MOLECULAR ECOLOGY RESOURCES
Molecular Ecology Resources (2010) doi: 10.1111/j.1755-0998.2009.02828.x

MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Development of novel chloroplast microsatellite markers to identify species in the *Agrostis* complex (Poaceae) and related genera

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Abstract

We needed a reliable way to identify species and confirm potential interspecific and intergeneric hybrids in a landscape level study of gene flow from transgenic glyphosate-resistant *Agrostis stolonifera* (Poaceae) to compatible relatives. We developed 12 new polymorphic chloroplast microsatellite markers to aid in identifying species recipient of transgenic pollen both within the *Agrostis* complex and the related genera *Polypogon.*

Keywords: *bentgrass,* creeping bentgrass, grasses, hybridization, primers, SSR

Received 20 *July 2009; accepted* 30 *November 2009*

Agrostis is considered one of the most difficult and complicated of the grass genera from a taxonomic perspective (Warnke 2003). Many *Agrostis* species, including *A. stolenifera, A. gigantea, A. capillaris,* and *A. castellana,* are cross compatible and form interspecific hybrids (Wipff & Fricker 2001). *Agrostis stolonifera* also produces intergeneric hybrids with several *Polypogon* species (Bjorkman 1960). The great challenge of identifying species and potential hybrids based on morphological characteristics has motivated the development of DNA markers to assist in confirming the identification of plants found in *situ.*

Chloroplast markers are useful for species determination and the study of hybrid evolution in plant taxa and seed dispersal because of the uniparental mode of transmission (Ennos *et al.* 1999). Powell *et al.* (1995) reported SSR variability in the chloroplast genome and suggested it may be a source of polymorphic markers for studies in plant population genetics and systematics. Chloroplast micro satellites (cpSSR) are now a high resolution tool for examining cytoplasmic variation in a wide range of species (Provan *et al.* 2001, 2004; McGrath *et al.* 2006). Our goal was to develop cpSSR markers that amplify in several *Agrostis* species and *P. monspeliensis* to assess maternal lineage of plants recipient of transgenic pollen, and confirm the formation of transgenic hybrids between *A. stolonifera* and compatible relatives.

The complete chloroplast genome sequence of *Agrostis stolonifera* (GenBank Accession no. NC_008591.1) was screened for the presence of mono- and dinucleotide repeats with a minimum of eight and five repeats, respectively, using *Phobos* 3.2.6. (Christoph Mayer, 2006-2009, http://www.rub.de/spezzoo/cm/cm_phobos.htm). A total of 52 cpSSR loci were identified. Primer pairs homologous to the flanking regions were designed for 32 cpSSR loci using Primer 3 (Rozen & Skaletsky 2000). Total genomic DNA was extracted from young leaves using the DNeasy 96 Plant kit (Qiagen). The PCR reaction mixture (15 uL) contained 2-5 ng of genomic DNA, 1x Phusion HF buffer, 200 uM each dNTP, 0.4 uM each primer, and 0.02 U uL^{-1} Phusion^{TM} High-Fidelity DNA Polymerase (Finnzymes). The PCR program consisted of: 2 min at 98°C, followed by 30 cycles of 10 sat 98°C, 20 s at 60-63 °C (primer-specific), and 20 s at 72°, with a final extension of 10 min at 72°, using a Cl000TM Thermal Cycler (Bio-Rad). Uniformity of PCR amplification products was confirmed by UV fluorescence after electrophoresis on 2% agarose gel with ethidium bromide. Screening of 32 primer pairs on *A. stolonifera* and *A. gigantea* revealed that 31 amplified in both species, but five gave multiple bands per plant and were discarded. From the remaining 26 primer pairs, 14 were chosen for fluorescent capillary analysis. The 14 selected labelled

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Table 1 Characterization of 14 chloroplast microsatellite markers for the grass *Agrostis stolonifera*. Position, amplicon expected size, repeat motif, and chloroplast gene and interspacer region associated are expressed relative to *A. stolonifera* chloroplast sequence (NC_008591.1)

Name	F sequence (5′–3′)	R sequence (5′–3′)	Genome position	Amplicon size (bp)	Repeat	T_a (°C)	Gene/intergenic spacer
Acp12	CATAATGCGCCGTTTCTATT	CAAGCAAGATTGGTTGGATT	36917	262	$(A)_7C(A)_{10}$	63	*psaB/rps14*
Acp18	CCGTTCCTTTTCTACCCAAT	TGGGTTTTCCTATGAGTGGA	3903	217	$(A)_{11}G(A)_8$	63	*matK/trnK*
Acp19	TTCCACAATGAAATCCAAAA	ATTGGATGGCAAAGATTTCA	19379	199	$(T)_{11}$	60	*trnC/rpoB*
Acp22	AACAAGTGACTCGAACTGTGTG	TCGTTCTTGATCGGAATCAT	56933	129	$(T)_{11}$	63	*rpl23/psaI*
Acp23	TAGGTTCAGGCGAGGTAGTG	AAATAAAAACCACCCTTCAATG	61542	147	$(A)_{11}$	63	*petA/psbJ*
Acp24	AACTCATCTGATTTCCGGTAGA	GGATTTACGAAAGGGTTGCT	65996	240	$(T)_{11}$	63	*rps18/rpl33*
Acp25b	TCCCTCCACTCGCTCTAAAA	GGGCCTTTTATGGCTGATCT	115028	261	$(AG)_6$	63	*ndhH* gene
Acp26b	CCTTCGTGGAGTCCCTTCTT	GGCCCAAAGGGTTAGCAATA	18312	153	$(A)_{12}$	63	*petN/trnC*
Acp27b	GCATCAGCATGTAGGTTCCA	AACACTTGCCTCCGATTGAC	41408	352	$(T)_{12}$	63	*psaA/ycf3*
Acp28b	GGTTTGGTGCTTTAGCAGGT	AGCTAGCCCAGCCTTTTTCT	65192	298	$(A)_{12}$	63	*psaJ/rpl33*
Acp29b	CGTCCGAAGGAATCAATGTT	CTGCTTGGCGTTGCATATTA	21167	396	$(A)_{13}$	63	*rpoB* gene
Acp30b	TCAATGGCCAACTCTCAGTG	TGACCAAAATGAACTCCTGCT	29956	223	$(A)_{13}G(A)_6$	63	*rps2/rpoC2*
Acp31	AAGGAAACATAGAGTCATAGCAAAT	CCTTGAGGTCTTTGCAATTC	46646	178	$(A)_{13}$	63	*trnT/trnL*
Acp32	CCCCACGATACAATGAATTT	TCCTCTGGACGCGTATAGTAA	57220	94	$(A)_{13}T(A)_6$	63	*rpl23/psaI*

Table 2 Allele sizes (bp) of the 12 newly developed polymorphic* cpSSR markers and number of unique haplotypes for nine *Agrostis* species and *Polypogon monspeliensis*

Species	N†	Acp12	Acp18	Acp19	Acp22	Acp23	Acp24	Acp26b	Acp27b	Acp28b	Acp30b	Acp31	Acp32	N_H‡
A. stolonifera	57	258, 259	213	195	125, 126	144	234, 235	148, 149	349, 350, 351	292, 293, 294	218, 219, 220	172, 173, 174	86, 87	7
A. gigantea	14	258	213, 214	193, 195	125, 126	142, 143, 145	233, 234, 235	145, 148, 149	348, 350	292, 293	218, 219	172, 173, 174, 193	87, 88	7
A. capillaris	16	258	213	191, 195	124, 125	142	232, 233, 234, 235	145	348	292, 293	216	189, 209	88	9
A. castellana	8	258	212	193, 195	123, 124	142	233, 234, 235	145	348	293, 294	216	191	88	5
A. scabra	6	258	214	192	125	143	235	147	350	291	219	173	92	1
A. canina	10	258	214	192	124, 125	143	235	148	350	293	218	175	91	2
A. exarata	6	257	212	192, 193	125, 126, 127	142	236, 237	147	348	292, 293	220, 221	172, 175, 176	90	4
A. vinealis	6	257	215	192	124	142	235	148	349	299	217	171	88	1
A. vinealis ssp. *trinii*	6	256	229	§	§	142	228	§	347	288	216	§	§	1
P. monspeliensis	10	257	213	§	125	143	237	145	348	291	218	168	89	1
Total	139	4	5	4	5	4	7	4	5	6	6	11	7	38

*Acp29b (394 bp) and Acp25b (258 bp) were monomorphic for all the species tested and are, therefore, omitted from the table.

†Number of individually tested plants for each of the species.

‡Number of unique haplotypes detected per species.

§No amplification.

primer pairs (Table 1) were tested on 139 plants representing nine *Agrastis* species and *Polypagon monspeliensis* (Table 2). PCR products were multiplexed in two sets 1:400-1:1000 dilution) and were screened by capillary electrophoresis using an ABI Prism® 3100 Genetic Analyzer. Genotypes were scored manually using ABI Prism® Genotypes version 3.7 NT (Applied Biosystems). All primer pairs amplified a product in all species tested except *A. vinealis* ssp. *trinii* and P. *monspeliensis* (Table 2). Twelve of the 14 markers were polymorphic, with between four and 11 alleles detected in the 10 species tested. Alleles were shared across species at several loci, but haplotypes were unique in all species tested (Table 2). The Acp18 and Acp30b loci repeat motifs in *A. stolonifera* are comparable to those of the *trnK* intron and *rpoC2/rps2* loci reported for rice, wheat, corn, and other grasses by Provan *et al.* (2004), which suggests the potential cross utility of the novel markers in additional species beyond those evaluated.

We demonstrated that the 14 cpSSR markers amplify in a wide range of *Agrostis* species and related genera, and that 12 are robust and polymorphic. Although the analysis of hybridization past the first generation requires a combination of chloroplast and nuclear markers (Reichman *et al.* 2006), the primers developed will be useful to identify maternal lineages that participate in the formation of interspecific and intergeneric F1 hybrids in the *Agrostis* complex. These novel cpSSR markers also can be used to estimate transgene movement via seeds. The cpSSR markers developed can be helpful to assist identification of some native vs. introduced *Agrastis* species, although this would need to be determined on a case by case basis due to the demonstrated interfertility of these species. Finally, these cpSSR markers will be a useful tool in *Agrastis* species breeding programs, with the potential to be applied to other genera as well.

Acknowledgements

Seeds were provided by M. C. Engelke from Texas AgriLife Urban Solutions Center and the U.S. National Plant Germplasm System.

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