Genetic diversity in wild and breeding populations and clones of *Eucalyptus urophylla* and *Eucalyptus grandis*

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Abstract

Brazil has a long history of intensive silviculture with *Eucalyptus* species, mainly *Eucalyptus urophylla* and *E. grandis*. However, breeding advances may reduce genetic diversity in bred populations. Nine microsatellite markers assessed genetic diversity in wild and improved populations of *E. urophylla* and *E. grandis*, and genetic similarity in nine widely planted clones. Four wild populations of *E. urophylla* were evaluated: Flores, Timor-Leste, Timor and other Islands, along with three improved populations. For *E. grandis*, one wild and one improved population were analyzed. Results showed higher genetic diversity in improved populations, possibly due to admixed composition from different provenances. Wild populations of *E. urophylla* formed two distinct groups. All clones were genetically similar to improved *E. urophylla* populations. Some clones are interspecific hybrids, contradicting their reported pedigree, but predominantly *E. urophylla*.

Keywords: *Eucalyptus*, genetic diversity, hybrid clones, microsatellite markers, tree improvement.

Introduction

The intensive cultivation of exotic species, including those from the *Eucalyptus* genus, has a long history worldwide, dating back to the 18th century (Martin and Quillet 1974), and has experienced significant expansion in recent times. In Brazil, this intensification began in the 20th century, specifically with *Eucalyptus urophylla* S.T. Blake and *E. grandis* Hills ex Maiden due to their good adaptation and rapid growth in various regions of the country (Schumacher and Vieira 2015; Silva et al. 2018, 2019). Among these species, *E. urophylla* is currently the most widely used, as it exhibits drought tolerance and resistance to various pests and diseases (Assis et al. 2015; Silva et al. 2019).

Despite the long history of eucalypt domestication in the Brazilian forestry (Ferreira and Santos 2015), eucalypt is a crop with a long reproductive cycle and the species have undergone only a few cycles of selection and recombination. Therefore, there is a high possibility that the breeding populations are still genetically close to the wild populations (Barros et al. 2023).

Hybridization is a commonly used technique in eucalypts breeding, as it allows the combination of desirable traits from species within the same subgenus (Scanavaca and Garcia 2021). Cloning has made it possible to propagate hybrid

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genotypes and clonal plantations have expanded throughout Brazil (Campinhos and Ikemori 1977; Assis and Mafia 2007; Palaudzyszyn Filho and Santos 2011). Thus, vegetative propagation combined with inter- and intra-specific hybridization has led to the development of superior cultivars, capitalizing on a single genotype's additive and non-additive genetic effects (Grattapaglia and Kirst 2008).

Hybridization and cloning continue to guide genetic improvement programs, but strategies that aim to achieve genetic gain without the loss of genetic diversity have gained prominence in eucalypt population improvement (Silva et al. 2018; Araujo et al. 2021, 2023; Barros et al. 2023). Genetic characterization within and among populations and species has become a more targeted focus (Mora et al. 2016; Lu et al. 2018), aiding population improvement for clonal selection.

Initially, population characterization was studied using morphological markers. However, molecular identification techniques, particularly molecular markers (Kirst et al. 2005; Ottewell et al. 2005) gained prominence, as they can also estimate similarities between individuals and populations. Due to their co-dominance, multi-allelic nature, and high polymorphism rate, microsatellites markers are excellent for estimating the levels of genetic diversity, population structure and relatedness etc (Payn et al. 2007, 2008; Zolet et al. 2017).

In genetic improvement programs, the selection of genotypes that present specific characteristics of commercial interest, such as greater growth vigor, resistance to diseases and pests, adaptation to specific environmental, causes the narrowing of the genetic base, which can lead to the loss of alleles. Genetic improvement populations with little or no genetic variation can become susceptible to biotic and abiotic stresses (Salgotra and Chauhan 2023). The loss of genetic variation can make the continuity of genetic improvement programs unfeasible, which leads to the need to introduce new materials to continue selection activities and capitalize on genetic gains. This can be the case of *Eucalyptus* breeding programs and the status of genetic diversity of such populations has been poorly investigated (Miranda et al. 2019). Therefore, it is essential to monitor genetic diversity throughout genetic improvement programs.

The objective of this study was to determine if there is difference in genetic diversity between wild and improved populations of *E. grandis* and *E. urophylla*, as well as the genetic similarity of nine commercial clones planted in the country. Our initial hypothesis is that in comparison to wild populations, selection in genetic improvement programs has led to the loss of genetic diversity. The alternative hypothesis is that in comparison to wild populations, selection in genetic improvement programs has not caused loss or even increased genetic diversity due to the mixture of genetic materials originating from different provenances.

Material and Methods

Studied populations

In this study, the best individuals from each population were genotyped, coming from the following sources:
a) Wild population of *E. grandis*- Gra-Wild: 253 individuals from wild populations of Coffs Harbor and Atherton. The collection description is in Miranda et al. (2019)
b) Improved population of *E. grandis*- Gra-PCMF: 990 individuals from a population deployed in 2009 at the Experimental Stations belonging to ESALQ/USP in the state of São Paulo, IPEF's experimental network. The populations were composed of 160 open-pollinated progenies from different populations (description in Miranda et al. 2019).

c) Wild populations of *E. urophylla*, described as,
c1) U-Flores: 25 individuals from a population established in Brazil in 1977, using seeds from the island of Flores–Indonesia, at altitudes between 600 and 1000 m;
c2) U-East Timor: 25 individuals from a population implanted in Brazil in 1977, from seeds from the macroregion of Bessi–Lao, Dili and Remexio, with altitudes between 1400 and 2200 m;
c3) U-Timor: 25 individuals from a population implanted in Brazil in 1980, from seeds of Lelogama, Timau, Debaha, Futusunam, Nautsuu, Kenkneno and Futumnasi;
c4) U-OtherIsland: 25 individuals from a population implanted in Brazil in 1980, from seeds from Adonara, Lomblen, Alor and Pantar islands.
d) Improved populations of *E. urophylla*, being:
d1) U-PCMF-A: 90 individuals from a population established in 2009 at the Forest Sciences Experimental Station in Anhembi, belonging to ESALQ/USP, as part of the IPEF experimental network. The population was composed of 167 open-pollinated families from different populations and from different private companies (description in Silva et al. 2019);
d2) U-PCMF-I: 79 individuals from a population established in 2009 at the Ilha Solteira Experimental Station, belonging to FEIS/UNESP, as part of the IPEF experimental network. The population was composed of 134 open-pollinated families from different populations and from different private companies (description in Silva et al. 2019);
d3) U-PCMF-L: 65 individuals from a population established in 2013 at the Experimental Farm in Lençóis Paulistas, belonging to Bracell S/A, as part of the IPEF experimental network. The population was composed of 130 open-pollinated families from different populations and from different private companies (description in Silva et al. 2019). These populations are located in the state of São Paulo, Brazil. Seed lots and selection of individuals were described in Silva et al. (2019).

e) Commercial clones, described as AEC144, CNB10, FIB0075, GG100, H13, IPB15, JAR2646, VER361 and VM04. Some of these clones were obtained from open-pollination. As *E. urophylla* and *E. grandis* are the most cultivated species in Brazil, there is the possibility that these clones are hybrids between these two species.

Microsatellite analysis

DNA extraction was performed following the CTAB protocol (Doyle and Doyle 1987) using fresh leaves, and

amplification of microsatellite markers was performed by PCR, as described by Brondani et al. (2006). Fluorescence detection was determined via capillary electrophoresis with the ABI 3100 XL automatic platform, as described by Faria et al. (2010, 2011). Nine microsatellite markers were used: EMBRA2, EMBRA3, EMBRA11, EMBRA12, EMBRA28, EMBRA63, EMBRA157, EMBRA204, and EMBRA333. These microsatellite markers, which exhibited good polymorphism for both species, were selected. The markers were obtained and described by Grattapaglia et al. (2015).

Genetic diversity and population structure

Statistical analyzes were performed using the R software (R Core Team 2022). The adegenet v.2 package (Jombart 2008) was used to estimate the total number of alleles per locus (*K*), the number of private alleles (*Pa*), mean observed (H_o) and expected heterozygosity (H_e) per population. The mean allelic richness (*R*) and fixation index (F_{IS}) were estimated using the hierfstat v.0.5.7 package (Goudet and Jombart 2020). The genetic differentiation among populations was estimated using the standardized statistic G_{ST}' (Hedrick 2005), $G_{ST}'=G_{ST}(1+H_S)/(1-H_S)$, where G_{ST} *and* H_S (mean genetic diversity within populations) were calculated using the FSTAT 2.9.3.2. software (Goudet 1995).

Genetic distance analyzes between trees were estimated using Goldstein et al. (1995) genetic distance with the poppr package, v. 2.9.3., and presented as a dendrogram, grouped by the UPGMA (Unweighted Pair Group Average) grouping method with 100.000 boostraps to evaluate the consistency of the nodes. The evaluation was also carried out through principal coordinate analysis (PCoA) using the distance matrix of Goldstein et al. (1995) of individual allele frequencies.

The genetic structuring analysis between the trees was carried out using Bayesian inference from the STRUCTURE v.2.3.4 software (Pritchard et al. 2000). The analyzes were performed with a burn-in of 500,000 and Markov and Monte Carlo Chains (MCMC) of 1,000,000 MCMC after burn-in and with 10 repetitions for each K value, with K ranging from 1 to 15. The number of K subpopulations that best determines the number of clusters used was the one implemented in the STRUCTURE Selector program (Li and Liu 2018), which is based on the indices defined by Puechmaille (2016).

Results

Among the *E. urophylla* populations, the breeding populations showed higher observed (H_o=0.792) and expected (H_e=0.868) heterozygosity, mean allelic richness (*R*=6.4) and number of private alleles (*Pa*=11), and lower inbreeding (F_{IS}=0.088) than wild populations, (H_o=0.728*;* H_e=0.818*;* *R*=5.8*;* *Pa*=9*;* F_{IS}=0.11) (Table 1). For *E. grandis*, the improved population also exhibited higher mean H_o (0.75), R (6.6), and number of private alleles (*Pa*=18), and lower F_{IS} than will populations (H_o=0.68; *R*= 6.4; *Pa*=3). Between species, *E. urophylla* showed higher H_o, H_e, and *R*, and lower F_{IS} than *E. grandis*. Clones displayed highest mean H_o (0.867) and H_e (0.865), and lower F_{IS} (-0.002).

For *E. grandis*, the genetic differentiation between all sampled populations (G_{ST}'= 0.775) was greater than between the breeding populations (G_{ST}'= 0.162), but lower than among the wild populations (G_{ST}'= 0.854) (Table 2). The genetic difference between the breeding populations and the clones was higher (G_{ST}'= 0.236) than between the breeding populations and the wild ones (G_{ST}'= 0.159), but smaller than between the wild ones and the clones (G_{ST}'= 0.86). For *E. urophylla*, the genetic differentiation between all sampled populations (G_{ST}'= 0.195) was greater than between the breeding populations (G_{ST}'= 0.082) and among the wild populations (G_{ST}'= 0.113). The genetic difference between the improved populations and the clones was smaller (G_{ST}'= 0.057) than between the improved populations and the wild ones (G_{ST}'= 0.094) and between the wild ones and the clones (G_{ST}'= 0.187). The commercial clones are the most genetically distant from the other populations. The separation between the *E. grandis* and *E. urophylla* populations was evident, as well as the distinction between the wild and breeding populations, which formed clusters (Figure 1).

Genetic differentiation is observed between the *E. grandis* and *E. urophylla*, but it is small among populations within the same species (breeding or wild). The clones grouped together between the species, indicating hybridization between them. It can also be observed more clearly that the clones are closely related to the population type and species. However, there is proximity between the wild and breeding populations (Figure 2).

With three clusters (K= 3), it was possible to observe the separation of the species and admixture within *E. grandis*. In the breeding population of *E. urophylla*, some individuals showed admixture with *E. grandis*. In fact, one of the clones exhibited greater similarity to *E. grandis*, while the others were more like *E. urophylla* (Figure 3).

All clones exhibited the influence of *E. urophylla*, and among them, all showed greater proximity to the improved populations, with the most prevalent being the population from Ilha Solteira (Table 3).

Table 1
Genetic diversity parameters estimated by species and improvement level.(Legend: *P< 0.01; n is the sample size; H_o and H_o are the mean observed and expected heterozygosity, respectively; F_{IS} is the mean fixation index; R is the mean allelic richness for five genotypes; Pa is the number of private alleles.)

Population	n	H_o	H_e	F_{IS}	R	Pa
E. urophylla	334	0.772	0.859	0.101*	-	-
E. urophylla: breeding	234	0.792	0.868	0.088**	6.4	11
• U-PCMF-A	90	0.801	0.859	0.067**	6.3	3
• U-PCMF-I	79	0.792	0.875	0.094**	6.5	7
• U-PCMF-L	65	0.778	0.854	0.09**	6.1	6
E. urophylla: wild	100	0.728	0.818	0.11**	5.8	9
• U-Flores	25	0.74	0.756	0.021	4.9	2
• U-East Timor	25	0.746	0.827	0.098**	6.0	1
• U-Other Island	25	0.738	0.835	0.116**	6.0	3
• U-Timor	25	0.688	0.813	0.154**	5.5	2
E. grandis	1247	0.736	0.89	0.173*	-	
E. grandis: breeding	990	0.75	0.882	0.149**	6.6	18
E. grandis: wild	253	0.68	0.881	0.228**	6.4	3
Clones	9	0.867	0.865	-0.002	6.2	2

Table 2
Genetic differentiation (G_{ST}, Hedrick 2005) among wild and breeding populations. Legend: U-wild = all wild populations of *E. urophylla*; U- breeding = all breeding populations of *E. urophylla*; *P< 0.05; G_{ST} between all *E. urophylla* populations = 0.236*; G_{ST} between all *E. urophylla* populations versus clones = 0.082*; G_{ST} between all *E. grandis* populations = 0.32*; G_{ST} between all *E. grandis* populations versus clones = 0.282*)

	U- breeding	U-wild	U-PCMF-I	U-PCMF-L	U-Flores	U-East Timor	U-Other Island	U-Timor	G-wild	Clones
U- breeding	0.082*	0.094*								0.057
U-wild		0.113*								0.187*
U-PCMF-A			0.042	0.052	0.266*	0.137*	0.141*	0.177*		0.095
U-PCMF-I				0.096*	0.205*	0.081*	0,107*	0.144*		0.029
U-PCMF-L					0.033*	0.023	0.003	0.005		0.107*
U-Flores						0.146*	0.097*	0.075*		0.331*
U-East Timor							0.033	0.071		0.156*
U-Other Island								0.041*		0.124
U-Timor										0.23*
G- breeding									0.159*	0.256*
G-wild										0.29*

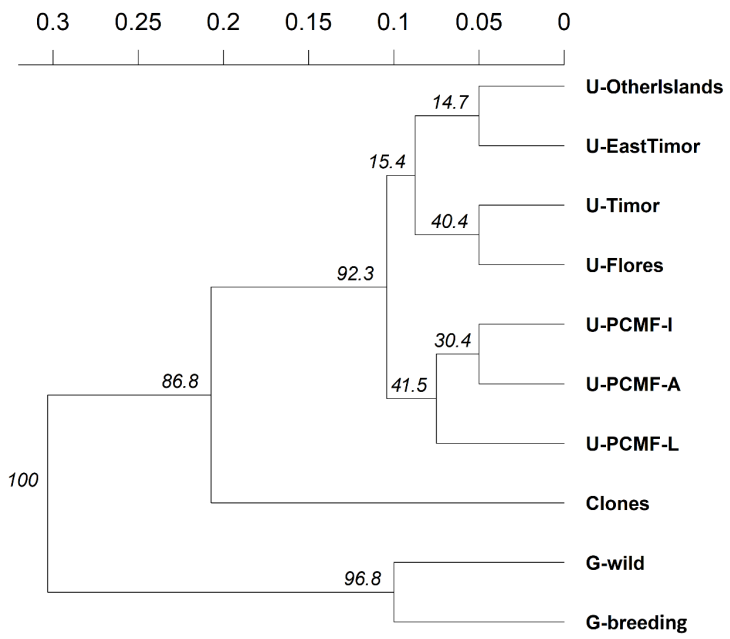

Figure 1
Dendrogram of populations based on Goldstein's genetic distance

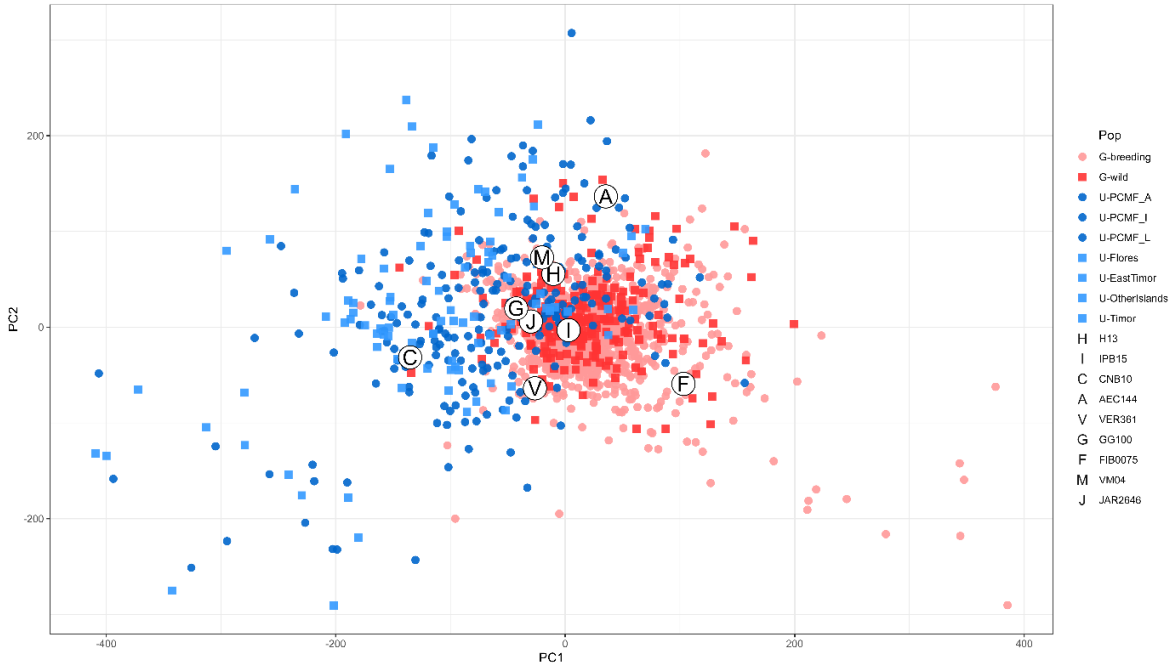

Figure 2
Genetic distances among samples of wild and breeding populations of *Eucalyptus* obtained from a principal coordinate analysis (PCoA).

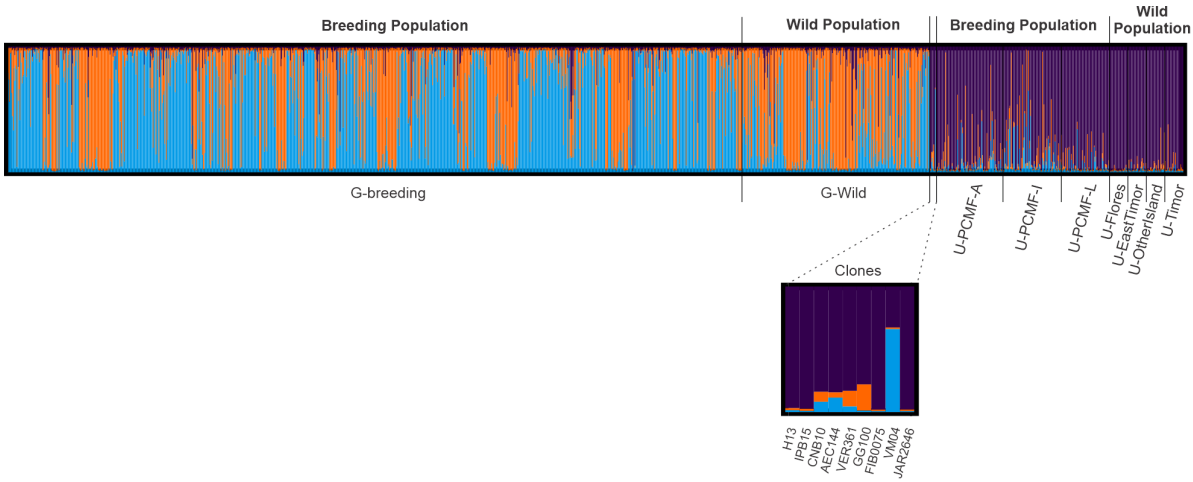

Figure 3
Barplot depicting the genetic composition of individuals with respect to the three clusters (K= 3) obtained in the best Structure model.

Table 3
Populations showing higher similarity with each of the nine clones. (Selection criteria Paetkau at al. (1995) and/or Rannala & Mountain (1997))

Clones	Populations	Similarity (%)
H13	U-PCMF-L	96.5
IPB15	U-PCMF-A	68.3
CNB10	U-PCMF-A	55.9
AEC144	U-PCMF-I	97.5
VER361	U-PCMF-I	96.3
GG100	U-PCMF-I	51.8
FIB0075	U-PCMF-L	77.3
VM04	U-PCMF-I	96.6
JAR2646	U-PCMF-L	79.5

Discussion

The results of genetic diversity indicated that the breeding populations of both *E. grandis* and *E. urophylla* exhibited higher observed heterozygosity and low inbreeding, demonstrating the genetic variability within these populations due to the mixture of different provenances. This highlights their importance as a genetic resource for the establishment of foundational populations in breeding programs for both species. The fixation index of the breeding populations was lower in both species, reinforcing the idea of mixing different origins within these populations. The breeding populations are composed of multiple origins with the purpose of achieving greater variability, including intra-specific hybrids (Assis et al. 2015). Wild populations may exhibit higher F values than breeding populations because the natural geographical barriers they face can limit gene flow between natural populations (Tipiama et al. 2007; Grattapaglia and Kirst 2008).

The allelic richness was higher in the populations of *E. grandis* compared to *E. urophylla*, surpassing the values reported for other species such as *E. cladoxylon* (1.47 in Buch and Thuma 2013; 1.54 in Mora et al. 2016). One of the reasons for this difference is the specificity of the SSR markers used, which have been previously tested in these species (Nevill et al. 2008). Another reason is the wide distribution of *E. grandis*, from latitude 18º to 32ºS (Boland et al. 2006), which occurs in two separate macro-populations, as observed in the results of this study, where two distinct genetic composition were detected within the species (Oliveira et al. 2023).

Despite the distinctions between wild and breeding populations not being entirely clear, clustering based on Nei's distance revealed some separation between these populations, although it had less bootstrap consistency compared to the differentiation observed between the species.

Among the wild populations of *E. urophylla*, no strongly distinct groups were observed, which is consistent with the description provided by Payn et al. (2008). They described that genetic differentiation among populations of *E. urophylla* was low across seven islands (F_{ST}=0.031); however, differentiation increased with geographic distance. Despite the absence of strong significant genetic distance or differentiation among wild populations, the findings indicate that in the improved populations of *E. urophylla*, the composition was structured with two distinct groups. The populations in Ilha Solteira originated from East Timor, akin to those in Anhembi, while in Lençóis Paulista, the provenance encompassed Timor.

The proximity of these populations within the breeding populations indicates that these specific origins are extensively utilized in breeding programs in Brazil. It is noteworthy that breeding populations consist of various populations that have undergone improvement in the country (Silva et al. 2018), potentially attributed to the better adaptability of these specific provenances in the region where the Brazilian forestry sector operates.

The clones are genetically close, forming a single group, in between the populations of *E. grandis* and *E. urophylla*. This is expected, as these cultivars are predominantly composed of admixtures of both species. However, it was observed that most clones have a greater contribution from *E. urophylla*, as indicated by pedigree records and highlighted in the literature (Assis et al. 2015). Our results particularly show that the nine clones are in genetically close to the Ilha Solteira breeding population. Nevertheless, one of the clones showed evidence of hybridization with *E. grandis*, contradicting its recorded pedigree. It should be noted that the clones were mostly obtained through open pollination or utilized parents obtained from open pollination, which could introduce errors in pedigree records due to gene flow occurring within distances of less than one km (Silva and Abrahao 2020).

All clones showed genetic similarity to the breeding populations of *E. urophylla*. On the other hand, clones VM04 and VER361 exhibited genetic similarity, or past hybridizations, with the breeding populations of *E. grandis*. The clone AEC144, which is the most planted clone in Brazil (Silva et al. 2021), showed a contribution from *E. grandis*, contradicting the recorded pedigree which register it as pure *E. urophylla*. These clones were obtained through open pollination and high-intensity mass selection. As open pollination naturally occurs in the base populations, spontaneous hybridization can occur unintentionally. Subsequently, the transgressive individuals are cloned (Assis et al. 2015), leading to the possibility of several clones being used without the correct annotation of species (Oliveira et al. 2023).

Conclusion

The analysis of genetic diversity revealed that the breeding populations exhibit greater genetic diversity compared to the wild populations of both species. *Eucalyptus grandis* demonstrated higher genetic variability than *E. urophylla*, likely due to its broader natural distribution. The studied clones exhibited different degrees of hybridization and genetic influences from both wild and breeding populations. Interestingly, the presence of *E. grandis* was observed in the clones, contrary to their expected pedigree annotations. This contamination of *E. grandis* was also observed in the breeding population of *E. urophylla*.

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