). All loci showed mild heterozygote excess (mean Het_{obs} = 0.84, mean Het_{exp} = 0.79, Table 4). When assessing all 5 markers, the probability of iden-

Table 4. *Acropora palmata*. Genetic variation of microsatellite markers. Data based on 14 genets pooled from Horseshoe, Little Grecian and Sand Island Reefs, Florida. The probability of identity (PI) gives a conservative estimate of the probability that 2 individuals sampled in the same population share a multilocus genotype by chance, not by descent (i.e. are clonemates, Waits et al. 2001). Combined PI after sequentially multiplying PI values over all loci. Biased: based on Paetkau & Strobeck (1994); unbiased: based on Kendall & Stewart (1977). Number of alleles sampled and gene diversities were calculated by FSTAT (Goudet 1995); observed (Het_{obs}) and expected (Het_{exp}) heterozygosities and PI calculated by Gimlet (Valiere 2002). Pop.: population; prob.: probability

Locus	No. alleles sampled	Gene diversity/ locus + pop.	Het_{obs}	Het_{exp}	Prob. of identity-biased	Prob. of identity-unbiased
166	10	0.876	0.93	0.85	0.039	0.010
181	5	0.604	0.64	0.58	0.233	0.177
182	9	0.852	0.93	0.82	0.050	0.016
192	8	0.879	0.79	0.84	0.043	0.015
207	11	0.882	0.93	0.85	0.036	0.007
Mean	8.6	0.819	0.84	0.79		
Combined					7.07×10^{-7}	2.85×10^{-9}

tity (PI) by chance was low (combined value of PI, Table 4). The 3 reefs differed markedly in genotypic diversity. At the 15 m scale, only 1 genet each was found at Horseshoe Reef (n = 20, Fig. 3A) and at Little Grecian Reef (n = 20, Fig. 2B), whereas Sand Island Reef (n = 31) had 8 genets (Fig. 3). An additional 4 genets were found at Sand Island Reef when the sample area was extended beyond 15 m (Fig. 2).

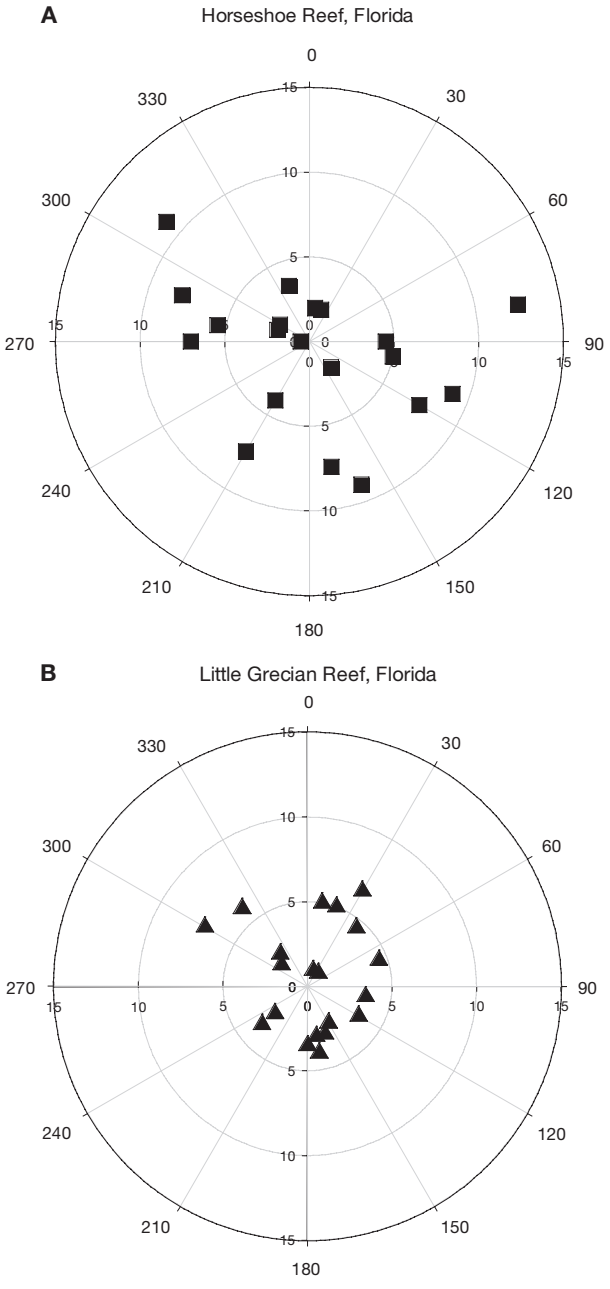

Fig. 3. *Acropora palmata*. Polar plots of populations at (A) Horseshoe Reef and (B) Little Grecian Reef. Only 1 genet was found on each of these reefs. Radial axis: distance in (m); angular axis: angle in degrees. Number of colonies = 20, in each case

DISCUSSION

Microsatellite markers

We report the development of 5 highly polymorphic microsatellite markers that demonstrate Mendelian inheritance patterns for the threatened Caribbean coral *Acropora palmata*. The Acroporidae have proven difficult subjects for microsatellite development (Marquez et al. 2000). Three labs used 5 methodological approaches to screen Pacific acroporid corals for 15 different di-, tri-, and tetra-nucleotide repeats (ACA, AG, AGTG, ATT, CA, CAAT, CAC, CGA, CT, GATT, GT, TA, TAG, TC, TG) (Marquez et al. 2000). In the face of the apparent paucity of microsatellite loci in the *Acropora* genome, we first determined which repeat type was relatively abundant using Southern blots. The observed differences in the abundance of probed sequences (AAT, AAC and AAG) between *A. palmata* and other organisms may explain why earlier attempts at microsatellite development failed. Furthermore, the AAC probe revealed a repetitive element that contains an AAC microsatellite (Fig. 1). Developing such loci would raise technical difficulties in subsequent DNA amplification: primers might amplify several distinct loci, or sequences may be found that are partly unique (in the flanking region of the repetitive element) and partly shared with other microsatellite loci (the repetitive element itself). Such problems arose twice in this project. First, 2 primer pairs designed for an AAT locus amplified more than 1 locus and second, we found that an intergenic spacer region between 2 highly repetitive histone genes, H2A and H4, contained an AC-rich motif. Due to the PCR-based enrichment method used in the protocol (Hamilton et al. 1999), the histone spacer sequences swamped the genomic library and rendered it useless for isolating single locus markers. Instead, directed by the Southern blots, AAT was targeted as the motif for microsatellite development.

Heterozygosity and the number of alleles per locus were high for the AAT-microsatellites developed (Table 4). Initial screening of *Acropora cervicornis* (n = 3) and the *A. palmata*/*A. cervicornis* hybrid (van Oppen et al. 2000a, Vollmer & Palumbi 2002) *A. prolifera* (n = 3) showed that the markers developed here for *A. palmata* also amplify the DNA of these congeneric species. The one exception is locus 192, which did not amplify the *A. cervicornis* individuals tested. Microsatellite primers commonly amplify in closely related species (Primmer et al. 1996, Primmer & Merila 2002), enhancing the cost- and time-effectiveness of their development, although such borrowed loci tend to show lower heterozygosity in populations and species other than those for which they were developed (Hutter et al. 1998).

Microsatellite motifs are not evenly distributed among genomes and may differ in their degree of polymorphism within genomes (Primmer et al. 1997). Several groups have been identified as depauperate in microsatellites (e.g. birds, Lagercrantz et al. 1993, Primmer & Ellegren 1998). Dinucleotides are commonly more polymorphic than tri- and tetranucleotide repeats. Of dinucleotides, CA is the most abundant (and variable, Bachtrog et al. 2000) repeat type in *Drosophila* and mammals (Katti et al. 2001). However, in *Arabidopsis thaliana* and yeast, AT is most abundant (Katti et al. 2001). Even among *Drosophila* species, differences in microsatellite abundance have been found (Warner & Noor 2000). Thus, the discovery of polymorphic microsatellite repeats in Caribbean acroporids does not necessarily contradict the apparent lack of microsatellites in Pacific acroporids. Interestingly, Chenuil et al. (1997) suggest that an under-representation of dinucleotides in barbel compared to other fish might result from selection pressure to eliminate DNA from polyploidized species via preferential loss of repeat units during slip-strand repair. Comparisons of motif abundance, cell size and DNA content between diploid and polyploid *Acropora* species (Kenyon 1997) might be enlightening.

Mendelian inheritance of markers

Co-dominant inheritance cannot necessarily be assumed for microsatellite markers (Dobrowolski et al. 2002). In addition, zooxanthellate DNA might have contaminated the genomic library we screened for coral markers, even though we had taken the precaution of using gamete-derived coral DNA for library construction. However, the controlled crosses performed here indicate that 5 of the 8 loci are host-specific and conform to Mendelian expectations. These 5 markers should prove useful for both inferring population connectivity and evaluating the contribution of asexual reproduction in this clonal organism. This information is crucial to assess the extinction threat faced by *Acropora palmata*.

Controlled crosses addressing the inheritance of a molecular marker have once been previously performed within *Acropora*. Hatta et al. (1999) demonstrated Mendelian segregation for the mini-collagen marker for a limited number of larvae (n = 11) in Pacific acroporids. Other authors have confirmed the Mendelian inheritance of allozyme markers for both hermatypic and ahermatypic corals when either one (Stoddart 1983, Ayre & Resing 1986) or both (Stoddart et al. 1988, Hellberg & Taylor 2002) parental genotypes were known. In the plant literature, such controlled crosses are a standard part of marker development (Jakse et al. 2001). Many broadcast spawners only reproduce once or a few times per year, at night and somewhat unpredictably. Thus, it is understandable that controlled crosses have been rare in the coral literature.

Selfed and triploid larvae

The controlled crosses yielded some unexpected genotypes that should not occur if larvae in *Acropora palmata* result solely from sexual outcrossing. Acroporids are known to be poor self-fertilizers (Fukami et al. 2003), and indeed this was the case here; crosses between sperm and egg from the same colony yielded far fewer larvae than were obtained from outcrosses (Table 2A). However, in crosses between different genets, high levels of selfed larvae were sometimes observed (Table 3). The number of selfed larvae produced appears to vary among egg donors (Table 3). Incomplete washing of eggs once egg-sperm bundles had broken apart probably left some self-sperm in the egg suspension, resulting in self-fertilized eggs (e.g. egg donor O, Table 3).

Triploid larvae were frequently observed in the offspring of egg donor R (Table 3). Three alternatives can be proposed for their origin. (1) Polyspermy occurred: haploid eggs were first self fertilized, and then fertilized by foreign sperm. We never observed 2 paternal alleles in the larvae, but rather always both maternal alleles. Thus, polyspermy without preceding self fertilization can be ruled out. (2) Asexual (mitotic parthenogenic) eggs could have been fertilized by a foreign sperm, resulting in a triploid organisms. However, only 4 of 287 diploid larvae show a genotype consistent with a mitotic, parthenogenic origin, making this explanation unlikely. (3) The retention of a polar body could explain the presence of both maternal alleles (meiotic parthenogenesis). Several coral species are reported to release polar bodies within 30 min of spawning (Szmant-Froelich et al. 1980, Harrison & Wallace 1990). We are not able to discriminate between retention of a polar body, self-fertilization or mitotic parthenogenesis as the origin of the second maternal allele.

The high proportion of triploid larvae may be artifactual, but is worth noting because the triploid larvae survived 80 h. The occurrence of both self-fertilized and triploid larvae, even at low percentages, underlines the necessity for genetic confirmation of larval origin when interpreting the fertilization success of acroporid crosses. Triploid larvae may be one route to developing variation in ploidy levels among *Acropora* species. Observation of triploid adults would lend further support to this hypothesis.

Application of markers in 3 adult *Acropora palmata* populations

The high heterozygosities of the 5 microsatellite markers we have developed result in a low probability that non-clonemates will share identical genotypes (Table 4); thus, we can distinguish genets with near-certainty. We applied the 5 Mendelian microsatellite markers to 3 *A. palmata* populations in the Florida Keys. Clonal maps were constructed from the physical location of the colonies recorded at the time of collection and overlayed with the genotypes (Figs. 2 & 3). While Little Grecian and Horseshoe reefs have very little (or no) clonal variation, Sand Island harbors many genets. The number of ramets per genet varies widely. Clonemates are clumped as would be expected if clonal propagation occurs through the breakage of branches and their subsequent re-attachment (Highsmith 1982, Coffroth & Lasker 1998).

The dominance of a single genet at Horseshoe Reef helps to explain previous failed fertilization studies. In 1999 and 2001, gamete bundles were collected at Horseshoe Reef during a vigorous spawning event and crossed. Survival rate was so low that no larvae could be settled (M. W. Miller & A. M. Szmant unpubl. data). This high larval mortality may have been a consequence of the lack of clonal variation at Horseshoe Reef.

The differences in levels of clonal diversity between reefs within a 15 km region raise management concerns. The total number of colonies (n = 93) genotyped here represented only 14 genets and only 1 genet each was found at Horseshoe Reef and Little Grecian Reef. Genets might differ in their susceptibility to bleaching (Edmunds 1994, Baird & Marshall 2002, McClanahan et al. 2004); thus, the dominance of a single genet at Horseshoe Reef and Little Grecian Reef could make populations at these localities highly vulnerable to disturbance, even though they have both coped with white band disease, bleaching, hurricanes and predators over the last 5 yr (Baums et al. 2003a,b; M. W. Miller, D. E. Williams, I. B. Baums unpubl. data).

CONCLUSION

The targeted enrichment protocol outlined here might help to circumvent problems encountered when developing microsatellites for scleractinian corals and other problematic taxa. The development of highly polymorphic, Mendelian markers for the threatened coral *Acropora palmata* will prove useful for the investigation of both clonal structure and population connectivity. Preliminary trials indicate that the markers developed here may also be useful in *A. cervicornis* and in the hybrid *A. prolifera*. Information on the genetic population structure of Caribbean acroporids is urgently needed because these corals are currently being evaluated as candidates for listing under the US Endangered Species Act (Diaz-Soltero 1999).

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Appendix 1. Chi-square analysis of larval genotypes observed from 5 *Acropora palmata* crosses at 5 loci. Genotypes are represented as 6 digit numbers. The first 3 digits refer to the paternal allele (bp) and the second 3 digits refer to the maternal allele. Cross G × D is between clonemates. Chi-square tests were carried out without applying a continuity correction. A simple Bonferroni correction involves dividing the cut off significance level ($p < 0.05$) by the number of tests carried out ($n = 50$). The new significance level then equals $p < 0.001$. Thus, none of the chi-square tests are significant (*$p = 0.006$ for locus 181 and D × Y, Y × D)

Locus	Cross	Parental genotype		No. of progeny	No. of larval genotypes observed				Expected ratio	χ^2 p-value
		Father	Mother							
166	R × D	164173	155161	22	8 (155164)	2 (155173)	8 (161164)	4 (161173)	1:1:1:1	0.18
	D × R	155161	164173	16	3 (155164)	4 (155173)	4 (161164)	5 (161173)	1:1:1:1	0.92
	R × D, D × R			38	11	6	12	9	1:1:1:1	0.54
	Y × R	170170	164173	17	11 (164170)	6 (170173)			1:1	0.23
	R × Y	164173	170170	28	10 (164170)	18 (170173)			1:1	0.13
	Y × R, R × Y			45	21	24			1:1	0.65
	Y × D	170170	155161	25	11 (155170)	14 (161170)			1:1	0.55
	D × Y	155161	170170	26	13 (155170)	13 (161170)			1:1	1
	D × Y, Y × D			51	24	27			1:1	0.98
	G × Y	155161	170170	24	13 (155170)	11 (161170)			1:1	0.68
181	R × D	156156	159162	22	12 (156159)	10 (156162)			1:1	0.67
	D × R	159162	156156	16	8 (156159)	8 (156162)			1:1	0.32
	R × D, D × R			38	18	20				0.75
	Y × R	156159	156156	17	15 (156156)	2 (156159)			3:1	0.66
	R × Y	156156	156159	28	22 (156156)	6 (156159)			3:1	0.21
	Y × R, R × Y			45	37	8				0.26
	Y × D	156159	159162	25	11 (156159)	8 (156162)	3 (159159)	3 (159162)	1:1:1:1	0.06
	D × Y	159162	156159	26	9 (156159)	10 (156162)	3 (159159)	4 (159162)	1:1:1:1	0.13
	D × Y, Y × D			51	20	18	6	7		0.00*
	G × Y	159162	156159	25	8 (156159)	10 (156162)	5 (159159)	2 (159162)	1:1:1:1	0.08

Appendix 1 (continued)

Locus	Cross	Parental genotype		No. of progeny	No. of larval genotypes observed				Expected ratio	χ^2 p-value
		Father	Mother							
182	R × D	142176	142173	22	7 (142142)	5 (142173)	4 (142176)	6 (173176)	1:1:1:1	0.67
	D × R	142173	142176	17	2 (142142)	8 (142173)	5 (142176)	2 (173176)	1:1:1:1	0.12
	R × D, D × R			39	9	13	9	8		0.68
	Y × R	158191	142176	18	2 (142158)	4 (142191)	6 (158176)	6 (176191)	1:1:1:1	0.49
	R × Y	142176	158191	28	9 (142158)	7 (142191)	4 (158176)	8 (176191)	1:1:1:1	0.57
	Y × R, R × Y			46	11	11	10	14		0.85
	Y × D	158191	142173	28	5 (142158)	8 (142191)	4 (158173)	11 (173191)	1:1:1:1	0.23
	D × Y	142173	158191	26	9 (142158)	6 (142191)	8 (158173)	3 (173191)	1:1:1:1	0.38
	D × Y, Y × D			54	14	14	12	14		0.97
	G × Y	158191	158191	27	9 (142158)	5 (142191)	9 (158173)	4 (173191)	1:1:1:1	0.17
192	R × D	163166	151160	23	8 (151163)	5 (151166)	7 (160163)	3 (160166)	1:1:1:1	0.46
	D × R	151160	163166	17	6 (151163)	0 (151166)	4 (160163)	7 (160166)	1:1:1:1	0.08
	R × D, D × R			40	14	5	11	10		0.24
	Y × R	144172	163166	18	6 (144163)	2 (144166)	5 (163172)	5 (166172)	1:1:1:1	0.57
	R × Y	163166	144172	28	9 (144163)	5 (144166)	8 (163172)	6 (166172)	1:1:1:1	0.70
	Y × R, R × Y			46	15	7	13	11		0.38
	Y × D	144172	151160	28	11 (144151)	3 (144160)	7 (151172)	7 (160172)	1:1:1:1	0.20
	D × Y	151160	144172	26	6 (144151)	6 (144160)	9 (151172)	5 (160172)	1:1:1:1	0.70
	D × Y, Y × D			54	17	9	16	12		0.38
	G × Y	151160	144172	25	5 (144151)	5 (144160)	5 (151172)	10 (160172)	1:1:1:1	0.11
207	R × D	185188	182197	12	6 (182185)	1 (182188)	4 (185197)	1 (188197)	1:1:1:1	0.11
	D × R	182197	185188	17	5 (182185)	6 (182188)	2 (185197)	4 (188197)	1:1:1:1	0.56
	R × D, D × R			29	11	7	6	5		0.41
	Y × R	173176	185188	17	5 (173185)	3 (173188)	4 (176185)	5 (176188)	1:1:1:1	0.89
	R × Y	185188	173176	28	5 (173185)	13 (173188)	5 (176185)	5 (176188)	1:1:1:1	0.08
	Y × R, R × Y			145	10	16	9	10		0.43
	Y × D	173176	182197	28	8 (173182)	8 (173197)	6 (176182)	6 (176197)	1:1:1:1	0.90
	D × Y	182197	173176	25	3 (173182)	8 (173197)	8 (176182)	6 (176197)	1:1:1:1	0.44
	D × Y, Y × D			53	11	16	14	12		0.77
	G × Y	182197	173176	26	6 (173182)	8 (173197)	7 (176182)	5 (176197)	1:1:1:1	0.54

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