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BRIEF COMMUNICATION

Isolation and characterization of eleven polymorphic microsatellite loci in *Aegiphila sellowiana* and their transferability

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Abstract

We isolated and characterized eleven polymorphic microsatellite loci for *Aegiphila sellowiana* an outcrossing pioneer tree species that is frequently used in reforestation programs of tropical riparian forests in Brazil. A total of 38 alleles were detected across a sample of 45 individuals of *A. sellowiana*, with an average number of 3.45 alleles per locus. The average polymorphic information content (PIC) was 0.430 and the observed (H_O) and expected (H_E) heterozygosity values varied from 0.156 to 1.000 and 0.145 to 0.730, respectively. Eight loci exhibited significant deviation from Hardy-Weinberg equilibrium ($P \leq 0.001$) and 32 pair combinations of loci showed significant linkage disequilibrium ($P \leq 0.001$). All 11 primers were tested for cross amplification in 12 species belonging to the family *Lamiaceae* and 5 species belonging to the related family *Verbenaceae*. The sequence and diversity information obtained using these microsatellites and their cross-transferability to other species of *Lamiaceae* as well as *Verbenaceae* will increase our understanding of genetic structures and species relationships within *Aegiphyla* and other genera of these families.

Additional key words: cross amplification, genetic diversity, *Lamiaceae*, microsatellite primers, *Verbenaceae.*

Aegiphila sellowiana Cham. of the *Lamiaceae* family (Rimpler *et al.* 1992, Wagstaff and Olmstead 1997, Wagstaff *et al.* 1998, Olmstead *et al.* 2000) is a deciduous, pioneer tree species that grows in different soil types and is found in secondary, pluvial, and semideciduous forests in Brazil. It is distributed in the Brazilian states of Rio de Janeiro, Minas Gerais and Paraná, occupying the semideciduous and pluvial forests (Lorenzi 2002). This species grows between 4 and 7 m in height with a trunk diameter 20 - 30 cm and is characterized by a light and soft wood that is commonly used to make furniture, boxes and shoes. Leaves are simple, opposite, with a size range between 18 and 28 cm in length. Flowers of *A. sellowiana* are honeyed with the flowering occurring during the months of December and January. Fruits are spherical drupes, produced in high amounts and mature from February to April. This species has great potential to stabilize and restore degraded, vulnerable ecosystems (Lorenzi 2002). Microsatellite markers are the preferred choice for certain genetic studies because they can easily detect co-dominant alleles, are highly reproducible, and have very high levels of polymorphisms (Weber and May 1989), which can be used in an immense variety of studies such as population genetics of native (Ruas *et al.* 2009) and cultivated plants species (Ma *et al.* 2010). Hence, this robust genetic

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Abbreviations: SSR - microsatellite or simple sequence repeat; CTAB - cetyltrimethylammonium bromide; PCR - polymerase chain reaction.
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marker system is valuable for developing markers in many species, but the requirement for flanking sequences of SSR motifs often limits their wider application. The current report presents 11 SSR markers developed for *A. selloviana* and their application to related species belonging to the families *Lamiaceae* and *Verbenaceae*.

Total genomic DNA was extracted from leaf tissue using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). Microsatellites were isolated using a hybridization-based capture methodology following the protocol described by Billotte *et al.* (1999) with $(CT)_8$ and $(GT)_8$ probes in the enrichment step. Briefly, approximately 5 ng of genomic DNA was digested with *Rsa*I and the blunt-ended fragments were ligated to the adaptors *Rsa*I-21 and *Rsa*I-25 (Edwards *et al*. 1996). Fragments containing CT and GT repeats were selected by hybridization to biotinylated oligonucleotides, complementary to the repetitive sequence and recovered by streptavidin coated magnetic beads (*Invitrogen Dynal*, Lillestrom, Norway). Microsatellite-rich fragments were amplified by PCR with *Rsa*I-21 primer (Peters *et al.* 2008), cloned into the *pGEM-T Easy Vector* (*Promega*, Madison, WI, USA) and then, transformed into *Escherichia coli* XL1 Blue MRF' super-competent cells (*Agilent Technologies*, Santa Clara, CA, USA). Plasmids from individual colonies were isolated and the sequence of the inserts was determined using the *ABI PRISM* terminator cycle sequencing kit and the *ABI 377* automated sequencer (*Applied Biosystems*, Foster City, CA, USA). Sequenced fragments were screened for microsatellites using the program *Gramene* markers database (Ware *et al.* 2002).

Sequences containing microsatellites that consisted of five or more repeats surround by a flanking region suitable for primer design were chosen for further study. Of 98 clones sequenced, 19 contained microsatellites but only eleven were suitable for primer design and PCR reactions (Table 1). Although the $(CT)_8$ and $(GT)_8$ oligomers were used for the precloning enrichment, other repeat motifs were also found in the cloned products (Table 1). Primer pairs complementary to sequences flanking the repeat elements were designed using the *PRIMER3* web interface software (Rozen and Skaletsky 2000). PCR amplifications were carried out across of 45 individuals from two different populations (30 individuals from Tamarana county 23°49'27"S and 51°10'00"W; 15 individuals from Nova Santa Bárbara county 23°39'27"S and 50°45'57"W) of *A. sellowiana*, 66.39 kilometers distant from each other. Reactions were performed in 0.025 cm^3 volume containing 1× PCR buffer, 1 U *Taq* DNA polymerase, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 8 pM forward and reverse primers, and 20 - 30 ng of template DNA. Cycling conditions consisted of 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at the locus-specific annealing temperature, 2 min at 72 °C, and a final

Table 1. Characterization of 11 polymorphic microsatellite loci genotyped in 45 individuals from two populations of the riparian species *A. sellowiana.* T_A corresponds to highest annealing temperature [°C], allele size indicates the range of observed alleles in bp, K is number of alleles, PIC is the polymorphic information content, H_O and H_E are observed and expected heterozygosities, *P*-value indicates significance of deviation from Hardy-Weinberg equilibrium.

Locus	Primer sequence 5´-3´	Repeat motif	T_A	Allele size	K	PIC	H_O	H_E	*P*-value
As18F-1	F: ACT GCC ACA AGC TCT CCA AG R: AGC CCA TTA ACA AAA ACA GG	$(CG)_1(CG)_4C$	60	170-260	3	0.287	0.400	0.332	0.1046
As87-1	F: TTC ATG GGA AGG TTC TGC AC R: TCA AAC TTG TTG CCG AAG AA	$(TA)_4$	60	180-250	2	0.133	0.156	0.145	-
As73	F: GGG GAT TGA CTT CAA CGA GA R: TTG TTC AAC CTG CAA ACC AA	$(GAA)_3$	60	170-200	2	0.375	1.000	0.506	0.0000001
As9F-1	F: GAT TTT TGG GGA TAG CAG GA R: ATG GGC AAC TGT ACG TGT GA	$(TG)_7(TA)_6TGTA(TG)_3$	59	160-180	4	0.539	1.000	0.618	0.0000001
As18F-2	F: CCG CAG ACT TAC ATG GTG AA R: AGC CCA TTC ACA AAA ACA GG	$(TG)_7$	60	190-220	2	0.375	1.000	0.506	0.0000001
As77	F: TCT TGC ATG TGA TTG GCA TC R: ATA TTT CGG CCC CAC TCT TT	$(AG)_{12}$	60	300-250	6	0.674	1.000	0.730	0.0000009
As 25F	F: CCG AAT AAA GAG AAA GCA GCA R: GGC ATC TTG TCC CTT CTT GT`	$(CA)_4CC(A)_{11}C(CA)_3(TACACA)_2(CA)_4$	60	250-300	5	0.596	1.000	0.660	0.0000001
As68	F: TCT TCA GGG AGG TGG TGA TT R: AGA TCC AAT GGC TCA TCG TT	$(GT)_7$	60	210-230	2	0.375	1.000	0.506	0.0000001
As84	F: TCC CGG AAG ACA TCA TTA GC R: TCC AAT AGT CCG ACC CAA TC	$(TG)_4$	60	220-260	5	0.649	0.689	0.705	0.0012
As93-1	F: GAT TCC GGC ATA CAC CAA AG R: AAT TTT TGG CCG GGT TTT AC	$(GT)_5$	60	170-200	3	0.406	0.956	0.525	0.0000001
As12F-1	F: ATG AGG GGA CAA AGG GTT TT R: GGT GGA GGA ATG TTG GAG AA	$TCTTC(CTT)_2(CT)_9$	59.5	160-200	4	0.324	0.333	0.348	0.2266

Table 2. Significant linkage disequilibrium of 32 pair combinations of 11 microsatellite loci of *A. sellowiana*.

Primers	As18F-1	As87-1	As73	As9F-1	As18F-2	As77	As25F	As68	As84	As93-1
As18F-1										
As87-1	-									
As73	+	+								
As9F-1	+	-	+							
As18F-2	-	-	+	+						
As77	-	-	+	+	+					
As25F	-	-	+	+	+	+				
As68	-	-	+	+	+	+	+			
As84	-	-	+	+	+	+	+	+		
As93-1	-	-	+	+	+	+	+	+	+	
As12F-1	-	-	-	-	-	-	+	-	-	-

Table 3. Cross-amplification test of eleven microsatellite loci throughout seventeen species of the *Lamiaceae* and *Verbenaceaea* families. '+' indicates clear amplifications; '-' indicates no amplification

Species	As18F-1	As87-1	As73	As9F-1	As18F-2	As77	As25F	As68	As84	As93-1	As12F-1
Lamiaceae											
Aegiphyla brachiata Vell.	+	-	+	+	+	+	+	-	+	-	+
Lavandula angustifolia Mill.	-	-	-	-	-	+	-	-	+	-	-
Clerodendrum thomsonae Balt.f. L.	-	-	+	-	+	-	-	-	-	-	-
Leonotis nepetifolia (L.) R. Br.	-	-	-	-	-	-	-	+	+	-	-
Leonurus japonicus L.	-	-	-	-	-	-	-	+	+	-	-
Plectranthus neochilus Schlechter L.	-	-	-	-	-	-	-	-	+	-	-
Plectranthus nummularius Briq.	-	-	-	-	-	-	-	-	-	-	-
Rosmarinus officinalis L.	-	-	+	-	-	-	+	-	-	-	-
Salvia splendens Selow *ex* Roem & Schult.	-	-	-	-	-	-	-	-	-	-	-
Salvia guarinitica A. St.-Hil.	-	-	-	-	-	+	+	-	-	-	-
Solenostemon scutellarioides (L.) Lodd	-	-	-	-	-	-	-	-	-	-	-
Vitex megapotamica (Spreng) Mold.	+	-	+	-	-	+	-	-	-	-	+
Verbenaceae											
Aloysia virgata (Ruiz & Pav.) Pers.	+	-	+	-	-	+	+	-	-	-	+
Citharexylum myrianthum Cham.	+	-	+	-	-	+	+	-	-	-	+
Duranta repens L.	+	-	+	-	-	+	+	-	-	-	+
Lantana sellowiana Link & Otto	+	-	+	-	-	+	+	+	-	-	+
Verbena hybrida Voss	-	-	-	-	-	-	-	+	+	-	-
Blanc sample	-	-	-	-	-	-	-	-	-	-	-

extension of 5 min at 72 °C. The amplified microsatellite products were visualized on a 5 % acrylamide: bisacrylamide (29:1) gel. Conditions and characteristics of the eleven microsatellite loci are given in Table 1. We used *Cervus* version *2.0* (Marshall *et al.* 1998) to estimate the number of alleles per locus (K), observed (H_O) and expected (H_E) heterozygosities and polymorphic information content (PIC). Deviations from Hardy-Weinberg equilibrium and Linkage disequilibrium were determined using *Genepop* version *1.2* software (Raymond and Rousset 1995).

The eleven polymorphic loci produced a total of 38 alleles, ranging from 2 (As87-1, As73, As18F-2, As68) to 6 (As77) with an average of 3.45 alleles per locus. The average PIC was 0.430 and the values of H_O and H_E varied from 0.156 to 1.000 and from 0.145 to 0.730, respectively (Table 1). Eight loci (As73, As9F-1, As18F-2, As77, As25F, As68, As84, and As93-1) deviated from expectations of Hardy-Weinberg equilibrium ($P \leq 0.001$) after Bonferroni correction for multiple comparisons (Table 1), while 32 pairs of loci (Table 2) exhibited significant linkage disequilibrium ($P \leq 0.01$). Because *A. sellowiana* is a very important species for the reforestation of degraded areas and has many compounds that have showed promising results in pharmaceutical trials it becomes very important and urgent to preserve the genetic variation still present in this species.

The 11 microsatellite loci developed for *A. sellowiana* were tested for cross-amplification in seventeen other species of 15 different genera that belong to the same (*Lamiaceae*) or related family (*Verbenaceae*) (Table 3). Advances in understanding the phylogeny of the

Lamiaceae, based on congruent plastid and nuclear DNA sequence data, have considered this family as a monophyletic group after several genera, including *Aegiphil*a, *Clerodendron* and *Vitex*, were transferred from *Verbenaceae* to *Lamiaceae* (Cantino *et al.* 1992). In the revised classification using cpDNA and *rbc*L sequences the genera *Aegiphil*a, *Clerodendron*, *Lavandul*a, *Leonotis*, *Leonurus*, *Plectranthus, Rosmarinus*, *Salvia*, *Solenostemon,* and *Vitex* were reclassified into the *Lamiaceae* family while the genera *Aloysia*, *Citharexylum*, *Duranta*, *Lantana*, and *Verbena* were concentrated into the *Verbenaceae* (Rimpler *et al.* 1992, Wagstaff and Olmstead 1997, Wagstaff *et al.* 1998, Olmstead *et al.* 2000). The cross-amplification tests comprised of 5 individuals from each of the twelve *Lamiaceae* species and of the five *Verbenaceae* species. Of the 11 microsatellite loci tested eight transferred to *Aegiphyla brachiata* and nine transferred to at least one of the other 11 *Lamiaceae* species. Five microssatelites were transferred to four of the five *Verbenaceae* species tested (Table 3). Primers As73 and As77 transferred to eight of the seventeen species, while the loci As87-1 and As93-1 did not transfer to any of them (Table 3). Our results show that besides the separation of these genera into two families they are related. Therefore, these loci may be applied for further studies of genetic diversity and population structure in *A. selloviana* and in species from different genera within the *Lamiaceae* and *Verbenaceae* families.

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