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Conservation and synteny of SSR loci and QTLs for vegetative propagation in four *Eucalyptus* species

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Abstract Conservation of microsatellite loci, heterozygous in *Eucalyptus grandis*, *Eucalyptus urophylla*, *Eucalyptus tereticornis* and *Eucalyptus globulus*, allowed us to propose homeologies among genetic linkage groups in these species, supported by at least three SSR loci in two different linkage groups. Marker-trait associations for sprouting and adventitious rooting ability were also compared in the four species. Putative quantitative trait loci (QTLs) influencing vegetative propagation traits were located on homeologous linkage groups. Our findings indicate high transferability of microsatellite markers between *Eucalyptus* species of the *Symphyomyrtus* subgenus and establish foundations for the use of synteny in the genetic analysis of this genus. Microsatellite markers should help integrate eucalypt genetic linkage maps from various sources. The availability of comparative linkage maps provides a basis of more-efficient use of genetic information for molecular breeding and evolutionary studies in *Eucalyptus*.

Keywords SSR · Mapping · QTLs · *Eucalyptus* · Synteny

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Introduction

Microsatellites (or SSRs) are stretches of di- to pentanucleotide repeats common in eukaryotic genomes (Tóth et al. 2000). A wide range of variation in the frequency of each class of SSR and in SSR repeat abundance have been reported in various species (Tóth et al. 2000). Mutation rate and mutation mechanism vary in different microsatellites (van Treuren et al. 1997; Provan et al. 1999) and between them and the rest of the genome (Schlötterer and Pemberton 1994).

Microsatellites have been used as genetic markers in plants. They are selectively neutral, inherited in a codominant Mendelian manner, relatively abundant, highly polymorphic, somatically stable and appear to be randomly distributed in the genome (Morgante and Olivieri 1993). Microsatellite loci are extremely valuable for fingerprinting, paternity identification, quality control of controlled crosses, estimation of outcrossing rates, monitoring of inbreeding, assessment of genetic differentiation and estimating gene flow (reviewed by Powell et al. 1996). Microsatellites are also a powerful tool in qualitative and quantitative trait analysis, marker-assisted breeding and screening of large insert libraries prior to cloning (Chen et al. 1997).

In contrast with their importance for intraspecific comparisons, SSR markers have been less used for phylogenetic reconstruction, mainly due to asymmetries in the mutation process and the degradation of repeats over time (Goldstein and Pollock 1997). The primary disadvantage of microsatellites is their high cost of development. This problem is exacerbated by the relatively low SSR abundance in plants compared to animals (Lagercrantz et al. 1993) and the fact that the most-abundant SSRs in many plant species (A-T dinucleotide) are difficult to isolate (Powell et al. 1996). Therefore, it is important to determine the extent to which the same SSR primers can be used across taxonomic boundaries, with related species.

The development and characterization of microsatellites have been reported in several tree species (reviewed by Brondani et al. 1998). However, few studies have

reported SSR linkage information and investigated the transferability of markers between species. In this work we evaluate the extent to which SSR primers developed from *Eucalyptus grandis* and *Eucalyptus urophylla* amplify orthologous loci in *Eucalyptus tereticornis* and *Eucalyptus globulus*, determine homeologies between genetic linkage groups and investigate consensus marker-trait associations in these species.

Materials and methods

Plant materials

Microsatellite genotyping was carried out on a *E. tereticornis* seed parent (clone TT Esc 87/90), a *E. globulus* pollen parent (clone GB MJ 6/90), and 73 F_1 progeny. Amplified fragment length polymorphism (AFLP) genetic linkage maps of the parent trees (based on the same progeny set) and procedures for DNA extraction have been reported elsewhere (Marques et al. 1998). Results were compared with SSR data of the *E. grandis* × *E. urophylla* family (parents and 94 F_1 progeny) described in Brondani et al. (1998). All species belong to the *Symphyomyrtus* subgenus and have 11 chromosomes. *E. grandis* and *E. urophylla* belong to the section *Transversaria*, *E. tereticornis* and *E. globulus* are included in the sections *Exsertaria* and *Maidenaria*, respectively (Eldridge et al. 1994).

SSR primer design

The development and characterization of the eucalypt SSR primer pairs used in this work was described elsewhere (Brondani et al. 1998; Brondani and Grattapaglia 2001). Two *E. grandis* and *E. urophylla* genotypes were used to develop genomic libraries enriched for $(AG)_n$.

Amplification of SSR marker loci

Polymerase chain reaction amplification of SSR loci was carried out in 96-well V-bottom plates. Each reaction contained 0.3 μM of primer, 1 U of *Taq* DNA polymerase, 0.25 mM of each dNTP, 10 mM of Tris-HCl pH 8.3, 50 mM of KCl, 1.0 mM of $MgCl_2$, DMSO (5.0%) and 7.5 ng of template DNA in a final 13-μl volume. Reactions were cycled in a MJ Research PT-100 Thermal Controller with a heated lid (96 °C for 2 min; then 29 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min; and finally 72 °C for 7 min). Reaction products of the initial screening and of loci EMBRA 55, SSR 23 and SSR 84 were separated in 3.5% Metaphor agarose (FMC Bioproducts) gels containing 0.1 μg/ml of ethidium bromide in 1 × TBE buffer (89 mM of Tris-borate, 2 mM of EDTA pH 8.3). These samples were flanked by a ϕ× 174 RF DNA/*Hae*III fragment ladder (Gibco BRL) and run at 120 V for 2 h. Reaction products of mapped SSR loci were separated on 8% denaturing polyacrylamide gels in 7.5 M urea and 0.8 × TBE buffer, visualized by silver staining (Bio-Rad silver staining kit). Samples were flanked by a 10-bp ladder (Gibco BRL) standard and run at 500 V for 2 h.

Mapping SSR marker loci

All 180 SSR primer pairs developed by Brondani et al. (1998; 2001) were screened to evaluate heterologous amplification of the SSR alleles in the *E. tereticornis* × *E. globulus* family (parents and six progeny). A subset of 40 primer pairs producing easily interpretable polymorphic fragments were selected for mapping. The segregation of alleles according to Mendelian ratios was analyzed in polyacrylamide gels using the χ^2 test ($\alpha = 0.01$ level). A cosegregation linkage analysis of the SSR and the AFLP marker data was carried out for each parental data set separately (two-way pseudo-testcross strategy) using the PGRI software (Liu 1997) as in Marques et al. (1998). Markers linked with a recombination frequency of $\theta \leq 0.25$ and $P \leq 0.00001$ were assigned to linkage groups.

Results

Transferability of microsatellite markers in *Eucalyptus*

Transferability of microsatellite loci was defined in terms of the success of the heterologous amplification of PCR products using the SSR primers developed from *E. grandis* and *E. urophylla* (Brondani et al. 1998). Of 180 SSR primer pairs tested in the *E. tereticornis* × *E. globulus* family, 140 cases yielded successful heterologous amplification products. These results indicate 78% transferability of SSR loci among eucalypt species in the *Symphyomyrtus* subgenus.

Linkage mapping of SSR marker loci in *Symphyomyrtus*

From the subset of 40 SSR loci, 34 were heterozygous in *E. tereticornis* and 34 in *E. globulus*. The inclusion of the SSR markers in the mapping analysis suggested the merging of linkage groups 2 and 14 in *E. tereticornis*. However, in most cases locus order was maintained. Usually, more than one SSR locus occurred per linkage group, validating the homeologies. The location of some SSR markers suggested new relationships between linkage groups in the genetic linkage maps of *E. tereticornis* and *E. globulus* (Table 1). As for the comparison between the four species, we report only the homeologies between linkage groups supported by at least three SSR loci in two different genetic linkage groups: 22 SSR markers in six linkage groups in *E. grandis*, 17 SSR markers in five linkage groups in *E. urophylla*, 21 SSR markers in eight linkage groups in *E. tereticornis* and 22 SSR markers in eight linkage groups in *E. globulus* genetic linkage maps (Table 1). Twelve SSR loci could be mapped in all four linkage maps.

Comparative marker-trait associations

Using the SSR suggesting synteny between the different genetic linkage maps, we compared the linkage group location of putative QTLs for sprouting and adventitious rooting ability detected in *E. grandis*, *E. urophylla*, *E. tereticornis* and *E. globulus* (Grattapaglia et al. 1995; Marques et al. 1999). We detected putative QTLs influencing vegetative propagation traits in homeologous linkage groups (Fig. 1). The most interesting case is the homeology between *E. grandis* linkage group 1 with a QTL of modest effect for sprouting, *E. urophylla* linkage group 1 with a putative QTL explaining the highest proportion of phenotypic variation in adventitious rooting

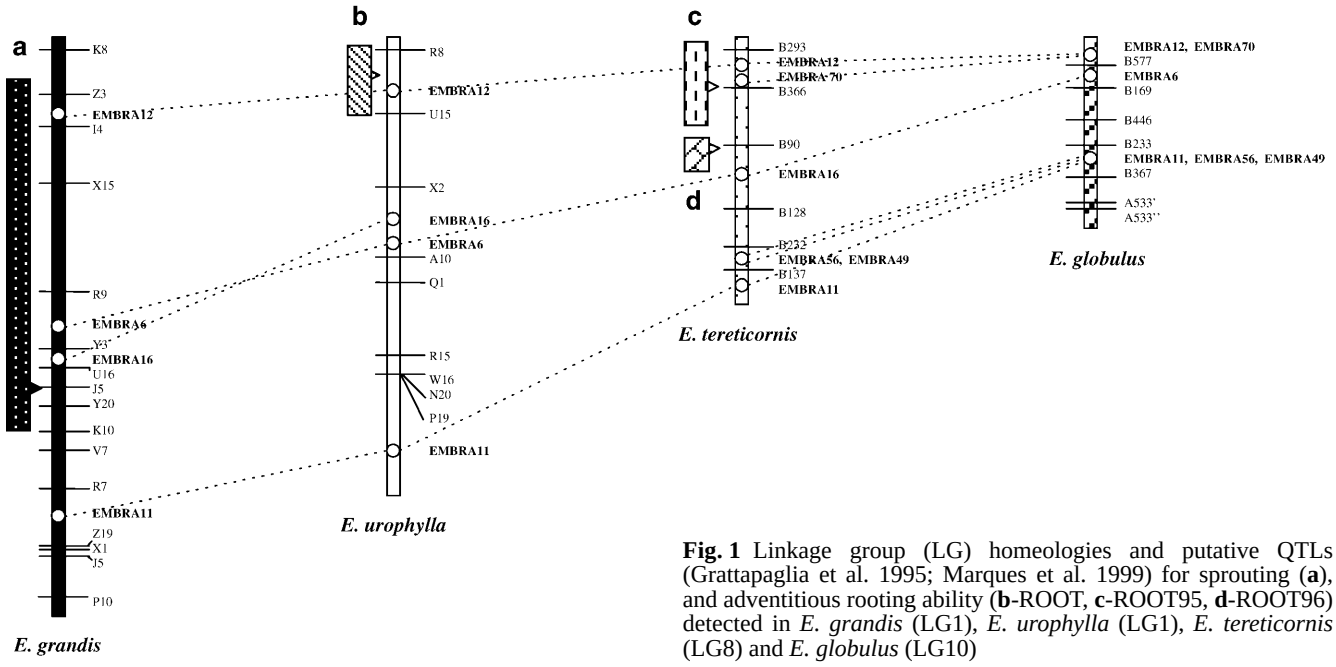

Fig. 1 Linkage group (LG) homeologies and putative QTLs (Grattapaglia et al. 1995; Marques et al. 1999) for sprouting (**a**), and adventitious rooting ability (**b**-ROOT, **c**-ROOT95, **d**-ROOT96) detected in *E. grandis* (LG1), *E. urophylla* (LG1), *E. tereticornis* (LG8) and *E. globulus* (LG10)

Table 1 Homeologies between linkage groups in *E. grandis*, *E. urophylla*, *E. tereticornis* and *E. globulus*. Linkage group references for *E. grandis* and *E. urophylla* as in Brondani et al. (1998) and *E. tereticornis* and *E. globulus* as in Marques et al. (1998). The SSR reference refers to Brondani and Grattapaglia (2001). "Unlinked #" refers to unlinked markers (clustered when marked with the same reference). At the bottom we summarize the number of linkage groups (LG) with at least three homeologous SSR and the total number of linkage groups in the genetic linkage maps

SSR reference	Linkage group in *E. grandis*	Linkage group in *E. urophylla*	Linkage group in *E. tereticornis*	Linkage group in *E. globulus*
EMBRA 6	1	1	8	10
EMBRA 11	1	1	8	10
EMBRA 12	1	1	8	10
EMBRA 56	1	1	8	10
EMBRA 70	1	1	8	10
EMBRA 55	2	2	2/14	15
EMBRA 68	2	2	2/14	15
EMBRA 43	2	–	2/14	–
EMBRA 5	5	5	10	6
EMBRA 37	5	5	10	6
EMBRA 9	5	5	Unlinked I	6
EMBRA 24	5	–	Unlinked I	6
EMBRA 8	6	–	1	1
EMBRA 50	6	–	1	1
EMBRA 28	6	–	1	1 and unlinked II
EMBRA 31	6	–	1	Unlinked II
EMBRA 7	–	9	5	3
EMBRA 13	9	9	–	4
EMBRA 17	9	9	–	4
EMBRA 18	9	9	12	4
EMBRA 10	10	10	4	14
EMBRA 61	10	10	4	16
EMBRA 40	10	10	4	Unlinked III
No. of LGs with SSRs	6	5	8	8
No. of LGs in maps	14	11	14	16

and *E. tereticornis* linkage group 8 with a smaller-effect putative QTL for adventitious rooting. The same genomic area in *E. tereticornis* is also responsible for the highest proportion of phenotypic variation in petrification and mortality (as defined in Marques et al. 1999).

Discussion

Transferability of microsatellite markers in *Eucalyptus*

Successful heterologous PCR-DNA amplification could be inversely related to the evolutionary distance between species (Steinkellner et al. 1997) and to the rate of evolution of the genomic region amplified (van Treuren et al. 1997). The successful amplification of 78% of the mi-

crosatellite loci across sections in the *Symphyomyrtus* subgenus loci indicates a high level of sequence conservation within the primer regions of the species tested.

Byrne et al. (1996) reported complete conservation of four SSR loci (developed from *E. nitens*) between three species (*E. globulus*, *E. grandis* and *E. camaldulensis*) within the *Symphyomyrtus* subgenus (sections *Maidenaria*, *Transversaria* and *Exertaria*, respectively). Van der Nest et al. (2000) also reported preliminary results of interspecific amplification of five SSR loci in *Eucalyptus*. Other studies have considered cross-species amplification, with varying degrees of primer-sequence conservation being demonstrated in *Pinus* (Fisher et al. 1998), *Quercus* (Steinkellner et al. 1997), *Populus* (van der Schoot et al. 2000) and among conifer species (Echt et al. 1999).

Linkage mapping of SSR marker loci in *Symphyomyrtus*

The location of some SSR loci suggested evidence for relatedness between genetic linkage groups in the maps of *E. tereticornis* and *E. globulus*, helping us to consolidate linkage groups. However, when PCR primers are transferred between species, the amplification of non-target sites is possible (Erpelding et al. 1996). Multiple copies of SSR loci could interfere with the determination of homeologous linkage groups. The presence of at least three SSR loci in two different genetic linkage groups support our inferences. Most of the indicated homeologies between *E. tereticornis* and *E. globulus* are further supported by earlier results of more than one 3:1 segregating AFLP markers per linkage group (Marques et al. 1998).

Integration of single-tree linkage information could lead to the construction of "species consensus maps" (Bucci et al. 1997) useful to saturate species-specific maps in targeted regions or to develop genetic linkage maps in species with a low level of polymorphism among parental lines (van Deynze et al. 1995). Maps based on common sets of markers constitute a basis for the comparison of genome organization and evolutionary change (Causse et al. 1994). Common markers could also be used in surveys of genetic variability, investigations on the consistency of QTL locations in different genomic backgrounds and prediction of the location of orthologous genes in other individuals (Harrington et al. 1997).

Comparative marker-trait associations

The putative QTLs reported in Marques et al. (1999) and Grattapaglia et al. (1995) represent the genes which show allelic variation with a phenotypic effect detectable in the crosses, progeny and environments studied. Very likely they do not comprise the entire set of genes affecting the traits studied. A number of authors have reported differences in the number of QTLs identified with environment (Paterson et al. 1991), age (Verhaegen et al. 1997) and sampling effects (Beavis 1994). Moreover, the confidence intervals for QTL map location in all four species were rather broad and we were not able to establish homeologies between all linkage groups. Despite this, putative QTLs associated with different vegetative propagation traits were located in homeologous linkage groups. This may indicate the presence of clusters of genes influencing different aspects of vegetative propagation, or the same major gene. Similarity in QTL locations between species might represent allelic variation in orthologous genes (Paterson et al. 1991).

The conservation of QTLs among species could provide many opportunities for plant breeders. Common QTLs from different species that affect the same character, and that have been tested in different environments and population structures, represent ideal targets for marker-assisted selection, fine mapping and map-based cloning (Grandillo and Tanksley 1996). Map-based cloning of orthologous genes could be easier from species with smaller and/or less complex genome sizes (Paterson et al. 1995). Besides providing a means to validate QTL existence (Paterson et al. 1991; Lin et al. 1995; Pereira and Lee 1995; Lagercrantz et al. 1996), the ability to conduct comparative high-resolution mapping experiments with QTLs should further help determine whether QTLs are single loci or clusters of tightly linked genes.

Prospects

The creation of index maps where SSRs constitute anchor points for specific regions of the genome of different species is possible in *Eucalyptus*; this work is a first contribution in that direction. The small nuclear genome and a low proportion of repetitive DNA of *Eucalyptus* facilitate genetic investigations (Grattapaglia and Bradshaw 1994). The construction of a eucalypt syntenic map would be a very useful tool both for breeding and for fundamental research purposes.

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