Genetic structure and diversity in wild and breeding populations of *Eucalyptus urophylla*

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Abstract

Eucalyptus urophylla S.T. Blake is a species of great commercial importance, especially in tropical regions, and it is the main eucalypts species cultivated in Brazil. This study evaluated the genetic diversity among and within seven populations of *E. urophylla* and estimated the genetic distance between individuals to draw inferences about the genetic structure between and within the sampled populations. For that, 19 microsatellite markers were genotyped in 254 individuals originating from four wild populations, introduced in Brazil, two breeding populations, and one population consisting of commercial clones. The wild populations of *E. urophylla* introduced in Brazil have high genetic similarity and the few generations of breeding have already generated significant differences in population structure between improved and wild populations. As expected, breeding populations are closer to commercial clones than wild populations. However, compared to wild populations, breeding populations exhibit greater genetic diversity as they originated from a mixture of provenances. The population formed by clones was the only one that showed a negative Wright fixation index, that is, heterozygosity was higher than expected for a population in Hardy-Weinberg equilibrium.

Keywords: *Population genetics. Genetic diversity. Clone. Microssatelite.*

Introduction

Intensive silviculture of exotic species started in Brazil in the 20th century and has been intensified mainly with species of the genera *Eucalyptus* and *Pinus* (Ferreira & Santos 1997). *Eucalyptus* stands out since it represents ~73 % of the forest plantations in the country, with *Eucalyptus urophylla* being the most widespread species (Assis, Abad & Aguiar 2015). From the 1970s onwards, this species was more intensively planted and bred in the country, due to its resistance to the fungus *Cryphonectria cubensis* that causes eucalypts canker (Ferreira 1989). Studies showed that this species presented a series of advantages and good productivity in several regions of the country (Silva, Brune, et al. 2019). Advantages of *E. urophylla* over *E. grandis*, which was the most planted *Eucalyptus* species in the 1970s, include higher tolerance to drought and resistance to several diseases and pests.

Because the cultivars planted in the country were subjected to only a few breeding cycles (3-5), they are probably still very close to wild populations. Therefore, the origin/provenance has a strong influence on the genotype performance, tolerance to biotic and abiotic stresses, and wood quality. The effect of provenance has been observed and reported for several species in different countries, such as in *Corymbia citriodora* sub *variegata* (Araujo et al. 2021; Silva et al. 2022), *Eucalyptus grandis* (Ferreira 2015; Silva, Marco, et al. 2019), *E. pellita* (Nieto et al. 2016), *E. pilularis* (Carnegie, Johnson & Henson 2004), including *E. urophylla* itself (Hodge & Dvorak 2015).

Eucalyptus urophylla is one of the few species of the genus occurring outside Australia (7 to 10°S and 122 to 127°E), limited to some islands located in north of Australia: Flores, Timor,

DOI:10.2478/sg-2022-0015
edited by the Thünen Institute of Forest Genetics

Wetar, Alor, Pantar, Lomblen and Adonara. Given the relatively limited geographic distribution, altitude is the main source of variation among *E. urophylla* populations. The altitude of the occurrence areas vary from sea level to 3000 m (Payn, Dvorak & Myburg 2007).

As genetic gain depends on the level of genetic diversity, it is important to characterize the genetic variation within and among *E. urophylla* populations available for breeding purposes. Molecular markers allow estimates of genetic variability between and within populations, in addition to the estimates of similarities between individuals and populations (Mora et al. 2017; Lu et al. 2018; Baldoni et al. 2020; Guimarães et al. 2019). With those estimates, breeders can infer about the genetic structure among populations. This is especially important for the establishment of heterotic groups, in case there is strong genetic structure among populations.

Due to their properties such as codominance, multiple allelism, high rate of polymorphism and transferability, microsatellites are very useful for population genetics studies. Specifically, they can be used to estimate the expected and observed heterozygosity; allelic richness; genetic structure and genetic distance of populations (Zolet et al. 2017; Soares 2014). In *Eucalyptus* populations, microsatellites have been used to estimate the degree of diversity and structuring (Costa et al. 2017; Payn et al. 2008).

The objective of this work is to evaluate the genetic diversity among and within wild populations, breeding populations and commercial clones of *E. urophylla* introduced and bred in Brazil.

Materials and methods

The populations of *E. urophylla* used in this study are as follows:

Wild populations

1. Flores population (A08A 032), introduced and planted in Brazil in 1977, consists of seeds harvested on Flores Island at altitudes between 600 and 1000 m.
2. East Timor population (A08B 033), also planted in 1977, originated from seeds collected in the macro-regions of Bessi – Lao, Dili and Remexio - (83 % from 420 m to 1400 m and 17 % from 1400 to 2200 m).
3. Timor population or West Timor (A08G 068), planted in 1980, from seeds harvested in Lelogama, Timau, Debaha, Futusunam, Nautsuu, Kenkneno and Futumnasi.
4. Other Islands (A08E 066), planted in 1980, from seeds harvested in Adonara, Lomblen, Alor and Pantar Islands.

The wild populations were introduced and planted in the municipality of Anhembi, São Paulo State of Brazil. They were planted in a farm belonging to the University of São Paulo. A total of 25 trees were selected from each of the studied wild populations.

Breeding populations

The two breeding populations studied consist of individuals originating from the following breeding programs.

1. PCMF-A Population (A05H 211) was implemented in the Anhembi Forest Science Experimental Station as part of the IPEF experimental network of the University of Sao Paulo (USP) in December 2009. It consists of 167 open-pollinated progenies that originated from diffe rent populations and were collected in different priva te companies (Suzano; Duratex; Jari; Aperam; Con pacel; Cenibra; Fibria). The description of seed lots and selection of individuals was performed as described by Pupin et al. (2015) and Silva et al. (2019).
2. PCMF-B Population (M1.058.13-F001) was implemen ted in the municipality of Lençóis Paulista, in January 2013. It consists of 130 open-pollinated progenies ori ginating from six populations of several sources, inclu ding seed lots from Brazilian companies, public institu tions and from abroad (Gerdau; FEENA; USP; Amcel; Mexico; Veracel). The description of seed lots and the selection of individuals are described in Silva et al. (2019).

The breeding populations are identified as PCMF-A and PCMF-B and are represented respectively by 79 and 65 individuals, which were selected by the highest predicted breeding values (Silva et., 2019).

Population of commercial cultivars (Clones)

This population consists of nine commercial clones, with the following identification: AEC144, CNB10, FIB0075, GG100, H13, IPB15, JAR2646, VER361 and VM04. It is worth mentioning that several of these clones were obtained via open pollination, with a high possibility of being hybrids of *Eucalyptus urophylla* x *grandis*, both species are planted and bred *Eucalyptus* species in Brazil.

DNA extraction and microssatelite genotyping

DNA extraction from leaves was performed following a protocol based on CTAB detergent (Doyle & Doyle 1987). Amplification of microsatellite markers was performed by PCR with the reagents composition described by Brondani et. al (2006) and the thermocycling protocol from Faria et. al (2011). Fluorescence detection was performed via capillary electrophoresis with the ABI 3100 XL automated platform, as described in Faria et al. (2010; 2011).

Nineteen microsatellite markers were used: EMBRA2; EMBRA3; EMBRA10; EMBRA11; EMBRA12; EMBRA21; EMBRA28; EMBRA32; EMBRA38; EMBRA45; EMBRA63; EMBRA128; EMBRA157; EMBRA204; EMBRA210; EMBRA681; EMBRA915; EMBRA1144; and EMBRA1349. These markers were obtained and described by Grattapaglia et. al (2015).

Diversity and genetic structure

The package adegenet v.2 (Jombart & Bateman 2008) from the R software (R Core Team 2022) was used for the statistical analysis. This statistical package allowed us to estimate the number of alleles (A), the number of private alleles (PA), observed

Table 1
Diversity parameters estimated per population.

POPULATIONS	*N*	*He*	*Ho*	*F*	*A*	*AR*	*PA*
Flores	25	0.745	0.741	0.005	149	5.802	6
East Timor	25	0.818	0.739	0.097	217	7.778	9
Other islands	25	0.817	0.720	0.119	206	7.683	7
Timor	25	0.796	0.672	0.156	180	6.947	5
PCMF-B	65	0.854	0.736	0.138	281	8.256	18
PCMF-A	79	0.856	0.775	0.095	310	8.417	30
Clones	9	0.822	0.848	-0.032	160	8.421	4
Mean/Average	-	0.8165	0.7456	-	216	7.98	11.3

where n: sample size; *He*: mean expected heterozygosity under Hardy-Weinberg equilibrium; *Ho*: observed mean heterozygosity; *N*: Fixation index; *A*: number of alleles; *AR*: mean allelic richness; *PA*: private alleles.

(Ho) and expected heterozygosity (He) per locus. The allelic richness (AR) and Wright's *F* statistics were estimated using the package hierfstat v 0.5.7 (Goudet & Jombart 2020).

To understand the genetic structure among individual trees and populations, genetic distances and Bayesian analyses of genetic structuring were performed.

Distances among wild and breeding populations were estimated using Nei's standard genetic distance (Nei, Tajima & Tateno 1983) available in the package poppr v. 2.9.3 (Kamvar & Grünwald 2021). To build the dendrogram, the distance matrix was analyzed using the UPGMA (unweighted pair-group method with arithmetic mean) clustering method. The consistencies of the dendrogram nodes were obtained through 100.000 bootstraps. The distance between trees was also evaluated using principal component analysis (PCA). This analysis was performed using the function indpca of package hierfstat, which uses the matrix of individual allelic frequencies. Genetic structuring analyses were performed using the Bayesian approach implemented in the Structure software (Pritchard, Stephens & Donnelly 2000). Structure calculates the likelihood of models varyingthe number of subpopulations (K) to identify the model that best explain the genotypic data. Analyses were performed with a burn-in of 500,000 Markov and Monte Carlo Chains (MCMC), 1,000,000 MCMC after burn-in, and 10 repetitions for each K value, with K ranging from 1 to 10. The number of subpopulations K that best fit the data was obtained with Structure Selector (Li & Liu 2018).

Results

Genetic diversity

The number of private alleles, the expected and observed heterozygosity, the number of total alleles and the allelic richness were higher in the breeding populations (PCMF-A and PCMF-B) than in the wild populations. This indicates greater genetic diversity in the breeding populations (Table 1).

Considering only wild populations, the genetic distance between individuals is not enough to discriminate them into groups corresponding to the populations of origin. The commercial clones are the most genetically distant from the other populations and the breeding populations are genetically "closer" to the clones compared to the wild populations (Figures 1 and 2).

Structure Selector analysis of Structure results indicates that K=4 is the number of clusters that best explains the whole data set, indicating possibly four genetically distinct groups. When considering only wild populations, the determined K value was equal to 2, indicating two groups (Figures 3 and 4).

Discussion

The results indicated higher genetic diversity in the breeding populations. This is probably due to the mixed provenances used to form the breeding populations, that could create intraspecific hybrids. Populations from different origins are generally mixed to generate base populations with higher genetic variability for breeding programs. Genetic gain is directly proportional to the variability available for selection. As such, the breeding success of any crop lies in efficiently identifying and incorporating genetic diversity and variability from alternative genetic resources, including wild materials, advanced breeding genotypes, and germplasm collections with elite or potential materials (Swarup et al. 2021). It is highlighted that all studied populations exhibited great genetic diversity compared to what has been seen for other *Eucalyptus spp* (Jones et al. 2002; Mora et al. 2017), with higher numbers of alleles for all studied loci.

In the literature, other *E. urophylla* studies report similar results. Tripiana et al. (2007) used ten microsatellite markers and obtained between 10 and 29 alleles per locus while He ranged from 0.51 to 0.72. Payn et al. (2008) used twelve microsatellite markers and reported from 6 to 56 alleles per marker while Ho varied between 0.737 and 0.610. These authors also found that the population of Timor had the highest diversity rate, similar to the result obtained in this work. The authors attributed this greater diversity to the large size of the Timor population and the possibility of having been the first island to be colonized. Population genetics theory shows that diversity

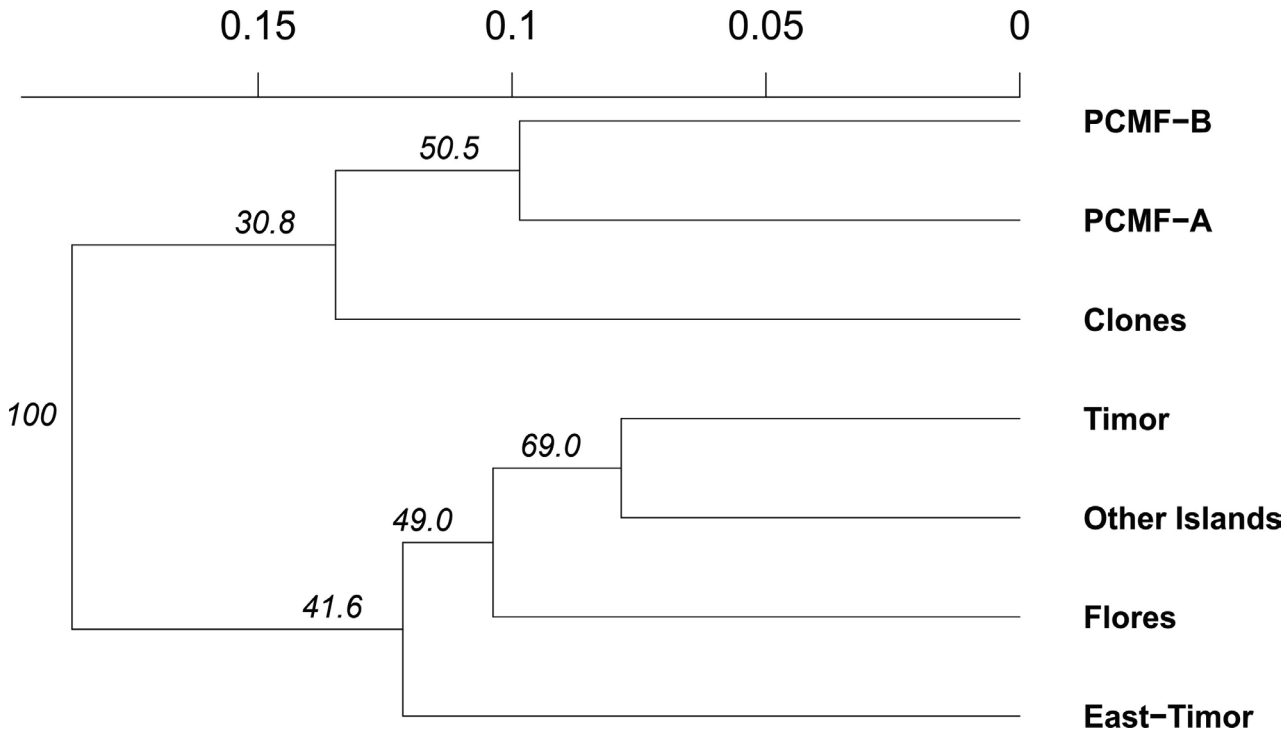

Figure 1
Dendrogram based on the Nei's distance of studied populations.

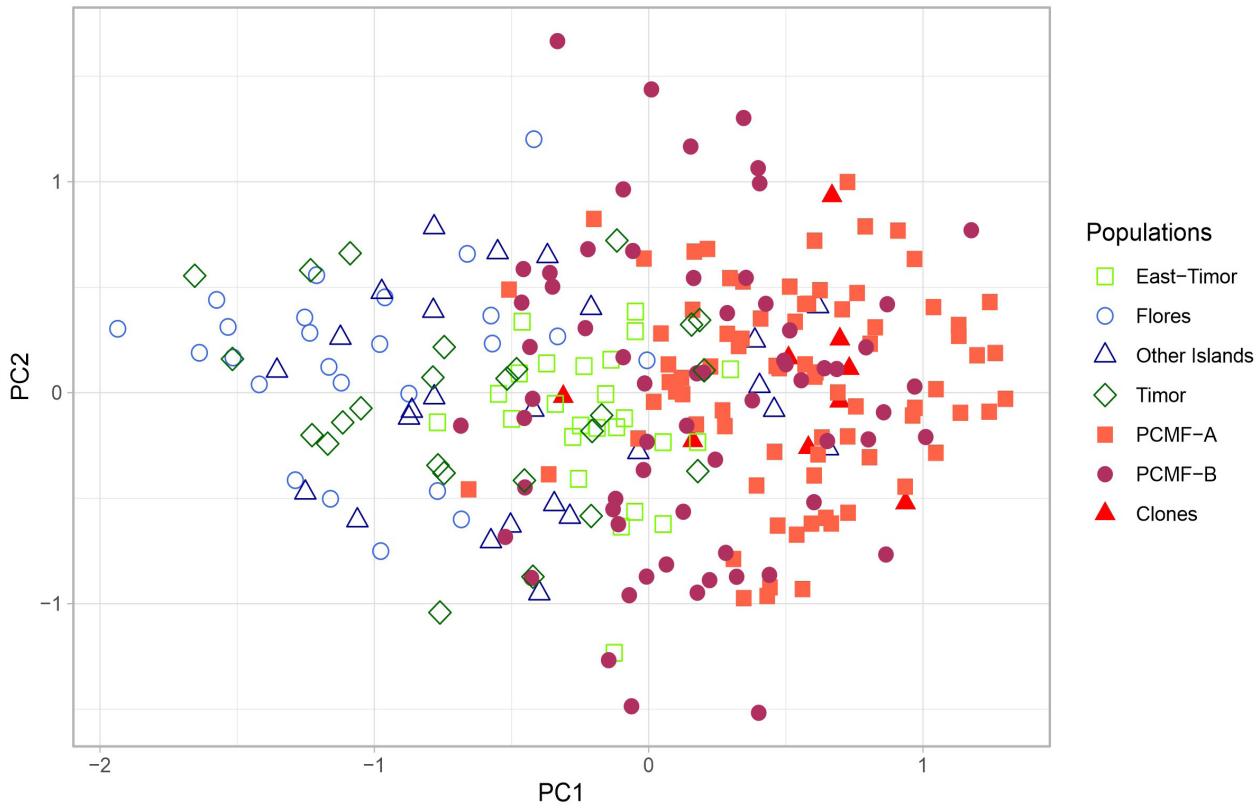

Figure 2
Principal component analysis (PCA) of individual allele frequency of all individuals.

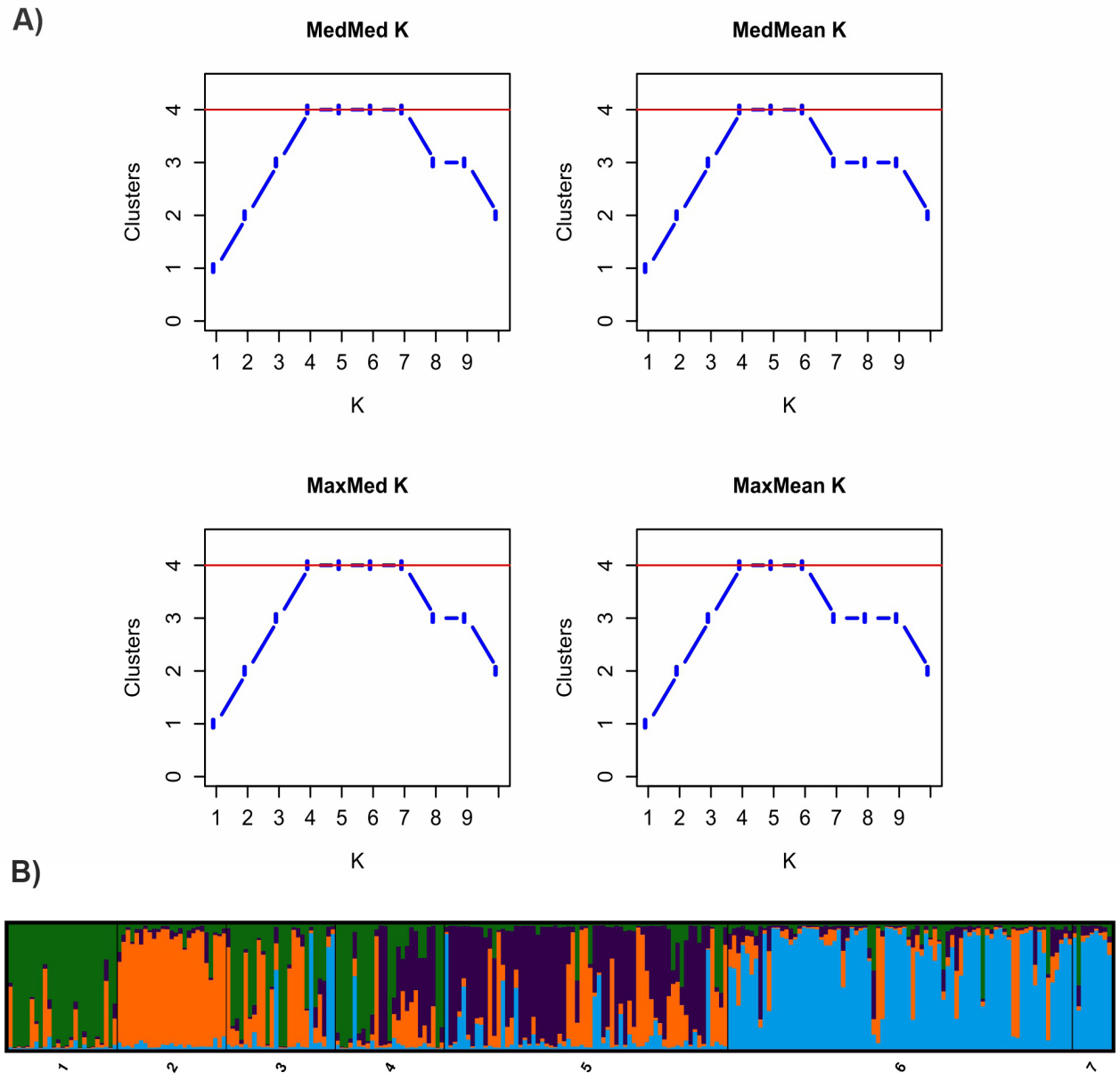

Figure 3
Structure Selector and Structure results for all wild and breeding populations. A) Structure Selector results indicating that the model with four clusters (K=4) best explains the genotypic data; B) Structure barplot indicating the likelihood for of each individual pertaining to each cluster. Each vertical bar correspond to a different individual and the four colors indicate the likelihood for each cluster. Numbers on the x-axis indicate the population of origin: 1: Flores, 2: East Timor, 3: Other Islands, 4: Timor, 5: PCMF-B, 6: PCMF-A, 7: Clones

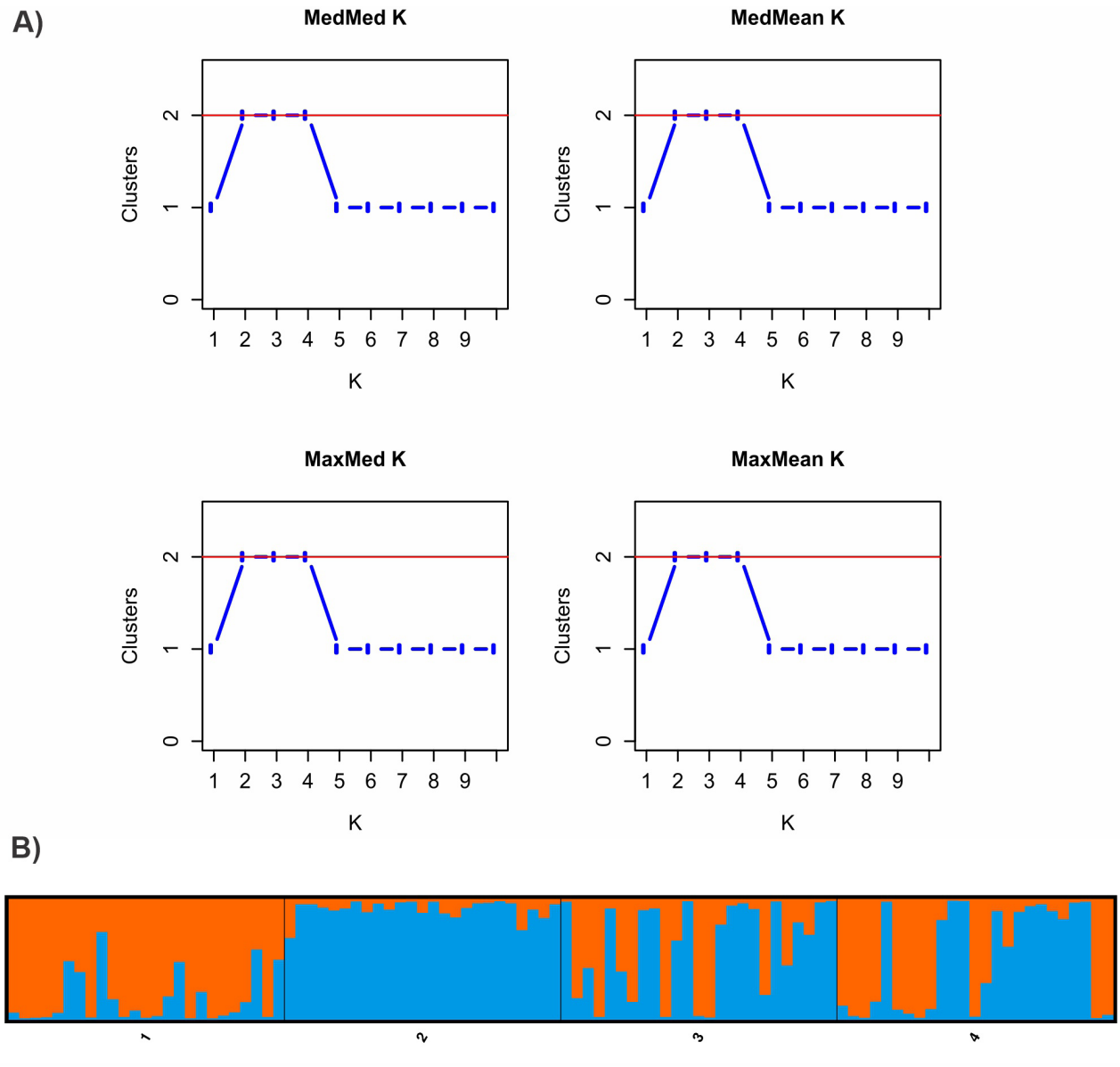

Figure 4
Structure Selector and Structure results for wild populations only. a) Structure Selector results indicating that the model with two clusters (K=2) best explains the genotypic data; b) Structure barplot indicating the likelihood for of each individual pertaining to each cluster. Each vertical bar correspond to a different individual and the two colors indicate the likelihood for each cluster. Numbers on the x-axis indicate the wild population of origin:1: Flores, 2: East Timor, 3: Other Islands, 4: Timor.

tends to be higher in older populations (Payn, Dvorak & Myburg 2007).

Tripiana et al. (2007) attributed this high rate of diversity to the evolutionary history of the species, that generated large effective population size, as well as its reproductive characteristics with pollen and seeds that can travel over long distances facilitated by animals (Hamrigk & Godt 1996; Mcgoldrick & Mac Nally 1998; Tripiana et al. 2007). However, it should be noted that the effective distance traveled is normally less than 850 m, with a considerable drop in the pollination rate at approximately 200 m, regardless of whether it is in exotic plantations or natural areas (Barbour, Potts & Vaillancourt 2005; Jones et al. 2008; Silva, Sebbenn & Grattapaglia 2015).

Despite not being possible to distinguish between wild populations, there is an indication of a correlation between geographic and genetic distances. Results obtained by Payn et al. (2008) show that *E. urophylla*, despite occurring on several islands, has only two genetic "groups", as also observed in this study. Perhaps the use of another type of marker would allow better differentiation among the studied populations. In a study with *Araucaria angustifolia*, SNPs generated more realistic estimates of genetic diversity and structure compared to SSR (Silva et al. 2020).

Although the breeding populations of *E. urophylla* are still in their early stages, with few breeding generations (Silva, Brune, et al. 2019), genetic differences with wild populations were observed. It must be considered that the breeding populations were crossed via open pollination and, therefore, other species and provenances may have being intercrossed with these *E. urophylla* populations. Gene flow studies show that, although at low rates, pollination may occur at distances close to 850m (da Silva & Abrahão 2021). Thus, the occurrence of spontaneous inter and intraspecific hybrids in breeding populations are possible, which can explain the genetic differences compared to the wild populations. However, this study did not aim at evaluating the occurrence of hybrids.

Breeding populations are formed by a mixture of provenances, thus increasing genetic diversity. It is worth noting that the choice of origin is fundamental for the success of breeding programs (Eldridge et al. 1994), as field performance can be strongly influenced by provenances. The selection must consider the interaction with the environment when working with very different environments (climate and soil), as performed with *E. urophylla* in Brazil (Silva, Brune, et al. 2019). Thus, populations with a broad genetic base can be used as a basis for different environments, but an adequate parental selection is necessary to clonally propagate and test individual genotypes for cultivar development and deployment.

The practical importance of maintaining a broad genetic base in the breeding population is directly related to long-term selection gains. Otherwise, as the genetic base narrows, productivity of the selected population will be less distant from the average population (Swarup et al. 2021). Therefore, the observed large genetic diversity in the breeding population of *E. urophylla* reflects the long-term breeding strategy effectively implemented by breeding programs in Brazil.

The population formed by commercial clones showed greater similarity with the breeding populations. Also, the clone population was the only one that showed greater than expected heterozygosity, under Hardy-Weinberg equilibrium. This is possibly a consequence of the system for obtaining cultivars in Brazil, which is strongly based on intra- or inter-specific hybridization, with subsequent cloning of the transgressive individuals (Assis, Abad & Aguiar 2015). Hybridizations increase the observed heterozygosity in the progeny.

Final considerations

The *Eucalyptus urophylla* populations present high genetic variability, especially, the breeding populations that were obtained by mixing different provenances.

The four wild populations of *E. urophylla* can be separated into two genetic groups.

The few breeding generations have already generated significant differences in the structure of the breeding population compared to the wild ones.

As expected, commercial clones are closer to breeding populations than to wild populations.

The commercial clones have higher heterozygosity than that expected for a population in Hardy-Weinberg equilibrium.

Acknowledgements

We wish to thank Amcel, Arborgen, Aperam, Bracell, CMPC, Dexco, Eldorado, Klabin, Montes del Plata, Suzano, Sylvamo, Vallourec, Veracel (companies which belong to the Genetic Improvement Cooperative Program - IPEF), University of São Paulo – USP/ESALQ, and FAPESP (15/15651-2) for providing information, leaf sample and financial support. EN and PHMS have been supported by research fellowship grants from the National Council for Scientific and Technological Development – CNPq, which they gratefully acknowledge (CNPq 302891/2019-6; 312338/2019-8).

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