

Pedigree reconstruction and genetic parameter estimation in a hybridization orchard of *Eucalyptus camaldulensis* and *E. urophylla*

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Abstract

Pedigree construction using molecular markers in open pollinated bulk seeds is proven to be one of the effective approaches in providing robust information about mating dynamics in the seed orchard. In addition, pedigree construction combined with phenotypic pre-selection of the progeny trees will eventually reduce the cost of genotyping and time. In the present study, we used highly informative microsatellite markers to construct half-sib and full-sib families from phenotypically pre-selected individuals for growth in an open pollinated hybridization seed orchard consisting of *E. camaldulensis* and *E. urophylla*. All the progenies were assigned with their respective female parents, while 84 % of the progenies were assigned with their candidate male parents. Both single locus (f_s) and multi-locus (f_m) outcrossing estimates revealed high level of outcrossing rate in the seed orchard. Intra-specific combinations in the progenies were found to be high compared to inter-specific hybrids. Variance components estimates using half-sib and full-sib based model revealed high heritability (h^2) for diameter at breast height (DBH) and height in the half sib model. We also found significant correlation between the breeding values estimated for parents and progenies in the half-sib and full-sib model using Best Linear Unbiased Prediction (BLUP) method. Present study demonstrates that molecular marker based pedigree construction combined with phenotypic pre-selection from open pollinated bulk seeds is an effective approach for identifying superior parents and studying mating dynamics in the seed orchard. In addition, breeding value estimates could eventually increase the efficiency and accuracy of forward, backward, and mixed selection models.

Keywords: *Eucalyptus*, seed orchard and parentage analysis

Introduction

For decades, forest tree breeders have employed a controlled crossing method to estimate genetic parameters, enabling the selection of superior clones for breeding purposes and the establishment of seed orchards. (White et al. 2007). However, number of controlled crosses needed for genetic analysis in a forest breeding program is usually high. Often this approach could be hampered due to reproductive phenology variation, parental fecundity and requires more resources. To overcome the constraints associated with the controlled cross approach, Lambeth et al. (2000) proposed the use of molecular markers for paternity assignment. This method aims to transform half-sibling families into full-sibling families within a polymix breeding population, facilitating the estimation of variance components and breeding values. Subsequently, Grattapaglia et al. (2004) utilized a similar strategy to develop a comprehensive pedigree from open-pollinated half-sib families in *Eucalyptus*. El-Kassaby et al. (2006) introduced 'Breeding without Breeding (BWB)' strategy in forestry, where he used DNA variation to reconstruct the half/full pedigree families from incomplete pedigrees. The authors also employed an animal model to estimate quantitative genetic parameters through the BLUP procedure, focusing on half-sib and full-sib families for selection purposes. BWB estimates variance components from both complete and incomplete pedigrees, whereas the earlier approaches (Lambeth et al. 2000, Grattapaglia et al. 2004) required complete pedigree information. Molecular marker-based pedigree reconstruction is widely utilized in animal breeding and is increasingly being adopted in forest breeding programs (Grattapaglia et al. 2004; Gaspar et al. 2009; Hansen et al. 2010; Doreksen et al. 2010; El-Kassaby et al. 2009; El-Kassaby et al. 2011; Lstibůrek, M et al. 2015 and Lee et al. 2022). This analysis of pedigree reconstruction is performed to enhance the existing tree improvement programs. (Klapste et al.

2017; Boufer et al. 2019). In addition, combination of BwB and phenotypic pre-selection appears to be an effective approach for selecting superior parents, estimating variance components and breeding values, due to the reduction in total number of trees to be genotyped and hence cost and time effective (Lee et al. 2022)

The genus *Eucalyptus* is the most widely cultivated hardwood, comprising over 700 species utilized for timber, pulp, and paper production across tropical, subtropical, and temperate regions. It covers an area exceeding 20.07 million hectares worldwide. India, Brazil, and China are the leading countries in eucalyptus cultivation (Global *Eucalyptus* Map 2009). The species *E. camaldulensis* is primarily grown in India, mainly due to the wide adaptability, fast growth and tolerance to salinity and drought (Ladiges et al. 1995). Majority (80 %) of the commercial *Eucalyptus* plantation in India are based on unimproved seeds (Griffin 2014). In general, seeds obtained from seed producing area are recommended for large scale plantation as they provide high quality seeds and serves as a good gene pool for breeding programs and also provide low cost option for the farmers (Varghese et al. 2009; Ladiges et al. 1995). The genetic quality of seeds derived from a seed orchard is significantly influenced by factors such as the mating pattern, outcrossing, inbreeding, and the rate of contamination. Hence understanding the mating system in seed orchard is essential in tree improvement program. The current study is aimed at identifying superior parents/progenies and understanding the mating system dynamics in a hybridization seed orchard of *E. camaldulensis* and *E. urophylla*. We used nuclear microsatellite markers for pedigree construction using bulk seeds, collected with unknown parentage. Results were used to evaluate parental reproductive success, selfing rate, contamination rate and effective number of contributing parents in the seed orchard. In addition, the reconstructed pedigree was further used to estimate breeding values for DBH and height in the parents and progenies using half/full sib BLUP model.

Materials and Methods

Plant materials

Open pollinated bulk seed was collected from the hybridization orchard established in ITC-LSTC research station, Mettupalayam (11°9' N, 77°56' E) India. The orchard was established during 2008, comprising of 33 *E. camaldulensis* and 13 *E. urophylla* parents. Clones used in this study are of second generation origin described by Kamalakannan et al. (2007, 2016). In brief, the parent trees of *E. camaldulensis* are chosen from a seedling seed orchard that was established using bulked seed sourced from over 500 selected trees representing various natural Australian provenances, including Petford, Emu Creek, Gilbert, Kennedy, Laura, Morehead Rivers, and Katherine. *Eucalyptus E. urophylla* parent trees are chosen from a progeny trial that was established using seeds collected from 100 selected trees from Timor Island, Indonesia. Fertility variation was low in the orchard. To avoid over representations of high fertile trees, equal quantity of seeds were mixed from each mother trees.

Open pollinated bulk seeds were collected from *E. camaldulensis* parents, and sown on sand media in green house and germinated. About 5 cm tall seedlings were transplanted into 6x4" size polybags containing Red Soil: Farm Yard Manure: Sand media in the ratio of 1:1:1. A total of 600 seedlings were selected based on seedling vigour (Height) and planted in 3 x 1.5 m spacing at Visakhapatnam, India (red sandy soil, 17.6800° N, 83.0200° E). At two years, 118 superior trees (out of 600) were selected based on diameter (DBH) and tree height.

DNA extraction and genotyping

Leaf samples were collected from 46 parental trees and 118 phenotypically preselected progenies. DNA was isolated using bead beater in conjunction with Xcelris plant gDNAmini kit (Ahmedabad, India). The quality and concentration of the extracted DNA was determined by 0.8 % agarose gel electrophoresis and a set of nine microsatellite markers were used for PCR amplification (Nagabhushana et al. 2011). Multiplex PCR amplification of microsatellite markers were performed using AmpliTaq® Gold DNA polymerase. PCR reactions were performed in a total volume of 10µl reaction, containing 10ng of genomic DNA, 0.4mM of each dNTPs and 5pmol of forward and reverse primers (upto three primer pair each), 1x-buffer, 1.5mM MgCl₂ and 0.25 unit of AmpliTaq Gold DNA polymerase (0.1µl). Amplification of the PCR reaction was carried out using the following cycling condition; initial denaturation of 94° C for 5 minutes followed by 35 cycles of denaturing at 94° C for 1 minute, annealing at 60° C for 45 seconds and extension at 72° C for 2 minutes followed by final extension 72° C for 7 minutes. Microsatellite primers were labelled with phosphoramidite fluorescent labels (6 FAM, 5'HEX, 5'ROX), which were further analyzed for automated fragment analysis on an ABI PRISM 3100 genetic analyzer. The resulting Electropherogram was analysed using peak scanner software (Thermo Fisher Scientific Inc.).

Analysis of genetic diversity

Genetic diversity parameters such as average number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He) and polymorphic information content (PIC) in the parents and progenies under Hardy-Weinberg equilibrium was estimated using CERVUS 3.4 (Marshall et al. 1998; Kalinowski et al. 2007).

Paternity analysis

Paternity and maternity analysis was conducted using standard maximum likelihood methods implemented in CERVUS 3.4 (Marshall et al. 1998; Kalinowski et al. 2007). Simulation analysis was carried out to determine the critical value of Delta (D) for each confidence in the paternity and maternity analysis. For simulations, we used 50,000 iterations; the proportion of mistyped loci at 0.01; confidence levels of 95 % (strict) and 80 % (relaxed). Firstly, the analysis was carried using *E. camaldulensis* as a putative mother trees for each seed-tree as the seeds were bulked only from *E. camaldulensis* parent. Maternity was assigned to a putative mother only when the D criterion associated with a 95 % confidence level. Similar analysis was carried

out to determine the paternity using all parents as candidate fathers. If the progenies that were not assigned to specific known candidate male parent, paternal trees were considered to be located outside the orchard and the progenies was not assigned to any parental candidate. Progeny array with more than five individuals assigned to putative *E. camaldulensis* mother tree were used to determine outcrossing rates. The multilocus, mixed mating model of MLTR 2.3 (Ritland, 2002) was used to determine multi-locus ($\hat{t}_m$), single-locus ($\hat{t}_s$) and bi-parental inbreeding ($\hat{t}_m - \hat{t}_s$) in the orchard. Standard error for mating system parameters were estimated based on 1000 bootstrap replicates.

Variance component and prediction of breeding values

A general linear mixed model was used to analyse the quantitative data using progeny array of more than two individuals assigned to maternal tree (Ronningen and Van Vleck, 1985). Two different models were used to estimate the variance components.

$$Y = Xb + Z1a + e \quad (1)$$

$$Y = Xb + Z1a + Z2f + e \quad (2)$$

where Y is a column vector containing the phenotypic values for the traits measured in the progenies; b is a fixed effect of overall mean; a is a random additive genetic effects of individual trees (progenies as well as parents); f is a random full-sib family effects and e is a normally distributed random error, the latter being assumed to be distributed independently of the random genetic effects. X, Z1 and Z2 are incidence matrices, which relate the observations in y to the effects in b, a and f. Restricted Maximum Likelihood was used to estimate variance components using ASReml program (Gilmour et al. 2006). Narrow sense heritability was calculated from the estimated additive genetic variance and the residual variance (REML, Henderson, 1984). BLUPs of parents and progenies breeding values were estimated from variance components using ASReml (Gilmour et al. 2006). Two models were analysed using two different pedigree file. In traditional half-sib model, we used known female parents, whereas in full-sib model, we used individuals with known paternal and maternal information. Pearson correlations were calculated for breeding values obtained from half/full sib models for parents and progenies. The accuracy of breeding values was estimated by correlation between BLUP estimated breeding values and true breeding value.

Results

Nine microsatellite markers used in this study were highly polymorphic displaying a total number of alleles ranging from 7 to 23 allele per locus with an average of 14.8 alleles per locus. The H_o and H_e ranged from 0.83 to 0.93 (average, 0.87) and 0.77 to 0.91 (average, 0.86) respectively, while PIC ranged from 0.72 to 0.93 (average, 0.85). Combined non-exclusion probabilities for the first parent, second parent and parent pairs are

4.03E-05, 2.4E-07 and 4.90E-12, respectively (Supplementary table S1).

Maternity analysis using CERVUS assigned maternity to all the progenies with 95 % confidence. All the progenies in the array displayed at least one of the maternal allele of the *E. camaldulensis* individuals in the orchard, confirming Mendelian inheritance of segregation, which also suggests no contamination of seeds. Pedigree reconstruction captured progenies for 17 out of 33 candidate mothers present in the orchard. Paternity analysis assigned male parent for 98 out of 118 progenies with 95 % confidence interval, which equals to 84 % of the progenies. If we consider the 98 seedlings fathered by male parents from the seed orchard, 27 trees (62 %) participated as pollen donors. Of these, 18 fathered more than one offspring. Each father tree had 1 to 16 partners on 17 maternal trees. Seed orchard parent EC214 is the most represented (37 maternal and 16 paternal) followed by EC212 (20 maternal and 8 paternal) and EC217 (6 maternal and 7 paternal). The maternal half-sib and full sib family size ranged from 1 to 37 and 1 to 6 respectively (Figure 1, Supplementary figure S1). The variation in the half-sib and full sib family size between the families could be due to the fertility difference between individual trees during the season/time of seed collection. Paternity analysis also revealed that 78 % of the full-sib families were due to intraspecific mating between *E. camaldulensis* x *E. camaldulensis* and remaining 22 % of the progenies resulted from interspecific hybridization between *E. camaldulensis* x *E. urophylla*. The low number of interspecific hybrids found in this study could be explained by presence of few *E. urophylla* (30 %) trees in the orchard compared to *E. camaldulensis* (70 %).

Maximum likelihood estimates of multi-locus outcrossing rate under the mixed-mating model was carried out using 1000 bootstraps with standard error of zero using six families, which had minimum of five progenies. Both average $\hat{t}_m$ and $\hat{t}_s$ estimates were close to one in the hybridisation seed orchard (Table 1). Estimates of bi-parental inbreeding ($\hat{t}_m - \hat{t}_s$) ranged from -0.37 to 0.03. Low or negative value of $\hat{t}_m - \hat{t}_s$ found in this study indicates mating among relatives is not a common occurrence (Gaiotto et al. 1997; Naito et al. 2005). A minimum maternal half-sib family size of more than two individuals was used for the variance component estimation. Descriptive statistics for DBH and height in the phenotypically pre-selected individuals was given in table 2. Using classical individual-tree additive model, analysis were conducted using half sib and full-sib families. Heritability of DBH and height was higher in the half-sib model compared to full-sib model (Table 3). Comparison of breeding values between two models for the maternal parents and progenies revealed significant correlation ($r=0.95-0.95$, $p=0.001$ - maternal; $r=0.84-0.89$, $p=0.001$ -offspring, Figure 2) Accuracy of predicted breeding values for DBH and height was similar in both the models (DBH 0.59 vs 0.62 and Height 0.59 vs 0.64, full-sib and half-sib models respectively).

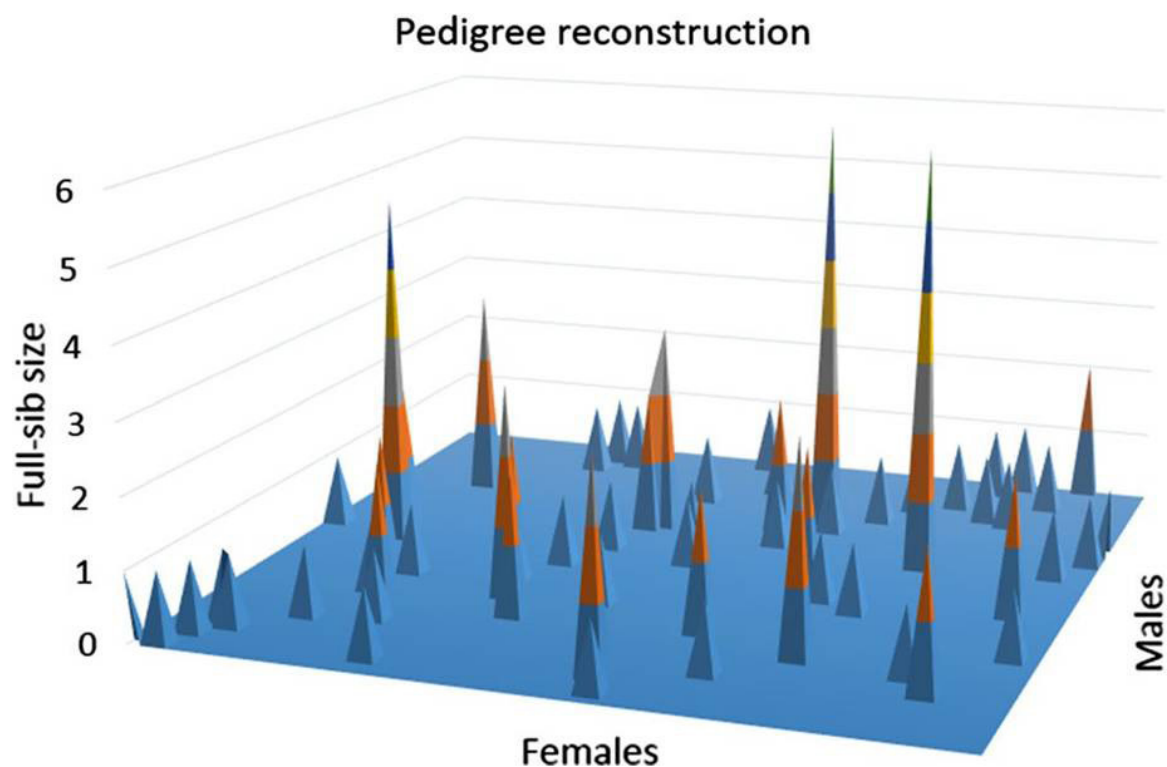


Figure 1

Mating dynamics in an *E. camaldulensis* and *E. urophylla* hybridization orchard revealed by pedigree reconstruction from random samples of progenies with unknown parental information using nine microsatellite markers. Each column represents the size of a unique full-sib family (vertical axis).

Table 1

Mating system parameter at family level in *E. camaldulensis* and *E. urophylla* hybridization orchard after pedigree reconstruction using microsatellite markers. ($\hat{t}_s$, single-locus outcrossing rate; $\hat{t}_m$, multi-locus outcrossing rate; $\hat{t}_m \hat{t}_s$, biparental inbreeding)

Family	Number of progeny	$\hat{t}_s$ (SE)	$\hat{t}_m$ (SE)	$\hat{t}_m \hat{t}_s$
Fam1	6	0.97 (0.03)	1 (0.001)	0.03
Fam2	5	1.11 (0.04)	1 (0.001)	-0.11
Fam3	6	1.10 (0.04)	1 (0.001)	-0.11
Fam4	37	1.37 (0.04)	1 (0.001)	-0.37
Fam5	17	1.17 (0.04)	1 (0.001)	-0.17
Fam6	5	1.11 (0.03)	1 (0.001)	-0.11

Table 2

Descriptive statistics of the DBH and height in the phenotypically pre-selected individuals at the age of two. (SD, standard deviation; CV, coefficient of variation)

Trait	No of trees	Mean	Minimum	Maximum	SD	CV%
DBH (cm)	118	6.09	4.52	8.09	0.69	11.6
Height (m)	118	6.39	5.00	8.00	0.50	8.2

Table 3
Estimates of variance components and narrow sense heritability for DBH and height in the half-sib and full-sib model

Source of Variations	DBH		Height	
	Half-sib	Full-sib	Half-sib	Full-sib
Additive	0.14	0.12	0.09	0.03
Error	0.38	0.44	0.19	0.19
Narrow sense heritability	0.27 (± 0.5)	0.23 (± 0.5)	0.32 (± 0.6)	0.13 (± 0.5)

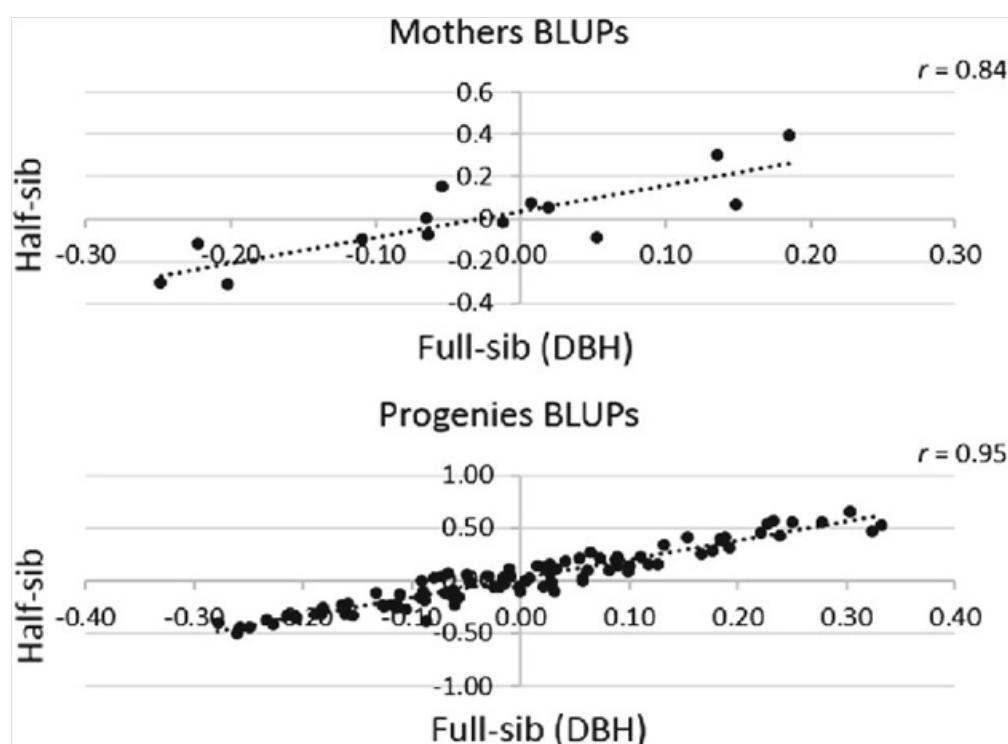


Figure 2
Relationship between breeding values estimated for DBH from reconstructed half-sib and full-sib pedigree.

Discussion

In this study we used nine microsatellite markers to construct the pedigree from the bulk seeds collected from open pollinated seed orchard of *E. camaldulensis* and *E. urophylla* trees. Controlled crosses and progeny testing are important components of tree breeding programs. The use of molecular markers for pedigree reconstruction from open-pollinated seeds eliminates the significant costs and labor associated with controlled crosses (El-kassaby et al. 2015). The traditional method for determining the most effective crosses or parental combinations necessitates the execution of hundreds of crosses within a diallel and half-diallel mating design (Isik, 2009). Whereas pedigree reconstruction and phenotypic pre-selection of individuals allowed us to identify best combinations of parent's

producing superior individuals without attempting a single cross and reduced the cost of genotyping and time. Furthermore, this method enabled the identification of the parent trees that play a significant role in the formation of both natural interspecific and intraspecific progenies. We used 46 parental trees present in the orchard to evaluate the mating design. Clone 214 showed good combining ability with majority of the *E. camaldulensis* trees in the orchard. Similarly clones 212, 217 and 172 showed good combining ability with specific *E. camaldulensis* and *E. urophylla* trees (Supplementary figure 1). All progenies assigned to clone 214 resulted from intraspecific hybridisation (*E. camaldulensis* x *E. camaldulensis*), whereas all progenies assigned to clone EC172 resulted from interspecific hybridization between *E. camaldulensis* x *E. urophylla* and few mother clones produced both interspecific and intraspecific

progenies. The number of full-sib families obtained using pedigree reconstruction approach is reported to be high compared to control crosses (El-Kassaby et al. 2011). El-Kassaby et al. (2015), used progenies from known mothers and random bulk samples (unknown parents), reconstructed 268 and 446 full-sib families respectively in Pine. The number of full sib families obtained in the current study was found to be 62 and was lower than the reports from the earlier studies (El-Kassaby et al. 2011, 2015). This could be due to few progenies (phenotypically pre-selected) used in the current study, however it is still considered as high compared to controlled crosses.

El-Kassaby et al. (2015) used both nuclear and chloroplast based DNA markers for the successful identification of maternal and paternal parents from open pollinated bulk seeds in Pine. In this study, we have used only nuclear DNA markers for both maternal and paternal analysis, because the bulk seeds were collected only from open pollinated *E. camaldulensis* mother trees. To identify the maternal parent, first we conducted maternity analysis using *E. camaldulensis* as a candidate mother tree, and then we conducted paternity analysis with both *E. camaldulensis* and *E. urophylla* as candidate fathers. It is important to note that the parents assigned for some of the full-sib families identified within *E. camaldulensis* could be true mother and father or vice versa. This is due to limitation of nuclear markers in distinguishing maternal and paternal information. However it has been reported that seed production is normally dominated by few maternal parents in the orchard (El-Kassaby and Askew 1991; El-Kassaby et al. 1989). In this study, seven out of 33 maternal parents (17 %) were produced 80 % of the phenotypically preselected individuals, with mother tree EC214 accounting for 34 % of this contribution. This 20/80 ratio was also observed in other studies (El-Kassabey et al. 2010; Lai et al. 2010). El-Kassaby et al. (2010) and Lai et al. (2010) reported about 77 % contribution from 20 % of the mother trees in a Douglas-fir seed orchard, in which a single mother tree contributed 36 % of the total seeds. In addition, the male contribution was found to be relatively high, about 80 % of the successful pollen were contributed by 50 % of fathers in the orchard (28/56), which is similar to the previous studies reported (El-Kassaby et al. 2010). These results suggest that both candidate mothers and fathers identified for full-sib families within *E. camaldulensis* parents could be the true mother and fathers for the respective full-sibs. Furthermore, this implies that the selection of phenotypically pre-selected individuals reflects the mating dynamics present within the orchard.

Eucalypt flowers are mainly pollinated by insects with a mixed mating reproductive system. We successfully assigned all offspring to their putative mother trees and 84 % of the progenies to their putative father trees. The unassigned progenies could be due to the fact that the true father is possibly not present in the candidate pool and contribution of pollens from outside the orchard, suggests that the seed orchard was not completely isolated from pollen contamination. It is also known that long distance pollen flow is quite common in insect pollinated species (Chase et al. 1996, Konuma et al. 2000). Pollen contamination rate found in this study is in agreement with the

previous studies reported in *Eucalyptus* (4.5 % to 46 %, Chaix et al. 2003, Grattapaglia et al. 2004, Jones et al. 2008, Grosser et al. 2008, Rao et al. 2008).

We found higher level of out crossing rate in the present study. Different levels of outcrossing rates were reported in *Eucalyptus*. Sato and Mori (1996) reported 87 % in *E. grandis* seed orchard, Moranetal. (1989) found 91 % in *E. regnans* seed orchard, Chaix et al. (2003) reported 96.7 % in *E. grandis* seed orchard and Gaiotto et al. (1997) obtained 90 % in *E. urophylla* natural population. Variation in outcrossing rate between populations of the same species is also common in mixed mating species (Coates & Sokolowski 1992). High level of outcrossing rate found in this study could be purely due to presence of self-incompatibility mechanism as previously reported in *Eucalyptus* or selection favoring outcrossed seeds (Ladiges et al. 1995).

Heritability and genetic correlation between the traits helps in estimation of expected genetic gain to design breeding programs. Heritability for growth traits were estimated in a number of *Eucalyptus* species (Raymond 2002; Hamilton and Potts 2008; Harrand et al. 2009; Silva et al. 2009). It was reported that heritability of growth traits were low compared to wood quality traits (Raymond and Apolaza 2004). Heritability estimates for DBH and height found in this study was similar to earlier reports in *Eucalyptus* (Hamilton and Potts, 2008, Lopez et al. 2002). The estimated additive genetic variance and heritability derived from half sib model was high compared to full sib model, suggesting over-estimation of additive genetic variance in half-sib model. However, significant correlation of breeding values among parents and offspring was observed in both the models. In addition, the prediction accuracy was found to be similar in both the models for DBH (0.56) and height (0.58). El-Kassaby et al. (2011) detected low correlation of breeding values between parents and progenies in the half-sib and halfsib + full-sib based model. High significant correlation found in this study could be due to low heterogeneity present in the experimental site or low number of individuals used in this study compared to El-Kassaby et al. (2011). Strong correlation observed among mother and progeny BLUPs estimated from the two models suggests that both models can be effective in forward selection. Even though both models produced similar results, full-sib model has more advantage compared to half-sib model. The half-sib model can produce breeding values only for maternal parents, which can hamper backward selection, whereas full-sib approach generates breeding values for all the parents and offsprings included in the study, which eventually increase the efficiency and accuracy of backward, forward and combined selection.

Genetic correlation is an important indicator for the selection of traits. Estimated genetic correlation between DBH and height was high in the half-sib (0.66) and full-sib (0.52) models. Genetic correlation observed in this study is similar to the previous studies reported in *Eucalyptus*. Hamilton and Potts (2008) reported average genetic correlation of 0.58 in *E. nitens*, Volker et al. (1990) reported 0.64 in *E. globulus* and 0.83 was reported in *E. urophylla* (Wei and Borralho 1998). Strong positive genetic correlation between DBH and height suggests that selection of

one trait will have positive correlation response to the other trait.

In conclusion, the construction of pedigrees using bulk seeds obtained from an open-pollinated seed orchard has been demonstrated to be an effective method for identifying parent trees that yield superior offspring and high-quality seed production. Bulk seeds encompass the entire seed-producing population, facilitating the assessment of both male and female reproductive success as well as mating dynamics within the orchard. Furthermore, phenotypic pre-selection used in this study, substantially reduced the resource utilization.

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Supplementaries

https://sciendoparsed.s3.eu-central-1.amazonaws.com/65da19aee59fb84ec211c619/sg-2024-0018_sm.pdf

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