Molecular characterization of selected *Eucalyptus* clones

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ABSTRACT: *In the present study, molecular characterization of 11 Eucalyptus clones was carried out using 20 Simple Sequence Repeat (SSR) markers to assess genetic variation. Among the clones, clone K-25 was found to be distinctly different from the others. Of the 20 SSR markers used, 14 were polymorphic, while six were monomorphic. The highest polymorphic information content (PIC) value was observed for the marker EMBRA59 (0.89), indicating its high discriminatory power. Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in NTSYS-pc version 2.02, resulting in the classification of the clones into two major clusters: Cluster I and Cluster II. Cluster I was further divided into two sub-clusters, Ia and Ib. Sub-cluster Ib was further subdivided into clades Ib1 and Ib2. Sub-cluster Ia comprised two genotypes, clones K-23 and K-68, while clade Ib1 included clones K-16, AP-7, G-22, K-24, K-28, and BCM-413, with K-24 and K-28 showing high similarity. Clade Ib2 consisted of clones K-43 and K-68. Cluster II was represented solely by clone K-25, indicating its distinct genetic identity. The observed genetic variation provides valuable insights for the development of improved hybrids and can be utilized to broaden the genetic base for commercial Eucalyptus cultivation.*

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1. INTRODUCTION

Eucalyptus, a member of the Myrtaceae family, is among the most widely cultivated tree species, originating from the Australian subcontinent (Brooker, 2000). This genus naturally thrives in latitudes ranging from 7°N to 43°39'S, predominantly in tropical and subtropical regions. With over 900 identified species (Chandorkar *et al.*, 2021), eucalyptus was first described by the French botanist L'Héritier in 1788, naming the species *E. obliqua*. The name "Eucalyptus" stems from the Greek words "eu," meaning "well," and "kalypto," meaning "to cover," referring to the flower's calyx that forms a protective cap (Gledhill, 2008). Eucalyptus is a diploid genus with a chromosome count of 2n = 22 and a relatively small genome size, ranging from 370 to 700 Mbp (Grattapaglia and Bradshaw, 1994). Its genome is characterized by minimal repetitive DNA, extensive diversity, and the capacity to produce large progeny groups, which has facilitated the development of linkage maps (Myburg *et al.*, 2003; Ferguson *et al.*, 2024). Globally, Eucalyptus species cover approximately 25 million hectares, making it the most extensively planted tree genus (FAO, 2021; Medeiros *et al.*, 2025). *Eucalyptus tereticornis* (Forest Red Gum) and *E. camaldulensis* (River Red Gum) are among the most widely cultivated species in India, valued for their fast growth, ecological adaptability, and economic utility. *E. tereticornis* is prized for its durable timber, fuelwood, and pulp, and is commonly used in agroforestry and land reclamation. In contrast, *E. camaldulensis* is highly tolerant to saline and degraded soils, with strong coppicing ability and rapid biomass production, making it ideal for pulpwood plantations and afforestation. It also supports honey production and yields essential oil with antimicrobial properties (Zhan *et al.*, 2022; Varghese *et al.*, 2024; Sabo *et al.*, 2019). Both species are central to clonal forestry efforts in India, with several clones developed for commercial and conservation use.

The productivity of natural forests in India averages less than 2 m^3 ha^{-1} annually, whereas Eucalyptus plantations achieve productivity rates of 6–10 m^3 ha^{-1} in forests and 15–20 m^3 ha^{-1} in farmlands (Kaur, 2021). However, declining productivity in Eucalyptus plantations has become a significant concern, emphasizing the importance of breeding programs aimed at enhancing productivity. These efforts rely heavily on exploiting genetic variation, which is a fundamental component of breeding success. The challenge with Eucalyptus lies in the limited genetic variability within its base population. Therefore, understanding the genetic variation of cultivars and their proper identification is essential. Grouping

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progeny based on similarities is also critical for selecting cultivars for crossing programs, particularly for traits of economic importance (Silva *et al.*, 2012). The substantial genetic variation found in forest tree species can also be used to trace the phylogeny of plants and their products, including timber and processed wood. In this context, molecular tools play an essential role. Clonal propagation has significantly enhanced the genetic gains of breeding programs by capturing both additive and non-additive effects into elite clones. Superior genotypes with high productivity are selected through genetic improvement programs, and cloning techniques are employed for large-scale propagation of elite clones (Grattapaglia and Kirst, 2008). However, errors, particularly in clone labelling, can occur during propagation, cultivation, and exchange processes (Li et al., 2011). Accurate clonal identification is therefore critical for vegetatively propagated trees and is essential for registering new cultivars (Finžgar *et al.*, 2023). Advances in molecular marker technology have proven effective in addressing these challenges. Although early markers like RAPD and AFLP were valuable, microsatellite markers (SSRs) have emerged as the most reliable for identifying Eucalyptus trees (Faria *et al.*, 2011; Negi *et al.*, 2025). While commonly used dinucleotide SSR markers provide robust discrimination, challenges arise when comparing multilocus profiles across laboratories. These issues can be mitigated by utilizing tetranucleotide and pentanucleotide repeat SSRs. This study aims to employ clonal fingerprinting and phylogenetic analysis to assess genetic variation in selected Eucalyptus clones.

2. MATERIALS AND METHODS

Experimental material and DNA extraction

The experimental material comprised eleven *Eucalyptus* clones, sourced from various forestry research institutions and industries. Clone K16 belongs to *Eucalyptus tereticornis* and was developed by the Kerala Forest Research Institute (KFRI). Clones K23, K24, K25, and K68, all belonging to *E. camaldulensis*, were also developed by KFRI. Among them, K23 have a high rooting success rate (80–90%), while K25 is notable for being the second most widely produced and planted clone in northern India, with good rooting ability (>90%) but a relatively higher mortality rate (60–70%). Additionally, clones K28 and K43, developed by KFRI, belong to *E. tereticornis*. Clone BCM413, developed by ITC Bhadrachalam, is a *E. camaldulensis* clone widely planted across north India. It is highly adaptable to unfavourable planting sites and shows good rooting. Despite its vulnerability to Cylindrocladium Leaf Blight, the clone exhibits a mortality rate of less than 50%. Clones AP7 and AP10, both derived from *E. tereticornis*, were obtained from West Coast Paper Mill, Karnataka. Lastly, clone G22, also a *E. tereticornis* clone, was selected by the Forest Department, Lalkuan, Uttarakhand. These clones were evaluated for molecular genetic variation using 20 SSR markers. The study was conducted at the Agroforestry Research Center, G.B. Pant University of Agriculture and Technology, Pantnagar, where the clones were planted in a randomized block design (RBD) with three replications, maintaining a spacing of 7m x 2m.

Genomic DNA was extracted from the young, tender leaves of all 11 clones using the CTAB (Cetyltrimethylammonium bromide) method as described by Saghai-Maroof *et al.* (1984), with slight modifications. To prevent the co-purification of secondary metabolites with DNA, 1% polyvinylpyrrolidone (PVP) (100 mg/g of leaf tissue) was incorporated during the isolation process. PVP was either added during leaf grinding or directly to the extraction buffer to ensure adequate DNA yield. The quality of the extracted genomic DNA was assessed by loading 2 µl of DNA, mixed with 4 µl of 6X DNA loading dye, onto a 0.8% agarose gel prepared in 1X TBE buffer. Electrophoresis was carried out at 80–100 volts for three hours, and the results were visualized under a UV transilluminator. DNA quantification was performed simultaneously by measuring the optical density (OD) of a 10 µl DNA sample diluted 100 times, using an Eppendorf™ UV Biophotometer. Absorbance was measured at 260 nm and 280 nm, with a blank setup using 2 µl of TE buffer. The 260/280 OD ratio was used to assess RNA or protein contamination, with DNA samples having a ratio of approximately 1.8 considered suitable for PCR reactions.

SSR Marker Selection

Twenty SSR markers, distributed over 11 linkage groups were selected from public domain, as previously defined in *Eucalyptus* linkage map by (Brondani *et al.*, 2006). The markers used in the present investigation are enlisted in table 1.

Polymerase Chain Reaction: PCR amplification was carried out in a 25 µl reaction mixture, which included 2.0 µl of genomic DNA at a concentration of 100 ng/µl, 2.5 µl of 1X Taq buffer [comprising 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$], 1.5 µl of dNTPs, 1 µl each of forward and reverse primers (0.6 µM each), 0.5 µl of Taq DNA polymerase (3 units/µl), and double-distilled water to make up the final volume. The PCR cycling conditions began with an initial denaturation step at 94°C for 5 minutes, followed by four cycles of denaturation at 94°C for 30 seconds, annealing at either 52°C or 57°C for 30

Table 1. List of SSR (microsatellite) primers and primer sequences used for Eucalyptus molecular characterization (EMBRA: Eucalyptus microsatellites from Brazil):

S.No.	Primer	Primer sequence (5' to 3')	
		Forward	Reverse
1	EMBRA 11	GCTTAGAATTTGCCTAAACC	GTAAAATCCATGGGCAAG
2	EMBRA 12	AGGATTTGTGGGGCAAGT	GTTCCCCATTTTCATGTCC
3	EMBRA 10	GTAAAGACATAGTGAAGACATTCC	AGACAGTACGTTCTCTAGCTC
4	EMBRA 186	GGAAGGCTTTGAGATAAC	ACCGAGACCAGTTGAGAG
5	EMBRA 180	ATCAACGACACATATGCAGC	GGATGCTTGGGTGATTGT
6	EGMA 30	AGTGCAGCACCTTTCAGACC	AAGATTGATTGCTAGATCAGTCACC
7	EMBRA 6	AGAGAATTGCTCTTCATGGA	GAAAAGTCTGCAAAGTCTGC
8	EMBRA 57	CCTTCTCTCTCTTGGAATAC	ATAGCCAGTGAAAGTGAGG
9	EMBRA 60	AACAGCAGTTGCTACACCAC	GAGCGAAAAGGAGAACACC
10	EMBRA 34	TCAAAACCCTCTCTCTCAT	AATAAACATTTTCTTTGAACAGA
11	EMBRA 71	GCGAATTTGCTAACAACC	GACACACCTCTACACACACC
12	EMBRA 37	CACCTCTCCAAACTACACAA	CTCCTCTCTCTTCACCATTC
13	EMBRA 45	GTCATTTGCACACAGTTTTC	AGTTCATAGAATGCAGAAAATG
14	EMBRA 8	CACAACTAAAAATCAAAACCC	AAAGAGCAGATTATTACAGAAGC
15	EMBRA 7	CACACCGTGTCAGTTAGC	AATAAGGAGGATTCCATGG
16	EMBRA 2	CGTGACACCAGGACATTAC	ACAAATGCAAATTCAAATGA
17	EMBRA 23	GGTTGTTTCATCTTTTCCATG	AGCGAAGGCAATGTGTTT
18	EMBRA 59	GTTGTGCATGGGCCTCTTG	CGACGGCCAGTGAATTGTAA
19	EMBRA 33	CAATTTGCATGTCCAGTTTG	GCAGAAGTTGATTGAAAGCA
20	EMBRA 5	ATGCTGGTCCAACTAAGATT	TGAGCCTAAAAGCCCAAC

seconds (depending on the primer), and extension at 72°C for 1 minute. This was followed by 34 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 1 minute, with a final extension step at 72°C for 5 minutes. The annealing temperature was primer-specific, and adjustments were made accordingly. For primers 1, 2, 3, 4, 5, 7, 11, 12, 13, 14, and 20, the annealing temperature was set at 52°C for the initial four cycles and 55°C for the subsequent 34 cycles. Meanwhile, primers 6, 8, 9, 10, 15, 16, 17, 18, and 19 required an annealing temperature of 57°C for the initial four cycles, followed by 55°C for the remaining 34 cycles. To accommodate these temperature changes for specific primers, touchdown PCR was utilized.

SSR Genotyping: Clear and reproducible SSR bands were analyzed for each lane by scoring their absence (0) or presence (1) to generate a binary matrix. Polymorphic Information Content (PIC) values for each marker were calculated in Microsoft excel (Microsoft Office 2021), using the formula provided by Botstein *et al.* (1980). The amplification data obtained from SSR markers were processed using the SIMQUAL subprogram in NTSYS-PC software version 2.02 (Rohlf, 1998) to compute Jaccard's similarity coefficient (Jaccard, 1908). This similarity coefficient was subsequently used to construct a dendrogram illustrating the genetic relationships among the ramets, employing the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) algorithm (Sneath and Sokal, 1973).

3. RESULTS AND DISCUSSION

The markers used in the study were distributed throughout the genome. Out of the twenty Simple Sequence Repeat (SSR) markers, only fourteen were polymorphic for the experimental clones under study, while the rest six were monomorphic. The polymorphic features of 20 SSR primer pairs are given in Table 2. EMBRA 11, EMBRA 60, EMBRA 71, EMBRA 37, EMBRA 45 and EMBRA 59 resulted in 2 amplified alleles, while EMBRA 12, EMBRA 10, EMBRA 186, EMBRA 180, EMBRA 6, EMBRA 8 and EMBRA 5 produced 1 amplified allele. EGMA 30 amplified 3 alleles. The bands obtained mostly varied in size from 100 bp to 200 bp. However, EMBRA12 did not amplify in maximum genotypes despite repeating the experiment and changing different annealing temperatures, thus referred to as null allele. Grewal *et al.* (2014) also did not observe any amplification for Populus species using the SSR

marker. Mutations at binding site of the targeted DNA sequence prevent annealing of primer which results in amplification failure during PCR reaction causing null alleles. They can also occur due to segmental aneuploidy, where chromosome with primer binding site can undergo certain deletion (Rico *et al.*, 2017). The inability to detect an allele in PCR-based genetic markers can occur due to several factors like, failure in primer binding caused by DNA sequence variations from the reference sequence or poor primer design or unequal amplification of alleles, with longer alleles sometimes failing to amplify. Also, insufficient DNA quantity, as variations in DNA extract quality can result in successful amplification at some loci but not others. Null alleles pose significant challenges, particularly in pedigree analyses or paternity testing, where they can lead to incorrect exclusion of a parent by misrepresenting a heterozygous genotype as homozygous. For several reasons, null alleles are a problem in the use of genetic markers. In the most common applications in pedigree analyses or paternity tests, they can cause the erroneous exclusion of one or both parents by implying the putative parent in a locus to be homozygous when in fact it is heterozygous for a null allele (Jahnke *et al.,* 2022). Some SSR markers exhibit high discriminatory power by revealing unique or rare alleles, aiding in genotype differentiation. Unique alleles are specific to a single genotype in a population, often resulting from unequal crossing over or replication slippage. Rare alleles, on the other hand,

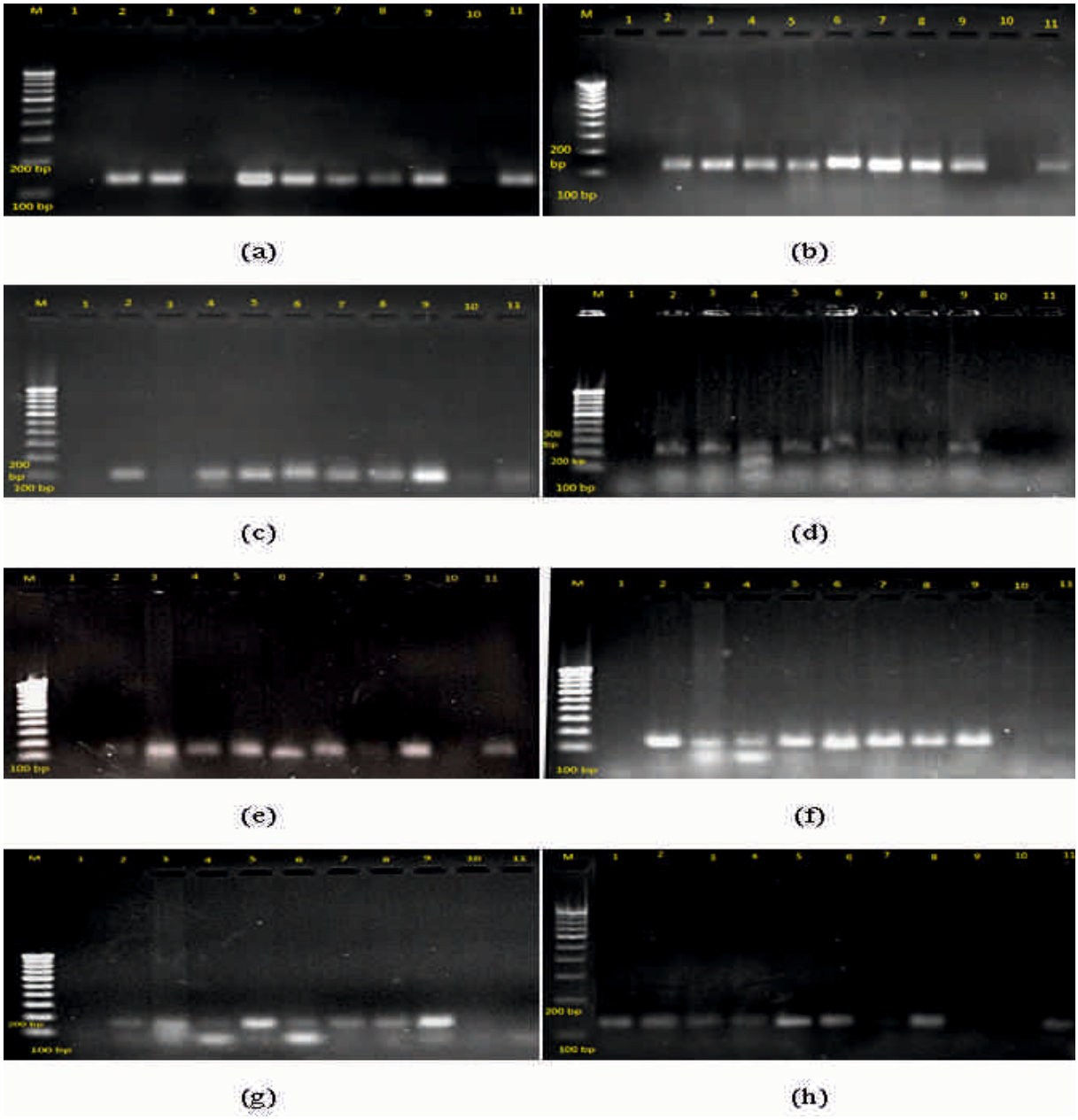

Figure 1 : Amplification shown by primers (a) EMBRA 12, (b) EMBRA 186, (c) EMBRA180, (d) EGMA 30, (e) EMBRA 6, (f) EMBRA 37, (g) EMBRA 45, (h) EMBRA 8

are found in only two or three genotypes. Unique alleles can serve as diagnostic tools, acting as genetic fingerprints to distinguish specific genotypes. As SSR markers at a locus can detect multiple alleles, each fragment generated corresponds to an allele at that locus. For instance, if a marker detects three alleles at a locus and one of them is found exclusively in a single genotype, it is classified as a unique allele. In this study, EGMA30 identified a unique allele in K-25, while EMBRA37 amplified rare alleles in K-25 and K-24, and EMBRA45 did so for K-24 and AP-7.

Polymorphic Information Content (PIC) value ranged from 0.32 to 0.89 (Table 2). The maximum PIC value was recorded for the primer EMBRA 59 (0.89) followed by EMBRA 60 (0.85), EMBRA 180 (0.59), EMBRA 45 (0.56). Polymorphism information content (PIC) or expected heterozygosity score is tantamount to 'gene diversity' as it provides an assessment of discriminating power of loci or locus, by considering not only the number of expressed alleles, but also their relative frequency. In the present study, the number of alleles amplified and PIC values obtained are comparatively lower than those reported in other studies (Kaur *et al.*, 2018; Kirst *et al.*, 2005). The probable reason for that may be the use of agarose gel electrophoresis or low primer number or nature of genotypes. PIC values of a primer also vary with the crop and the set of genotypes used. Lower PIC value may be the result of closely related genotypes and higher PIC values may be the result of diverse genotypes. There are several factors affect the polymorphic information content (PIC) value, including the genetic variation of the germplasm, the number of SSR loci and clones examined, the characteristics of the SSR repeats, and the allele detection technique used, such as agarose gel or PAGE (Li *et al.*, 2024). In the present study, although fewer markers were analysed, the substantial genetic variation among the clones resulted in a high PIC value. The SSR markers used were also highly informative despite their limited number, highlighting the critical role of both the quality and quantity of SSR loci in determining the PIC value. In Eucalyptus, a diploid and cross-pollinating species, single alleles were observed at several loci in some clones, potentially due to homozygosity or the limited resolution of agarose gel for detecting allelic variation.

Similarity and Cluster analysis among Eucalyptus clones: The genetic relationship among different clones was detected through Jacquard's similarity coefficient, depicted in Table 3. The similarity coefficient among the clones ranged from 0.28 to 0.90 where the minimum similarity was between clone K-25 and clone AP-10 and also between clone K-68 while maximum similarity coefficient (0.9) was observed between clone K-28 and clone K-24. The dendrogram (Figure 2), constructed using the UPGMA clustering method, grouped the 11 *Eucalyptus* clones into two major clusters—Cluster I and Cluster II—at a similarity coefficient threshold of 0.69. Cluster I, the larger of the two, was further subdivided into two sub-clusters: Ia and Ib. Sub-cluster Ib was subsequently divided into two clades: Ib1 and Ib2. Sub-cluster Ia consisted of two genotypes, K-23 and K-68, indicating notable genetic divergence from the remaining clones. Clade Ib1 included six clones—K-16, AP-7, G-22, K-24, K-28, and BCM-413—with K-24 and K-28 showing a high degree of genetic similarity. Clade Ib2 contained clones K-43 and K-68. Cluster II was represented exclusively by

Table 2: Polymorphic information content (PIC) of Eucalyptus clones by SSR markers

Name of Primer	No. of alleles amplified	Allele size range	PIC
EMBRA 11	3	150-210 bp	0.32
EMBRA 12	2	150-160 bp	0.47
EMBRA 10	2	170-180 bp	0.59
EMBRA 186	3	140-165 bp	0.33
EMBRA 180	2	100-110 bp	0.59
EGMA 30	3	100-250 bp	0.68
EMBRA 6	2	150-155 bp	0.47
EMBRA 60	2	120-150 bp	0.85
EMBRA 71	2	550-600 bp	0.79
EMBRA 37	3	140-155 bp	0.43
EMBRA 45	2	140-160 bp	0.43
EMBRA 8	2	140-150 bp	0.47
EMBRA 59	3	150-200 bp	0.89
EMBRA 5	2	150-160 bp	0.70

Table 3: Similarity coefficient based on DNA amplification of eleven Eucalyptus genotype estimated by Jaccard's coefficient

Clones	K-23	K-16	BCM413	K-25	K-24	AP-7	G-22	K-43	K-28	AP-10	K-68
K-23	1										
K-16	0.47	1									
BCM-413	0.42	0.76	1								
K-25	0.33	0.57	0.52	1							
K-24	0.42	0.85	0.71	0.52	1						
AP-7	0.33	0.85	0.71	0.52	0.71	1					
G-22	0.42	0.85	0.71	0.42	0.71	0.80	1				
K-43	0.61	0.76	0.71	0.33	0.71	0.71	0.80	1			
K-28	0.33	0.76	0.71	0.42	0.90	0.71	0.80	0.71	1		
AP-10	0.85	0.52	0.38	0.28	0.47	0.38	0.57	0.57	0.47	1	
K-68	0.66	0.61	0.66	0.28	0.66	0.57	0.66	0.76	0.66	0.61	1

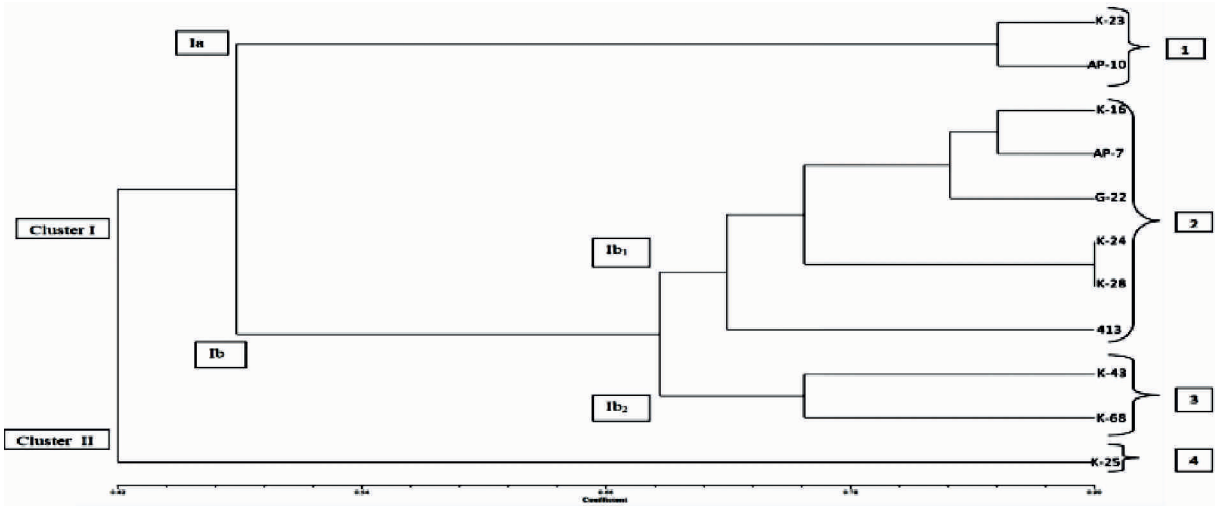

Figure2: Dendrogram for 11 Eucalyptus clones based on genetic distance

clone K-25, highlighting its distinct genetic identity compared to the rest of the population. These results clearly indicate that clone K-25 is genetically the most divergent among the studied clones, followed by clones K-23 and K-68, which also exhibit considerable variation. These findings are consistent with previous studies, such as those by Kaur *et al.* (2018), who assessed 16 *Eucalyptus tereticornis* and *Eucalyptus grandis* clones using SSR and EST markers, and Torres-Dini *et al.* (2021), who examined the genetic diversity and structure of 107 elite clones—including 80 *E. grandis* and 27 *E. globulus*—using 17 microsatellite loci. Another study by Patturaj *et al.* (2021) offers valuable insights into the genetic diversity of *Eucalyptus* species and reports the development of validated SSR markers for genetic mapping in *E. camaldulensis* and related populations. The findings are consistent with the present study, further reinforcing the effectiveness of SSR markers in assessing genetic variation within Eucalyptus clones. The limited genetic diversity observed in the present study is consistent with earlier findings in *E. benthamii* breeding programs in Brazil, which also reported a narrow genetic base and signs of inbreeding (Ferreira *et al.*, 2024).

4. CONCLUSION

Genetic variation existing in Eucalyptus can be further exploited in it's breeding and improvement programs. With the help of DNA paternity testing, one can retrospectively select better combining parents. Categorization of Eucalyptus clones into different clusters is helpful for tree breeders for intra-specific hybridization studies, developing new hybrid clones and improving seed orchards. Moreover, diverse clones could be used for intra-specific hybridization.

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