Applications in Plant Sciences 2017 5(3): 1600141

Primer Note

Microsatellite markers for population studies of the salt marsh species *Juncus roemerianus* (Juncaceae)[1]

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- *Premise of the study: Juncus roemerianus* (Juncaceae) is a foundational species and ecosystem engineer of salt marshes in the Gulf of Mexico. These ecosystems provide coastal flood attenuation, nurseries for important species, and other ecosystem services, but are experiencing significant decline. Nuclear microsatellite markers were developed for *J. roemerianus* to study genetic diversity and population structure for conservation and restoration efforts.
- *Methods and Results:* Illumina NextSeq high-throughput sequencing was used to develop a panel of 19 polymorphic microsatellite markers that were tested across individuals from three populations on the Gulf Coast. All markers were polymorphic, with observed and expected heterozygosities ranging from 0.212 to 0.828 and from 0.362 to 0.873, respectively. Allelic richness ranged from two to 13 alleles per locus with an average of 5.737.
- *Conclusions:* The 19 microsatellite markers are useful for population studies throughout the range of *J. roemerianus*. Three loci cross-amplified in the related taxon *J. effusus.*

Key words: ecosystem engineer; Juncaceae; *Juncus effusus*; *Juncus roemerianus.*

Black needlerush (*Juncus roemerianus* Scheele; Juncaceae) is a clonal, gynodioecious macrophyte found in salt marshes from the mid-Atlantic in Maryland and Delaware to the western coast of the Gulf of Mexico in Texas (Godfrey and Wooten, 1979). The species has a high salt tolerance and dominates areas of low tidal flux, such as the Gulf Coast, forming large monotypic stands through sexual and clonal reproduction (Eleuterius, 1984). *Juncus roemerianus* is an ecosystem engineer and forms the foundation of the salt marsh, creating habitat for other marsh species by accumulating and stabilizing sediment (Pennings and Bertness, 2001). Genetic diversity of foundation species has an elevated importance in maintaining ecosystem health and resiliency in monotypic ecosystems such as salt marshes (Reusch and Hughes, 2006; Hughes et al., 2008). Restored macrophyte populations with higher genetic diversity are more resilient and have greater overall restoration success (Reynolds et al., 2012). Across the Gulf Coast, *J. roemerianus* habitat has been fragmented by human development and is vulnerable to future losses and degradation from pollution and sea level rise. Information on the genetic diversity and population structure of *J. roemerianus* is essential for salt marsh conservation.

While transplant studies suggest the existence of distinct populations of *J. roemerianus*, no molecular population genetic studies have been conducted on the species (Eleuterius, 1989). We address this need by developing and characterizing 19 microsatellite markers for *J. roemerianus* suitable for population studies. Microsatellites are highly variable and useful in characterizing the scale of population structure necessary for successful restoration and management.

METHODS AND RESULTS

Microsatellite markers were developed using an Illumina NextSeq sequencing system (Illumina, San Diego, California, USA). Genomic DNA was extracted from a leaf sample collected from the same site as the voucher specimen at the Grand Bay National Estuarine Research Reserve (NERR) in Moss Point, Mississippi, USA, using a QIAGEN DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany) (Appendix 1). Library preparation was completed using a KAPA LTP Library Preparation Kit (KAPA Biosystems, Wilmington, Massachusetts, USA) for Illumina platforms following the manufacturer's protocol. Reads totaling 872,449 sequences were paired by name using Geneious version 8.1.2 (Kearse et al., 2012) and archived in the GenBank Sequence Read Archive (SRR5076849). Illumina TruSeq adapters and bases with an error probability limit above 0.04 were trimmed, and de novo assembly was performed on sequences greater than 150 bases. Unused reads were extracted to MSATCOMMANDER version 1.0.8beta (Faircloth, 2008) and queried for microsatellite loci. MSATCOMMANDER identified 4237 loci with perfect repeats of 3–6 nucleotides using default minimum lengths and melting temperatures, and combining loci less than 50 bp apart. Five hundred and two loci had unique sequences surrounding the repeats with sufficient length for primer design. We selected 96 primer pairs

[1]Manuscript received 14 November 2016; revision accepted 3 January 2017.

The authors thank E. T. Jones for assistance during field collection and laboratory experimentation and J. Feura for assistance with field collection at the Grand Bay National Estuarine Research Reserve (NERR). The authors thank the University of Georgia Graduate School for funding awarded to H.R.T. and acknowledge resources provided by the Warnell School of Forestry and Natural Resources, the University of Georgia Genomics Facility, the Grand Bay NERR, and Apalachicola NERR.

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doi:10.3732/apps.1600141

with a pair penalty assigned by Primer3 (Rozen and Skaletsky, 1999) below six that had a diversity of repeat lengths and nucleotide motifs. One primer for each locus was tagged with either a CAG (CAGTCGGGCGTCATCA) or M13 (GGAAACAGCTATGACCAT) sequence addition to the 5′ end. Identical nucleotide matches between the 3′ end of the tag sequence and the 5′ end of the locus-specific primer were not duplicated. The corresponding primer for each locus was tagged with a GTTT PIG-tail (Schable et al., 2002).

Amplification through PCR was performed on individual loci in 10-μL reactions containing 0.05 μM CAG (CAGTCGGGCGTCATCA)–tagged or M13 (GGAAACAGCTATGACCAT)–tagged locus-specific primer, 0.5 μM GTTT PIG-tailed locus-specific primer (both from Integrated DNA Technologies, Coralville, Iowa, USA), 0.45 μM fluorescently labeled CAG- or M13-tagged primer, 0.125 mM dNTPs, 0.1 μg/μL bovine serum albumin (both from New England Biolabs, Ipswich, Massachusetts, USA), 15 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 units AmpliTaq Gold DNA polymerase (all from Applied Biosystems, Foster City, California, USA), and 1–10 ng of template DNA. Fluorophores used to label CAG- and M13-tagged primers included VIC, PET, NED (Applied Biosystems), and FAM (Integrated DNA Technologies) (Table 1). Thermal cycling parameters were: 95°C for 2 min; 25 cycles of 95°C for 3 min, 60°C for 30 s, and 72°C for 20 s; then 25 cycles of 95°C for 3 min, 52°C for 30 s, and 72°C for 20 s; with a final extension of 72°C for 5 min. Two microliters of PCR product was diluted in 50 μL USB nuclease-free water (Affymetrix, Santa Clara, California, USA). A 3730*xl* DNA Analyzer (Applied Biosystems) at the Georgia Genomics Facility and GeneScan 500 LIZ Size Standard (Applied Biosystems) were used to analyze amplicon sizes. GENEMAPPER version 4.0 (Applied Biosystems) was used to score allele sizes.

The 96 primer pairs were initially tested for amplification in two individuals from the Grand Bay NERR, and 48 amplified and were polymorphic. These 48 primer pairs were subsequently screened for amplification consistency and polymorphism using 24 individuals that were collected throughout the Grand Bay NERR (Table 2). Nineteen loci consistently amplified, did not significantly deviate from Hardy–Weinberg equilibrium, and had a frequency of null alleles below 0.1 (Table 1). The 19 loci were then tested across two additional populations on the Gulf of Mexico from the Apalachicola NERR and Choctawatchee Bay area to ensure consistent amplification across the range in which the species is dominant (Table 2). No clonal replicates were used to test the microsatellite markers. Three loci consistently cross-amplified in 24 samples of the related taxon *J. effusus* L., collected from Perdido Bay, Alabama, USA (Table 3).

Allelic data from GENEMAPPER was formatted for analysis using GMCONVERT (Faircloth, 2006). CERVUS version 3.0.7 (Kalinowski et al., 2007) was used to calculate allelic richness, observed heterozygosity, expected heterozygosity, deviations from Hardy–Weinberg equilibrium, and frequency of null alleles (Table 2). GENEPOP version 4.2 (Raymond and Rousset, 1995) was used to calculate linkage disequilibrium. No loci exhibited linkage disequilibrium across or within populations following sequential Bonferroni correction except one pair of loci (Jr03 and Jr29) in the Choctawhatchee Bay

TABLE 1. Traits and thermal cycling conditions for 19 microsatellite loci for *Juncus roemerianus*.[a]

Locus[b,c]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	GenBank accession no.
Jr01[V]	F: [d]GGGTACGTGCGAATTTCCAG	$(AAAG)_9$	226–268	KX398592
	R: [e]AGCACATTCTTCAGCCCTTG			
Jr02[F]	F: [d]CTCGGTGAAGGCGGTTTC	$(AAAG)_8$	213–237	KX398593
	R: [f]TTCTTTCAATCCCTGCCCAG			
Jr03[P]	F: [d]ACACCTTACAGACGGGCATC	$(AATT)_8$	112–128	KX398594
	R: [f]CGACATAGTAAATTGTGCCCAG			
Jr05[P]	F: [f]CCTCTCCATGTTAGCCCTTTC	$(AAAT)_9$	255–271	KX398595
	R: [d]AGAGTCGATTTGTTTGGCACG			
Jr12[N]	F: [f]CTCTCCTCCGCTTCTGTTCC	$(ACT)_{10}$	200–215	KX398596
	R: [d]AGGGCTTCACTATCCCACTTC			
Jr13[F]	F: [d]AGCAAAGGTGAAGTCGGAGG	$(AAC)_{10}$	175–193	KX398597
	R: [f]ATCCGCTCTCACCGTACAC			
Jr16[P]	F: [d]CGGTGCAGGTTTGGATTCAG	$(AAG)_{11}$	192–207	KX398598
	R: [f]GGATCCTGATTTCAAGCGCC			
Jr19[F]	F: [d]GATCAGGGAGGAGGATTCGG	$(AGG)_{13}$	156–183	KX398599
	R: [f]CTCCAACTCCTCCGCCAG			
Jr29[N]	F: [d]AACTTGACAAGCGAACAGGC	$(AAT)_{16}$	139–154	KX398600
	R: [f]TTTGACTAGACAACACCACCC			
Jr33[V]	F: [d]GTTGGGCCTAAACTCTTCCC	$(AAT)_{16}$	179–218	KX398601
	R: [f]CCTCTGCAACGATCTCAACG			
Jr41[F]	F: [d]AACCCTCCCTTCTCAAACCC	$(AAG)_{23}$	168–204	KX398602
	R: [f]TTCTTGACCCGGTCCTTCTG			
Jr42[N]	F: [d]GCTCTCTTTACTGCTTGCG	$(ACTGG)_8$	168–208	KX398603
	R: [f]TGGTAGATAGGCCCGGATTG			
Jr46[V]	F: [e]TCAACATGTCTCCACCCTCC	$(AAAAT)_9$	157–197	KX398604
	R: [d]CCGACAGTTTACATGTGAAGC			
Jr58[N]	F: [e]TCACTCTGGTCAAGGTTTAGGG	$(AAATC)_6$	149–175	KX398605
	R: [d]CCGACGACTGCAATCTCAAC			
Jr72[F]	F: [e]AGTGGGCATTATCTTATCACCG	$(AAAT)_8$	333–341	KX398606
	R: [d]GGCCGTTGTTGGAGTTTG			
Jr73[V]	F: [e]TCTACGTGAGCTACAGTTTCAC	$(AGG)_{11}$	159–180	KX398607
	R: [d]GTAACTTGGCTGCGGTGC			
Jr80[F]	F: [f]CCAGAAATGAGCACGCTGAAG	$(AAAAG)_7$	133–148	KX398608
	R: [d]CATGGGCTTGAGAAACCC			
Jr86[P]	F: [f]CCGTGAAGTGTGGCCTTTG	$(AGCAGG)_6$	160–187	KX398609
	R: [d]ATCCTTGGACGGCTCTGATC			
Jr87[V]	F: [f]ATATATTCGGCCCAGCTCGG	$(ACCTG)_6$	304–314	KX398610
	R: [d]CCACGTGAAGAGACCGATC			

[a]Values are based on 66 samples from the northeastern Gulf of Mexico in North America located in eastern Mississippi and Florida (N = 20–24).
[b]Fluorophore used to label M13- and CAG-tagged primers: [F] = FAM, [V] = VIC, [N] = NED, [P] = PET.
[c]Thermal cycling conditions for all loci were set at two annealing temperatures, 60°C for 25 cycles and 52°C for 25 cycles.
[d]GTTT tag addition to 5′ terminus.
[e]CAG tag (CAGTCGGGCGTCATCA) addition to 5′ terminus.
[f]M13 tag (GGAAACAGCTATGACCAT) addition to 5′ terminus.

TABLE 2. Genetic diversity metrics for three populations of *Juncus roemerianus* located in the northern Gulf of Mexico.[a]

	Grand Bay NERR (N = 24)			Apalachicola NERR (N = 20)			Choctawhatchee Bay (N = 22)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
Jr01	5	0.625*	0.57	7	0.6	0.641	4	0.818	0.735
Jr02	3	0.458	0.393	2	0.1	0.097	3	0.381	0.33
Jr03	3	0.542	0.582	5	0.5	0.518	4	0.636	0.678
Jr05	2	0.458	0.403	3	0.45	0.422	2	0.727	0.507
Jr12	3	0.625	0.664	3	0.15	0.229	3	0.455	0.65
Jr13	5	0.542	0.462	5	0.5	0.687	5	0.773	0.724
Jr16	5	0.583	0.691	4	0.7	0.688	4	0.591	0.576
Jr19	3	0.5	0.526	5	0.7	0.679	5	0.636	0.512
Jr29	5	0.625	0.594	5	0.7	0.672	5	0.636	0.72
Jr33	6	0.833	0.816	7	0.8*	0.808	9	0.85	0.855
Jr41	10	0.958	0.887	6	0.65	0.629	5	0.818	0.779
Jr42	6	0.542	0.566	4	0.55	0.696	4	0.864	0.692
Jr46	3	0.417	0.434	4	0.2	0.191	4	0.727	0.552
Jr58	4	0.458	0.414	4	0.4	0.645	4	0.773	0.591
Jr72	2	0.583	0.507	2	0.1	0.097	2	0.318	0.274
Jr73	5	0.292	0.27	7	0.8	0.767	5	0.636	0.729
Jr80	2	0.333	0.454	1	0	0	3	0.273	0.246
Jr86	4	0.792	0.691	4	0.7	0.721	5	0.818	0.758
Jr87	3	0.375	0.318	3	0.55	0.559	3	0.455	0.54

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of samples; NERR = National Estuarine Research Reserve.

[a] Geographic coordinates for populations are: Grand Bay NERR = 30°21.865′N, 88°26.246′W; Apalachicola NERR = 29°44.177′N, 84°53.094′W; Choctawhatchee Bay = 30°24.069′N, 86°13.834′W. Populations were collected from eastern Mississippi and the panhandle of Florida in the United States. Note that the coordinates provided here are a centroid of the samples collected for this study from each population and do not match the coordinates given for individual samples in Appendix 1. The centroid coordinates are provided here to more accurately describe the location of each population, as multiple voucher specimens were collected from some populations.

* Significantly deviated from Hardy–Weinberg equilibrium after a sequential Bonferroni correction (P = 0.05).

population. Allelic richness ranged from 2–13 alleles per locus with an average of 5.737. The panel of 19 microsatellites had a combined nonexclusion probability of identity of 1.009×10^{-15}.

CONCLUSIONS

The 19 polymorphic nuclear microsatellite markers are useful for investigating genetic diversity and population structure in *J. roemerianus* for conservation and restoration efforts. The markers provide sufficient resolution to identify clonal replicates and to examine the roles of clonal and sexual reproduction in natural populations of *J. roemerianus*.

TABLE 3. Characteristics of three primers that cross-amplified in the related species *Juncus effusus*.

Locus	Allele size (bp)	T_a (°C)
Jr05	263	60/52
Jr46	170–180	60/52
Jr73	129–489	60/52

Note: T_a = annealing temperature.

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Appendix 1. Voucher specimen information for the three populations of *Juncus roemerianus* and one population of *J. effusus*.[a]

Species	Collector no.	Location	Population	Geographic coordinates
J. roemerianus Scheele	Mark Woodrey 1	Moss Point, MS, USA	Grand Bay NERR	30°21.761′N, 88°27.023′W
J. roemerianus	Mark Woodrey 2	Moss Point, MS, USA	Grand Bay NERR	30°22.229′N, 88°24.429′W
J. roemerianus	Mark Woodrey 3	Moss Point, MS, USA	Grand Bay NERR	30°23.712′N, 88°23.981′W
J. roemerianus	Hayley Tumas 3	East Point, FL, USA	Apalachicola NERR	29°40.303′N, 84°51.101′W
J. roemerianus	Hayley Tumas 4	East Point, FL, USA	Apalachicola NERR	29°40.300′N, 84°51.106′W
J. roemerianus	Hayley Tumas 2	Santa Rosa Beach, FL, USA	Choctawhatchee Bay	30°23.918′N, 86°13.771′W
J. effusus L.	Hayley Tumas 1	Lillian, AL, USA	Perdido Bay	30°25.947′N, 87°24.843′W

Note: NERR = National Estuarine Research Reserve.

[a]Herbarium vouchers are deposited at the University of Georgia Herbarium (GA), Athens, Georgia, USA.