Theor Appl Genet (2004) 109:192–199
DOI 10.1007/s00122-004-1617-9

ORIGINAL PAPER

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Retrospective selection of elite parent trees using paternity testing with microsatellite markers: an alternative short term breeding tactic for *Eucalyptus*

Received: 25 July 2004 / Accepted: 26 January 2004 / Published online: 5 March 2004

Abstract The conventional way to drive modifications in old forest tree seed orchards is to establish progeny trials involving each parent tree and then evaluate its contribution to the performance of the progeny by estimating its general and specific combining ability (GCA and SCA). In this work, we successfully applied an alternative parent selection tactic based on paternity testing of superior offspring derived from a hybrid seed orchard established with a single *Eucalyptus grandis* seed parents and six *E. urophylla* pollen parents. A battery of 14 microsatellite markers was used to carry out parentage tests of 256 progeny individuals including two independent samples of selected trees and one control unselected sample, all derived from 6-year-old forest stands in eastern Brazil. Paternity determination was carried out for all progeny individuals by a sequential paternity exclusion procedure. Exclusion was declared only when the obligatory paternal allele in the progeny tree was not present in the alleged parent tree for at least four independent markers to avoid false exclusions due to mutation or null alleles. After maternity checks to identify seed mixtures and selfed individuals, the paternity tests revealed that approximately 29% of the offspring was sired by pollen parents outside the orchard. No selfed progeny were found in the selected samples. Three pollen parents were found to have sired essentially all of the offspring in the samples of selected and non-selected progeny individuals. One of these three parents sired significantly more selected progeny than unselected ones ($P \leq 0.0002$ in a Fisher exact test). Based on these results, low-reproductive-successful parents were culled from the orchard, and management procedures were adopted to minimize external pollen contamination. A significant difference ($P<0.01$) in mean annual increment was observed between forest stands produced with seed from the orchard before and after selection of parents and revitalization of the orchard. An average realized gain of 24.3% in volume growth was obtained from the selection of parents as measured in forest stands at age 2–4 years. The marker-assisted tree-breeding tactic presented herein efficiently identified top parents in a seed orchard and resulted in an improved seed variety. It should be applicable for rapidly improving the output quality of seed orchards, especially when an emergency demand for improved seed is faced by the breeder.

Communicated by D.B. Neale

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Introduction

Eucalypts are the most widely planted hardwood trees in the world. Estimates made in 2000 (FAO 2000) indicated that the eucalypt plantation area was globally greater than 17.8 million hectares, with India being the largest planter with 8.0 million hectares—mostly in extensive low-productivity plantations—followed by Brazil with 3.0 million hectares of mostly intensively cultivated clonal plantations of industrial forests reaching average productivities of 45–60 m^3/ha per year (Mora and Garcia 2000). Elite hybrid clones consisting of *Eucalyptus grandis* and *E. urophylla* are extensively used by the cellulose and paper industry because of its wood quality, rapid growth and high volumetric yield (Bertolucci et al. 1995).

In Brazil, eucalypt genetic improvement programs were initiated in the early 1970s. The main purposes of the breeding programs were to match the most adapted

species to the existing environmental conditions and to provide sufficient plant stock of reasonable genetic quality (Brune and Zobel 1981). Several *E. grandis*, *E. urophylla* and *E. saligna* seed orchards were established on the basis of initial results of available provenance/progeny trials. Furthermore, due to the early observation of hybrid superiority for growth coupled to resistance to eucalypt canker caused by *Cryphonectria cubensis* (Bruner) Hodges, hybrid seed orchards of *E. grandis* × *E. urophylla*, were also established, thereby generating the so-called Urograndis seed variety (Campinhos 1980; Vigneron 1991; Eldridge et al. 1993). These open-pollinated seed orchards often involved the deployment of one or a few self-incompatible or male-sterile *E. grandis* trees as female parents and a number of selected *E. urophylla* as male parents to ensure enough pollen pressure (Bertolucci et al. 1995).

Clonal propagation of elite trees started in the 1980s followed the initial breeding/testing/selection efforts. This new technology, involving the establishment of true clonal stands through vegetative propagation via rooted cuttings, was developed and applied on a wide scale, thereby providing significant productivity and uniformity gains (Campinhos 1980; Brandão et al. 1984) and consequently reducing the demand for seedlings in the establishment of new forests. However, recent increases in the industrial demand for wood have forced forest companies to rapidly expand planting areas and establish partnership programs with farmers. The only way to fulfill this immediate challenge has been to use seeds from first-generation breeding orchards to complement the production of rooted cuttings. The development of improved seed varieties has thus become necessary again in the context of current breeding programs, both by establishing new orchards with trees of high general and specific combining ability (GCA and SCA, respectively) and by restructuring old ones.

The conventional way to drive modifications in old seed orchards is to establish progeny trials to evaluate the contribution of the parent to the performance of its progeny by SCA estimation. This approach requires a long time to achieve its purpose (Namkoong et al. 1988). An alternative way to estimate the superiority of the parent trees of a seed orchard could be attained by a marker-assisted breeding tactic: carrying out DNA-based paternity tests of superior progeny trees in forest stands established with open-pollinated seeds from the orchard. Parents that display a low frequency of superior offspring could be culled from the orchard, thus practicing a backwards selection that would result in an improved seed variety involving exclusively trees of higher GCA and SCA (Ribeiro et al. 1998; Grattapaglia 2000).

A similar approach, termed PMX/WPA (polymix breeding with parental analysis) was evaluated as an alternative solution to full-sib crosses in pine breeding programs (Lambeth et al. 2001). As pointed out by those authors, although PMX breeding is easy, provides good estimates of breeding values and allows the testing of a larger number of parental combinations, pedigree control is lost. Instead of using a single pollen for each cross, the concept then proposed by Lambeth et al. (2001) was to use PMX breeding involving many male parents followed by paternity analysis of progeny with microsatellites, thus allowing full pedigree control.

Similar to the situation in humans (Hammond et al. 1994) and domestic animals (Glowatzki-Mullis et al. 1995), the high degree of multi-allelism and the clear and simple codominant Mendelian inheritance of microsatellites provide an extremely powerful system for the unique identification of *Eucalyptus* individuals in parentage testing, particularly when individuals are expected to be related (Brondani et al. 1998). In the study reported here, we applied a marker-assisted breeding tactic to an old *E. grandis* × *E. urophylla* seed orchard with the specific objectives of verifying the differential reproductive success of six male parents and potentially identifying those with higher SCA with the maternal tree to develop an improved seed variety.

Materials and methods

Seed orchard

The seed orchard studied was established in 1982 in Aracruz (latitude 19°49", longitude 40°16") in Espirito Santo State, Brazil by Aracruz Celulose S.A. Six *Eucalyptus urophylla* plus trees identified as being canker-free were selected from provenance/progeny trials of open-pollinated, half-sib families originally collected on the Bessi-Lau, Flores and Timor Islands in Indonesia. These six trees were clonally propagated by grafting and constituted the male parents in the seed orchard. A single *E. grandis* plus tree selected from a progeny trial of open-pollinated, half-sib families originally collected in Atherton (Australia) was used as the female parent. This particular female parent was selected due to its good growth performance and putative self-incompatibility that was deduced from its very low seed set in controlled self-pollinations (Bertolucci et al. 1995). The seven parent trees were clonally replicated and planted in a 6×6 spacing in 267 hexagonal plots totaling 7.5 ha, with each of the *E. grandis* maternal tree surrounded by the six pollinator trees. The orchard was surrounded by a 300-m wide belt of native tropical forest to provide isolation from any pollen coming from nearby eucalypt plantations. Seeds were only collected from the *E. grandis* maternal tree.

Selection of progeny individuals

Sampling of progeny individuals was carried out in 6-year old stands established with seeds derived from the seed orchard studied. Two independent samples of 72 selected trees each were taken from two different forested areas—SSA selected sample A and SSB selected sample B. Individual trees with a circumference at breast height (CBH) above one standard deviation from the mean (mean = 55±15 cm) were selected, reaching a selection intensity of approximately 1:200. A control sample of 72 random non-selected (RNS) trees was also taken to allow a comparative analysis with the selected tree samples. To verify potential trees derived from selfing of the maternal tree, we also investigated a sample of ten trees with stunted growth and a sample of 30 trees that displayed symptoms of chlorotic leaves. Total genomic DNA was extracted from adult leaf tissue following the protocol described by Grattapaglia and Sederoff (1994).

Microsatellite genotyping

Forty-seven microsatellite markers developed by Brondani et al. (1998, 2002) were screened for polymorphisms among the seven parent trees in order to identify a battery of markers with higher information content for the proposed study. Microsatellite marker amplification and detection were performed as described earlier (Brondani et al. 1998). The amplified products were separated on 4% denaturing polyacrylamide gels, stained with silver nitrate (Bassam et al. 1991) and sized by comparison to a 10-bp DNA ladder standard (GibcoBRL, Gaithersburg, Md.) on a computer screen. Allele sizes were estimated using the software SEQAID II (Rhoads and Roufa 1990), taking into consideration the expected allelic series in basepairs for the locus. Multiplex loading in the same gel was carried out for up to three microsatellite loci simultaneously.

Paternity testing

Gels were scanned, and scoring was carried out manually on a computer screen. Multi-locus genotypes were determined for all seven parent trees and the five samples of progeny individuals (SSA, SSB, RNS, stunted and chlorotic). Maternity of the *E. grandis* tree was first checked, and seed contaminants identified. Paternity determination was carried out for all progeny individuals by a sequential paternity exclusion procedure. Exclusion was declared only when the obligatory paternal allele in the progeny tree was not present in the alleged parent tree for at least four independent markers to avoid false exclusions due to mutation in the microsatellite marker. Paternity was ultimately declared when the alleged parent tree shared the obligatory paternal allele at all of the microsatellite markers tested. Because the number of potential pollen parents was limited, paternity declaration was deterministic and did not involve the calculation of a paternity index using likelihood-ratio methods. Progeny that were not assigned to a specific pollen parent were classified either as selfs or as having been derived from outside pollen contamination.

Paternity in selected versus non-selected trees

For each pollen parent tree that effectively sired progeny individuals, a two-tailed Fisher exact probability test (Fisher 1934) was used to test the null hypothesis that the proportion of sired progeny individuals is not significantly different between the selected and non selected offspring samples.

Improvement of productivity with parental selection

The male parents displaying the lowest reproductive success were eliminated from the orchard in the aim of producing a new improved seed variety. Realized gain in mean annual increment (MAI) (m^3/ha per year) was evaluated by means of a comparative inventory between stands established with seedlings derived from the original orchard (78 stands) and seedlings derived from the improved orchard following the culling of the worst parents (18 stands). These forest stands were 2–4 years old and had an average size of 20 ha with 1,111 trees per hectare; they were located at uniform sites in Bahia State. Student's *t*-probability test was used to test for significant differences in MAI between the old and the new variety.

Results

Out of the 47 microsatellite markers screened, a battery of 14 markers was ultimately selected and used throughout the study (Table 1). On the basis of multi-locus microsatellite data, paternity testing tables were set up for each progeny individual, and a sequential exclusion procedure was applied (Table 2). In each progeny population sample, the analysis of 14 microsatellite markers allowed immediate identification of: (1) progeny from other seed sources (*E. grandis* mother excluded); (2) progeny derived from outside pollen contamination (all six fathers excluded); (3) selfed individuals (only maternal alleles); (4) progeny that belonged to one of the six pollen parent (Table 3). Approximately 29% of the offspring was sired by pollen parents located outside of the orchard, indicating the ineffectiveness of the seed orchard isolation zone. No selfed progeny were found in the selected samples, which confirmed self-incompatibility of the seed donor parent. However, 6 of the 72 randomly taken trees (8.3%) and eight of the ten stunted trees (80%) were selfs. The sample of chlorotic trees analyzed indicated that these were sired either by outside pollen (Table 3) or by pollen tree no. 6 (Table 4).

Pollen parent trees nos. 3 and 5 displayed indistinguishable multi-locus genotypes at all 14 microsatellite markers (data not shown). Analyses were repeated three

Table 1 Microsatellite markers used in the paternity testing of progeny individuals. The linkage group is as determined in Brondani et al. (2002) (*nd* not determined)

Microsatellite marker	Forward primer 5′–3′	Reverse primer 5′–3′	Observed heterozygosity	Number of alleles	Linkage group
Embra 6	AgAgAATTgCTCTTCATggA	gAAAAgTCTgCAAAgTCTgC	0.50	5	1
Embra 10	gTAAAgACATAgTgAAgACATTCC	AgACAgTACgTTCTCTAgCTCA	0.83	7	10
Embra 11	gCTTAgAATTTgCCTAAACC	gTAAAATCCATgggCAAg	0.17	4	1
Embra 16	CAACgTTCCCCTTTCTTC	ATgTTAggCCAAACCCAg	0.67	7	1
Embra 21	ACAAgggAAACTTgATCg	ggAACCgAACATAgCAAg	1.00	7	10
Embra 22	gCACATgCACACACggTTg	AAggCCAgTggTCgTgAgTC	0.67	7	11
Embra 27	ATAACCACACCAATCTgCA	TATAgCTCgAACgCTCAAC	1.00	6	2
Embra 30	TTAgTTgAATCCAACCATTg	TATATAAggTgCAAATAATAAA-Cg	1.00	7	8
Embra 37	CACCTCTCCAAACTACACAA	CTCCTCTCTCTTCACCATTC	0.83	9	5
Embra 40	AAAgTATCTCCACgCTTCAT	TCCCAATCATgATCTTCAg	1.00	9	10
Embra 49	ATTATTggTTCATATTgAAAACC	AgATAgAgATTgAgTgAgACCC	0.67	9	3
Embra 52	TAATCAgCATTAgCgAAAgA	CgTATATgTTCAgAgTCAATCC	0.67	9	7
Embra 53	ATTAgCTTTTCTgTAACCCg	gAATggACAAgCTCTgATg	1.00	7	8
Embra 131	ACTTAACATCTATACATATTTg	TgTCCTATCTggCTCA	1.00	6	nd

Table 2 Example of paternity testing of a selected progeny individual with six microsatellite markers. Alleles were numbered from the smallest to the largest sized allele. At each locus, the maternal allele is indicated in italic and the obligatory paternal allele is indicated in bold and underlined. Pollen parent tree no. 6 was declared the father of individual 46

EMBRA locus	Mother tree	Progeny individual 46	1	2	3	4	5	6
16	*4*/*5*	**2**/*5*	1/3	1/1	1/2	4/4	**2**/4	**2**/3
37	1/*2*	*2*/**6**	7/7	**6**/9	5/8	8/8	7/8	**6**/6
6	*3*/5	**1**/*3*	3/5	4/4	4/4	3/4	4/4	**1**/4
131	*4*/*4*	**2**/*4*	1/6	1/6	1/5	3/6	1/5	**2**/5
27	1/*3*	*3*/**6**	2/5	2/4	2/4	5/5	4/4	4/**6**
10	3/*5*	*5*/**7**	5/5	2/5	1/5	5/5	1/4	5/**7**

Table 3 Parentage determination in the five progeny samples

Progeny sample	Number of individuals	Non-maternity (seed mixture)	Sired by outside pollen parents	Selfed	Sired by orchard pollen parents
Selected-plus trees (SSA)	72	1	17	0	54
Selected-plus trees (SSB)	72	4	23	0	45
Non-selected trees (RNS)	72	14	22	6	30
Chlorotic trees	30	1	11	0	18
Stunted trees	10	0	0	8	2
Total	256	20	73	14	149

Table 4 Number of offspring sired by each pollen parent in the progeny samples from the seed orchard

Progeny sample	Pollen parents					
	1	2	3	4	5[a]	6
SSA—Selected sample A	32	11	0	0	-	11
SSB—Selected sample B	29	1	1	0	-	14
Non-selected trees	5	8	0	0	-	17
Chlorotic trees	0	0	0	0	-	18
Stunted trees	0	2	0	0	-	0
Total	66	22	1	0	-	60

[a] Pollen parents nos. 3 and 5 are the same tree

times with leaf samples collected from different clonal ramets; these confirmed the results. A probability of a random match between trees nos. 3 and 5 was estimated to be 1 in 89 billion using allele frequency estimates for these loci for *E. grandis* and *E. urophylla* (R. Brondani and M. Kirst, unpublished results).

Pollen parents nos. 1, 2 and 6 sired 148 out of the 149 offspring sired with orchard pollen. Pollen parents nos. 3 and 5, actually the same tree, sired only a single offspring in SSB, and parent no. 4 did not sire any offspring (Table 4). For parents nos. 1 and 6, significant differences were found in the proportions of progeny individuals in both selected samples versus the non-selected sample of trees. However, while pollen tree no. 1 sired significantly more selected progeny than non-selected ones in both SSA and SSB (P=0.0002 and 0.00005 respectively), pollen tree no. 6 sired significantly more offspring in the non-selected tree sample for both comparisons (P= 0.00141 and 0.03365, respectively) (Table 5). For pollen tree no. 2, no significant difference was observed between the proportions of sired offspring in SSA versus RNS (P= 0.58942), but significantly more RNS offspring were sired as compared to SSB (P=0.00221) (Table 5). A similar trend was observed when the test was carried out analyzing the outside pollen contribution—i.e. either the outside pollen contribution sired more non-selected offspring in SSA (22 vs. 17, Table 3) (P=0.00028), or no difference was detected in SSB (P=0.09132) (Table 5).

For practical breeding purposes, since parents nos. 1, 2 and 6 displayed significant reproductive success and effectively contributed to the generation of superior hybrid trees, they were kept in the orchard. Parents nos. 3, 4 and 5 did not contribute to the generation of superior offspring and were thus eliminated. A significant difference (P 0.01) in volume growth was observed between forest stands produced from orchard seeds before and after culling the worst parents (Table 6). The new seed variety (MAI = 44.5±5.8 m^3/ha per year) resulted in forest stands that were on average 24.3% more productive in volume growth than the original seed variety (MAI = 35.8±2.3 m^3/ha per year).

Table 5 Results (P-values) of Fisher exact contingency tests for binomial proportions of sired progeny individuals in the selected and non-selected offspring samples for the different pollen sources

Pollen source	SSA—Selected sample A	SSB—Selected sample B
Pollen tree no. 1	0.00020	0.00005
Pollen tree no. 2	0.58942	0.00221
Pollen tree no. 6	0.00141	0.03365
Outside pollen	0.00028	0.09132

Table 6 Number of commercial stands (average 20 ha, with 1,111 trees/ha) submitted to inventory. Mean annual increment (MAI) in cubic meters per hectare per year is given as the mean and standard deviation for seed varieties produced before and after elimination of parents with a low reproductive success for revitalization of the orchard

Seed variety	Number of stands	MAI (mean)	MAI (standard deviation)
Old (before culling)	78	35.8	2.3
New (after culling)	18	44.5	5.8

Discussion

A battery of 14 polymorphic markers was selected to carry out parentage tests of 256 progeny individuals. Maternity checks were carried on all individuals and, following the exclusion of seed mixtures and selfed individuals, paternity tests were carried out. Markers were specifically selected to allow easy scoring in polyacrylamide gels and to maximize allelic differences between the group of five pollen parents and between them and the maternal parent. This was possible as the five pollen parents were unrelated and the maternal parent belonged to a different species. We also specifically looked for markers for which all of the parents, maternal and pollen parents, were heterozygous so as to avoid the occurrence of null alleles that could result in false paternity exclusions (Moller 1995). A relatively large number of alleles were observed for the microsatellite markers used in the study, and for 6 out of the 14 markers all the parents were heterozygous, thereby allowing a significant discrimination power and confidence in the parentage testing (Table 1).

We also were interested in markers for which the maternal parent did not share alleles with any of the six pollen parents. Out of the 14 markers, three had this configuration. Based on these three markers the analysis of maternity versus non-maternity—i.e. seed mixture—was very fast and efficient. Progeny individuals that did not have any of the two maternal alleles were declared to result from the seed mixture. These loci were also very useful for determining the presence of selfed progeny individuals. The presence of homozygous loci in the maternal tree would also be efficient for the maternity checks, however null alleles could lead to false maternity exclusions.

The analysis of pollen contamination in the orchard was based on multiple paternity exclusions when the six pollen parents were tested. In this procedure it was essential to have genotypes for several loci to declare non-paternity with absolute confidence with a minimum of three excluding loci to avoid false exclusions due to mutations (Gunn et al. 1997). Di-nucleotide repeat microsatellites have been reported to have higher mutation rates following the step-wise mutation model (Valdes 1993) than longer repeat motifs in humans (Brinkmann et al. 1999) and, more recently, in maize (Vigouroux et al. 2002). Because no data are available yet on the frequency of null alleles for *Eucalyptus* microsatellites and, in fact, for the vast majority of plant species, our premise was that such a behavior would be the case for *Eucalyptus* as well. Although the most informative six markers for which all trees were heterozygous could be sufficient, we used an over-abundance of marker loci in the paternity testing to avoid false exclusions due to the inheritance of mutated alleles from the pollen parent to progeny individuals.

When we excluded the offspring resulting from pollen contamination and took into consideration the fact that pollen trees nos. 3 and 5 were clonal (i.e. identical), a total of 149 progeny individuals were tested against the five unique pollen parents, making a total of 745 paternity tests. In these 745 paternity tests, a putative occurrence of a null allele was observed at locus EMBRA6 where pollen parent no. 1 was apparently homozygous. However, 28 out of a total of 66 offspring lacked the obligatory paternal allele. At all other loci offspring and pollen parent shared the paternal allele correctly. This result strongly suggests the occurrence of a null allele in pollen parent no. 1 that, as expected, was transmitted to approximately half (28/66=42%) of its progeny. This locus was not considered in the paternity analysis for pollen tree no. 1.

A total of 745×14= 10,430 allelic transmission analyses were carried out in the paternity testing. There were five putative occurrences of paternal mutations—i.e. where the obligatory paternal allele was different, usually by a single dinucleotide step, from the allele observed in the pollen parent. Again, at all other loci, a full consistency of obligatory paternal allele was observed. The resulting mutation frequency estimate of 4.8×10^{-4} is very similar to recent estimates in maize where the mutation rate per generation was estimated to be 7.7×10^{-4} for microsatellites with dinucleotide repeat motifs (Vigouroux et al. 2002).

When the inconsistencies due to mutations and null alleles were not counted, the sequential procedure of paternity exclusion proved to be a very efficient and accurate method to quickly exclude non-fathers at several marker loci. By this approach, once a pollen parent was excluded in at least three loci, the remaining paternity tests were only carried out with the non-excluded fathers. Typically, however, all non-fathers were excluded in the analysis of the first five more informative loci. The proposed approach is therefore very efficient and should not demand the analysis of a large number of loci. It is obvious that as the number of mother trees and pollen parents increases and the relatedness of these trees also increases, confident paternity testing will require an increased number of polymorphic loci to reach conclusions as multiple males are often found to be genetically compatible with each offspring tested, even when the probability of excluding an unrelated male is high. In such

complex parentage testing situations, the deterministic approach used in this study is usually not possible, and likelihood based paternity inference methods become necessary (Meagher 1986; Marshall et al. 1998; Chaix et al. 2003).

A significant proportion of the analyzed offspring, approximately 29%, was sired by pollen parents located outside of the orchard. Campinhos et al. (1998) estimated a lower contamination rate of 14% in the same orchard based on isozyme markers, possibly due to limited informative polymorphism that would allow correct parental discrimination or simply due to a sampling effect. Outside pollinator trees did not contribute significantly to the generation of superior offspring individuals (Table 5), although one cannot preclude the possibility of some occasional superior trees deriving from pollen outside the orchard. Heavy pollen contamination has been observed in a number of studies with wind-pollinated conifer species (El-Kassaby and Ritland 1986; Harju and Nikkanen 1996; Pakkanen et al. 2000; Moriguchi et al. 2002), typically reducing the expected gains from seed orchards. For eucalypts, a number of studies have estimated the preferential mating system in natural populations and seed orchards using isozymes (e.g. Moran et al. 1989) and, recently, a complex pattern of mating was described in an *E. regnans* seed orchard in Australia. Gene dispersal was influenced by crop fecundity and orchard position of the mother trees with approximately 50% of effective pollen gametes coming from males more than 40 m away from mother trees, indicating that insect pollinators are efficient promoters of cross-fertilization (Burczyk et al. 2002). Furthermore, in a recent outcrossing rate study carried out in an *E. grandis* orchard in Madagascar, a pollination rate from outside the seed orchard of 39.2% was estimated based on six microsatellite markers (Chaix et al. 2003). The results from our study also indicate that in our exotic conditions the 300-m-wide belt of native tropical forest maintained around the orchard to provide genetic isolation has not been effective in preventing insect-mediated gene flow from nearby eucalypt plantations.

Pollen parents nos. 1, 2 and 6 displayed the highest male reproductive success, siring 148 out of the 149 offspring analyzed. All three pollen parents were thus maintained in the seed orchard. Pollen trees nos. 3, 5 and 4 did not father any offspring and were subsequently excluded. The absence of offspring from these parents is probably due to asynchronous flowering in relation to the maternal tree as well as temporal variation in the flower crop, suggesting that physical proximity between eucalypt tree crowns not necessarily implies successful mating in a seed orchard. Sampling effects may also have contributed to a certain extent. Differential male reproductive success was also observed in a recent *E. grandis* seed orchard study, where only 199 out of 349 potential male trees in the seed orchard contributed to the pollination of 440 offspring and at a very variable siring rate (Chaix et al. 2003).

Pollen tree no. 1 sired significantly more selected progeny than non-selected ones in both samples. For the other two pollen trees, either no significant difference was observed between the proportions of sired offspring in the selected versus non-selected samples or, in the case of pollen tree no. 6, significantly more non-selected trees were sired (Table 5). These results indicate a higher SCA and flowering synchrony of pollen tree no. 1 with the maternal tree, ensuing the immediate establishment of a bi-clonal seed orchard composed exclusively of pollen tree no. 1 and the *E. grandis* maternal tree.

Although a bi-clonal seed orchard could have been established, involving only pollen tree no. 1 and the *E. grandis* seed parent, due to spatial distribution of the remaining pollen donors and seed parent ramets and with the objective of maintaining a slightly larger effective population size, it was decided to leave all three pollen parents and to cull only those that clearly did not contribute to the generation of offspring. It seems therefore unreasonable to have accrued a 24% gain in volume growth leaving the same pollen donors, i.e. nos. 1, 2 and 6. However, besides culling the worst parents, a significant improvement was made in the silvicultural management of the orchard so as to minimize external pollen contribution and to improve flowering of the remaining pollen donors. Intensive fertilization regimes were applied to the orchard so as to stimulate abundant flowering, and a number of bee cages were introduced in the seed orchard so as to keep the bees from flying longer distances to harvest pollen and nectar. These measures were likely successful in minimizing external pollen contribution and at the same time increasing pollen contribution from the pollen donors in the orchard. Besides the revitalization of the orchard, it was also noted that the number of grafted ramets of pollen donors nos. 1, 2 and 6 was not balanced due to tree death since the time that the seeds used to establish the commercial plantation studied were harvested. The census number of ramets of pollen donor no. 1 was slightly larger that of pollen donor nos. 2 and 6, possibly due to its more adapted growth and vigor. A potentially larger contribution of pollen parent no. 1 to the new seed crop from the orchard also contributed to the increased volume growth.

As expected, no selfed progeny individuals were found among the selected trees. However, selfs were found in the random non-selected trees at a rate of 8.3%, which is in agreement with the selfing rates typically estimated in eucalypts (Griffin and Cotterill 1988; Gaiotto et al. 1997). This same selfing rate should therefore be expected even in a biclonal seed orchard. Not surprisingly, however, 80% of the stunted trees were selfs, thereby confirming the inbreeding depression effect on growth typically observed in controlled selfing experiments in eucalypts (Griffin and Cotterill 1988; Hardner and Potts 1995). Finally, no selfs were found in the sample of chlorotic trees. The paternity testing results suggest a specific association between pollen parent no. 6 and the appearance of leaf chlorosis, although 11 out of the 30 chlorotic trees were sired by outside pollen parents. The establish-

ment of a more efficient barrier to outside pollen contamination should minimize the manifestation of this symptom.

Our study demonstrates that a marker-assisted breeding tactic involving parentage testing with microsatellite markers can efficiently and rapidly be applied to existing seed orchards with the specific objectives of verifying the differential male reproductive success of pollen parents and identifying those that successfully generate superior offspring. It should be pointed out, however, that the tactic applied in this study represents an alternative measure in situations where time is the critical issue, the breeder is faced with an emergency demand for improved seeds from existing orchards and no data are available on progeny testing of trees but rather only on commercial plantations derived from the seed orchard. This tactic is certainly no substitute for a well-conducted progeny trial followed by estimation of parental breeding values for backwards selection. We have shown, however, that this retrospective selection procedure coupled to improved management of the seed orchard did in fact result in an improved seed variety displaying a significant gain in volume growth over the average performance of the seed lots derived from the original unselected orchard.

This short-term marker-assisted breeding tactic should be applicable to the improvement of old seed orchards currently supplying planting stock of other forest tree species. Although this demonstration experiment was carried out on a relatively small sample of parent trees, given an adequate screening and selection of a battery of informative microsatellite markers, sufficient power of discrimination can easily be attained to resolve paternity and maternity of offspring derived from orchards consisting of several tens of unrelated parents. Relatedness among the orchard parents could be a problem when a limited number of markers are available. Lambeth et al. (2001), after genotyping a set of 45 parental trees at seven chloroplast and three nuclear microsatellite loci, found that more markers would be necessary for unambiguous paternal determinations of progeny from a complete pollen mix due largely to relatedness in the pine population studied.

Finally, this tactic, as opposed to the conventional approach of estimating predicted gain based on controlled pollination and progeny trials, measures realized gain in an operational setting, which is a function not only of the SCA of pairs of parents but also of the effective reproductive success of mating between them, which is often a much more critical variable when large supplies of improved seeds are desired.

Acknowledgements We would like to acknowledge Rosana P.V. Brondani for her valuable advice in the genotyping phase. This work was supported by the Brazilian Ministry of Science and Technology—PADCT—FINEP Competitive Grants in Biotechnology to D.G.; Ministry of Education CAPES with a Master fellowship to V.J.R. and the Brazilian National Research Council-CNPq with a research fellowship to D.G.

References

Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Ann Biochem 196:80–83

Bertolucci FL, Resende GD, Penchel R (1995) Produção e utilização de híbridos de eucalipto. Silvicultura 51:12–16

Brandão LG, Campinhos E, Ikemori YK (1984) Brazil's new forest soars to success. Pulp Pap Int 26:38–40

Brinkmann B, Klintschar M, Neuhuber F, Huhne J, Rolf B (1999) Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat. Am J Hum Genet. 64:1473–1474

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D (1998) Development and mapping of microsatellite based markers in Eucalyptus. Theor Appl Genet 97:816–827

Brondani, RPV, Brondani C, Grattapaglia D (2002) Towards a genus-wide reference linkage map for Eucalyptus based exclusively on highly informative microsatellite markers, Mol Gen Genomics 267:338–347

Brune A, Zobel B. (1981) Genetic base populations, gene pools and breeding populations for Eucalyptus in Brazil. Silvae Genet 30:146–149

Burczyk J, Adams WT, Moran GF, Griffin AR (2002) Complex patterns of mating revealed in a Eucalyptus regnans seed orchard using allozyme markers and the neighbourhood model. Mol Ecol 11:2379–91

Campinhos E (1980) More wood of better quality through intensive silviculture with rapid growth improved Brazilian *Eucalyptus*. Tappi 63:145–147

Campinhos EN, Peters-Robinson I, Bertolucci FL, Alfenas AC (1998) Interspecific hybridization and inbreeding effect in seed from a *Eucalyptus grandis* x *Eucalyptus urophylla* clonal orchard in Brazil. Gen Mol Biol 21:369–374

Chaix G, Gerber S, Razafimaharo V, Vigneron P, Verhaegen D, Hamon S (2003) Gene flow estimation with microsatellites in a Malagasy seed orchard of *Eucalyptus grandis*. Theor Appl Genet 107:705–712

Eldridge KG, Davidson J, Hardwood C, Van Wyk G (1993) *Eucalypt domestication and breeding*. Oxford Science Publ, Oxford University Press, Oxford

El-Kassaby YA, Ritland K (1986) The relation of outcrossing and contamination to reproductive phenology and supplemental mass pollination in a Douglas-fir orchard. Silvae Genet 35:240–244

FAO (2000) Global forest resources assessment 2000—main report. FAO Forestry paper http://www.fao.org/forestry/fo/fra/main/index.jsp

Fisher RA (1934) Statistical methods for research workers, 5th edn. Oliver and Boyd, Edinburgh

Gaiotto FA, Bramucci M, Grattapaglia D (1997) Estimation of outcrossing rate in a breeding population of *Eucalyptus urophylla* s.t. Blake with dominant RAPD and AFLP markers. Theor Appl Genet 95:842–849

Glowatzki-Mullis ML, Gaillard C, Wigger G, Fries R (1995) Microsatellite-based parentage control in cattle. Anim Genet 26:7–12

Grattapaglia D (2000) Molecular breeding of Eucalyptus—state of the art, operational applications and technical challenges. In: Jain SM, Minocha SM (eds) Forestry sciences, vol 64. Molecular biology of woody plants. Kluwer, Dordrecht, pp 451–474

Grattapaglia D, Sederoff R (1994) Genetic linkage maps of *Eucalyptus grandis* and *E. urophylla* using a pseudo-testcross mapping strategy and RAPD markers. Genetics 137:1121–1137

Griffin AR, Cotteril PP (1988) Genetic variation in growth of outcrossed, selfed and open-pollinated progenies of *Eucalyptus regnans* and some implications for breeding strategy. Silvae Genet 37:124–131

Gunn PR, Trueman K, Stapleton P, Klarkowski DB (1997) DNA analysis in disputed parentage: the occurrence of apparently

false exclusions of paternity, both at STR loci, in the one child. Electrophoresis 18:1650–1652
Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R (1994) Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am J Hum Genet 55:175–189
Hardner CM, Potts BM (1995) Inbreeding depression and changes in variation after selfing *Eucalyptus globulus*. Silvae Genet 44:46–54
Harju AM, Nikkanen T (1996) Reproductive success of orchard and non-orchard pollens during different stages of pollen shedding in a Scots pine seed orchard. Can J of For Res 26:1096–1102
Lambeth C, Lee B-C, O'Malley D, Wheeler N (2001) Polymix breeding with parental analysis of progeny: an alternative to full-sib breeding and testing. Theor Appl Genet 103:930–943
Marshall TC, Slate J, Kruuk LE, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–55
Meagher TR (1986) Analysis of paternity within a natural population of *Chamaelirium luteum*. 1. Identification of most-likely male parents. Am Nat 128:199–215
Moller A, Wiegand P, Brinkmann B (1995) Observation of null alleles apparently due to deletions. Int J Legal Med 108:90–92
Mora AL, Garcia CH (2000) Eucalypt cultivation in Brazil. Brazilian Society of Silviculture, Brazil
Moran G, Bell JC, Griffin AR (1989) Reduction in levels of inbreeding in a seed orchard of *Eucalyptus regnans* F. Muell, compared with natural populations. Silvae Genet 38:32–36
Moriguchi Y, Taira H, Tsumura Y (2002) Gene flow of seed orchard in *Cryptomeria japonica* d.don using microsatellite markers. In: Plant Anim Genome 10. San Diego, abstr 562
Namkoong G, Kang HC, Brouard JS (1988) Tree breeding: principles and strategies. Springer, Berlin Heidelberg New York
Pakkanen A, Nikkanen T, Pulkkinen P (2000) Annual variation in pollen contamination and outcrossing in a *Picea abies* seed orchard. Scand J For Res 15:399–404
Rhoads DD, Roufa DJ (1990) Seqaid II 3.80. Molecular Genetics Laboratory, Kansas State University, Manhattan, Kan.
Ribeiro VJ, Brondani RPV, Ciampi AY, Grattapaglia D (1998) Paternity determination of superior open pollinated progenies of *Eucalyptus* based on microsatellite markers [abstr. J24]. Genet Mol Biol 21:212
Valdes AM, Slatkin M, Freimer NB (1993) Allele frequencies at microsatellite loci: the stepwise mutation model revisited. Genetics 133:737–749
Vigneron P (1991) Creation et amelioration de varietes d'hybrides d'Eucalyptus au Congo. In: Intensive forestry: the role of *Eucalyptus*. Proc IUFRO Symp. Durban, South Africa, pp 345–360
Vigouroux Y, Jaqueth JS, Matsuoka Y, Smith OS, Beavis WD, Smith JS, Doebley J (2002) Rate and pattern of mutation at microsatellite loci in maize. Mol Biol Evol 19:1251–1260