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ARTICLE

Using of relatedness and heritability in a *Eucalyptus benthamii* trial for conservation and breeding

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Abstract: *We evaluated the genetic diversity, coancestry and heritability of an E. benthamii trial. The 115 individuals were genotyped (13 SSR) and had their height and diameter at breast height (dbh) measured. Heritability was estimated using the RR-BLUP and the pairwise kinship coefficient method. An average of nine alleles per locus was observed. The expected heterozygosity (0.655) was similar to the observed heterozygosity, with the estimated inbreeding (0.02) being low. The group coancestry (0.051) demonstrates that the trees are related to some degree. The trees were clustered in five groups using the Evanno's method. The average kinship within each group ranges from 0.042 to 0.082. The heritability estimated by RR-BLUP was low. The heritability estimated using the kinship coefficients is moderate, reaching estimated genetic gains of 14% for dbh. After knowing how genetic groups are distributed within the population, strategies for collecting, conserving, and using these germplasm resources can be performed.*

Keywords: *Clustering method, frost-tolerant, inbreeding, kinship*

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INTRODUCTION

Some eucalypt species are frost tolerant, showing high productivity in subtropical areas with a high probability of frost. In this aspect, one of the most attractive species is *Eucalyptus benthamii* Maiden & Cambage (Han et al. 2020). In Brazil, *E. benthamii* is the most adapted frost-tolerant eucalypt species (Santarosa et al. 2014). Its wood is mainly used for fuelwood, including charcoal and firewood (Kellison et al. 2013). However, the lack of information about the genetic material introduced into Brazil is one of the limiting factors to the development of this species' breeding programs. *E. benthamii*'s natural habitat is narrow, being found only in a restricted area southwest of Sydney in Australia, making the species vulnerable to extinction (Butcher et al. 2005). Currently, the efforts in the region have been to protect and study the forest remnants (Baccarin et al. 2015). Tambarrussi et al. (2022), when studying the reproductive system of the species, observed in nine progenies outcrossing rates ranging from 0.990 to 1.0.

The genetic variability of tree trials can be measured using genetic markers by parameters such as number of alleles per locus (*A*), observed heterozygosity (H_0) and expected heterozygosity (H_e) in Hardy-Weinberg equilibrium, as performed by Allendorf et al. (2013). H_0 is the main factor responsible for the short-term response to selection, and *A* is the main factor responsible for the long-term response (Vilas et al. 2015). Inbreeding levels can be measured by the fixation index (*F*). In general, outcrossing rates are higher and inbreeding levels are lower in seed orchards than reported for natural populations, showing that population structure affects outcrossing rates in parental populations and inbreeding levels in offspring generations (Porth and El-Kassaby 2014). However, selection can result in increased biparental inbreeding in advanced generations of breeding programs, which can limit greater genetic gains (Jones et al. 2006). So, it is critical to monitor the coefficient of relatedness between pairs of individuals or populations of tree breeding programs.

Genetic markers also have been used to measure populations' heritability (Sumathi and Yasodha 2014). A critical feature of marker-based heritability estimation methods is the need to measure the actual relatedness variance (Rodríguez-Ramilo et al. 2007). The estimation of quantitative genetic parameters in small populations is generally limited by the accuracy and completeness of the available pedigree information (Bérénos et al. 2014). Bérénos et al. (2014) suggest that the relatedness information used in marker-based heritability estimates can potentially remove this limitation and lead to less biased and more accurate parameters. The same authors compared different methods based on molecular data to estimate quantitative genetic parameters and observed that it is necessary to choose the best statistical approach according to how the kinship is structured in the target population.

Genetic parameters, such as heritability, can also be estimated by breeding values predicted through phenotypic and pedigree data, using the mixed model methodology. In this case, the kinship matrix (A matrix) is obtained through the expected value of the proportion of identical loci by descent (Gay et al. 2013). The objective of this work was to apply SSR markers in a *E. benthamii* trial to understand the kinship structure within this population. The information regarding the relatedness will be used to estimate quantitative genetic parameters for the trial promoting a better use the species genetic resources in breeding programs.

MATERIAL AND METHODS

Study population

The trial was the first active germplasm collection of *E. benthamii* in Brazil. It was established by Embrapa Florestas in 1988 at the city of Colombo in southern Brazil. The seeds were provided by CSIRO[1] (Australia) and originated from a mix of 10 trees located at Wentworth Falls (NSW). It has been managed for seed production, providing cuttings and seeds for commercial planting. The initial number of 443 trees was reduced to 199 by a selective thinning based on volume traits in 1995. Nowadays, there are 115 remaining individuals. The population is at an elevation of 1,027 m above sea level in a Cfb climate (Köppen 1936), fully humid with warm temperature. The average annual rainfall is 1,638 mm. The average maximum temperatures in the warmer and colder months are 28 °C and 19 °C, respectively. Frost was observed in the coldest days of the year (INMET 2018).

Data collection

All the 115 individuals had their diameter at breast height (dbh) and height measured in 2017. For the genetic analyses, cambium samples were collected from all the individuals. Genomic DNA was extracted using a CTAB-sorbitol based method (Inglis et al. 2018). The detection and quantification of DNA were performed using the NanoDrop spectrophotometer (Thermo Fisher Scientific) to study the gene expression. The samples were then diluted to a final concentration of 5.0 ng μL^{-1} to run PCR reactions for genotyping 13 microsatellite loci using primers previously reported (Butcher et al. 2005). PCRs were carried out using 5.0 ng of DNA, 1 unit of Taq polymerase, buffer 1x, 0.25 mg bovine serum albumin, 0.28 µM of each primer in 8.0 µL reaction. PCR products were multiplexed in duplexes and triplexes according to fluorochrome labelling and size range for injection in 3730 DNA Analyzer. Allele call was conducted using Gene Mapper software (Thermo Fisher Scientific).

1 The Commonwealth Scientific and Industrial Research Organization (*CSIRO*) is an independent agency of the *Australian* Federal Government responsible for scientific research in *Australia*

Genetic analysis

Population genetics analyses of the genotyped trees were carried in the GDA software (Lewis and Zaykin 2001). The allele frequency (*A*), expected (H_e) and observed (H_o) heterozygosis and fixation index (*F*) were estimated. H_e was calculated based on Brown and Weir (1983): $H_0 = 1 - \Sigma p_{ii}$, where p_{ii} is the observed frequency of homozygotes on the *i* allele. The fixation index (*F*) was estimated as $F = 1 - (H_o/H_e)$ (Wright 1965). The coancestry coefficient (θ_{xy}) was calculated by the estimate of the pairwise kinship coefficient described by Ritland et al. (1996). The kinship estim0.5/ates were calculated using Jackknife resampling among loci in the SPAGeDi 1.5 software (Hardy and Vekemans 2002). The effective population size (N_e) was calculated as proposed by Sebbenn (2003), following Cockerham (1967) assumptions: $N_e = 0.5/\theta_{xy}$.

The pairwise θ_{xy} between trees and mean population was calculated according to Ritland et al. (1996), and standard error were calculated using Jackknife resampling among loci, in SPAGeDi 1.5 software (Hardy and Vekemans 2002). The effective population size (Ne) was calculated as proposed by Cockerham (1967): $N_e = 0.5/\theta$, where θ_{xy} is the group coancestry. A Bayesian model-based clustering method was used to cluster the individuals in groups according to kinship. This clustering was performed using the STRUCTURE software version 2.3 (Pritchard et al. 2000). The method uses genotypic data to determine the number of distinct genetic clusters (*K*) among the sample locations and estimates individual assignment probability to each cluster (Evanno et al 2005). Twenty replicate runs (100,000 Markov Chain Monte Carlo step burn-in plus an additional 100,000 runs) were performed for each value of K. For the clustering analyses, only the individuals that show a significant kinship ($\phi_{ij} \geq 0.1$) with at least one other individual from the population were considered. The tested numbers of clusters (*K*) were from 1 to 11. Results were summarized using STRUCTURE Harvester version 0.6.6 (Earl and vonHoldt 2012), generating a plot of the mean value of L(K) (ln likelihood of data) at each *K*. The analysis points the most likely number of clusters by identifying the highest L(K).

Heritability estimates

The pairwise kinship was used to calculate individual heritability ($\hat{h}_2$) using a marker-based method proposed by Ritland (1996), where the calculation of $\hat{h}_2$ was given by the equation: $\hat{h}_2 = \frac{C_{ZR}}{2V_r}$, where V_r is the actual variance of relatedness among all pairs *i* and C_{ZR} is the sample covariance between phenotypic similarity (Z_i) and estimated relatedness (R_i). Among related individuals the phenotypic similarity is written as: $Z_i = \frac{(Y_i - U)\,(Y'_i - U)}{V}$. For *i* pair, Y_i is the value of trait in the first individual, Y'_i the value of trait in the second individual, *U* is the mean of the trait and *V* is the population variance (Ritland 1996). The genetic gain (*GG*%) obtained through the selection was calculated as $GG\% = h^2\,(\overline{X}_s - \overline{X}_0)$; where $\overline{X}_s$ is mean the of the selected population, and $\overline{X}_0$ is the mean of the original population. The genetic gain was calculated for all possible selection intensities.

The markers had their effects estimated by fitting all the allelic effects simultaneously using the random regression best linear unbiased predictor (RR-BLUP). The RR-BLUP assumed that the markers effects were random. The variance parameters were assumed to be unknown and were estimated by restricted maximum likelihood (REML). The linear mixed model $y = X_b + W_m + e$ was fitted to estimate the effects of markers, where *y* is the vector of phenotypic data (deregressed additive genetic values), *b* is the vector of fixed effects, *m* is the vector of random effects of markers and *e* refers to the vector of random residuals. *X* and *W* are the incidence matrices for *b* and *m*. The mixed model equation for genomic prediction of the marker's effects (*m*) via the RR-BLUP method is: $\begin{bmatrix} X'X & X'W \\ W'X & W'W + I\,\frac{\sigma^2_e}{(\sigma^2_g/n_Q)} \end{bmatrix} \begin{bmatrix} \hat{b} \\ \hat{m} \end{bmatrix} = \begin{bmatrix} X'y \\ W'y \end{bmatrix}$, where σ^2_g is total genetic variation and n_Q is the number of loci. The σ^2_g was estimated by REML from phenotype. The total genomic breeding value of individual j is given by $VGG = \hat{y}_j = \sum_i w_{ij}\,\hat{m}_i$, where W_i is equal to 0 corresponding to the genotype *m*, or 1 corresponding to the genotypes *Mm* and *MM*. The amount of n_Q equals $n_Q = 2\sum_i^n p_i\,(1 - p_i)$. The genomic breeding values were used to compute the narrow-sense heritability as described by Resende et al. (2012) using the Jackknife cross-validation method. The approach used was the "leave-one-out". A single individual from the population was used as the validation set, and the remaining individuals as the estimation or training set. This process was repeated 115 times, using each time a different set of individuals for estimation and one different individual for validation, and all individuals had their phenotypes predicted and validated. The method was performed using rrBLUP package in R (Endelman 2011), as described previously (Resende et al. 2012). To use information

from the SSR markers in this technique it was necessary to transform the genetic information into a binary code of 0 and 1. For each individual a "0" was added when it did not show the corresponding allele and one "1" when it showed the correspondent allele. Thus, each individual has a code indicating the presence or absence of all observed alleles in the population.

RESULTS AND DISCUSSION

For the total population, 122 alleles were found. The mean of alleles per locus was nine, ranging from three to 17 alleles (Table 1). The observed heterozygosity (H_0) ranged from 0.342 to 0.79 and the expected heterozygosity (H_e) ranged from 0.352 to 0.859. The fixation index (*F*) ranged among loci from -0.214 to 0.262, with a mean of 0.023 not significantly different from zero. The genetic diversity of the studied population was relatively high compared to genetic parameters of the natural populations. In the restricted natural area of occurrence of this species, the number of alleles ($\overline{A}$ = 10.4) and the indexes of heterozygosity (H_e = 0.739 and H_0=0.630) are very similar to those found in the stand (Butcher et al. 2005). Even though only ten adult trees were used as a seed source for the population establishment, the stand represents a significant part of the total species diversity.

Table 1. Descriptive genetics of the microsatellite loci used for individuals of *Eucalyptus benthamii*, with number alleles (*n*), expected heterozygosity (H_e), observed heterozygosity (H_o) and fixation index (*F*)

Locus	n	H_e	H_o	*F*
eg61	6	0.342	0.352	0.023
embra06	14	0.613	0.834	0.262
embra10	12	0.567	0.734	0.225
embra11	14	0.790	0.653	-0.214
eg126	5	0.664	0.575	-0.159
eg67	17	0.785	0.859	0.082
es157	7	0.624	0.726	0.137
eg91	8	0.670	0.692	0.028
en06	9	0.761	0.681	-0.123
eg99	10	0.781	0.790	0.006
es140	8	0.455	0.448	-0.023
eg128	3	0.639	0.650	0.011
eg84	9	0.700	0.732	0.038
Mean	9.38	0.645	0.671	0.023

The high diversity levels observed for this population might be attributable to the gene flow among individuals within the population from which it originates. This scenario may suggest that this population was originated by random crossbreeding between unrelated parents (Randall et al. 2016). On the other hand, the diversity indicators in this study were lower than those of *Eucalyptus* species with a more widespread natural occurrence area. Breeding populations and seed orchards of *Eucalyptus* species such as *E. dunnii* (Poltri et al. 2003), *E. globulus* (Jones et al. 2006), *E. grandis* (Chaix et al. 2003), and *E. urophylla* (Silva et al. 2018) showed higher expected heterozygosity. In general, breeding populations composed of selected trees usually show a reduced genetic diversity (Sumathi and Yasodha 2014). For each new breeding cycle, the genetic diversity will be reduced (Jones et al. 2006). The stand exhibited a high value of diversity compared to the species' natural populations. Also, the trees in this trial survived several annual frosts. After two selective thinning operations, the remaining trees are selected genotypes that can be used in breeding programs aiming at frost tolerance and fast growth.

The trial originated from a mix of 10 trees, and this population was initially composed of 10 open-pollinated families. So, considering the calculated N_e (Table 2), we can conclude that all the ten original individuals (families) are still represented in the population. Considering the kinship between pairs of individuals, we observed higher probabilities of grouping them into two, three, four, and five groups (Figure 1). As we are trying to set progeny families in this population, we focus on the cluster of five groups (Figure 1). So, considering the existence of five groups in this population, the average relatedness between individuals of the same group and in relation to individuals of the other groups was estimated (Table 2). The results showed a higher level of kinship between individuals in the same group, suggesting that these groups may probably contain trees of the same family.

Neophytou et al. (2022) following STRUCTURE analysis used the solutions for K = 2, 4 and 6 for further investigation considering the selection of the Douglas Fir trees for breeding purposes. Noormohammadi et al. (2015) through STRUCTURE analysis identified 9 distinct population groups, while K-Means clustering suggested 2-3 major genetic subgroups in the present germplasm. They intend to use the obtained results to establish a better hybridization and selection plan for cotton. Rao et al. (2008) also used clustering to identify promising accession of *Jatropha curcas* with favorable traits for future establishment of elite seedling seed orchard for hybridization programs.

Table 2. Average pairwise kinship coefficients within clusters and cross-cluster pairings, rank with the phenotypic means per cluster for each trait and genetic parameters estimated by marker-based pairwise kinship and breeding values (rrBLUP) in an *E. benthamii* population

	Average kinship		**Phenotypic mean per cluster**				
	Within cluster	**Cross-clusters**	**Rank**	**Cluster**	**DBH** (cm)	**Cluster**	**Height** (m)
C1	0.082	0.008	1	C3	1.99	C4	44.13
C2	0.062	0.009	2	C4	1.83	C2	43.04
C3	0.046	0.011	3	C2	1.77	C3	42.50
C4	0.042	0.010	4	C5	1.68	C1	41.52
C5	0.046	0.011	5	C1	1.65	C5	40.67

Trait	σ^2_a	σ^2_y	σ^2_e	h_2	N_e	θ_{xy}	h_2'
dbh	3.17	104.02	100.85	0.03			0.22
Height	0.35	23.00	22.00	0.02			0.17
Population					9.80	0.051	

Additive genetic variance (σ^2_a), phenotypic variance (σ^2_y), error variance (σ^2_e), heritability estimated by rrBLUP (h_2), effective population size (N_e) mean pairwise kinship coefficient (θ_{xy}) and heritability estimated using pairwise kinship coefficient (h_2').

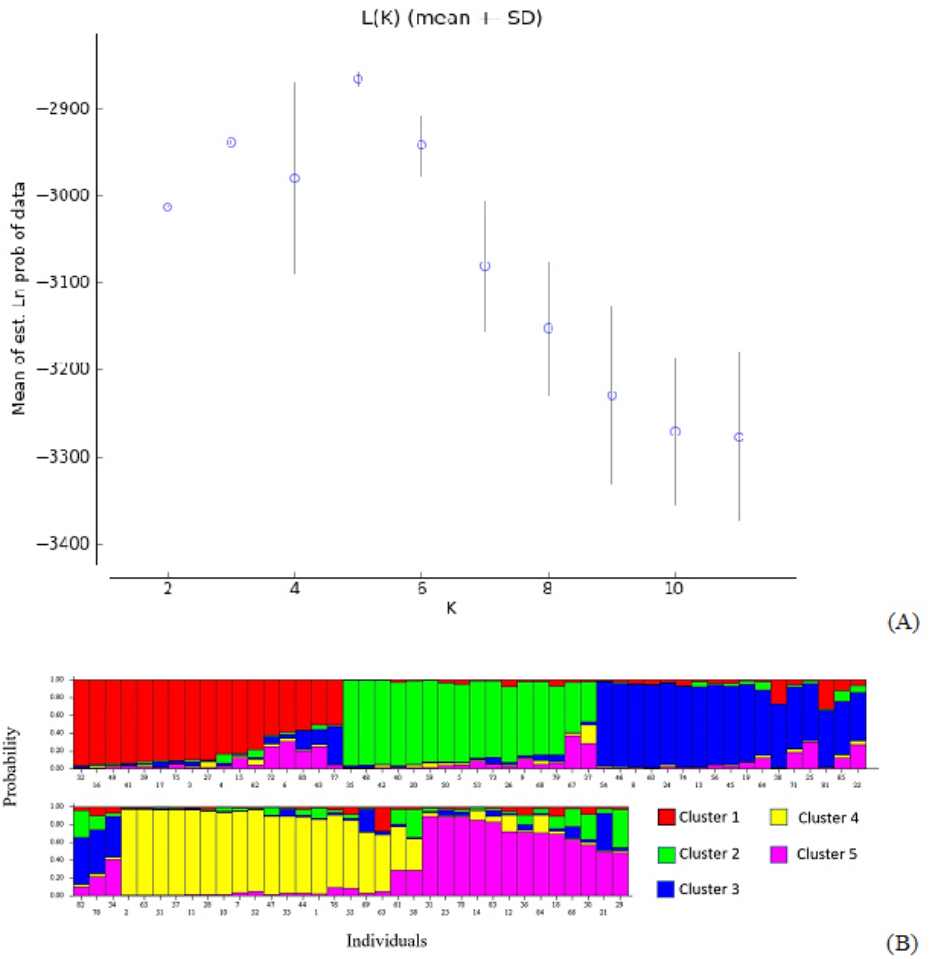

Figure 1. Mean value of L(K) (ln likelihood of data) corresponding to each number of clusters simulated in a population of *E. benthamii* (A) and the Bayesian-based analysis of population structure for *K* = 5, L'(K)= 191.61 and ΔK= 22.28 (B).

Regarding the average performance of the individuals for wood volume production, the individuals of clusters 2, 3, and 4 had a higher average performance than those of clusters 1 and 5 (Table 2). The estimated pairwise kinship coefficient shows that the individuals in the population have a considerable relatedness. And the marker-based heritability estimated was very disparate from that one found through RR-BLUP. The heritability estimated by the kinship method was considerably higher than that obtained by RR-BLUP for the dbh trait. The genetic gains were calculated based only on the heritability estimated by the kinship method. It was observed that higher genetic gains are obtained for dbh than for height, reaching around 14% (Figure 2).

João Gaspar et al. (2009) estimated the θ_{xy} coefficient of the families present in a *Pinus pinaster* progeny trial originating from collected seeds and studying how deviations from the standard assumption of θ_{xy} = 0.125 affect heritability estimations. They concluded that in the trial, the associated error in heritability estimates due to the inclusion of full-sibs, when assuming a standard coefficient of relationship among open-pollinated sibs of 0.250, was low and that this result is robust with respect to the number of families sampled, given the unbiased estimates of average relationship among offspring within sib families. Considering the origin of the population and the calculated effective population size, we can verify that the kinship between individuals must be structured mainly in families of half-sib progenies. It was possible to group the individuals with a kinship slightly lower than half-sibs (θ_{xy} =0.125) using the clustering method. It indicates that most individuals in each cluster must share at least one common parent. As the seeds that originated this population were collected from only ten trees of one provenance, part of them probably share a common mother and different fathers.

The RR-BLUP and the marker-based procedures (using kinship coefficients) should estimate similar values for heritability. In this study, the marker-based heritability was the most accurate. For RR-BLUP, Marchal et al. (2016), using 313 SRR markers and G-BLUP method, observed high heritability values for 478 crosses in an *Elaeis guineensis* (oil palm) population (ranging from 0.23 to 0.57). In these cited studies, the number of individuals sampled and marker density are higher than those in the present study. Fritsche-Neto et al. (2012) also found reliable results with heritability ranging from 0.14 to 0.24 for tropical maize using eighty SRR markers. In consensus, the method accuracy tends to get higher as the individual of the training population gets larger (Jannink 2010). It is presumable that the number of individuals in the training population and markers were insufficient to produce an accurate prediction.

Genetic markers provide information about relatedness between individuals of unknown pedigree, making it possible to estimate a kinship matrix based on average values of relatedness. Silva et al. (2015) compared the results obtained for heritability based on the kinship and concluded that the Ritland's (1996) method is the most robust. The authors argued that the method allows a greater adequacy of the data in the model used. However, the RR-BLUP procedure can be more accurate than the one based on relatedness, because it effectively captures the actual kinship matrix performed and not an average kinship matrix associated with the pedigree, like the second procedure (Arcia et al. 2011). According

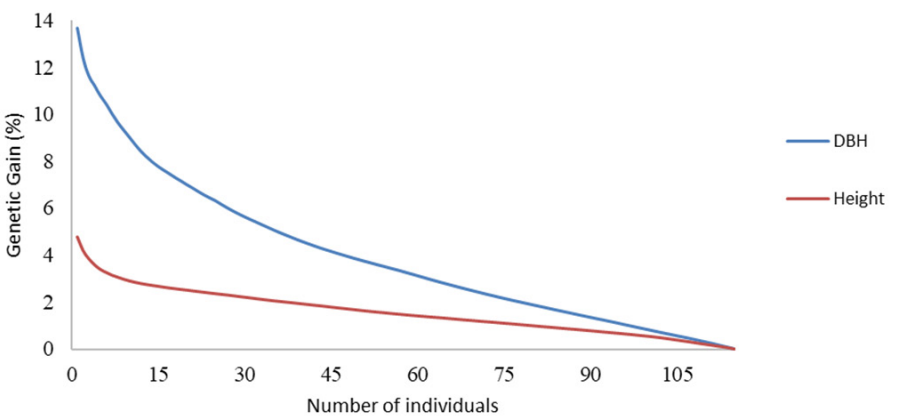

Figure 2. Genetic gain percentages for diameter at breast height (DBH) and height considering different selection intensities for an *E. benthamii* trial.

to Munoz (2014), RR-BLUP is the method that best explores the mendelian sampling segregation occurring during the gamete origin as it directly evaluates the associated DNA at each locus of all polygenic traits. Therefore, it captures the exact kinship matrix and not a pedigree-matched relatedness matrix.

The heritability estimated through kinship shows a potential to achieve considerable genetic gain using short-term selection methods. The results from this study could be used to direct crossing between pairs of unrelated individuals to minimize inbreeding. Highly productive individuals from different groups can be used in controlled pollinations aiming at the production of seeds with higher genetic quality for wood production, thus exploring the potential of heterosis in breeding programs (Bessega et al. 2015). In selected populations, it is easier to manage the maintenance of genetic variability by knowing how genetic groups are distributed within the population (Schwartz and Mckelvey 2009). So, it is possible to develop strategies for collecting, conserving, and using these germplasm resources.

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