GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Microsatellite in *Aeschynomene falcata* (Leguminosae): diversity, cross-amplification, and chromosome localization

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Genet. Mol. Res. 13 (4): 10390-10397 (2014)
Received April 8, 2014
Accepted August 8, 2014
Published December 4, 2014
DOI http://dx.doi.org/10.4238/2014.December.4.34

ABSTRACT. *Aeschynomene falcata* is an important forage species; however, because of low seed production, it is underutilized as forage species. *Aeschynomene* is a polyphyletic genus with a challenging taxonomic position. Two subgenera have been proposed, and it is suggested that *Aeschynomene* can be split in 2 genera. Thus, new markers, such as microsatellite sequences, are desirable for improving breeding programs for *A. falcata*. Based on transferability and *in situ* localization, these microsatellite sequences can be applied as chromosome markers in the genus *Aeschynomene* and closely related genera. Here, we report the first microsatellite library developed for this genus; 11 microsatellites were characterized, with observed and

expected heterozygosities ranging from 0.0000 to 0.7143 and from 0.1287 to 0.8360, respectively. Polymorphic information content varied from 0.1167 to 0.7786. The departure from Hardy-Weinberg equilibrium may have resulted from frequent autogamy, which is characteristic of *A. falcata*. Of the 11 microsatellites, 9 loci were cross-amplified in *A. brevipes* and *A. paniculata* and 7 in *Dalbergia nigra* and *Machaerium vestitum*. Five of these 7 cross-amplified microsatellites were applied as probes during the *in situ* hybridization assay and 2 showed clear signals on *A. falcata* chromosomes, ensuring their viability as chromosome markers.

Key words: Cross-amplification; Fluorescence *in situ* hybridization; Forage; Microsatellite

INTRODUCTION

Aeschynomene falcata (Poir.) DC. (Leguminosae) is an important forage species with high persistence, resistance to intensive grazing, and tolerance to low-fertile soils. However, the seeds are not produced in sufficient quantities for marketing because of low fertility (Jones et al., 2000). Productivity may be increased through plant breeding techniques, which may contribute to the development of molecular markers, which are useful for assaying genetic variation. Microsatellite markers, or simple sequences repeats (SSRs), are effective for planning crosses in breeding programs (Ruiz and Asins, 2003; Flajoulot et al., 2005). In addition to their low cost, SSRs are hypervariable, present codominant heritage, show reproducible results, have chromosome-specific locations, and show high transferability (Kalia et al., 2011).

SSR cross-amplification is a potential source of variation for evolutionary and taxonomic studies, with the SSR transfer rate corresponding to taxonomic distance (Ellis and Burke, 2007; Pinheiro et al., 2009). This approach may be helpful for studying the Dalbergia clade, in which *Aeschynomene* genus is included. The Dalbergia clade position in the Papilionoideae phylogeny is well-understood (Cardoso et al., 2012); however, its internal resolution has not been well-defined. Among its taxonomic problems is the genus *Aeschynomene*, which is split into 2 groups (Ribeiro et al., 2007), including *Aeschynomene* subgen. *Ochopodium* (monophyletic with *Dalbergia* and *Machaerium*) and *Aeschynomene* subgen. *Aeschynomene*. Some authors have suggested that the subgen. *Ochopodium* should be placed in another genus. Morphological, molecular, and chromosome markers have been applied for the Dalbergia clade, but the resolution of *Aeschynomene* remains obscure (Klitgaard and Lavin, 2005; Ribeiro et al., 2007; Queiroz and Cardoso, 2008; Polido, 2013). Taking advantage of the chromosome-specific location and transferability of SSR, we developed new chromosome markers using the microsatellite library as a source of probes for *in situ* localization. This approach was previously applied for Orchidaceae (Begum et al., 2009), in which 3 new chromosome markers were identified and used to determine species relatedness to the *Dendrobium* genus.

We developed and characterized a microsatellite library for *A. falcata* (subgen *Ochopodium*) for future applications in both breeding systems and taxonomy studies. Cross-amplification, as well as *in situ* hybridization, were tested in *A. brevipes* Benth., *A. paniculata* Willd. ex Vogel (both subgen. *Ochopodium*), *Dalbergia nigra* (Vell.) German ex Benth., and *Machaerium vestitum* Vogel.

MATERIAL AND METHODS

Plant collection DNA extraction

Individuals from 2 populations of *A. falcata* and from one population of *A. brevipes*, *A. paniculata*, *D. nigra*, and *M. vestitum* were collected in São Paulo State and Mato Grosso do Sul State. Specimen vouchers were deposited in the UEC and CGMS Herbarium. Additional information for the populations and voucher are shown in Table 1. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Table 1. Taxa used in this study.

Species	Geographical location	Latitude	Longitude	Voucher No.
Aeschynomene falcata	Passos, Minas Gerais State, Brazil	20°49'17"S	46°37'01"W	UEC 148660
	Porto Murtinho, Mato Grosso do Sul State, Brazil	21°41'50"S	57°41'17"W	CGMS 19860
A. brevipes	Porto Murtinho, Mato Grosso do Sul State, Brazil	21°41'50"S	57°41'17"W	CGMS 19828
A. paniculata	Campinas, São Paulo State, Brazil	22°48'53"S	47°05'46"W	UEC 169750
Dalbergia nigra	Campinas, São Paulo State, Brazil	22°48'53"S	47°04'02"W	UEC 169748
Machaerium vestitum	Campinas, São Paulo State, Brazil	22°48'53"S	47°04'02"W	UEC 169749

CGMS = Herbarium of the Universidade Federal de Mato Grosso do Sul; UEC = Herbarium of the Universidade Estadual de Campinas

Microsatellite-enriched library construction and sequencing

A microsatellite-enriched genomic library was obtained for *A. falcata*, according to Billotte et al. (1999). Enrichment was performed using a hybridization-based capture with $(GT)_8$ and $(CT)_8$, biotin-linked probes, and streptavidin magnetic-coated beads. A total of 56 selected recombinant colonies were bidirectionally sequenced using an automated ABI PRISM 377 sequencer (Applied Biosystems, Foster City, CA, USA) with T7 and SP6 primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA). Sequences were aligned and edited using the Seqman program (DNASTAR, Madison, WI, USA). Microsatellite regions were identified and characterized using the program Gramene (Temnykh et al., 2001). As a criterion for SSR selection, sequences showing at least 5 dinucleotide repeats, 4 trinucleotide repeats, and 3 tetra-, penta-, and hexanucleotide repeats were selected. The primer pairs were designed using the PrimerSelect software (DNASTAR) and microsatellite loci were amplified by polymerase chain reaction performed in a 20 µL total volume containing 15 ng template DNA, 1X PCR buffer, 2 mM $MgCl_2$, 8 mM dNTPs, 0.04% bovine serum albumin, 0.5 mM each forward and reverse primer, and 0.5 U *Taq* DNA polymerase. A Bio-Rad C1000™ Thermal Cycler (Hercules, CA, USA) was used for the amplification reaction with the following program: an initial denaturation at 94°C for 2 min; 2X 10 cycles (-1°C per cycle) at 3 different temperatures of touchdown, 65°-55°C, 60°-50°C, and 55°-45°C (94°C for 1 min, 65°C, 60° or 55°C for 1 min, and 72°C for 2 min), followed by 18 cycles (94°C for 1 min, 55°C, 50° or 45°C for 1 min, and 72°C for 2 min), and a final extension of 72°C for 5 min. Amplification products were analyzed by electrophoresis on 3% agarose gels stained with ethidium bromide and were genotyped using a 6% denaturing polyacrylamide gel stained with silver nitrate (Creste et al., 2001) and the genotyper Advance FS96. Because of the low number of individuals of 2 populations of *A. falcata* (Minas Gerais State, 12 individuals; Mato Grosso do Sul State, 3 individuals), the 2 different populations

were statistically analyzed together. Analysis of expected and observed heterozygosities were performed using the TFPGA software (Miller, 1997), the polymorphism information content was determined using Pic Calculator (Kemp, 2002), and Hardy-Weinberg equilibrium tests for each locus were calculated using Genepop available online (Rousset, 2008).

Fluorescence *in situ* hybridization (FISH)

Roots from all material were pretreated in 0.002 M solution of 8-hydroxyquinoline for 20-24 h at 10°C and then fixed in 3:1 absolute ethanol: glacial acetic acid (v:v) for 20-24 h at room temperature; samples were stored at -20°C.

The chromosome preparation and the *in situ* hybridization followed the method described by Moraes et al. (2012). Five SSR were used as probes: Aesfal.1 (monomorphic), Aesfal.8 (monomorphic in one region/polymorphic in another region), Aesfal.10 and Aesfal.13 (2 polymorphic regions each), and Aesfal.11 (polymorphic). The probes were labeled by nick translation (Roche, Basel, Switzerland) using Cy-3 (Invitrogen, Carlsbad, CA, USA). After 1 h incubation in 1% RNase, slides were treated with pronase-E for 10 min and fixed in 4% paraformaldehyde for 10 min. *In situ* hybridization was performed at 77% stringency and slides were mounted with DAPI-Vectashield (Vector Labs, Burlingame, CA, USA). The slides were analyzed and photographed under a Leica DM2500 fluorescent microscope (Solms, Germany) coupled with a DFC360 FX camera and images were acquired using the LAS 3.6 software (Media Cybernetics, Inc., Rockville, MD, USA).

RESULTS AND DISCUSSION

Microsatellite sequences-polymorphism and transferability

A total of 23 primer pairs were designed and 11 were amplified in *A. falcata* (Table 1), 7 of which were polymorphic. A total of 38 alleles were identified, ranging from 1 to 7 alleles per locus, with a mean of 3.45. The observed and expected heterozygosities ranged from 0.0000 to 0.7143 and from 0.1287 to 0.8360, respectively, while the polymorphism information content varied from 0.1167 to 0.7786 (Table 2). Hardy-Weinberg Equilibrium was calculated for 6 of the 11 SSR developed and deviation from the equilibrium was detected, which was likely caused by the autogamous crossing of this species.

Polymorphism and heterozygosity

Of these 11 amplified loci, 9 were amplified in *A. brevipes* and *A. paniculata*. Of these loci, 6 amplified in both *D. nigra* and *M. vestitum*, plus another unique locus in each species (Table 3). The 11 markers and cross-amplification for the other 2 genera, *Dalbergia* e *Machaerium*, confirmed that all would be useful for future breeding programs.

Two microsatellite loci (Aesfal.8 and Aesfal.10) showed more than 1 region of amplification. Aesfal.8 locus presented a monomorphic region (approximately 147 base pairs) for all species, whereas a polymorphic region was present only in the *A. falcata* sample. The Aesfal.10 locus presented 2 polymorphic regions, 1 exclusive for *A. falcata* and the other observed only in related species.

Table 2. Genetic characterization of 11 microsatellite loci amplified for *Aeschynomene falcata* (Poir.) DC. (N = 15).

Primer	Primer sequences (5'-3')	Repeat motif	Ta (°C)	Allele sizes (bp)	A	H_O	H_E	PIC	HWE	GenBank accession No.
Aesfal.1	F: GGCCGCACTAGTGATTCTCTTG R: TTTGTTACAGTCATGGCAGGTG	$(TGG)_5$	TD55-45	172	01	-	-	-	-	JX870529
Aesfal.3	F: TGCAGGCAAAACATAGAGC R: TTCGTCACTTCATTCACTTCAC	$(AAAAC)_3$	TD55-45	205-211	02	0.2000	0.5080	0.3705	0.103	JX870530
Aesfal.5	F: AGGGAAGGGAGGGAATGGAGAG R: CCCGCAAAAACAAACACAGAGT	$(GT)_{15}$	TD65-55	196-206	07	0.3846	0.8031	0.7406	0.007	JX870531
Aesfal.7	F: CAACAGCCTCCTCCCACACC R: AGGCCTCCCACGCATCAG	$(AC)_9$	TD55-45	300	01	-	-	-	-	JX870532
Aesfal.8	F: AGAAGAAGCGGATGATGGAGAA R: CTGTTGAAAGTGCGTAGG	$(GAA)_4$	TD60-50	249-261	05	0.2667	0.7448	0.6739	0.000	JX870533
Aesfal.10	F: TTATACAGCTGGAGCAAAACAT R: GCAAGTAAAAGTCGAGAAAGAG	$(GAA)_6$	TD65-55	154-157	02	0.0000	0.1287	0.1167	-	JX870534
Aesfal.11	F: CTTCCTCATTGTTTGTGTCTAC R: GAGTGTGGCGGTGAGTCT	$(GT)_8$	TD60-50	198-220	07	0.3333	0.7862	0.7245	0.027	JX870535
Aesfal.13	F: ATTGCTGGCTATGGGTTATT R: GATGAGGTCCGCAAAAGA	$(GT)_5$	TD60-50	305	01	-	-	-	-	JX870536
Aesfal.14	F: CGGACTTCATGGGGTTCTA R: CCGGATAAATGAGGGTGAG	$(AC)_{10}$	TD60-50	254-266	05	0.0000	0.6805	0.6005	0.000	JX870537
Aesfal.16	F: CTGCGCCCTCTCCCTCATTC R: ACTCTAGTCCTGGCGTGGTTGC	$(CA)_5$	TD65-55	150-193	06	0.7143	0.8360	0.7786	0.001	JX870538
Aesfal.20	F: CGCCAATGAGAGAACTGAGA R: AACATGCTTATGATTGATGAAA	$(CT)_5$	TD55-45	240	01	-	-	-	-	JX870539

N = number of individuals tested; A = number of alleles per locus; Ta = annealing temperature; TD = touchdown; H_O = observed heterozygosity; H_E = expected heterozygosity; (-) = not applicable; PIC = polymorphism information content; HWE = Hardy-Weinberg equilibrium.

Table 3. Microsatellite loci of *Aeschynomene falcata* tested for cross-amplification in 14 individuals of four species.

Primer	*Aeschynomene brevipes* (N = 5)	*Aeschynomene paniculata* (N = 5)	*Dalbergia nigra* (N = 2)	*Machaerium vestitum* (N = 2)
Aesfal.1	+	+	+	+
Aesfal.3	-	-	-	-
Aesfal.5	+	+	-	-
Aesfal.7	+	+	+	+
Aesfal.8	+	+	+	+
Aesfal.10	+	+	+	+
Aesfal.11	-	-	-	-
Aesfal.13	+	+	+	+
Aesfal.14	+	+	-	+
Aesfal.16	+	+	+	+
Aesfal.20	+	+	+	-

N = number of individuals tested; "+" = successful amplification; "-" = failed amplification.

In situ localization

Two of the 5 SSR sequences tested (Aesfal.1 and Aesfal.11) showed a clear signal on the *A. falcata* chromosomes, each SSR labeling 1 chromosome pair (Figure 1). The remaining 3 SSR sequences were likely below the method limit of detection, preventing the signal visualization. The sensibility limit for *in situ* location is approximately 1 kb (Schwarzacher and Heslop-Harrison, 2000) and because microsatellites could be repeated hundreds of times, this value can be above 1 kb, but is frequently below this value. All SSR tested were cross-amplified in *A. brevipes*, *A. paniculata*, *D. nigra*, and *M. vestitum*; however, it was not possible to visualize the sequences on the chromosomes, likely because the sequences were below the method detection capabilities.

In *Dendrobium*, SSR sequences can also be transferred to close genera, as observed in this study, but the SSR mapped were likely larger than those mapped here as 3 SSR probes from *Dendrobium moschatum* (Buch.-Ham.) Sw were detected after FISH in close species, as well as in close genera (1 SSR) [*Nageliella angustifolia* (Booth ex Lindl.) Ames *et* Correl, *Oncidium sphacelatum* L. and *Phalaenopsis lueddemanniana* var. *purpurea* (Rchb.f.) Sweet.] (Begum et al., 2009).

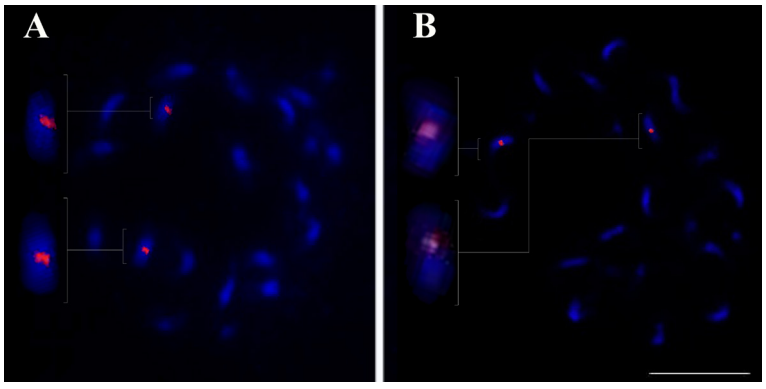

Figure 1. Microsatellite mapping through *in situ* hybridization in *Aeschynomene falcata* chromosomes. **A.** SSR Aesfal.1 and **B.** SSR Aesfal.11. Barr in B corresponds to 10 µm.

CONCLUSIONS

We developed the first microsatellite library for *Aeschynomene*, with specific sequences, amplifying only *Aeschynomene* species, broadly distributed sequences, and the *Dalbergia* and *Machaerium* genera. This new SSR library can be used for breeding programs and as a source of new chromosome markers, which is a novel approach in molecular cytogenetics. However, the SSR sequences applied were localized by *in situ* hybridization only in *A. falcata*, indicating that they were exclusive chromosome markers. Future studies mapping new SSR probes may reveal new chromosome markers; together with currently used probes, these markers will enable chromosome characterization of *Aeschynomene* and close genera.

ACKNOWLEDGMENTS

Research supported by Fundação de Amparo à Pesquisa de São Paulo (FAPESP, Project #2011/22215-3 to A.P. Moraes and #2010/19132-6 to E.R. Forni-Martins) and Conselho Nacional de Pesquisa (CNPq, Project #471708/2010-0). The authors thank F.M. Alves and R.T. Queiroz for sample collection.

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