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Brief Report

New Microsatellite Markers for the Model Coral Species *Stylophora pistillata* from Eilat, the Red Sea

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Abstract: Nineteen microsatellite loci, obtained by the whole genome sequencing approach, were developed and validated for the 'smooth cauliflower' coral *Stylophora pistillata*, a widespread Indo Pacific branching coral species. A sample size of 40 colonies collected at five reef sites along the northern Gulf of Eilat, the Red Sea, were genotyped, revealing loci reproducibly and suitable outcomes for wide applications, including population genetic studies. The 19 new microsatellite loci in this sample were composed of 4–20 alleles/locus, of which 10 microsatellites are highly polymorphic (≥10 alleles/locus). The observed and expected heterozygosity ranged between 0.289 and 0.957 (mean 0.597) and 0.101 and 0.911 (mean 0.726), respectively, and the Fixation Index (F), which also indicates the inbreeding coefficient, ranges between −0.174 and 0.569 (mean 0.207). The polymorphic information content (PIC) ranges between 0.100 and 0.904 (mean 0.699). This new set of microsatellite loci will be employed for population genetics studies as for identifying the distribution of various genotypes within *S. pistillata* chimeras.

Keywords: *Stylophora pistillata*; microsatellites markers; next generation sequencing; Gulf of Eilat; population genetics

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1. Introduction

The 'smooth cauliflower' coral *Stylophora pistillata* (Esper 1797), a common and widespread Indo Pacific branching coral species of the family Pocilloporidae, has served from the late 1980s as a model coral reef species for a wide range of basic and applied studies (Figure 1) [1]. As a key species in many Indo Pacific reefs, the decades-long accrued studies on *S. pistillata's* distributions and its reproductive mode (e.g., [2]) have led researchers to investigate other biological and ecological facets of this species such as population dynamics, population genetics, and larval dispersal on small and large geographic scales [3–9], as well as studies focusing on active reef restoration, where *S. pistillata* has being suggested as the preferred model species for evaluating the population dynamics parameters (e.g., [10–16]).

Clearly, the employment of microsatellite alleles, or other genetic markers, on various *S. pistillata* populations, on small recruits, and larvae under field scenarios, may further reveal connectivity and genetic flow processes and patterns [3,4,8]. Yet, as there are no detailed population genetics studies that use highly polymorphic markers, the development of efficient and inexpensive population genetics tools is of prime importance. A single study [17] has developed a panel of 17 specific microsatellites loci for population and phylogeographic studies in *S. pistillata* from the central Red Sea. While only three loci in this set of microsatellite loci are highly polymorphic (≥10 alleles/locus), the usefulness of the available microsatellites has been demonstrated by the identification of various genotypes within *S. pistillata* chimeras [8,18], yet not in population genetics analyses.

For the improved understanding of *S. pistillata* population genetic structures, and the evaluation of connectivity between sites throughout the Indo Pacific region, there is a need

for additional highly polymorphic microsatellite markers. Using a whole genome sequencing approach through new generation sequencing (NGS) technology, this study presents the development of 19 microsatellite loci new for *S. pistillata* colonies from the northern Gulf of Eilat, the Red Sea, of which 10 loci are highly polymorphic (≥10 alleles/locus), all revealing clean and reproducible amplifications.

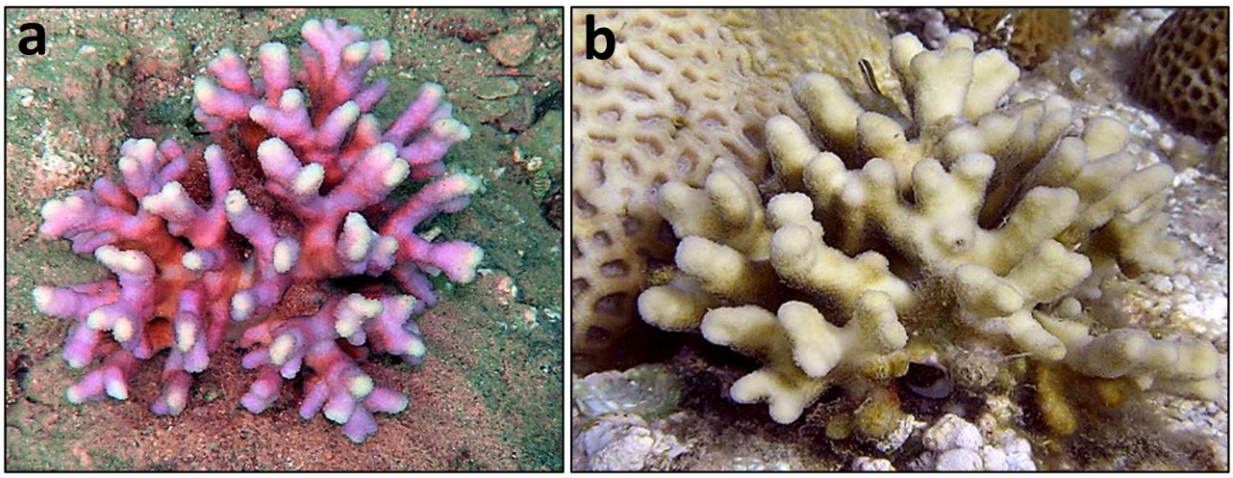

Figure 1. *Stylophora pistillata* (Esper 1797) colonies (**a**,**b**) form the Gulf of Eilat (phots: E. N. Rachmilovitz).

2. Materials and Methods

2.1. Genomic DNA Extraction and NGS Sequencing

A colony of *Stylophora pistillata* was collected from the Israeli northern Gulf of Eilat (N 29°32′34.843″ E 34°58′24.88″, 12 m depth), brought to the laboratory at the National Institute of Oceanography at Haifa, Israel and was inserted in a 17 L running sea water aquarium at 25 °C for 8 days under dark conditions (covered with aluminum foil) to reduce the numbers of algal symbionts. Then, small branch samples from the branch tips were clipped off, placed in one 1.5 mL vial containing 240 µL of lysis buffer [19], homogenized, and the DNA was extracted with equal volumes of phenol/chloroform/isoamyl-alcohol, 240 µL, 25:24:1 ratio). The vials were vortexed (10 min), centrifuged (10 min at 14,000× *g* 4 °C), and then the aqueous solution was transferred to new vials and equal volumes of chloroform/isoamyl-alcohol (24:1 ratio) were added, mixed, and centrifuged again. The upper phases, containing the DNA, were treated with 2 µL of RNAse A (10 mg/mL) to remove RNA contaminations (30 min, 37 °C), followed by phenol/chloroform extractions. The upper phases were transferred to new vials and two volumes of 100% ethanol were added for DNA precipitation. DNA was spooled out from each vial using a glass capillary and washed by dipping in 70% ethanol. The DNA was dried and resuspended in 50 µL of sterilized double distillated water (DDW). The integrity and the quantity of the genomic DNA was checked on a 0.8% agarose gel and NanoDrop 2000 (Thermofisher, Waltham, MA, USA).

The coral genomic DNA was checked for the existence of algal symbionts DNA by using rbcL primers following Amar et al. [20]. A total of 6 µg (20 µL, 300 ng/µL) of the uncontaminated, purified coral genomic DNA was sent to Macrogen Inc. (Seoul, Republic of Korea) for sequencing and assembling using the Roche 454 titanium GS-FLX plus Titanium pyrosequencing platform [21,22].

2.2. Primers Design for Microsatellites

The sequencing reads were screened for short nucleotide tandem repeats (STRs, di-, tri-, and tetranucleotides, >8 repeats), using MSATCOMMANDER version 0.8.1 software [23]. The software primer3 imbedded in MSATCOMMANDER was used to design sets of forward and reverse primers, flanking the STR repeats which will be used for PCR amplifica-

tions. One primer in each set included a M13 short sequence (GGAAACAGCTATGACCA, Table 1). The additional four primers of the M13 sequence were labeled with either VIC, FAM, NED, or PET fluorescent dyes (e.g., PET-GGAAACAGCTATGACCA, ThermoFisher). The sets of three primers (forward, revere, and labeled M13) were used for PCR amplifications of the genomic DNA samples.

Table 1. Characteristics of novel microsatellite loci developed for *S. pistillata*: locus name, forward and reverse primer sequences (5′ to 3′); RM = repetitive motive, NA = number of alleles; AR = alleles range (bp); AN = GenBank accession numbers.

Name	Forward Primer	Reverse Primer	RM	NA	AR	AN
SP_1	GCCTCGAATGCGGTGAATG	GGAAACAGCTATGACCATAAATGGAGG TCCCGTCTCC	(AAGT)11	14	132–318	OP851549
SP_2	GGAAACAGCTATGACCATGGGTGGGTTAATAGAGGGC	TGGGCTGGTGGTTATCAGG	(CT)14	6	379–397	OP851536
SP_3	GGAAACAGCTATGACCATGTTAGGCTCCGTGCAAAGC	GGTCAGGGTCTGAAGGCTC	(AG)10	7	168–194	OP851539
SP_4	GGAAACAGCTATGACCATGGCTTCAGTTTACAAGCGTC	AAGTACACGAGCCCACAAG	(GTA)18	4	125–159	OP851534
SP_5	GGAAACAGCTATGACCATGCCAAAGTGGAACCGAGTG	TACCTGCTGTGCGAAATGC	(GAT)10	7	450–469	OP851542
SP_6	GGAAACAGCTATGACCATAAGATGGGCTCAAGGTCCG	CAAGCAACTTAGGCAGGGC	(GTTC)10	20	352–399	OP851547
SP_7	AACTTCTCACTCGTGCCAG	GGAAACAGCTATGACCATCAAGGAA GAGGCCGAATGG	(GA)13	9	240–256	OP851532
SP_8	GAGGCTGAAGACAATGGCG	GGAAACAGCTATGACCATGTGGTG TATTGGTATCACAGC	(GA)18	7	156–175	OP851537
SP_9	GGAAACAGCTATGACCATGATAGAGTGCGGCCTATTGC	GCGCAAGGAGACAATACAGG	(CTCA)15	12	172–225	OP851546
SP_10	GGAAACAGCTATGACCATAAACGCCATTAAGCTCCGC	AGCAGGGCCAGCACTTAC	(AGGT)10	17	369–437	OP851548
SP_11	GGAAACAGCTATGACCATGCAAATGGCAGGATTGACTG	TCTGGGACGGAGAAGCAAC	(TGA)12	11	247–267	OP851544
SP_12	GGAAACAGCTATGACCATGTGACGTGCGTTTCAAGCC	CAGCCTGTTATGACGTCGG	(CA)10	14	213–241	OP851535
SP_13	AGAGGCAGCGAAGGAATTG	GGAAACAGCTATGACCATGTGT CCGAAATCTGACGGG	(GTCT)14	11	182–229	OP851541
SP_14	GGAAACAGCTATGACCATCTGCTTCAAAG GATAACTAGGTC	CGCACCAAACGTGCTAACG	(CTT)13	5	311–336	OP851550
SP_15	GGAAACAGCTATGACCATGAGGGCCACAAGTTCATTAGC	CAGCCACACTACCAGTTGC	(ACTC)16	18	134–318	OP851545
SP_16	GGAAACAGCTATGACCATAACAACCTG TGTAAGCTTTGG	AGAGCCATAAGCGGGATGG	(CTT)12	14	250–286	OP851533
SP_17	GGAAACAGCTATGACCATGTCGGTGT ATTGCTGTACC	GTCACTGAAATATGAACAAAGACCG	(AT)10(AG)15	18	199–271	OP851540
SP_19	TGGTGAGTGGGAAATAGGTTTG	GGAAACAGCTATGACCATGGTGCC AATTACCTTGGGC	(GTT)11	8	359–392	OP851551
SP_20	GGAAACAGCTATGACCATCAAAGCCGTCCTCACAACC	GGCTGAGCATCTTTCCCTTG	(GTT)12	4	319–329	OP851543

2.3. Stylophora Pistillata Sampling

Tissue samples of 40 *S. pistillata* colonies were collected by SCUBA from 4 reef sites at the Israeli Gulf of Eilat. During the dive, a branch tip (about 1 cm^3) of a mature colony (estimated diameter greater than 15 cm) was clipped with electrician clippers and placed in 5 mL vials with its surrounding sea water. Eight samples were collected at the Kisoski beach (29°32′49.98″ N, 34°57′14.78″ E) at depths down to 12 m. From the underwater observatory beach (29°30′12.57″ N, 34°55′7.55″ E), 8 samples were collected at depths down to 12 m. At the Lighthouse beach (29°30′2.76″ N, 34°54′58.89″ E), 16 samples were collected at a shallow dive down to 12 m and an additional set of 8 samples was collected from a 26 m to 40 m depth. All the samples were transferred, separately, into 1.5 mL vials containing 200 µL of lysis buffer, 40 µL of sodium perchlorate, and 240 µL of phenol chloroform isoamyl alcohol (PCI) [19], homogenized, and extracted with 240 µL of phenol/chloroform. The DNA was extracted as described above, except for the last steps where the DNA samples

were precipitated in 100% ethanol, centrifuged at 14,000× *g*, 4 °C, 30 min, washed twice with 70% ethanol, re-suspended in DDW, and kept in a cold room until they were used.

2.4. Microsatellite Amplification and Analysis

Microsatellite alleles for each DNA sample were amplified according to [24] in 20 µL of the total volume containing 2 µL of total DNA (diluted 1:50 with DDW), three primers, the forward and reverse primers and the fluorescence M13 primers (different dyes were used for different reactions, 0.8 pmol of each primer, Table 1), and 10 µL of ready-to-use commercial *Taq* polymerase mixture. The PCR conditions were as follows: 94 °C (5 min), followed with 30 cycles at 94 °C (30 s), 56 °C (45 s), and 72 °C (45 s), an additional 8 cycles of 94 °C (30 s), 53 °C (45 s), and 72 °C (45 s), and a final extension at 72 °C for 10 min. The fluorescent-labeled PCR products were examined in an agarose gel (1.5%). Positive PCR products were analyzed in a sequence analysis system (Applied Biosystems ABI PRISM 3100 Genetic Analyzer; the University of Cambridge, the UK) as follows: 0.25 µL of each amplification product were mixed with 0.4 µL of LIZ size standard (MepMarker DY632, 50–500 bp, BioVenture Inc. Murfreeboro, TN, USA) and 8.6 µL of HiFi Formamide (ThermoFisher). The fluorescent amplification products were scored using the genotyping software GeneMapper and Peak Scanner Version 1.0 Software (Applied Biosystems).

2.5. Data Analysis

The raw data generated by the microsatellites genotyping process were analyzed and binned using an Excel Macro, AutoBin 0.9, written in Microsoft Visual Basic (VBA) by Franck Salin (INRA Pierroton-UMR BIOGECO). The binned alleles of each locus were further analyzed using Micro-cheker [25], a software for scoring errors and potential null alleles. GenAlex6.5 [26] was used for calculating the observed heterozygosity (Ho), expected heterozygosity (He), Fixation index (F), allele numbers, and frequencies. Polymorphic information content (PIC) by locus was estimated by Cervus version 3.0.7 [27]. Hardy–Weinberg equilibrium and Linkage disequilibrium were calculated using "Genepop on the web" (https://genepop.curtin.edu.au/index.html, accessed on 10 December 2022, [28]), GeneAlEx6.5 and Alequin ver. 3.5 [29].

3. Results

3.1. Next Generation Sequencing Results

Sequencing using high-throughput technology resulted in 153,916 reads with an average read length of 732 bp (total of 112,686,846 bp). Most read sizes were distributed between 600 and 950 bp. The sequence reads were assembled into 16,060 contigs and 82,743 singletons.

3.2. Scanning for Short Tandem Repeats and Primers Design

The scanning of the 153,916 sequences reads with MSATCOMMANDER software for reads with short tandem repeats (STRs) resulted with 10606 reads (ca 7%). Restricting the STR screening for reads containing >10 repeats of di, tri, and tetra-nucleotides revealed a total of 2060 sequences (879 sequences of di-nucleotides, 910 sequences of tri-nucleotides, and 271 sequences of tetra nucleotides), of which 20 sets of forward and revers primers were designed for 20 reads (7, 7, and 6 sequences for di, tri, and tetra-nucleotides, respectively; Table 1) and were used for the PCR amplification of the 40 DNA samples of the corals collected in Eilat.

3.3. Microsatellites' Statistical Analysis

The 20 novel microsatellites primers were employed on the 40 *S. pistillata* DNA samples from the Gulf of Eilat. The new set of the microsatellite's loci revealed 4 to 20 alleles/locus and 10 loci that contain ≥10 alleles/locus. Polymorphic information content (PIC) ranged between 0.100 (4 alleles) and 0.904 (20 alleles, mean 0.699) (Table 2).

Table 2. Allelic diversity of *S. pistillata* microsatellite loci. Heterozygosity, F statistics, and polymorphism by population and Chi-square tests for Hardy–Weinberg equilibrium for codominant Data. N = number of samples; NA = number of alleles; Ho = observed heterozygosity = no. of heterozygote/N; He = expected heterozygosity = 1 − Sum pi^2; uHe = unbiased expected heterozygosity = (2N/(2N − 1)) × He; F = Fixation index = (He − Ho)/He = 1 − (Ho/He); PIC = polymorphic information content; Signif = ns = not significant, ** $p < 0.01$, *** $p < 0.001$.

Locus	N	NA	Ho	He	uHe	F	PIC	HW Prob	Signif	Null Present
SP_1	26	14	0.462	0.703	0.716	0.343	0.687	0.000	***	yes
SP_2	38	6	0.289	0.488	0.494	0.407	0.462	0.000	***	yes
SP_3	40	7	0.475	0.758	0.767	0.373	0.718	0.000	***	yes
SP_4	38	4	0.053	0.101	0.103	0.481	0.100	0.000	***	yes
SP_5	37	7	0.757	0.789	0.800	0.041	0.757	0.004	**	no
SP_6	40	20	0.875	0.911	0.922	0.039	0.904	0.000	***	no
SP_7	38	9	0.579	0.802	0.812	0.278	0.776	0.001	**	yes
SP_8	40	7	0.525	0.766	0.775	0.314	0.729	0.000	***	yes
SP_9	23	12	0.957	0.815	0.833	−0.174	0.800	0.561	ns	no
SP_10	40	17	0.925	0.846	0.857	−0.093	0.833	0.993	ns	no
SP_11	36	11	0.806	0.818	0.829	0.015	0.798	0.000	***	no
SP_12	40	14	0.875	0.799	0.809	−0.095	0.781	0.925	ns	no
SP_13	39	11	0.667	0.817	0.827	0.184	0.793	0.005	**	yes
SP_14	32	5	0.375	0.654	0.665	0.427	0.590	0.000	***	yes
SP_15	40	18	0.750	0.888	0.900	0.156	0.879	0.051	ns	yes
SP_16	36	14	0.556	0.859	0.871	0.353	0.844	0.000	***	yes
SP_17	38	18	0.658	0.875	0.886	0.248	0.864	0.000	***	yes
SP_19	40	8	0.550	0.595	0.603	0.076	0.533	0.072	ns	no
SP_20	32	4	0.219	0.507	0.515	0.569	0.439	0.000	***	yes
Mean	36.474		0.597	0.726	0.736	0.207	0.699			

The basic statistical analysis of the coral population (Table 2) with the 19 microsatellite loci using GenAlex6.5 (one marker, No. SP_18 GenBank accession numbers OP851538, failed to amplify and was excluded from further analysis) revealed observed heterozygosity ranging from 0.289 to 0.957 (mean 0.597 ± 0.057) and expected heterozygosity ranging from 0.101 to 0.911 (mean 0.726 ± 0.044). The Fixation index (F), which is also an estimate of the inbreeding coefficient, ranged between -0.174 and 0.569 (mean 0.207 ± 0.049). Fourteen loci show a significant deviation from the Hardy–Weinberg equilibrium ($p < 0.01$ or $p < 0.001$) and 12 loci show signs of a null allele (Table 2). Four pairs (SP_10/SP_1, SP_5/SP_1, SP_5/SP_19, and SP_13/SP_7) out of 171 paired loci comparisons show a significant linkage disequilibrium.

4. Discussion

In this study, we developed a new set of molecular markers, 19 new and highly polymorphic microsatellites loci for the branching coral species *Stylophora pistillata* (Esper 1797) from Eilat, by using a whole genome sequencing approach with the Roche 454 titanium GS-FLX plus Titanium pyrosequencing platform. The microsatellites markers developed for this study will be used, together with the former developed microsatellites [17], for detailed biological studies on this species, such as identifying the distributions of various genotypes within *S. pistillata* chimeras [30]. The new set of microsatellite loci further improves ongoing

studies on the *S. pistillata* population genetics, aiming to determine the levels of population genetics differentiation and gene flow among colonies of *S. pistillata* in the northern Gulf of Eilat. The current new set of microsatellite loci may further improve the *S. pistillata* population genetics analyses due to the appearance of 10 highly polymorphic loci, each containing $\geq$10 alleles/locus. Yet, the preliminary analysis of the 40-sample colony collected in this study indicates a deviation from the Hardy–Weinberg equilibrium, probably due to the presence of null alleles, an outcome that should be considered in future studies.

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