IMPROVED MANAGEMENT OF MALAGASY *EUCALYPTUS GRANDIS* SEED ORCHARDS USING MICROSATELLITES AND PATERNITY ASSIGNMENT

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CHAIX G, VIGNERON P, RAZAFIMAHARO V & HAMON S. 2010. Improved management of Malagasy *Eucalyptus grandis* seed orchards using microsatellites and paternity assignment. Open-pollinated *Eucalyptus grandis* seed orchards have been established in Madagascar. Pollen contamination, mating between relatives and an imbalance in gametic contributions affect the efficiency of seed crop quality in seed orchards on which genetic improvement strategies are based. A set of six microsatellite markers was used to carry out parentage tests on 600 progenies harvested from 30 trees in a seed orchard and to identify open-pollinated mating designs, progeny pedigrees and a high genetic admixture in the seed orchard. The trial, which was planted with those progenies and monitored from 9 to 37 months revealed substantial inbreeding depression effects on growth traits due to selfing. Also observed were quite similar allele distributions for the seed orchard and the pollen cloud. This study demonstrated that marker-assisted breeding by parentage analysis based on microsatellites could improve efficiency in open-pollinated seed orchards used as a breeding population and genetic gain could be expected, along with sufficient genetic stability, during selection cycles.

Keywords: Breeding programme, parentage tests, paternity assignment, gene flow, Madagascar

CHAIX G, VIGNERON P, RAZAFIMAHARO V & HAMON S. 2010. Pengurusan kebun biji benih *Eucalyptus grandis* yang lebih baik di Madagascar menggunakan mikrosatelit serta umpukan paterniti. Kebun biji benih *Eucalyptus grandis* pendebungaan terbuka telah ditubuhkan di Madagascar. Pencemaran debunga, proses mengawan antara pokok dan sumbangan gamet yang tidak seimbang mempengaruhi keberkesanan kualiti biji benih tanaman di kebun biji benih yang menjadi asas dalam penggubalan strategi penambahbaikan genetik. Satu set penanda mikrosatelit diguna untuk menjalankan ujian keturunan bagi 600 progeni yang dituai daripada 30 pokok di kebun biji benih. Penanda mikrosatelit itu juga dapat mengenal pasti reka bentuk pengawanan pendebungaan terbuka, salasilah progeni dan bahan tambah genetik yang tinggi di kebun biji benih. Tapak yang ditanami progeni tersebut dipantau selama sembilan hingga 37 bulan. Keputusan menunjukkan bahawa pendebungaan sendiri mengakibatkan kemerosotan pembiakbakaan dalam yang ketara terhadap ciri pertumbuhan. Juga, taburan alel hampir sama bagi kebun biji benih dan awan debunga. Kajian ini menunjukkan bahawa pembiakan berbantu penanda melalui analisis keturunan berdasarkan mikrosatelit dapat menambah baik keberkesanan kebun biji benih pendebungaan terbuka yang diguna sebagai populasi pembiakan. Selain itu, gandaan genetik dijangka akan berlaku bersama-sama kestabilan genetik semasa kitaran pilihan.

INTRODUCTION

In Madagascar, open-pollinated seed orchards were created 10 years ago to meet the market demand for eucalyptus seeds, amounting to around 150 to 250 kg annually. Such seed orchards are used both as breeding populations and seed sources (Lebot & Ranaivoson 1994, Chaix & Ramamonjisoa 2001). For such species and open-pollinated seed orchards, checking progeny composition is crucial because there are three main factors involved in mating patterns: (1) selfing and its consequences of inbreeding depression, (2) pollen pollution from outside, and (3) composition disequilibrium due to differential fertility between pollen donors.

Eucalyptus grandis, an insect-pollinated species, has a mixed mating system. Offsprings are subject to inbreeding depression like most forest population species, especially conifers (reviewed in Williams & Savolainen 1996). They are characterised by a substantial genetic load. In breeding programmes, based on the recurrent scheme and open-pollinated seed orchards, relatedness among individuals can rapidly increase (Durel *et al.* 1996). Outcrossing preserves the population from homozygosity and deleterious genes remain in heterozygous state and enhance the value of trees by heterosis. Mating with relatives or selfing create homozygosity which adversely affects fitness.

Eucalyptus inbreeding depression has been reported to affect various traits, namely, lower seed set (Hodgson 1976, Griffin *et al.* 1987, Sedgley *et al.* 1989, Tibbits 1989, Hardner & Potts 1995), lower seed germination (Eldridge 1978, Eldridge & Griffin 1983) and lower field growth and survival (Hodgson 1976, Wyk 1977, 1981, Eldridge & Griffin 1983, Potts *et al.* 1987, Griffin & Cotteril 1988, Hardner & Potts 1995, Hardner & Tibbits 1998).

The breeding theory, based on the recurrent scheme, requires an equal gametic contribution to the seed crop from each individual in the orchard. Pollen contamination can affect genetic gain which will be jeopardised if non-selected trees contribute to the seed crop. The most frequently reported consequences are a decrease in the theoretical genetic gain and a greater difference between the expected genetic values of variety and the surrounding stands.

Managing the quality and quantity of pollen is important for attributing genetic gain estimations and also for practical management of gene pools. The seed orchard has two objectives which are to maximise breeding progress and preserve genetic diversity. In that context, it is important to know not only the relative outcrossing/selfing rate in breeding populations, but also how much selfing occurs. The degree of selfing can be used to adjust the coefficient of additive genetic variance which is estimated from open-pollinated families when the assumption of a complete half-sib relationship is not met.

Molecular tools and analyses are available to assist breeders. Microsatellite markers are increasingly being used to study gene flow (e.g. Gaiotto *et al.* 1997, Dow & Ashley 1998, Streiff *et al.* 1999, Gerber *et al.* 2000, 2003, Chaix *et al.* 2003, Kirst *et al.* 2005, Jones *et al.* 2008) and paternity assignment enables parental identification and determination of the mating type. The aim of this study, based on an *E. grandis* seed orchard and a progeny test using microsatellite markers, paternity analysis results and growth in the field, was to estimate the efficiency of open-pollinated seed orchards in terms of variety quality and the stability of genetic diversity.

MATERIALS AND METHODS

Plant material

Open-pollinated seeds were collected in 1998 from 30 mother trees with 20 offspring per family (600 in total) in the seedling seed orchard (SSO) described by Chaix *et al.* (2003). This Malagasy *E. grandis* SSO contains 12 open-pollinated progenies corresponding to four Australian provenances and one Malagasy provenance. Plantlets were selected at random and a trial was established in May 2004 near Brickaville, Toamasina province, in eastern Madagascar (19° 01' S, 48° 56' W, about 50 m asl). The mean annual air temperature was 24 °C and annual rainfall was 2300 mm.

A five-tree plot design, with four replicates of the 30 half-sib families was used. The initial spacing between trees was 3 × 4 m (825 trees ha^{-1}) and the stand covered 0.8 ha. The trial was buffered by two rows of *E. grandis* trees.

Paternity assignment and genetic diversity comparison

The paternity assignment model developed by Gerber *et al.* (2003) was used with the approach described by Chaix *et al.* (2003). Plants were genotyped using six microsatellite markers (Embra06, Embra10, Embra11, Embra13, Embra15, Embra19) published and described by Brondani *et al.* (1998) and Chaix *et al.* (2002). Results of the paternity analysis helped to identify pollen donors and pedigree were deduced.

Four mating types or inbreeding levels were deduced from the paternity assignment and knowledge of the two parents for each progeny: selfed (group a), half-sib matings (group b), within-provenance matings (outcrossed progenies but with an unknown degree of relatedness, group c) and between-provenance matings (strict outcrossing progenies, group d). The haplotype of the male parents of all progenies was inferred from the paternity assignment results. Pollen clouds from both outside and inside the SSO were deduced by subtracting the female contribution from SSO multilocus genotype.

Statistical analysis

Relationships between mating types and height, circumference/bark at breast height (1.3 m), bole volume, which were all measured and calculated at 9, 12, 23, 31 and 37 months after planting, were examined using progeny trial data. The initial experimental design was elaborated to analyse family effects. In addition, to limit environmental effects and consider mating effects, transformations of variables were carried out. The experimental protocol was regarded as completely randomised.

The following linear model was fitted using the SAS GLM procedure (SAS 1989–1996, Windows version 6.12) to study the family and mating effects:

$$Y_{ijk} = \mu + F_i + MT_{j/i} + \varepsilon_{ijk} \qquad (1)$$

where

Y_{ijk} = the trait value of the tree in the i^{th} family in mating type j in family i
μ = the overall experimental value
F_i = the effect attributed to the i^{th} family
$MT_{j/i}$ = the effect associated with the j^{th} mating-type hierarchy in the family
ε_{ijk} = the residual of Y_{ijk} not accounted for by any of the preceding terms.

All effects, except for the overall mean, were considered to be random effects with variances σ^2_F, σ^2_{MT} and σ^2_E. Mean multiple comparisons by *t*-tests were performed after variance analysis.

Inbreeding depression values (ID) for the different variables and for the different ages were estimated by

$$ID = 1 - S/O$$

where

S = the performance of selfed progenies or half-sib mating progenies (groups a and b)
O = the performance of out-crossed progenies (group d).

RESULTS AND DISCUSSION

Progeny typology

Among the 600 progenies derived from the 30 mother trees, 321 and 279 were sired respectively by 175 pollen parents located inside the orchard and unknown pollen parents located outside the SSO (Figure 1). Of the 321 progenies, for which a paternity analysis was conducted to identify the open-pollinated mating design, 18 occurred through selfing (group a), corresponding to an outcrossing rate of 97% for all progenies. Of the 303 unselfed progenies, three groups were distinguished according to the degrees of parental relationship, namely, 38 progenies (11.8%) from half-sib matings (group b), 67 progenies (20.9%) from within-provenance matings (group c) and 198 progenies (61.7%) from strict outcrossing matings (group d).

No distinction could be done for progenies issued from full-sib matings which might be included in group b. In group c, several undefined degrees of parental relationships were included and not distinguished. In group d, we considered crosses between unrelated parents (as strict outcrossing) because the provenances were isolated at least 14 km (from 14 to 220 km) from each other. On the whole, the progenies were classified into four categories according to inbreeding levels.

The 161 fathers identified for the 321 progenies confirmed strong genetic admixture in the SSO. Earlier results from the same orchard showed that individual parental contributions to the pollen cloud were weak, well balanced and independent of tree development or their origins; on the other hand, there were no dominant pollen donors, no mating units and pollination success was independent of distance (Chaix *et al.* 2003). These results were contradictory to those obtained elsewhere showing that matings were not random in relation to the pollination distance for *E. regnans* (Burczyk *et al.* 2002) or *E. grandis* seed orchard (Jones *et al.* 2008). This might be due to ecological condition differences, the quality of foraging pollinators or the limits of the stand.

Mating types and growth values

Significant family and cross effects were recorded for growth traits (height, circumference at breast

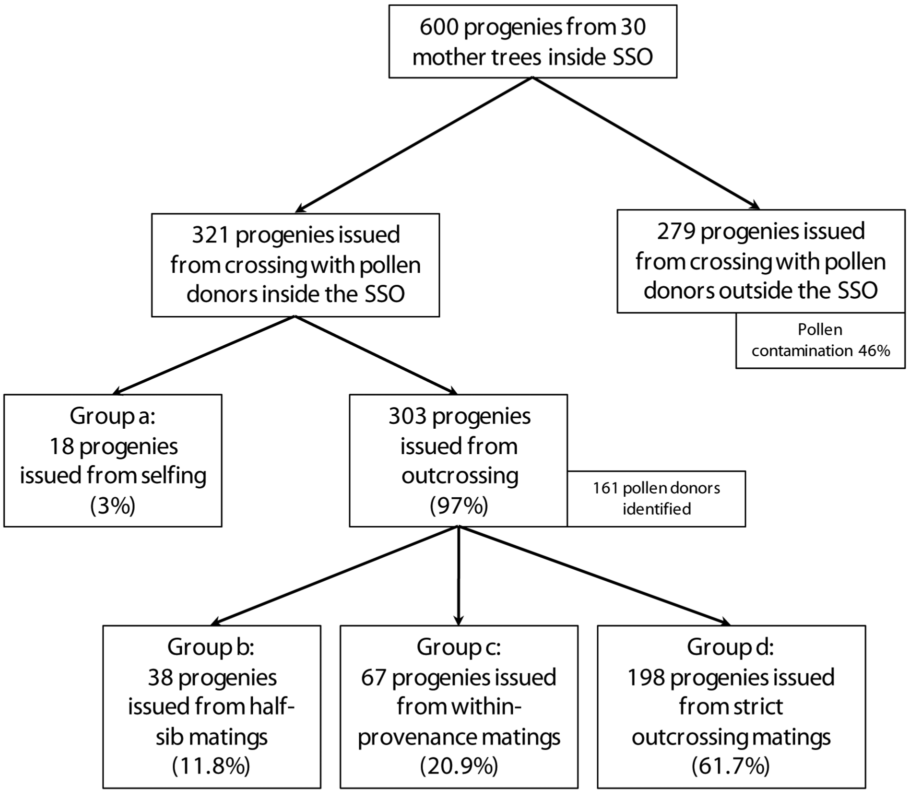

Figure 1 Pedigree identification of the 600 progenies collected inside the seedling seed orchard

height and bole volume from 9 to 37 months) in the progeny trial, apart from a lack of family effect on bole volume in the early stages (9 months) (Table 1). The family and type of crossing effects decreased from 31 to 37 months because several trees measured at 31 months died.

Overall, there were highly significant differences between selfed progenies and all the other mating structures (Figure 2). For the selfed progenies, circumferences at breast height of half-sib matings from 23 to 37 months and the bole volume at 31 months were significantly lower compared with progenies issued from the other mating types. At 37 months, the mean values of circumference at breast height were significantly different for the different groups of mating progenies. There were no significant effects on height, except at 31 months between the within-provenance mating progenies and those from outcrossing (Figure 2).

Inbreeding depression effects were estimated from 9 to 37 months respectively for the selfed progenies (Figure 3a: inbreeding coefficient F = 0.5) and for the half-sib matings (Figure 3b: F = 0.125). The mean inbreeding depression effects due to selfing reached 20, 35–40 and 55% respectively for height, circumference at breast height and bole volume. Except for height growth, the inbreeding depression effects seemed to be independent of age. Except for the early stage (9 months), the mean inbreeding depression values due to half-sib matings were between 5 and 15%. These values were lower than for selfing and were rarely significant, as shown in Figure 2.

Inbreeding depression effects in cross-fertilising forest species, especially for *Eucalyptus*, are greater in selfed trees. There is abundant literature showing that selection pressures on these genotypes are expressed in the field phase, depending on the species and the environmental conditions (Hodgson 1976, Wyk 1977, 1981, Eldridge & Griffin 1983, Griffin *et al.* 1987, Potts *et al.* 1987, Griffin & Cotteril 1988, Moran *et al.* 1989, James & Kennington 1993, Hardner & Potts 1995, Burgess *et al.* 1996, Durel *et al.* 1996, Hardner & Tibbits 1998, Butcher & Williams 2002, Wang *et al.* 2004).

The high inbreeding depression effects for selfed trees may decrease the variety quality and contribute to a reduction in genetic diversity in the breeding population. However, outcrossing approaching 97% for random sample progenies was reported by Chaix *et al.* (2003). Undoubtedly, culling at the nursery stage would increase

Table 1 Analysis of variance results (variance ratio and significance level) for height, circumference at breast height and bole volume from 9 to 37 months in the family trial

Growth trait	F	df	9 months	12 months	23 months	31 months	37 months
Height	F_{family}	28	1.62 (0.0289)	2.18 (0.0009)	2.48 (< 0.0001)	2.51 (< 0.0001)	1.97 (0.0035)
	$F_{\text{mating (family)}}$	44	2.13 (0.0002)	2.12 (< 0.0001)	2.02 (< 0.0001)	2.27 (< 0.0001)	1.75 (0.0042)
Circumference at breast height	F_{family}	28	-	2.11 (0.0014)	2.35 (0.0003)	2.71 (< 0.0001)	2.59 (< 0.0001)
	$F_{\text{mating (family)}}$	44	-	2.15 (< 0.0001)	2.33 (< 0.0001)	2.20 (< 0.0001)	2.31 (< 0.0001)
Bole volume	F_{family}	28	1.48 (0.0622)	1.86 (0.0069)	2.37 (0.0002)	2.75 (< 0.0001)	2.25 (0.0005)
	$F_{\text{mating (family)}}$	44	2.10 (0.0002)	1.95 (0.0008)	2.36 (< 0.0001)	1.90 (0.0012)	1.85 (0.0018)

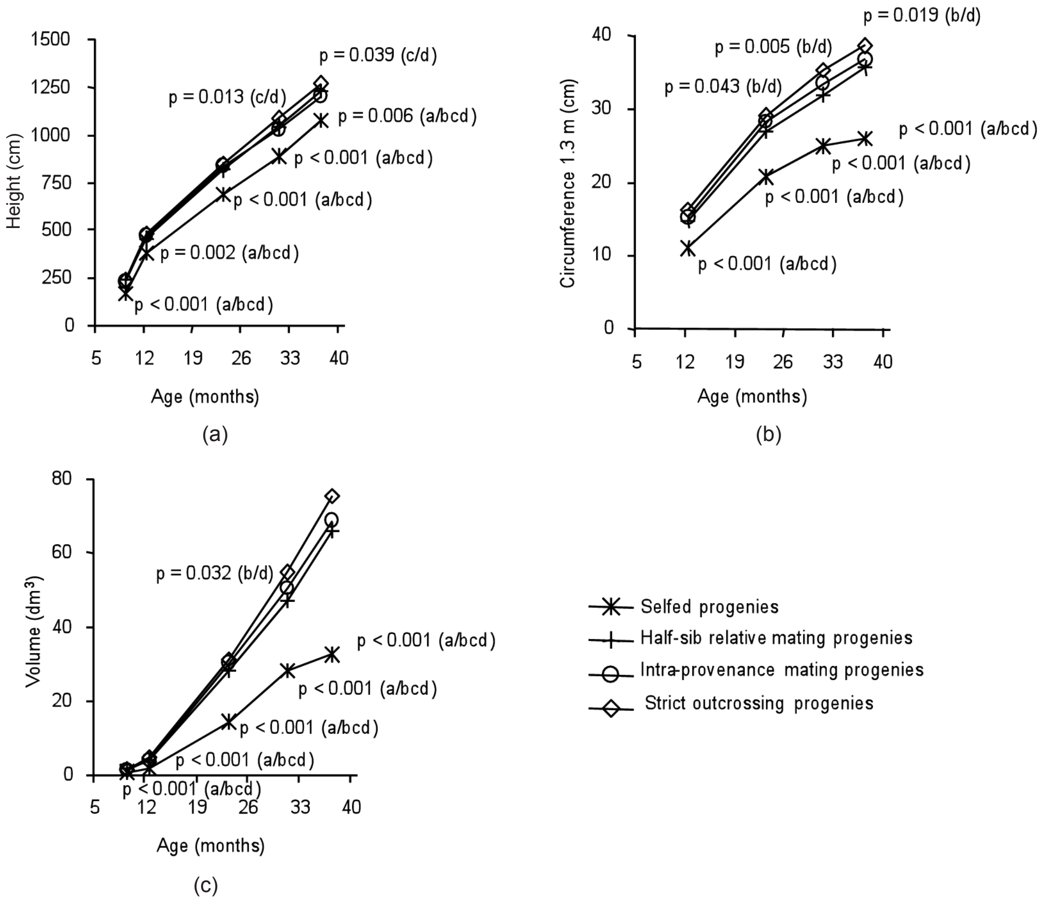

Figure 2 Mean comparisons for growth traits and mating types from 9 to 37 months: (a) height, (b) circumference at breast height and(c) volume of mating progenies

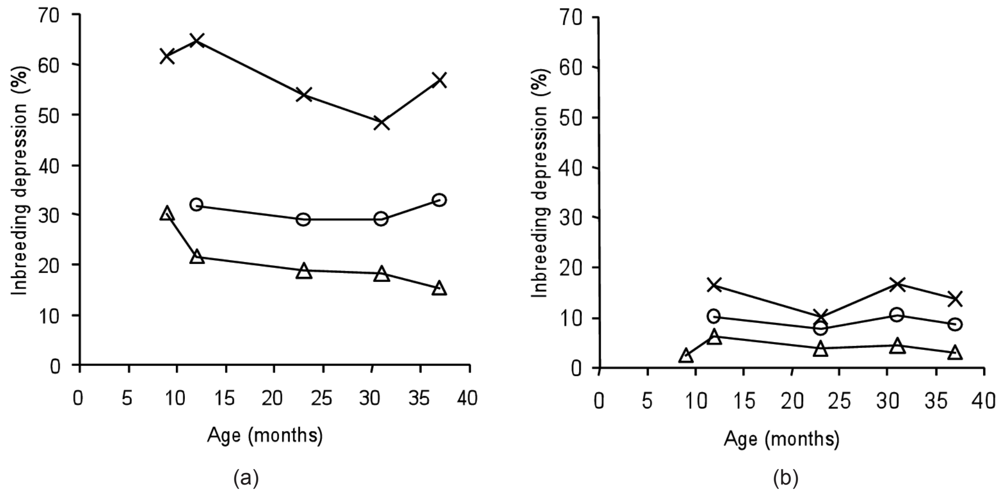

Figure 3 Inbreeding depression effect (%) from 9 to 37 months on height (—Δ—), circumference at breast height (—o—) and bole volume (—×—) due to (a) selfed progenies (F = 0.5) against unselfed progenies and (b) half-sib mating progenies (F = 0.25) against unselfed progenies

outcrossing rate and, according to their growth due to selfing effects, natural and artificial selections would eliminate the inbreeds during the first phase under field conditions.

It has been reported that depression levels of 40 and 15% were observed for trees from selfed trees and full-sib matings respectively, but there was no effect for half-sib matings (Wyk 1981) and these results were similar to those obtained in this study. Hodgson (1976) reported the same results but with a 31% depression effect on height in two-year-old *E. grandis* trees. The consequences of those inbreeding depression effects were probably insignificant for the evolution of genetic diversity because heterozygosity will be restored after the next generation and enhanced by the high genetic admixture in the open-pollinated SSO. The growth of trees with a low inbreeding level, derived from full- or half-sib matings, was less affected probably because there was a high level of heterozygosity in cross-fertilising species. Although the inbreeding effect on variety quality was not significant for the quality produced by the SSO, we nevertheless expect that those mating types could have consequences for the degree of inbreeding in breeding populations over several selection cycles.

Genetic diversity comparisons between pollen clouds and SSO

The haplotype of the male parent of offspring was inferred by subtracting the female contribution from its multilocus genotype. Two populations were deduced, namely, the SSO pollen cloud with 321 gametes and the pollen cloud constituted by fathers from outside the SSO with 279 gametes. The allelic distributions between the two pollen clouds and the SSO (600 gametes) were compared.

The allele distributions for six microsatellite comparisons between the total pollen cloud and the SSO population and inside/outside pollen clouds are shown in Figure 4. For all loci, allele distributions were quite similar. The observed differences concerned rare alleles in the parental population and progenies respectively. There were no significant differences between pollen clouds and the observed variations corresponded to rare alleles from the outside pollen cloud.

In terms of genetic diversity between the two generations, results showed that there was no loss because allele patterns were very similar and weak variations mainly involved alleles at a low frequency in the parental population. These results are encouraging because the sample of 321 progenies, harvested from 30 mother trees, amount to less than 10% of the parental population. Under such conditions, the stability of genetic diversity over several generations is guaranteed in commercial or selection use when harvested from more mother-trees.

The pollen contamination rate was lower than that observed in seed orchards or plantations of wind-pollinated species (Dow & Ashley 1998, Streiff *et al.* 1999, Pakkanen *et al.* 2000, Buiteveld *et al.* 2001) but similar to that for insect-pollinated species (Lee 2000, Konuma *et al.* 2000, Kameyama *et al.* 2000). The comparison between the parental population and progeny sample showed that the allele distributions were similar. Nevertheless, the use of highly polymorphic microsatellites was sufficient for within-species discrimination between provenances. A population of *E. robusta* was located nearest the SSO but another *E. grandis* SSO, which was partially planted with the same provenances, was situated 400 m away but had allele diversity close to the studied SSO. Consequently, it was assumed that most of the contamination came from that *E. grandis* SSO.

Managing genetic diversity in SSO

Our study demonstrates that marker-assisted breeding by parentage analysis with microsatellites, as expected from phenological observations (Chaix *et al.* 2007), can be efficient for managing breeding populations.

With the same tools, Grattapaglia *et al.* (2004) proposed an approach which achieved an average gain of 24.3% in volume by verifying differential male reproductive success, identifying those that successfully generated superior offspring and culling those parents in a hybrid seed orchard that displayed low reproductive success. The tactic we applied could be used to keep only outcrossing progenies to build up breeding populations for the next SSO plantations. It is better to cull undesirable individuals (selfed and related progenies) during the nursery stage prior to planting. This can be improved by genotyping with sequencer genotyping combined with microsatellite primer amplification by multiplexing (Chaix *et al.* 2002).

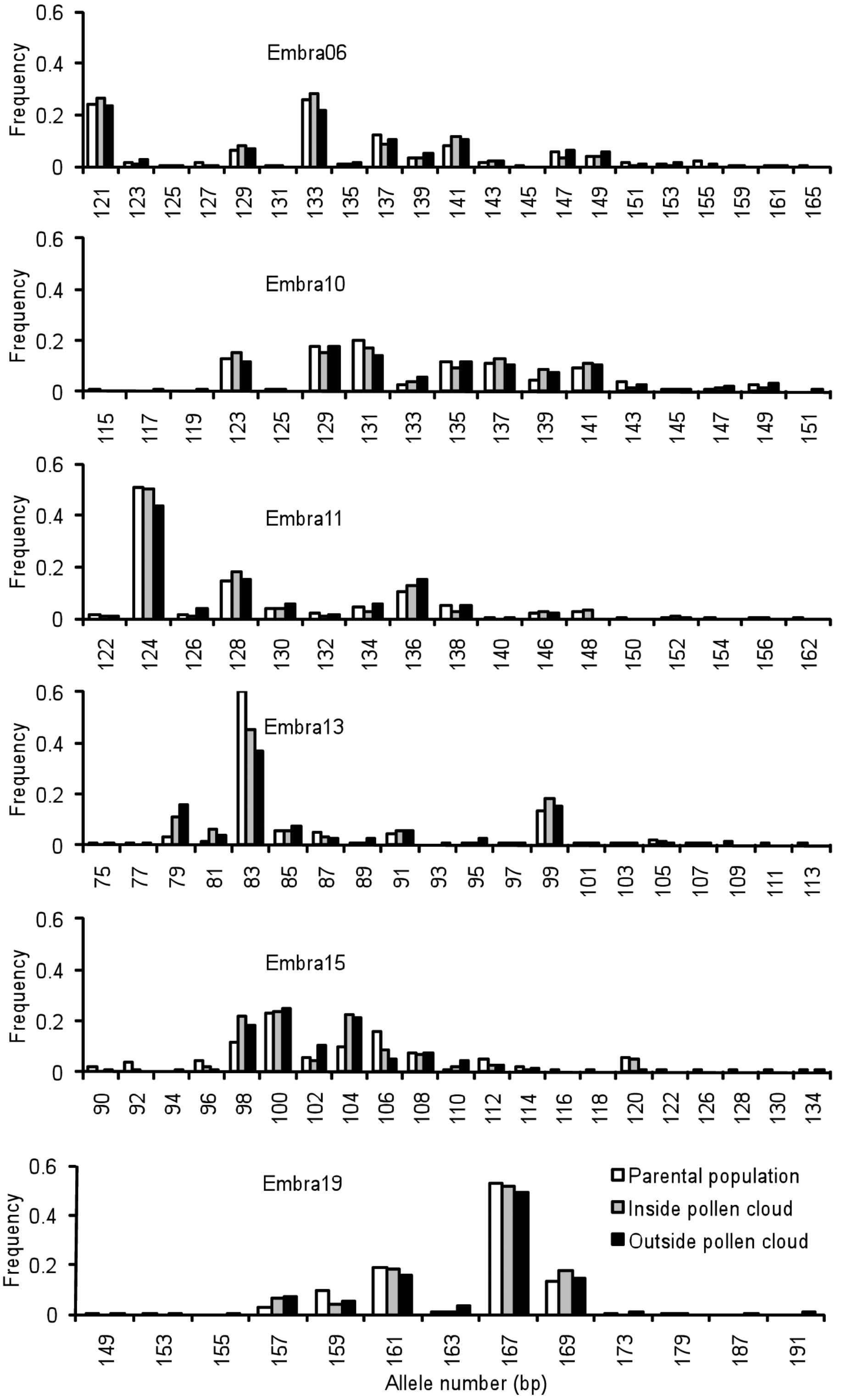

Figure 4 Allele frequency comparisons for the six EMBRA microsatellites loci (Embra06, Embra10, Embra11, Embra13, Embra15, Embra19), from Brondani *et al.* (1998), in the parental population, inside pollen cloud and outside pollen cloud; bp = base pairs

CONCLUSIONS

This study which was monitored from 9 to 37 months revealed high inbreeding depression effects on growth traits due to selfing. In terms of allele variability between the two generations, distributions between the SSO and the pollen cloud were quite similar, suggesting a good admixture of the pollen cloud and stability of genetic diversity.

Our study demonstrates that marker-assisted breeding by parentage analysis with microsatellites can be efficient for managing breeding populations. According to the high genetic admixture in the SSO demonstrated by parental analysis we expect genetic gain and stable genetic diversity over selection cycles.

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