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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR A TROPICAL SEAGRASS, *SYRINGODIUM FILIFORME* (CYMODOCEACEAE)[1]

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- *Premise of the study:* A total of 17 polymorphic microsatellite markers were developed for the tropical Atlantic seagrass *Syringodium filiforme* (Cymodoceaceae), enabling analysis of population genetic structure in this species for the first time.
- *Methods and Results:* The 17 primers amplified di- and trinucleotide repeats revealing two to eight alleles per locus among the South Florida populations tested. In the analysis of two populations from the Florida Keys (Florida, USA), observed heterozygosity ranged from 0.063 to 0.905, although sampling was from relatively closely located populations so heterozygosity is expected to be higher across larger spatial scales. Multiplex PCRs consisting of two 6-plex and one 5-plex reactions were developed to maximize genotyping efficiency.
- *Conclusions:* We present here 17 polymorphic markers that will be useful for the study of clonality and population structure of *S. filiforme*, a marine plant that forms extensive habitat throughout the tropical Atlantic and Caribbean.

Key words: Caribbean; clonality; Cymodoceaceae; population genetics; seagrass; *Syringodium filiforme.*

Syringodium filiforme Kütz. (Cymodoceaceae) is one of three dominant seagrasses native to the tropical Atlantic. It is widely distributed and habitat forming, often growing intermixed with other species and sometimes in dense, monospecific meadows (Green and Short, 2003). *Syringodium filiforme* is capable of both recruitment from seed and clonal propagation through rhizome expansion (Kendrick et al., 2012). The relative contribution of each strategy is difficult to determine, but may be resolved using polymorphic microsatellites to accurately identify clones and examine genetic structure of populations (Arnaud-Haond et al., 2005). Primers for microsatellite loci have been developed for the only other congener, *S. isoetifolium* (Asch.) Dandy (Matsuki et al., 2013; Wainwright et al., 2013). The evolutionary distance between species of *Syringodium* Kütz. (Les et al., 1997) renders these markers unlikely to be suitable for use in *S. filiforme*, thus new markers were isolated. The microsatellite markers developed here will enable assessment of genetic diversity and population structure of *S. filiforme* throughout its range.

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METHODS AND RESULTS

Genomic DNA was extracted from leaf tissue of *S. filiforme* from eight samples collected in the Indian River Lagoon, Florida, USA (27.19298°N, 80.18067°W) using a DNeasy Plant Kit (QIAGEN, Valencia, California, USA) according to manufacturer's protocols. Approximately 250 ng of DNA from each sample was pooled to create a final solution containing 2 µg of total genomic DNA. Microsatellite loci were isolated by GenoScreen (Lille, France) following the methods described by Malausa et al. (2011). Libraries were prepared by mechanically fragmenting genomic DNA and probing for the following microsatellite repeats: TG, TC, AAC, AAG, AGG, ACG, ACAT, and ACTC. Enriched fragments were amplified, purified, and quantified. Sequencing using 454 GS-FLX Titanium chemistry (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) returned a total of 4410 distinct sequences containing microsatellite motifs. QDD software (Meglécz et al., 2010) was used to design 51 primer pairs. Primers were synthesized (Invitrogen, Carlsbad, California, USA) with M13 tails (5′-TGTAAAACGACGGCCAGT-3′) preceding the 5′ end of the forward primer sequences to facilitate cost-efficient fluorescent labeling of PCR products (Schuelke, 2000). To improve genotyping accuracy, PIG-tails (5′-GTTTCT-3′) were added to the 5′ end of the reverse primer sequences (Brownstein et al., 1996).

Primers were initially tested using DNA from eight samples from two populations, Florida Bay (vouchers deposited at the State Herbarium of South Australia [AD]: AD267896, AD267897; geo-reference: 25.12575°N, 80.78830°W) and Tampa Bay (27.65007°N, 82.67941°W). Given the close proximity of locations and unambiguous taxonomy, vouchers were only collected from one Florida Bay sampling site representing the entire South Florida region. DNA was extracted as above and gradient PCR performed containing the following reagent amounts in each 15-µL reaction: 1.5 mM $MgCl_2$, 1.3 mM dNTPs, 6.7 µg/µL bovine serum albumin (BSA), 0.5 units Bioline Immolase DNA polymerase and 1.50 µL Bioline ImmoBuffer (Bioline, Taunton, Massachusetts, USA). Primer quantities included 0.6 pmol of the M13-labeled forward primer, 2.4 pmol each reverse primer and the fluorolabeled M13 primer (5′-FAM, 5′-NED, or 5′-VIC; Invitrogen), and 1.0–3.0 ng·µL^{-1} of DNA template. PCR cycling conditions were 95°C for 10 min, 35 cycles of 45 s at 95°C, 30 s at an annealing temperature gradient of 52–58°C, and 30 s at 72°C, with final extension at 72°C held for 5 min. PCR products were visualized on a 1.5% agarose

TABLE 1. Characteristics of 17 microsatellite loci developed for *Syringodium filiforme*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
SYF-02	F: ACGAAGAAGAGGAGGAAGAGG	$(GAG)_{10}$	108–117	60	KM359149
	R: GTGTGGAATTGCAAGGAGGT				
SYF-03	F: ACAAATGATGACTAAGGAGGGG	$(TC)_{12}$	244–258	60	KM359150
	R: CTTATTAAGAATTAGTGCTCTTATGGC				
SYF-04	F: GGTGGTCGTACTTTTCAGGC	$(GTT)_{11}$	139–169	60	KM359151
	R: CAAAGCCAAGCAAACCTCAT				
SYF-06	F: ATCAATTCTTTGCCGATCC	$(AG)_{10}$	196–236	60	KM359152
	R: GAGGAGGATGATCTTGACCG				
SYF-12	F: TGAAACTGTTGAAGTGGAGGTG	$(TC)_{10}$	206–218	60	KM359153
	R: GCTGCAATTGTAAACCCCATT				
SYF-14	F: CCCTTGCATCGACACTATCC	$(TTC)_{11}$	134–166	60	KM359154
	R: CCCACTAAAAGAACATACGTACTGAA				
SYF-15	F: TTAGTTTCACTTTTCTGGCCG	$(CTT)_{17}$	281–293	60	KM359155
	R: GAACAGAAGCATGCAGTCTAGC				
SYF-16	F: CAAACATGGGCAGAGAAACA	$(TC)_{10}$	155–157	60	KM359156
	R: TGATCATGGACCTCAGGGA				
SYF-19	F: AAATCTCAAATCATCCTACGCA	$(TC)_{12}$	139–147	60	KM359157
	R: CGTCTGAAGGGTAGGTGTGG				
SYF-20	F: CCAACTCTGGCTGGCTGT	$(CT)_{10}$	180–198	60	KM359158
	R: GTTTCTGTCGGTATGTTCGAGCCATATT				
SYF-22	F: GAAAGGCCTCCATTCCATTT	$(AGG)_9$	146–161	60	KM359159
	R: TCGATTCTCCTCTTTCTTCGAC				
SYF-25	F: CCGTCGTAAGGACAAGGAAG	$(GAC)_9$	94–106	60	KM359160
	R: AGCAAAACAGCGTTCATGG				
SYF-29	F: GGACGAAATGAATGGTACGC	$(GA)_9$	215–223	60	KM359161
	R: GATTCTCACCTCGGAGCAAC				
SYF-37	F: TAGTCATTGCTCTATTGGGGTG	$(TTC)_8$	240–246	60	KM359162
	R: TGTGATTGTTAAGCCGAAAGG				
SYF-40	F: TCGTTGATCATAGAAGTCTCATCTC	$(TC)_8$	114–116	60	KM359163
	R: CCATAAAGGTAGCCCACACC				
SYF-49	F: TAAGAGGAACGACCCCATCA	$(GAC)_8$	140–143	60	KM359164
	R: CCTTGTCCTCCTCGTCTTCA				
SYF-51	F: TAGGGTTGTAGGAGCCAGGA	$(GA)_8$	164–174	60	KM359165
	R: GGGTGACTAGAAGCATTGCC				

Note: T_a = annealing temperature.
[a]Reverse primer sequences, and therefore also size range, do not include the optional PIG-tail (5′-GTTTCT-3′) sequence.

gel where a single band of approximate expected size according to locus primer design signified successful amplification. To determine polymorphism, PCR products were analyzed by capillary electrophoresis on a MegaBACE 1000 (GE Biosciences, Pittsburgh, Pennsylvania, USA) with an internal ET-ROX 400 Size Standard (GE Biosciences). A final set of 17 microsatellite loci were found to be polymorphic in *S. filiforme* (Table 1).

The high number of polymorphic loci enabled the development of multiplex PCR panels in which several loci are amplified in a single reaction, increasing genotyping efficiency while minimizing costs. Using the software Multiplex Manager version 1.2 (Holleley and Geerts, 2009), two 6-plex and one 5-plex multiplexes were designed, and new fluorescently labeled forward primers synthesized (Invitrogen; Table 2). Multiplex PCRs were conducted using a Type-it Microsatellite Multiplex PCR Kit (QIAGEN) in 10-μL reactions containing 5 μL of Type-it PCR Multiplex Master Mix, 0.5 μL of 2 μM primer mix (Table 2), and 1–5 ng of template DNA. PCR conditions were set to the manufacturer's optimized cycling protocol (Type-it Microsatellite Multiplex PCR Kit, QIAGEN).

To assess the genetic diversity of selected loci, two populations of *S. filiforme* were screened from the Florida Keys in Florida, USA: the Elbow Reef (25.12960°N, 80.26537°W; n = 36) in the Upper Keys and the Sluiceway (24.82836°N, 80.87138°W; n =34) in the Middle Keys. Samples were collected at least 2 m apart from an area of approximately 50 × 50 m. Fragment analysis of PCR products was performed on a capillary-based 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, California, USA) with an internal ET-ROX 500 Size Standard at the Georgia Genomics Facility (University of Georgia, Athens, Georgia, USA).

A total of 70 individual shoots sampled from two populations in the Florida Keys were screened, and 37 unique multilocus genotypes were identified. The probability individuals sharing the same genotype were derived via separate

TABLE 2. Suggested multiplex PCR panels for *Syringodium filiforme* using fluorescent labels FAM, NED, and VIC, and primer stock concentration of 100 μM.

Locus	Label	Volume (μL)[a]
Panel Syf-A		
SYF-25	VIC	5
SYF-19	FAM	12
SYF-02	NED	7.5
SYF-20	NED	7.5
SYF-15	NED	7.5
Panel Syf-B		
SYF-40	FAM	10
SYF-51	FAM	10
SYF-16	NED	10
SYF-04	VIC	7.5
SYF-12	VIC	7.5
SYF-03	VIC	7.5
Panel Syf-C		
SYF-29	FAM	10
SYF-14	VIC	6
SYF-49	NED	5
SYF-22	NED	7
SYF-06	NED	7
SYF-37	NED	8

[a]The volume listed is for both forward and reverse primers to be added to each panel with water to a total volume of 500 μL.

TABLE 3. Summary genetic statistics for two populations of *Syringodium filiforme* screened with the newly developed microsatellites.

	Elbow Reef (n = 36)				Sluiceway (n = 34)				Tampa Bay and Florida Bay (n = 8)[a]
Locus	A	H_o	H_e	P[b]	A	H_o	H_e	P[b]	A
SYF-02	3	0.714	0.547	0.173	3	0.813	0.579	0.069	3
SYF-03	5	0.762	0.722	0.800	3	0.875	0.675	0.478	3
SYF-04	5	0.762	0.754	0.759	2	0.688	0.514	0.309	2
SYF-06	6	0.905	0.792	0.627	3	0.625	0.685	0.609	3
SYF-12	3	0.714	0.675	0.772	2	0.125	0.121	1.000	3
SYF-14	8	0.810	0.808	0.470	3	0.875	0.595	0.042	3
SYF-15	3	0.381	0.361	0.381	3	0.625	0.675	0.277	2
SYF-16	1	—	—	—	1	—	—	—	2
SYF-19	4	0.714	0.618	0.192	2	0.467	0.370	0.528	2
SYF-20	4	0.571	0.632	0.186	3	0.750	0.548	0.215	2
SYF-22	3	0.619	0.577	1.000	2	0.375	0.315	1.000	2
SYF-25	3	0.619	0.633	0.579	3	0.250	0.232	1.000	2
SYF-29	2	0.095	0.093	1.000	2	0.563	0.417	0.256	2
SYF-37	2	0.095	0.093	1.000	2	0.375	0.387	1.000	2
SYF-40	1	—	—	—	1	—	—	—	2
SYF-49	2	0.143	0.136	1.000	2	0.063	0.063	—	2
SYF-51	1	—	—	—	1	—	—	—	3

Note: A = number of alleles; H_e = unbiased expected heterozygosity; H_o = observed heterozygosity.

[a] The number of alleles observed in the Tampa Bay and Florida Bay populations has also been provided to show that additional alleles are detected across larger spatial scales among the small number of preliminary screening samples.

[b] P values for deviation from Hardy–Weinberg equilibrium.

sexual events (P_{gen}) and the probability of clonal identity (P_{sex}) were calculated in GenClone2.0 (Arnaud-Haond and Belkhir, 2007). The highest values observed for P_{gen} and P_{sex} were $1.07 \cdot 10^{-5}$ and $1.27 \cdot 10^{-4}$, respectively. The loci SYF-16, SYF-40, and SYF-51 were fixed for single alleles although they were polymorphic in the initial screening runs. All of the other 14 loci were polymorphic, and allelic diversity was A = 2–8 (Table 3). Heterozygosity was only analyzed for genets, the presumptive genetic individuals based on unique multilocus genotypes, to avoid bias due to overrepresentation of a single clone. Observed heterozygosity ranged from 0.063 to 0.905 and unbiased expected heterozygosity ranged from 0.063 to 0.808. Deviation from Hardy–Weinberg equilibrium was not observed for any locus ($P > 0.05$). Linkage disequilibrium among loci was tested using GENEPOP web version 4.2, using Fisher's method (Raymond and Rousset, 1995; Rousset, 2008), and was detected between loci pairs SYF-2 and SYF-6, SYF-6 and SYF-20, and SYF-2 and SYF-14 ($P < 0.05$), but is most likely an artifact of sampling relatively closely related populations.

CONCLUSIONS

The 17 polymorphic microsatellite loci and multiplex PCR panels developed in this study will facilitate population genetic analyses of the extensive meadows of *S. filiforme* found throughout tropical Atlantic marine ecosystems. The allelic diversity detected will allow assessment of clonality, genetic diversity, and population genetic structure. Fixed alleles observed in the populations in the Florida Keys are probably due to the relatively close proximity of the sampling locations, as all loci were found to be polymorphic when comparing fewer samples from the Tampa Bay and Florida Bay populations. Clonal plant populations existing within geographical extremes of their range may exhibit higher clonality and less diversity, which may be true for *S. filiforme* in subtropical latitudes (Billingham et al., 2003). Therefore, it is expected that sampling more broadly across the species range will increase the number of alleles detected. The available microsatellite loci encourage future study of population connectivity and gene flow in *S. filiforme* that will further contribute to the management and conservation of this species.

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