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Research Article

Transference of microsatellite markers from *Eucalyptus* spp to *Acca sellowiana* and the successful use of this technique in genetic characterization

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Abstract

The pineapple guava (*Acca sellowiana*), known in portuguese as the goiabeira-serrana or “Feijoa”, is a native fruit tree from southern Brazil and northern Uruguay that has commercial potential due to the quality and unique flavor of its fruits. Knowledge of genetic variability is an important tool in various steps of a breeding program, which can be facilitated by the use of molecular markers. The conservation of repeated sequences among related species permits the transferability of microsatellite markers from *Eucalyptus* spp. to *A. sellowiana* for testing. We used primers developed for *Eucalyptus* to characterize *A. sellowiana* accessions. Out of 404 primers tested, 180 amplified visible products and 38 were polymorphic. A total of 48 alleles were detected with ten *Eucalyptus* primer pairs against DNA from 119 *A. sellowiana* accessions. The mean expected heterozygosity among accessions was 0.64 and the mean observed heterozygosity 0.55. A high level of genetic diversity was also observed in the dendrogram, where the degree of genetic dissimilarity ranged from 0 to 65% among the 119 genotypes tested. This study demonstrates the possibility of transferring microsatellite markers between species of different genera in addition to evaluating the extent of genetic variability among plant accessions.

Keywords: Feijoa sellowiana, genetic diversity, goiabeira-serrana, pineapple-guava, transferability.

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Introduction

The pineapple guava (*Acca sellowiana*, synonym *Feijoa sellowiana*), known in portuguese as the goiabeira-serrana or “Feijoa”, is a native of the Brazilian southern plateau with secondary dispersion in Uruguay (Mattos, 1990; Thorp and Bieleski, 2002). Due to the uniqueness of its flavor, the economic importance of the pineapple guava is steadily increasing on the world market (Thorp and Bieleski, 2002) and it is an attractive commercial alternative for farmers in southern Brazil (Mattos, 1990).

Although the pineapple guava can be found on the European market or in the countries in which adapted cultivars are active (*e.g.* New Zealand, Colombia and the USA) as yet there are no improved cultivars in Brazil, its greatest center of diversity. However, there is an *A. sellowiana* Active Germplasm Bank (AGB) located at the São Joaquim Experimental Station (Estação Experimental de São Joaquim (EPAGRI), São Joaquim-SC, Brazil) in the town of São Joaquim in the Brazilian state of Santa Catarina. This germplasm bank contains 119 *A. sellowiana* accessions from several regions of Brazil and other countries, and it is possible to use directly an accession as a clone or to develop a cultivar by means of genetic breeding methods in order to scale up commercial production

The genetic variability of this species is normally high at the center of origin, and information on such variability is essential for *A. sellowiana* conservation, breeding and commercial production. In general, specific phenotypes of discreet variation are used as morphological markers. However, a limited number of morphological markers have been identified for this species (Nodari *et al.*, 1997), which are frequently affected by dominance and epistatic gene interactions, environmental effects and pleiotropy. To overcome such problems, molecular markers can be used to help genetic characterization and breeding (Nodari *et al.*, 1997; Brondani *et al.*, 1998, 1997).

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Among the classes of molecular markers available to identify variation at DNA level, the microsatellites, or simple sequence repeats (SSRs), are considered ideal markers for genetic studies because they combine several suitable features: (i) co-dominance; (ii) multiallelism; (iii) high polymorphism, allowing precise discrimination even of closely related individuals; (iv) abundance and uniform dispersion in plant genomes; and (v) the possibility of efficient analysis by a rapid and simple polymerase chain reaction (PCR) assay (Morgante and Olivieri, 1993; Rafalski and Tingey, 1993; Sharma *et al.*, 1995; Brondani *et al.*, 1998). In addition, for the amplification of microsatellite loci, a knowledge of their DNA sequence is required, and this is an expensive and time consuming process (Zucchi *et al.*, 2003). However, the approach of using enriched libraries with repetitive sequences has been very successful in developing SSRs at a reasonable cost (Zane *et al.*, 2002).

The ability to use the same microsatellite primers in different plant species, called transferability, depends on the extent of sequence conservation in the primer sites flanking the microsatellite loci and the stability of those sequences during evolution (Choumane *et al.*, 2000; Decroocq *et al.*, 2003; Zucchi *et al.*, 2003). It has been shown that closely related species are more likely to share microsatellite priming sites than more distantly related ones, but it is possible to transfer functional microsatellite primers even from more distantly related species (Lorieux *et al.*, 2000).

Because there are no microsatellites available for *A. sellowiana*, the *Eucalyptus* spp. primers of microsatellite loci (Brondani *et al.*, 1998) can be used as an alternative to find similar regions on the *A. sellowiana* genome, since they belong to the same family (Zucchi *et al.*, 2003).

Thus, the objectives of the work described in this paper were to evaluate the transferability of microsatellite markers from *Eucalyptus* to *A. sellowiana* (both members of the Myrtaceae) and to characterize the genetic variability present in the Active Germplasm Bank (AGB) of this species.

Material and Methods

Genetic material

The 119 accessions tested shown in Table 1 were obtained from the pineapple guava Active Germplasm Bank (AGB) located at the São Joaquim Experimental Station (Estação Experimental de São Joaquim - EPAGRI, São Joaquim-SC, Brazil). Most of the accessions came from the Brazilian state of Santa Catarina, although a few accessions came from other countries (Table 1).Samples of DNA were obtained following the protocol developed by Doyle and Doyle (1987). The extracted DNA was quantified in agarose gel (Sambrook *et al.*, 1989) and diluted to 3 ng μL^{-1} for further use in the amplification reactions. Leaf DNA from *Eucalyptus grandis* was used as a control.

Microsatellite markers and DNA isolation

For amplification in *A. sellowiana* we used 404 primer pairs developed by Brondani *et al.* (1998) for the *Eucalyptus* complex *E. grandis* x *E. Urophylla* and they were obtained from the Genetics and Biotechnology unit of the Brazilian agricultural company Embrapa (Empresa Brasileira de Pesquisa Agropecuária-Recursos Genéticos e Bio-

Table 1 - Accession number and origin of the 119 accessions from the Active Germoplasm Bank of Goiabeira-serrana located at the São Joaquim/Epagri Experimental Station in the Brazilian state of Santa Catarina. All the Brazilian cities are located in the state of Santa Catarina.

Country, city of isolation and accession number			
Brazil			Other counties
Bom Jardim: 370, 371, 372, 373, 373B, 374, 376, 527	Lages: 228, 229, 246, 247, 249, 250, 259, 276, 276B, 276-20A, 278-1, 278-2, 294, 301, 306B, 321, 326B, 331, 332, 337, 401	Urupema: 233, 234, 238, 239-2, 240, 242, 244, 390, 392	Israel: 459, Israel*
Caçador: 66, 511, 512, 522	Palmas: 159-27, 159-30	Vacaria: 902, 903	New Zealand: 451, 454, 456, 457, 457A, 457B
Campos Novos: 85	Papanduvas: 755	Vargem Bonita: 804, 805, 805-2	Unknown origin: 438
Curitibanos: 80, 735A, 735B, 735	Ponte Alta152-24, 732, 732B, 740		Uruguai: 441
Frei Rogério: 79	São Joaquim: 103, 110, 117, 118, 119, 120, 124, 260, 277, 300, 358, 359, 360, 366, 369, 387	Videira: 50, 50-2, 53, 53B7, 59-30, 91, 98A, 98B, 101, 101PR, 132, 135, 152-12, 333, 393, 509, 526, 528	USA: 452-Califórnia, 453-USA
Fraiburgo: 148, 501, 502B, 504, 519, 521	Tangará: 141		
Iomerê:150B			
Lebon Régis: 138, 707, 711, 712, 716			

*Unspecified source.

tecnologia, Brasilia, DF, Brazil). Polymerase chain reaction (PCR) amplification of the microsatellite markers was performed in 96-well plates containing a 13 μL reaction volume composed of buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 5% (w/v) dimethyl sulfoxide, 9 ng of template DNA, 0.3 μM of each primer, 0.02 mM of each dNTP (Invitrogen) and one unit of *Taq* DNA polymerase (Invitrogen). Amplifications were performed using an MJ Research PT-100 thermal controller adjusted to the following conditions: 96 °C for 2 min, then 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min followed by a final elongation step at 72 °C for 7 min.

The screening of the pairs of primers was done in two steps. The first step employed the DNA of two *A. sellowiana* plants and one control *E. grandis* plant, the amplification products being visualized on 1.5% (w/v) agarose gel. In the second step, the pairs of primers showing positive amplification were confronted with an additional group of eight *A. sellowiana* genotypes to detect polymorphism and 10 selected primers were then utilized to analyze the genetic variability in the 119 *A. sellowiana* accessions, the amplification products being separated on 6% (w/v) denaturing polyacrylamide gel. A 100 bp DNA ladder was used as a molecular weight reference to estimate the sizes of the amplification products. The gels were stained with silver nitrate, as described by Creste *et al.* (2001).

Data analysis

The genetic diversity characterization potential of the primers was based on allele frequency estimates of the mean observed heterozygosity (Ho), mean expected heterozygosity (He) (Nei, 1978) and the number of alleles per locus for the AGB accessions. These estimates were obtained using the BIOSYS-1 program (Swofford and Selander, 1989). In addition, a dendrogram was plotted from an unbiased genetic similarity matrix (Nei, 1978) grouped by the unweighted pair group method with arithmetic mean (UPGMA) of Sneath and Sokal (1973).

Results

Of the 404 primer pairs tested we found that 180 (44.5%) amplified visible products in *A. sellowiana*. Furthermore, 38 (9.4%) primer pairs allowed the detection of clear bands and easy fragment recognition for eight *A. sellowiana* genotypes, generating an average of 1.5 alleles per locus. Satisfactory amplification products were obtained using an annealing temperature of 56 °C.

When we screened the 119 accessions with ten selected polymorphic primer pairs we detected 49 alleles, varying from 120 bp to 320 bp. The quality of the amplification products is shown in Figure 1.

The number of detected alleles per locus ranged from 2 to 9, averaging 4.9 alleles per locus, with the EMBRA 26 marker being the most polymorphic one (Table 2). The ten

Table 2 - Sequence of the 10 used pairs of primers developed for *Eucalyptus* genera, allele size range, number of alleles per locus (A), observed heterozygosity (H_o) and expected heterozygosity (H_e) of amplified microsatellite loci in *Acca sellowiana*.

Primer*	Sequence (F- forward/ R- Reverse)	Allele size range (bp)	A	H_o	H_e
EMBRA 26	F-CAT GAG TTA CTG CAA GAA AAG R- ACA GCC AAA AAC CAA ATC	155-320	9	0.600	0.868
EMBRA 69	F-TGT GTT CTC GGT TTC AAA ACT R- TGT GAA GTG ATG CGA AGC	200-290	5	0.434	0.758
EMBRA 85	F- CAC CTC TCC AAA CTA CAC AA R- CTC CTC TCT CTT CAC CAT TC	140-300	7	0.685	0.831
EMBRA 99	F- AAT ACA ATT GAG GGG TCT C R- ACC AAA AAC AAA TGT CGT	230-250	3	0.120	0.499
EMBRA 108	F- CGG TTA CTT GCT TCA TTC G R- GTA CGG ATG GGT GGA CAC	150-160	2	0.505	0.442
EMBRA 123	F- AGA ACC CTC TAT AAA ACC CC R- GGG CTA GAC ATG ATG GAG	180-300	5	0.813	0.656
EMBRA 148	F- TGG ATG CTG TTC TCA TCC T R- GGG TTT CTT TGT GAA ACG A	200-320	6	0.702	0.695
EMBRA 166	F- CTG GTC AAC GTC CGA AAG R- ATG CTG CAG AGG GCA TAA	130-300	5	0.781	0.724
EMBRA 265	F- TAT CAC TGG CAG GAC GCA R- ACC GAC GCC GAT AAG AGA	200-250	5	0.602	0.652
EMBRA 267	F- GAC CTC CGC TTC AAC GAT R- GTG CGA GAC CCT CAA TTC TA	120-140	2	0.274	0.275
Average	-	-	4.9	0.551	0.640

*The primers were those reported by Brondani *et al.* (1998) and were obtained from the Genetics and Biotechnology unit of the Brazilian agricultural company Embrapa (Empresa Brasileira de Pesquisa Agropecuária-Recursos Genéticos e Biotecnologia, Brasilia, DF, Brazil).

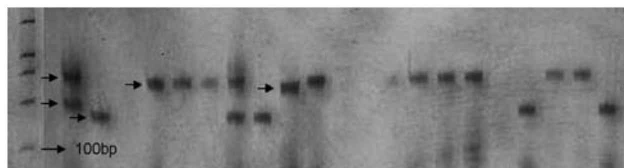

Figure 1 - Profile of the PCR products of 21 *Acca sellowiana* accessions from the Active Germoplasm Bank. The products were amplified by the *Eucalyptus* primer EMBRAPA 123 and resolved on silver stained polyacrylamide gel. Left line: 1 kb Plus DNA Ladder (Invitrogen).

Table 3 - Origin of the plant accessions presenting alleles with a low frequency ($f < 0.05$). All Brazilian cities are located in the state of Santa Catarina.

Local Brazil	Primer*	Local Brazil (cont)	Primer*
Bom Jardim		Ponte Alta	
527	EMBRA 26	740	EMBRA 26
372	EMBRA 123	732	EMBRA 26
		152	EMBRA 108
Caçador			
511	EMBRA 26	São Joaquim	
522	EMBRA 69	103-110	EMBRA 26
511	EMBRA 123	119-120	EMBRA 85
		277	EMBRA 108
Campos Novos			
85	EMBRA 26	Vargem Bonita	
		805	EMBRA 69
Curitibanos		805	EMBRA 123
735	EMBRA 26		
		Videira	
Fraiburgo		101	EMBRA 26
521	EMBRA 26	101-333	EMBRA 69
501	EMBRA 85	50-91-526	EMBRA 85
501-502	EMBRA 123	50	EMBRA 108
Lages		Other countries	
246-247	EMBRA 26	Israel,	
229	EMBRA 26	Unspecified source	EMBRA 26
276	EMBRA 69	Unspecified source	EMBRA 108
250-276	EMBRA 85		
326	EMBRA 108	New Zealand	
246-249	EMBRA 123	456-457	EMBRA 108
332	EMBRA 265		
		Unknown origin	
		438	EMBRA 85

*The primers were those reported by Brondani *et al.* (1998) and were obtained from the Genetics and Biotechnology unit of the Brazilian agricultural company Embrapa (Empresa Brasileira de Pesquisa de Agropecuária-Recursos Genéticos e Biotecnologia, Brasilia, DF, Brazil).
Local: Country, city of origin and accession number.

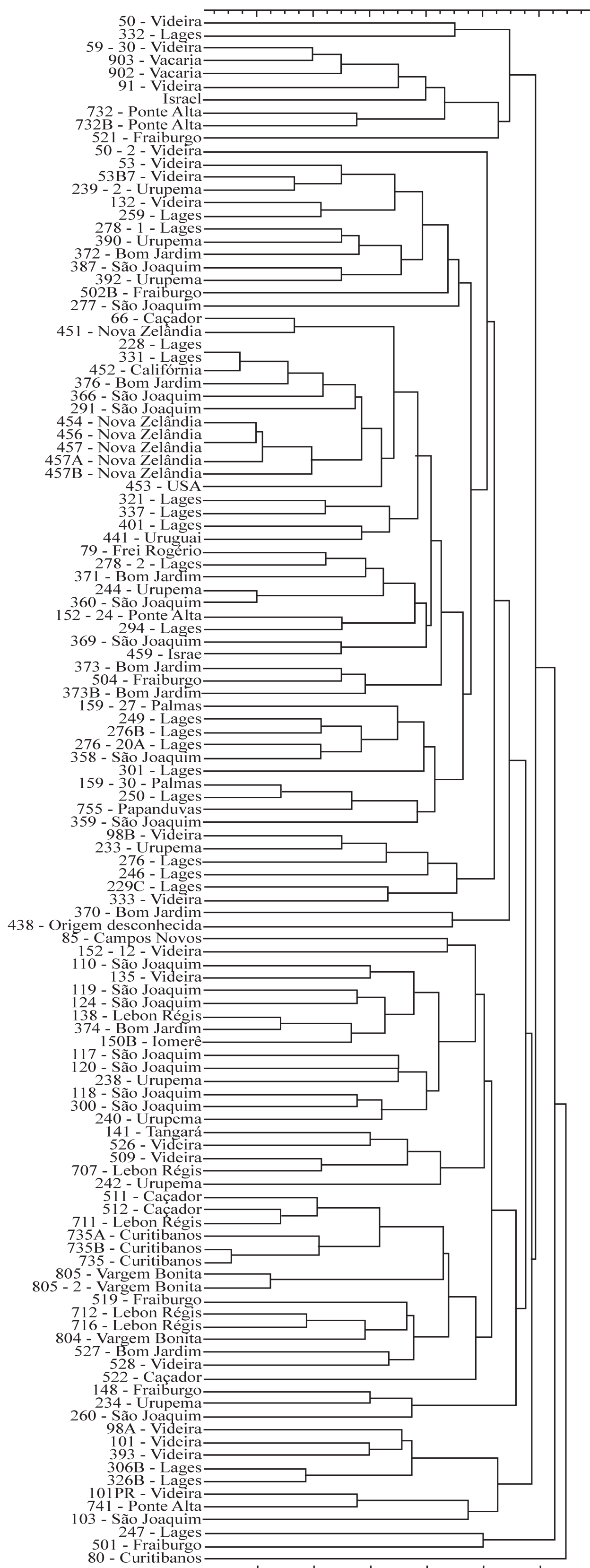

Figure 2 - Unweighted pair group method with arithmetic mean (UPGMA) dendrogram reflecting the genetic similarities, based on 10 microsatellite loci, among 119 accessions from the *Acca sellowiana* Active Germplasm Bank.

pairs of primers used were able to detect low frequency alleles (≤ 0.05), which were distributed in accessions of different origins (Table 3).

The mean expected heterozygosity among loci was He = 0.640, while the mean observed heterozygosity was Ho = 0.551 (Table 2). In the dendrogram (Figure 2), the 119 accessions were distributed in different groups and the degree of genetic similarity ranged from 35% to 100%. Two sub-groups contained two accessions with 100% similarity, one sub-group consisting of accessions 228 and 331 from Lages and the other sub-group consisting of the New Zealand accessions 456 and 457. On the other hand, three accessions formed two sub-groups, one of which contained accession 80 from Curitibanos (37.5% similarity with the other 117 accessions) and the other accession 247 from Lages plus accession 501 from Fraiburgo (35% similarity with the other 117 accessions), these two sub groups being very different from the other sub-groups. Besides this main feature of grouping, the other sub-groups did not reveal any special structure, except one, which included 7 out of 9 accessions from outside Brazil. Interestingly, the two genotypes from Israel were located outside the group that included accessions from the USA, New Zealand and Brazil.

Discussion

Considering the time-consuming and expensive process of microsatellites isolation (Powell *et al.*, 1996), we took advantage of the availability of *Eucalyptus* primer sequences and used them in *Acca sellowiana*. Our study demonstrated the transferability of microsatellite markers from *Eucalyptus* to *Acca* across different genera belonging to the same family (Myrtaceae). This demonstration of transferability means that future genetic studies can be carried out, this marker type being extremely useful due to its ease of use and high amount of information generated. Because of these features, microsatellites are considered as useful molecular markers in plant breeding, and are widely used for cultivar fingerprinting, paternity testing and genome mapping.

The transferability across related species and genera makes these markers very powerful for comparative genetic studies (Szewc-Mcfadden *et al.*, 1996; Smulders *et al.*, 1997 Roa *et al.*, 2000). A high cross-species conservation of microsatellite loci within genera has been reported in tree species such as *Citrus* (Kijas *et al.*, 1995), *Prunus* (Cipriani *et al.*, 1999; Dirlewanger *et al.*, 2002; Wünsh and Hormaza, 2002), *Elaieis* (Billote *et al.*, 2001), *Picea* (Hodgetts *et al.*, 2001), *Pinus* (Shepherd *et al.*, 2002; Liewlaksaneeyanawin *et al.*, 2004), *Olea* (Olive) (Sefc *et al.*, 2000), *Malus* (Coart *et al.*, 2003) and *Eucalyptus* (Marques *et al.*, 2002).

However, a cautious approach is required when comparing similar PCR products obtained across different species, since various factors can cause size homoplasy. Over long periods of evolution, the interspecific allelic differences at one locus are often more complex than simple changes in repeat number. Products amplified in different species might include mutation, rearrangements and duplications in the flanking region and/or changes in the number of repeats (Peakall *et al.*, 1998).

Microsatellite transferability has also been confirmed to occur between species from different genera. In the work described in this paper we have demonstrated that primer pairs developed for *Eucalyptus* were able to amplify in the *A. sellowiana* genome. Zucchi *et al.* (2003) used a sample from the same set of *Eucalyptus* complex primer pairs to test their transferability to other Myrtaceae species such as *Eugenia dysenteria* and found that of the 356 microsatellite primer pairs tested it was possible to transfer 10, representing a transferability of 2.8%. Interestingly, none of the microsatellite primer pairs transferred to *Eugenia dysenteria* by Zucchi *et al.* (2003) coincided with the primers transferred to *A. sellowiana* in our study. Although it is premature to make inferences about relatedness before obtaining further evidence, by considering these results together it can be hypothesized that there is more similarity between *Acca* and *Eucalyptus* than between *Eugenia* and *Eucalyptus*. According to Palop *et al.* (2000), microsatellites loci are more likely to be amplified in closely related species.

The number of primer pairs transferred from *Eucalyptus* to *A. sellowiana* (44.5%) can be considered very high in comparison to other studies (Padian *et al.*, 2000). In addition, at least 26% of the primer pairs transferred were able to detect polymorphism among the 119 *A. sellowiana* genotypes screened, which can be considered to be a high level in light of the fact that in the study by Zucchi *et al.* (2003) cited above with 10 pairs of *Eucalyptus* primers transferred to *E. dysenteria* the level of polymorphism was only 2%.

It is relevant to mention the existence of a large amount of genetic variability among the *A. sellowiana* accessions. Besides supporting such a conclusion, the presence of alleles with a low frequency in accessions of different origins suggests that the genetic variability is dispersed across locations. Genotypes with a low level of similarity in comparison with others were also found in this study. This high amount of genetic variability is no surprise, since the *A. sellowiana* germplasm bank contains a collection of 119 representatives from 14 locations distributed across southern Brazil. Most of the accessions have agronomic importance, since they express one or more agronomic traits that can be further integrated into breeding programs.

The high value of expected heterozygosity in comparison with observed heterozygosity among the accessions in the *A. sellowiana* germplasm bank indicates that heterozygote deficit is present in the germplasm bank accessions. However, this deficit is relatively low and the heterozy-

gosity is high, which suggests the existence of high genetic diversity in the natural populations from which the *A. sellowiana* accessions were collected.

The dendrogram which we obtained based on 10 loci showed two sub-groups consisting of two accessions each, with 100% of similarity. At the other extreme, three accessions showed a low degree of similarity (from 35.0 to 37.5%) with the other 117 plant accessions. These results agree with the high values of heterozygosity and the 48 alleles detected in the Active Germplasm Bank. It is important to mention that the accessions from New Zealand and the United States were included in a sub-group, indicating the narrowing of the genetic base for these accessions and a substantial degree of relationship among them.

This study has demonstrated the transferability of *Eucalyptus* spp. microsatellite markers to *Acca sellowiana*, which belong to the same family (Myrtaceae) but are distinct genera. Because the *Eucalyptus* microsatellite loci were able to detect the existence of a large amount of genetic variability among the *A. sellowiana* accessions they can be used for genetic characterization of both accessions and natural populations, knowledge of which helps to accelerate not only the establishment of appropriate conservation strategies but also marker assisted selection, the selection of parents for controlled crosses and the monitoring of the segregation of genomic regions of agronomic interest in segregating progenies. In addition, the *Eucalyptus* primers can be used for genetic studies in other Myrtaceae species for which there are no species-specific microsatellites available.

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