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Notes and Discussion Piece

Characterization of Microsatellite Loci for an Endangered Plant, Warea amplexifolia.

ABSTRACT.—Clasping Warea (*Warea amplexifolia*) is a critically endangered plant species endemic to Florida. It is threatened due to habitat loss through urban development and poor land management, resulting in habitat fragmentation and small population sizes. To aid recovery strategies, information is needed on the genetic diversity among and within populations. We identified fourteen microsatellite loci that were polymorphic in *W. amplexifolia* and in the related *W. sessilifolia,* and nine loci that were polymorphic in *W. cuneifolia*. In *W. amplexifolia*, the number of alleles per microsatellite locus varied between four and 15, with observed heterozygosity in the range of 0.10 to 0.73. These newly identified markers will be used to inform recovery efforts for *W. amplexifolia* and may also be useful for studies of the population genetics of closely related taxa.

INTRODUCTION

The genus *Warea* in the mustard family (Brassicaceae) contains four species, all of which are found in Florida. Research on this group is limited and the phylogenetic relationships among *Warea* species is currently unknown. Three *Warea* species are listed as state-endangered, and two are also federally endangered and endemic to Florida's upland habitats, primarily sandhill. Florida's xeric sandhill is part of the longleaf pine (*Pinus palustris*) -wiregrass (*Aristida stricta* var. *beyrichiana*) ecosystem that was predominant across the presettlement southeastern United States. Longleaf pine-wiregrass ecosystems supported hundreds of rare and endemic species (Sorrie and Weakley, 2006) and held an exceptionally high ground cover biodiversity (Kirkman *et al.* 2004); more than 40 species were documented in a single m^2 of sandhill (Landscope America, 2015). However, less than 3% of the original acreage of this ecosystem remains due to the effects of land conversion and logging. Much of what remains is fragmented, fire-suppressed, and teeming with invasive species, resulting in greatly reduced levels of biodiversity (U. S. Fish and Wildlife Service (USFWS, 1999).

Clasping Warea, *Warea amplexifolia,* (Nutt.) Nutt. is an annual that is both federally and state listed as endangered. It is endemic to sandhill habitat along the northern third of the Lake Wales Ridge in Central Florida, and persists in well-managed areas with bare ground and reduced canopy cover (Chafin, 2000). Preliminary data suggests a variety of pollinators visit the species, but it is unclear whether flowers provide nectar, pollen, or both. Recent surveys suggest an ongoing decline of this species with only ten small populations remaining, primarily on private lands (Peterson *et al.*, 2018). Federal recovery objectives include introducing diverse and self-sustaining populations on protected lands and preservation of the remaining genetic diversity within an *ex situ* collection (USFWS, 1999). A subsequent federal review of this species recommends genetic studies to help inform these recovery objectives (USFWS, 2007). Therefore, it is imperative to determine the level of genetic diversity remaining in natural populations to identify populations of greatest conservation concern and potential source populations for re- introduction efforts. Genetic markers developed for *W. amplexifolia* could also be used to assess genetic diversity and to inform conservation strategies in other species of *Warea*.

MATERIALS AND METHODS

Genomic DNA of *W. amplexifolia* was extracted from leaf samples taken from two individuals using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). An Illumina paired-end shotgun library was prepared by shearing 1 ug of DNA (Illumina TruSeq DNA Library Kit, San Diego, CA) and using a multiplex identifier adapter index. DNA was sheared using a Covaris S220 (Woburn, MA). Hi Seq 2000 (Illumina) sequencing was performed to generate 100-bp paired-end reads. PAL_FINDER_v0.02.03 (Castoe *et al.*, 2012) was used to examine five million reads and to extract reads containing microsatellite repeats. Primer3 (ver. 2.0.0; Rozen and Skaletsky, 1999) was used for primer design. Loci that occurred only once in five million reads were selected as potential candidates. Primers from 53 loci of the 4409 identified were randomly selected for further examination. One primer of each pair was modified by adding an

TABLE 1.—Characteristics of 14 microsatellite loci in *Warea amplexifolia*. T_a is the annealing temperatures for the touchdown protocol. Number after parenthesis for repeat motif indicates the number of repeats

Locus	Primer sequence (5′–3′)[a]	Repeat motif	Allele size range (bp)	T_a (C)
Waam23	F: *TCCTGTGGTGACTTTAGCTGC	$(ATT)_{17}$	297–330	60/50
	R: GACAGACTGTTAAAGAAACAGGCG			
Waam25	F: GTCCACTAGTTAAGAACAATTGCATCG	$(ATT)_{17}$	221–308	55/45
	R: *CAGTTCTAATGGATGCTCATCACC			
Waam31	F: *GGTCGTGCGGAGAGTTAGG	$(ATT)_{15}$	280-301	60/50
	R: AACGTTAGTGCTGGTGCTGC			
Waam33	F: *GGAGTCGATGAGGAAGACGG	$(ATT)_{14}$	441–459	59/49
	R: TTTGGAAGAGCAAGACGAGG			
Waam35	F: *TCTTTCATTGTCTCCAATAACTCTCC	$(TTC)_{14}$	315–441	61/51
	R: CGCTTCAAGTCTGAGCAACC			
Waam43	F: *CCTTCGATAATTGCAGGTTGG	$(ATT)_{13}$	375–402	61/51
	R: TTCTTGCTTTCCTGGAACCG			
Waam44	F: *GATGAAGGAAACGTCAGCAGG	$(ATT)_{13}$	241–307	61/51
	R: CCATGAATCGTCGTAATCGC			
Waam52	F: *TTGAGTCCTATCGAATGCGG	$(ATT)_{12}$	486–504	60/50
	R: AAGGCTGAAGGAATTGGTGG			
Waam57	F: *AACCAGCTCACTCCTTCTCCC	$(ATT)_{12}$	352–376	60/50
	R: TCTTCTTCCTCATCAGACCTGC			
Waam58	F: *CTCGCTTCTTCCTCTGCTCC	$(ATT)_{12}$	226–274	60/50
	R: GGAGGTGTTTGATGGGTGC			
Waam59	F: GCGGAGATTGACAGGAAAGG	$(ATT)_{12}$	283–313	60/50
	R: *CATCGCTGCTATCGTCATCC			
Waam61	F: *CTTCAAGTCATTAAAGAGTGGCG	$(TTC)_{12}$	373–597	60/50
	R: AAATCAATGCGGGTCAACG			
Waam62	F: *TTTATCTTAATCATAAGAGTTGGTGGG	$(TTC)_{12}$	332–407	60/50
	R: CCACTGGTCACTCTTCTAACACC			
Waam71	F: GATTCCGGTGACATAAGGAGC	$(TTC)_{11}$	243–252	60/50
	R: *AGAACAGAGTGGTCCTCGGG			

[a] Sizes of alleles in base pairs including CAG tag with location shown with an asterisk

engineered sequence to the 5′ end (CAG tag 5′-CAGTCGGGCGTCATCA-3′) to allow for the addition of a fluorescently labeled, third primer during amplification (6-FAM, VIC, or NED; Applied Biosystems, Culver City, CA).

Microsatellite primer pairs were tested in 20 samples of *W. amplexifolia* from a single, natural population using the touchdown amplification protocol as outlined in Ricono *et al.* (2015). Amplified products were run on an ABI3730XL sequencer (Applied Biosystems) and scored for size manually using GeneMapper (Applied Biosystems). *Warea amplexifolia* is diploid and microsatellite loci were readily scorable (Warwick *et al.*, 2009). Levels of polymorphism for each locus were assessed by examining the number of alleles, observed heterozygosity, and expected heterozygosity. Tests for Hardy-Weinberg equilibrium and linkage equilibrium were performed using Arlequin 3.5 (Excoffier and Lischer, 2010). Microchecker (Van Oosterhout *et al.*, 2004) was used to test for the presence of stuttering, large allele drop-out, and null alleles. Five to 15 samples were tested for amplification and polymorphism in two related species *W. cuneifolia* and *W. sessilifolia* to determine the utility of *W. amplexifolia* primers for cross-species amplification. Leaves for this study were collected from the wild and deposited at Bok Tower Gardens, Lake Wales, Florida. Sequences of paired-end reads are available from

TABLE 2.—Genetic diversity of 14 microsatellite loci developed from *Warea amplexifolia* and the number of alleles found in cross-species amplification with closely related taxa

	W. amplexifolia (n = 20)			*W. cuneifolia* (n = 5–13)	*W. sessilifolia* (n = 6–15)
Locus	A[a]	H_o	H_e[b]	A[a]	A[a]
Waam23	4	0.35	0.38	3	3
Waam25	15	0.73	0.95	1	6
Waam31	7	0.65	0.77	6	7
Waam33	5	0.5	0.63	7	5
Waam35	11	0.73	0.76	2	5
Waam43	7	0.1	0.88*	3	6
Waam44	12	0.4	0.73	6	9
Waam52	6	0.65	0.76	–	6
Waam57	8	0.7	0.81	5	4
Waam58	8	0.53	0.75	6	6
Waam59	5	0.5	0.71	4	4
Waam61	9	0.5	0.87	1	11
Waam62	12	0.55	0.89*	1	8
Waam71	4	0.5	0.49	1	4

A = number of alleles found; H_o = observed heterozygosity, H_e = expected heterozygosity; n = number of individuals genotyped

[a] Includes individuals from different locations to sample diversity of the species. Dash in the column for number of alleles indicates that the primer did not amplify DNA for that species

[b] Tests based on samples from a single location to minimize effects of population structure. Loci that showed Hardy-Weinberg disequilibrium, after Bonferroni correction for multiple tests (P = 0.004), are indicated with an asterisk

NCBI Bioproject PRJNA557477. All *Warea amplexifolia* in the paper are from Lake County, central Florida, all *W. cuneifolia* are from Jackson, Liberty, or Gadsden Counties in the panhandle of Florida, and all *W. sessifolia* are from Liberty, Calhoun, or Washington Counties, also in the panhandle of Florida. Bok Tower Gardens coordinates are: 27°56′14.17″n, 81°34′38.57″W.

RESULTS AND DISCUSSION

Fourteen of the 53 primer pairs (Table 1) were amplifiable and exhibited polymorphism in *W. amplexifolia* and *W. sessilifolia* (Table 2). Nine of the 14 loci were polymorphic in *W. cuneifolia* (Table 2). The number of alleles per locus varied between four and 15 for *W. amplexifolia*, one and seven for *W. cuneifolia*, and three and 11 for *W. sessilifolia*. Within *W. amplexifolia*, all loci were in linkage equilibrium relative to each other; however, two loci were out of Hardy-Weinberg equilibrium due to an excess of homozygotes (Waam43 and Waam62; Table 2). Stuttering or large allele drop-out were not indicated in Microchecker. Instead the analysis suggested Hardy-Weinberg disequilibrium for the two loci could be due to the presence of null alleles. An alternative explanation for an excess of homozygotes could be self-fertilization (Moore *et al.* 1998); however, self-fertilization is expected to impact all loci similarly and that is not what we detected in our data. The possibility of self-fertilization in populations of *W. amplexifolia* will be further investigated using these markers in all known populations.

Steps in the restoration of longleaf pine-wiregrass ecosystems in Florida include vegetation monitoring, invasive species removal, regular prescribed fire, and re- introduction of native species, such as *W. amplexifolia*. The overall objectives of the *W. amplexifolia* recovery plan include the protection

of self-sustaining populations and increasing the knowledge base regarding seed germination, seedling recruitment, seedling survival, pollinator behavior, population structure, and genetic diversity. Information about the genetic diversity and relatedness among populations is needed to fulfill these recovery goals. The fourteen loci developed in this study will be used to assess levels of diversity within and among populations of *W. amplexifolia* and to detect relatedness or historical gene flow with other *Warea* species. These microsatellite primers are the first developed for the genus *Warea*, and will likely have broad utility for the study and conservation of other endangered members of this genus, such as *W. carteri* and *W. cuneifolia*.

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JESSICA L. EMOTO, Tabor College, Hillsboro, Kansas 67030. ANGELA RICONO, University of Minnesota, Minneapolis, 55455. CHERYL L. PETERSON, Bok Tower Gardens, Lake Wales, Florida 33853, AND CHRISTIN L. PRUETT[1], Ouachita Baptist University, Arkadelphia, Arkansas 71998.
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[1] Corresponding author: E-mail: pruettc@obu.edu