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BRIEF COMMUNICATION

Genetic diversity of *Eucalyptus* hybrids estimated by genomic and EST microsatellite markers

F.B. CUPERTINO, J.B. LEAL, R.X. CORRÊA and F.A. GAIOTTO*

Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna km 5, Ilhéus 45662-000, *Bahia, Brazil*

Abstract

The knowledge of breeding impacts on the genetic diversity of hybrids of *Eucalyptus* is crucial to the exploration of genetic resources. We estimated genetic polymorphic parameters of 112 hybrids of *Eucalyptus* spp. using 10 genomic simple sequence repeats (SSR) markers and 10 expressed sequence tags (EST) microsatellite markers. According to Student´s *t*-test, there were no significant differences between genomic SSR and EST-SSR markers. Our results also revealed high polymorphism in the hybrids analyzed, indicating that both markers are appropriate for use in genetic breeding programs.

Additional key words: allelic diversity, eucalypt, SSR.

Eucalypts are native to Australia, Papua New Guinea, and Philippines. The subgenus *Symphyomyrtus* (*Myrtaceae*) includes the most economical important species of *Eucalyptus* such as *Eucalyptus grandis, E. urophyla*, *E. camaldulensis* and *E. globulus* (Eldridge *et al*. 1994). There is a high demand for eucalyptus wood due to its use in several industrial sectors such as paper and cellulose production, coal, resin, sawing industry, latex, cosmetics, and essences (Campinhos 1999).

Traditional breeding programs of many *Eucalyptus* species have aimed at the exploration of genetic resources and the selection of phenotypes (Poke *et al*. 2005). In order to potentialize and accelerate economic gains resulting from genetic improvement, hybridization is largely used to combine desirable characteristics that cannot be obtained in pure species, besides providing heterosis or hybrid vigor (Eldridge *et al*. 1994).

PCR-based molecular markers have been extensively used on genetic diversity, parentage, and linkage mapping studies in *Eucalyptus* species (Marcucci-Poltri *et al*. 2003, Kirst *et al.* 2005, Brondani *et al*. 2006). Microsatellite markers or simple sequence repeats (SSR) are considered a powerful molecular tool for the characterization of genetic variability in tree species. These markers show multiallelic polymorphism, codominance, and have a wide distribution in the genome (Powell *et al*. 1996). They are more abundant in noncoding regions of the genome than in transcribed regions (Varshney *et al*. 2005). Microsatellites present in expressed regions of the genome (expressed sequence tags, EST) can be easily obtained due to the availability of a large number of sequences deposited in online databases such as *GenBank*. EST-SSR have some advantages over genomic SSR as they can be transferred between species of the same genus, once expressed regions are more conserved (Varshney *et al*. 2005).

The knowledge of genetic variability in populations submitted to genetic improvement can be crucial for the use of genetic resources and the identification of molecular patterns (fingerprinting) of the genotype of interest, which can lead to the better efficiency of breeding programs (Marcucci-Poltri *et al*. 2003). Moreover, genetic diversity is desirable for any long-term culture improvement, reducing the susceptibility to inbreeding depression (Kantartzi *et al*. 2009). In view of such need and the availability of genomic SSR and EST-SSR markers, we analyzed allelic diversity in hybrids of *Eucalyptus* spp. with genomic and EST

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Abbreviations: EST - expressed sequence tags; PIC - polymorphic information content; SSR - simple sequence repeats.
* Corresponding author; fax: (+55) 73 3680 5226, e-mail: gaiotto@uesc.br

microsatellite markers. We also established a comparison between these markers using population genetic parameters and indices of discriminatory power among related individuals.

A total of 112 samples of *Eucalyptus* spp. was randomly collected from progenies of 14 hybrid full sib families. These families were obtained by crosses between *E. grandis*, *E. urophylla*, *E. dunni*, *E. globulus,* and *E. camaldulensis* planted in an experimental field in the states of Bahia and Minas Gerais, Brazil. Genomic DNA was extracted from the leaves according to Ferreira and Grattapaglia (1998). Twenty microsatellite loci (10 genomic SSR and 10 EST-SSR) were amplified with primers previously developed for *E. grandis* and *E. urophylla* (Mamani and Lourenço, unpublished data). The genomic SSR loci were developed by the construction of genomic libraries and EST-SSR loci were originated from expressed sequence tags (EST) libraries available in a database. The PCR mix (0.013 cm^3) contained 7.5 ng of genomic DNA, 250 μM dNTPs, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 2.25 mM $MgCl_2$, pH 8.3), 2.5 g dm^{-3} BSA, 0.2 μM of each primer [one dye-labeled primer (6-FAM, HEX, or NED dyes) and one unlabeled primer], and 1 U of Taq DNA polymerase. Amplifications were performed using the *GeneAmp 9600* PCR system (*Applied Biosystems*, Foster City, CA, USA) thermocycler, using the following amplification cycle: 96 °C for 2 min; 30 cycles at 94 °C for 1 min, the primer-specific annealing temperature for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Loci were amplified in single and duplex systems and PCR products were run in duplex and tetraplex systems in electrophoresis gels, in at least two independent experiments. For each multiplex set, equal amounts of PCR product were diluted and 0.002 cm^3 of each was mixed with 0.003 cm^3 of *LB-ROX* mix (0.0025 cm^3 of loading buffer 1:5 deionized formamide and 0.0005 cm^3 of an internal fluorescent labeled DNA standard). An automated sequencer *ABI 377* (*Applied Biosystems*) was used for electrophoresis with a virtual D filter. Global Southern method and fluorescent peak amplitude thresholds of 50 were used for sizing with *Genescan® v. 3.1* and *Genotyper® v2.5* softwares (*Applied Biosystems*). The average number of alleles per locus (A) and polymorphic information content (PIC) were estimated and compared between genomic SSR and EST-SSR markers using the Student´s *t*-test (*SPSS* program *v. 10*). To show the discriminatory power of these two types of markers, the identity probability (I) and the paternity exclusion probability (Q) were calculated. Linkage disequilibrium for all loci was tested for the two sets of microsatellite markers at $P < 0.05$. Primer transferability was tested for all loci.

All 20 microsatellite markers successfully amplified in the samples studied, showing that they were efficiently transferred to *Eucalyptus* hybrids. According to Brondani *et al.* (2006), microsatellite transferability across species of the subgenus *Symphyomyrtus* can vary from 80 to 100 %. High transferability of SSR markers can occur primarily in those species in the same subgenus as

Table 1. Genomic SSR and EST-SSR loci used for genotyping in *Eucalyptus* hybrids. Repeat motifs, amplified fragment size, number of alleles (A), polymorphic information content (PIC), identity probability (I) and paternity exclusion probability (Q) are presented.

	Locus	Motif	Size [bp]	A	PIC	I	Q
Genomic SSR	Embra604	$(GAAA)_{16}$	215-259	17	0.8830	0.0235	0.7775
	Embra623	$(GAG)_9$	205-237	14	0.9217	0.0315	0.6611
	Embra627	$(CT)_{18}$	225-255	15	0.8488	0.0381	0.7179
	Embra632	$(CTT)_{10}(CT)_{15}$	216-260	20	0.9139	0.0137	0.8318
	Embra645	$(AG)_{11}$	190-228	15	0.8866	0.0236	0.7807
	Embra646	$(GA)_{17}$	140-164	12	0.8679	0.1391	0.7459
	Embra648	$(TC)_{13}$	149-196	12	0.8531	0.0368	0.7231
	Embra651	$(AG)_{17}$	92-118	12	0.8544	0.0359	0.7262
	Embra665	$(GA)_{13}$	128-156	14	0.8443	0.0412	0.7082
	Embra679	$(AG)_{11}$	257-296	18	0.8477	0.3514	0.7177
EST-SSR	Embra844	$(TC)_{20}$	192-237	27	0.9253	0.2793	0.8552
	Embra915	$(CCCT)_5$	201-235	20	0.9065	0.0157	0.7751
	Embra941	$(CT)_{16}$	231-256	16	0.8839	0.0235	0.7785
	Embra945	$(AG)_{11}$	90-109	9	0.5344	0.2501	0.3399
	Embra949	$(CT)_{16}$	265-286	9	0.7709	0.0858	0.5855
	Embra950	$(CTCG)_4$	156-199	30	0.9397	0.0067	0.8819
	Embra954	$(CTGC)_4$	128-191	26	0.8968	0.0191	0.8059
	Embra979	$(CT)_{17}$	375-409	19	0.8498	0.0383	0.7182
	Embra1213	$(CT)_9$	82-109	10	0.8607	0.0356	0.7298
	Embra1445	$(AG)_6$	99-147	31	0.9131	0.0136	0.8321

reported by Marques *et al.* (2002) and Akkak *et al.* (2009), although it can also occur, in lower proportion, between species in different subgenera (Jones *et al*. 2001).

The 20 microsatellite loci revealed 346 alleles and an average number of 17 alleles per locus. This is considered an elevated number in view of the hyper-variability found in each locus (Table 1). Comparing the estimates of genetic parameters from genomic SSR and EST-SSR (Table 1), the average number of alleles was clearly higher for EST-SSR markers, although the difference was not statistically significant, according to Student´s *t*-test ($P > 0.05$) (Table 2). All 10 genomic microsatellite were highly polymorphic, with polymorphic information content (PIC) higher than 0.84. EST-SSR markers were also very polymorphic and only two of them showed PIC values lower than 0.80 (Table 1). PIC is commonly used to estimate how polymorphic is a marker locus and values higher than 0.8 indicated that they are extremely informative for any kind of genetic studies. There were not significant differences for the average PIC values between these two markers (Table 2).

Table 2. Comparison of average number of alleles (A) and polymorphic information content (PIC) for genomic SSR and EST-SSR markers.

	Parameters	Average	SD	*t*-test	*P*-value
A	genomic SSR	14.90	2.730	-1.593	0.146
	EST-SSR	19.70	8.590		
PIC	genomic SSR	0.872	0.028	0.559	0.590
	EST-SSR	0.848	0.120		

Some comparative studies report that the genetic variability parameters, are in general, higher when estimated by genomic SSR than by EST-SSR (Cho *et al*. 2000, Peleg *et al*. 2008), due to the high conservation of coding sequences. Thus, EST-SSR markers tend to exhibit lower polymorphism when compared with genomic SSR (Varshney *et al.* 2005). In our study, we verified no significant differences even if EST-SSR were slightly more polymorphic than genomic SSR. There was no relationship between the number of nucleotide repeats, the number of alleles, and the level of polymorphism in the *Eucalyptus* hybrids studied. Perfect trinucleotide repeats $(GAG)_9$ (Embra623) and composite repeats $(CTT)_{10}(CT)_{15}$ (Embra632) showed the highest PIC values found in genomic SSR markers (Table 1), while the highest values of genetic content for EST-SSR were found within di-$(TC)_{20}$ (Embra844) and tetranucleotide repeats $(CTCG)_4$ (Embra950) (Table 1). Some previous studies reported a positive correlation between genetic diversity and the number of repeats (Cho *et al.* 2001, Liewlaksaneeyanawin *et al*. 2004). The number of alleles detected and the length of the SSR motifs are also correlated (Gupta *et al.* 1996).

Two other genetic parameters were estimated to evaluate the potential of two sets of microsatellite markers to discriminate related individuals (Table 1). The identity probability (I) varied from 0.0137 to 0.3514 on genomic SSR loci, while on EST-SSR loci it varied from 0.0067 to 0.2793. According to the exact test ($P < 0.05$), all genomic SSR and EST-SSR are in linkage equilibrium. Thus, the combined values of identity probability of 10 loci (2.4×10^{-14} for genomic SSR and 5.2×10^{-15} for EST-SSR) revealed that the chance of finding two individuals with the same genotype in a population is practically zero ($I < 0.00$) using either sets of microsatellite markers. The probability of paternity exclusion (Q) varied from 0.6611 to 0.8318 on genomic SSR and from 0.3399 to 0.8819 on EST-SSR (Table 1). The combined Q value of 10 loci was higher than 0.9999 for both sets of microsatellites, indicating the probability of 99.99 % of either set of markers exclude correctly a random nonparent tree in a population. These results were similar to that reported by Kirst *et al*. (2005), confirming the potential of these markers for the identification of related individuals, parentage testing and genetic diversity studies on *Eucalyptus* species.

We observed high allelic diversity among hybrids of *Eucalyptus* spp. in genomic SSR regions as well as in EST. Hybrid species, besides having higher genetic diversity, provide some advantages in breeding program as they combine the best traits of interest of both parental species. The 20 microsatellite markers analyzed here were highly polymorphic and are considered adequate for QTL mapping. In conclusion, there were no significant differences between genomic SSR and EST-SSR markers for the number of alleles (A) and the polymorphic information content (PIC) values, which showed high levels of polymorphism. Both sets of markers revealed great discriminating power among hybrids and can be used in studies that involve genetic and QTL mapping.

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