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Development and cross-species amplification of microsatellite loci for *Puccinellia maritima*, an important engineer saltmarsh species

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ABSTRACT. The grass *Puccinellia maritima* is an important saltmarsh ecosystem engineer exhibiting wide morphological variation, which is partially genetically determined. Nevertheless, nothing is known about its population genetics or how neutral genetic variation is distributed throughout its geographical range. Here, we describe 12 polymorphic microsatellites pooled into two multiplexes for this octoploid species. Assessment of 24 samples from three populations revealed 4 to 29 alleles per locus, with variation in allele presence and abundance between populations. The transferability of these markers is reported based on their cross-amplification in six other *Puccinellia* species of different ploidy levels.

Key words: Microsatellite; Multiplex; Polyploidy; Saltmarsh; *Puccinellia maritima*

INTRODUCTION

Saltmarshes are coastal ecosystems characterised by the presence of salt-tolerant vegetation that is frequently flooded by sea tides (Nottage and Robertson, 2005). Over past centuries, the extent of these coastal ecosystems has been substantially reduced for several reasons, including land reclamation for agriculture, urbanisation, and coastal squeeze between sea defences and rising sea levels (Adam, 2002). Restoration programs have been implemented throughout Europe to counteract this loss.

Puccinellia maritima is a perennial and octoploid saltmarsh plant species (Scott and Gray, 1976). This species is often the dominant plant of the early successional stages of European saltmarshes. *P. maritima* is considered as an engineer species due to its ability to accumulate and stabilise sediments, thereby creating suitable habitats for the establishment of other saltmarsh plants (Langlois et al., 2001, 2003). This species shows great morphological variation between populations either along the coastline or along the tidal gradient. This variation has been shown to be partially genetically determined through classical genetic studies (Gray et al., 1979; Gray and Scott, 1977, 1980; Gray, 1985). However, the distribution of genetic variation within and between populations of *P. maritima* is poorly understood, whilst gene flow and genetic structuring at regional scales remains unknown, largely due to the lack of variable genetic markers for this species.

Here, we describe 12 newly developed microsatellite markers that will enable further study of the genetic diversity and structure of this species at both local and regional geographic scales, and allow us to develop a greater evidence base for saltmarsh restoration and management.

MATERIAL AND METHODS

Ten *P. maritima* individuals from five populations were sampled across the United Kingdom (Walborough: 51°19'N, 2°59'W; Chalkdock: 50°48'N, 0°52'W; Lepe: 50°47'N, 1°21'W; Abbotts Hall: 51°47'N, 0°51'E; Goosemoor: 50°40'N, 3°27'W). Genomic DNA was extracted from silica-dried leaves using the ISOLATE Plant DNA Mini Kit (Bioline, London, UK). An equimolar DNA solution was prepared from the 10 samples and sent to Genoscreen (Lille, France) for development of a microsatellite-enriched library using eight different probes (TG, AAC, AGG, ACAT, TC, AAG, ACG, ACTC), and sequencing by 454 GS FLX Titanium (Roche Applied Science, Meylan, France) according to methods described in Malausa et al. (2011).

The resulting reads were analysed with the QDD2 software (Meglécz et al., 2010) to detect microsatellite loci and design primers for each of them. Default parameters of the software were used, apart for the maximum length of polymerase chain reaction (PCR) products for primer design, which was set to 400 bp. The selection criteria used for choosing candidate primers for testing were adapted from Lepais and Bacles (2011). First, microsatellites containing the AT motif were discarded due to the difficulty of their amplification (Temnykh et al., 2001), and only di- or tri-nucleotide microsatellites were selected. Candidate primers were grouped into six classes of 50 bp according to the expected size of their PCR products (90-140, 140-190, 190-240, 240-290, 290-340, and >340 bp). Four primers from each size class were selected for a first screening of 24 loci. This selection was based on statistics given by

QDD2, with "A" design with the lowest penalty score being selected whenever possible. Two additional screenings of 24 loci were each conducted by preferentially selecting candidates in the size classes for which less reliable loci were found in the previous screening. Our objective was to obtain a sample of primer pairs spread across size classes that would allow us to maximise the number of loci that could be included in each multiplex PCR (Lepais and Bacles, 2011). Sequence data for each tested locus were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KC588847 to KC588900. Each primer pair was run in simplex PCR format using an M13 tailed primer protocol (Schuelke, 2000). PCRs were carried out in a final volume of 10 µL containing 1X Type-it Multiplex PCR Master Mix (Qiagen, Manchester, UK), 0.05 µM M13-forward primer, 1 µM reverse primer, 1X M13-fluorescent dye using four different dyes (6-FAM, HEX, TAMRA, ATTO 565; MWG Operon, Ebersberg, Germany), and approximately 20 ng genomic DNA. PCR cycles were performed on a DNA engine Tetrad® 2 Peltier Thermal cycler (Bio-Rad, Hertfordshire, UK) with a starting step of 5 min at 95°C followed by 32 cycles of 30 s at 95°C, 90 s at 60°C, 30 s at 72°C, and then 8 cycles of 30 s at 94°C, 45 s at 53°C, 45 s at 72°C, and finishing with a final elongation step of 10 min at 72°C. PCR products labelled with different fluorescent dyes were pooled and analysed in an ABI 3730xl sequencer (Applied Biosystems, Warrington, UK). Fragment data were analysed with Peak Scanner (Applied Biosystems). The clarity and exploitability of the signal was assessed by running PCR on seven samples from three populations. Primer pairs showing no amplification, too much stutter, or amplifying monomorphic loci were discarded.

The selected primers were checked for multiplex compatibility using Multiplex Manager (Holleley and Geerts, 2009), and used to design multiplex assays. Multiplex PCRs were carried out in a final volume of 10 µL containing 1X Type-it Multiplex PCR Master Mix (Qiagen), variable concentrations of each fluorescently labelled primer (see Table 1), 0.5X Q-solution (Qiagen), and 20 ng genomic DNA. The PCR cycle was 5 min at 95°C followed by 32 cycles of 30 s at 95°C, 3 min at 62°C, 30 s at 72°C, and a final elongation step of 30 min at 60°C.

DNA of eight individuals from three populations (Walborough, Goosemoor, and Nigg Bay: 57°44'N 4°2'W) was amplified with the two designed multiplexes. Alleles were scored using STRand (Veterinary Genetics Laboratory, University of California, http://www.vgl.ucdavis.edu/informatics/strand.php/) with allele binning performed using MsatAllele (Alberto, 2009) modified by M. Vallejo-Marin to allow binning of more than two alleles per locus per individual. Population genetic studies for polyploid species remain challenging since some common statistics, such as expected heterozygosity or deviations from Hardy-Weinberg equilibrium, cannot be computed for polyploids. Consequently, since *P. maritima* is octoploid (Scott and Gray, 1976), SPAGeDi (Hardy and Vekemans, 2002) was used to determine the number of alleles per locus and the number of private alleles per locus by specifying the ploidy level.

Cross-species amplification was tested in six *Puccinellia* species differing in ploidy level. Genomic DNA from 10 individuals of *P. vahliana* (2x, five from Ny-Ålesund, Svalbard, Norway; five from Ringhorndalen, Svalbard, Norway), eight individuals of *P. angustata* (6x, three from Innerholmen, Svalbard, Norway; five from Björndalen, Svalbard, Norway), and two individuals of *P. svalbardensis* (6x, Innerholmen, Svalbard, Norway) was extracted from silica-dried material using the CTAB protocol (Murray and Thompson, 1980). Genomic DNA from two individuals of *P. convoluta* (2x or 4x, one from Tavira, Algarve, Portugal, and one from Alvor, Algarve, Portugal), one individual of *P. festuciformis* (6x, Quinta do Lago, Algarve, Portugal), and one individual of *P. stenophylla* (10x, Alvor, Algarve, Portugal) was

extracted from dry herbarium samples using the ISOLATE Plant DNA Mini Kit (Bioline) according to manufacturer instructions.

RESULTS AND DISCUSSION

The sequencing strategy yielded 13,253 reads (average length = 284.73 bp, SD = 117.67 bp) and 936 microsatellite locus candidates. Of the 72 screened primer pairs, 60 were monomorphic or yielded unreliable bands. Twelve primer pairs showed a clear, repeatable, and polymorphic signal across different samples, and were compatible with multiplex PCR amplification. Two multiplex assays of six primers each were constructed (Table 1).

Table 1. Characteristics of the 12 microsatellite primers developed for *Puccinellia maritima*.

Multiplex	Locus	Repeat motif	Allele size range (bp)	Primer sequences (5'→3')	Primer (nM)	Dye	GenBank accession No.
1	Pm29	$(AAC)_6$	132-165	F: CATCCTCGAGAGGGAGAAA R: ACACATATCAGCCCTCGGT	200	FAM	KC588874
	Pm61	$(AAC)_6$	252-336	F: GAATCATGTGCGAACCTGTG R: ATCTTCAGCAATGCCTGGAT	200	FAM	KC588894
	Pm26	$(AC)_7$	109-115	F: TGGGGACATCGAAATGGTAT R: TCAAATAGCTGCTGGGAACC	100	HEX	KC588871
	Pm65	$(AC)_7$	226-314	F: ATCGTAGGAGATGCACGCTT R: CGCCAGGAGCTGTTAAATGT	200	HEX	KC588896
	Pm10	$(AAC)_8$	222-234	F: TCAGCTCAAACTCTCAGGCA R: ACCAAGCTCACCAATCAACC	400	TAMRA	KC588856
	Pm19	$(AG)_9$	312-352	F: GCAGGTTTGATAGAGGCAGG R: TGGTAACCTAGCGAGCAGTG	400	TAMRA	KC588865
2	Pm27	$(AAG)_{14}$	91-208	F: ATCATTGGCCTCTCGTTGTC R: AGTGTTGGGCGTATAGGCTG	400	FAM	KC588872
	Pm25	$(AGG)_6$	99-117	F: CTAGTTGCAGCCATGGGATT R: CCGGAACCATTAGAAGACGA	100	HEX	KC588870
	Pm34	$(AAC)_9$	171-309	F: TGGCAAATTTACACCACGAA R: GCAAGCAATGAAAACACGAA	100	HEX	KC588877
	Pm23	$(AAC)_{10}$	337-352	F: CTTGTTTGGGACTGAAAGGC R: GACCAGCACGGCATATGTTA	100	HEX	KC588869
	Pm39	$(AG)_9$	243-299	F: TTTCGGTCATTAGGATTCGC R: AAGGCCTGGCTAGATGTGAA	400	TAMRA	KC588880
	Pm12	$(AGG)_6$	213-225	F: GGGTGACTGGGGTGATAAGA R: AATCCACGAATTTCCACCAA	200	ATTO-565	KC588858

Annealing temperature of each multiplex, Ta = 62°C.

The number of alleles per locus spanned from 4 to 29. The maximum number of alleles for an individual at a single locus was eight, which is in accordance with the ploidy level of this species. The number of private alleles varied from 16 to 22 across all loci from eight individuals per population across the three sampled populations (Table 2). The high level of polymorphism and the frequent private alleles demonstrated the efficiency of these loci for studying relatedness between individuals and differentiation between populations.

The results of cross-species amplification differed between species (Table 3). Apart from Pm23, most of the loci amplified in at least one other species. Cross-amplification was most successful in *P. vahliana*, *P. angustata*, and *P. svalbardensis*. However, it should be noted that DNA from *P. convoluta*, *P. festuciformis*, and *P. stenophylla* was extracted from dry herbarium samples, and that amplification from fresh sample DNA may give superior results.

Table 2. Characteristics of the 12 microsatellite loci described for *Puccinellia maritima* across 3 populations.

Locus	Nigg Bay (N = 8)			Walborough (N = 8)			Goosemoor (N = 8)			Overall (N = 24)	
	N_A	N_I	PA	N_A	N_I	PA	N_A	N_I	PA	N_A	N_I
Pm10	3	2-3	0	5	3-5	0	5	2-5	0	5	2-5
Pm12	3	1-3	1	3	1-3	0	3	1-3	0	4	1-3
Pm19	9	1-6	2	10	4-5	5	9	2-4	3	17	1-6
Pm23	4	1-4	2	2	1-2	1	3	1-3	0	6	1-4
Pm25	4	1-4	1	3	1-3	0	5	1-3	1	6	1-4
Pm26	4	2-4	0	3	1-3	0	4	1-3	0	4	1-4
Pm27	15	4-7	7	10	1-3	2	19	2-5	7	29	1-7
Pm29	6	2-3	1	8	2-5	3	4	1-3	0	9	1-5
Pm34	13	3-8	3	12	3-6	2	10	3-6	2	18	3-8
Pm39	8	2-5	2	9	1-6	2	16	1-5	8	21	1-6
Pm61	5	1-3	0	6	2-4	1	7	3-5	1	8	1-5
Pm65	5	2-5	0	6	2-4	0	6	3-5	0	6	2-5
Overall	79	-	19	77	-	16	91	-	22	133	-

N_A = number of alleles; N_I = number of alleles per individual; PA = number of private alleles.

Table 3. Cross-species amplification of the 12 microsatellite loci designed for *Puccinellia maritima* in other *Puccinellia* species.

Locus	*P. vahliana* (N = 10)			*P. angustata* (N = 8)			*P. svalbardensis* (N = 2)			*P. convolute* (N = 2)			*P. festuciformis* (N = 1)			*P.stenophylla* (N = 1)		
	S	N_A	NP	S	N_A	NP	S	N_A	NP	S	N_A	NP	S	N_A	NP	S	N_A	NP
Pm10	+	2	10	+	2	8	+	3	2	+	7	2	-	0	0	+	1	1
Pm12	+	1	5	+	1	4	+	2	2	-	0	0	-	0	0	-	0	0
Pm19	+	4	5	-	1	3	+	3	2	-	0	0	-	0	0	-	0	0
Pm23	-	0	0	-	0	0	-	0	0	-	0	0	-	0	0	-	0	0
Pm25	+	1	5	+	2	4	+	2	2	+	3	2	-	0	0	-	0	0
Pm26	+	2	10	+	2	8	+	2	2	+	1	1	-	0	0	-	0	0
Pm27	+	1	9	+	2	7	+	1	2	-	0	0	-	0	0	-	0	0
Pm29	+	2	10	+	2	8	+	4	2	+	4	2	-	0	0	-	0	0
Pm34	+	1	5	-	1	1	+	1	2	+	2	2	-	0	0	-	0	0
Pm39	-	0	0	-	0	0	+	2	2	-	0	0	-	0	0	-	0	0
Pm61	+	1	5	+	3	4	+	1	2	+	1	1	-	0	0	-	0	0
Pm65	+	1	5	-	1	3	+	2	2	+	1	1	-	0	0	-	0	0

S = amplification success; N_A = number of alleles across all individuals amplified; NP = number of samples positively amplified. The amplification was considered as successful when half or more of the samples showed amplification.

In conclusion, the 12 polymorphic microsatellite loci described here will be highly useful to study the population genetics of *P. maritima* across a broad range of applications in molecular ecology and habitat restoration. Moreover, cross-amplification of these markers demonstrates their utility for research in congeneric species, such as *P. svalbardensis*, a rare endemic plant from the island of Svalbard, Norway.

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