Genetic diversity of eucalypts for germplasm conservation in Forest Area with the Special Purpose of Mount Bromo, Karanganyar, Indonesia

RAHAYU[1,♥], FATIMAH[2], JAMAL WIWOHO[3], SUNNY UMMUL FIRDAUS[3], PUJIYONO[3], MARIMIN[4], DWI PRIYO ARIANTO[1], AHMAD PRAMONO[1]

[1]Faculty of Agriculture, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax. +62-271-663375,
♥email: rahayu@uns.ac.id
[2]Indonesian Center of Biotechnology and Genetic Resources, Ministry of Agriculture. Jl. Tentara Pelajar 3A, Bogor 16111, West Java, Indonesia
[3]Faculty of Law, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia
[4]Faculty of Cultural Studies, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia

Abstract. *Rahayu, Fatimah, Wiwoho J, Firdaus SU, Pujiyono, Marimin, Arianto DP, Pramono A. 2021. Genetic diversity of eucalypts for germplasm conservation in Forest Area with the Special Purpose of Mount Bromo, Karanganyar, Indonesia. Biodiversitas 22: 4223-4235.* As a repository of a gene pool, eucalypts germplasm enriches biodiversity, maintains ecosystem sustainability, and aids in conservation. Therefore, this study aims to analyze the genetic diversity of eucalypts (*Corymbia* and *Eucalyptus*) for the development of germplasm conservation in Forest Area with the Special Purpose (KHDTK) Bromo Forest, Karanganyar, Indonesia. In this study, 14 simple sequence repeat (SSR) markers were used to assess the genetic diversity among 20 accessions (*Corymbia* and 5 *Eucalyptus* species) from Central and West Java. Subsequently, the genetic parameters were measured and a phylogenetic tree was constructed. The result showed that the SSR markers have high variability, although they belong to different genera. Furthermore, the genetic diversity showed 49 alleles with an average of 3 alleles per locus, while the polymorphism information content (PIC) values were 0.55. There were 4 SSR markers (EMBRA13, EMBRA8, EMCRC11, and EMBRA2) with high PIC value, while the gene diversity (H*e*) of *Corymbia* and 5 *Eucalyptus* showed a low level of genetic diversity. The genetic relationship and population structure were divided into genera *Corymbia* and *Eucalyptus*. For further application, the eucalypt cultivated in the KHDTK Bromo Forest can contribute as a reference set and 14 SSR markers as a potential marker in combination with morphological characterization to generate a database for germplasm management and conservation.

Keywords: *Corymbia citriodora, Eucalyptus deglupta, E. globulus, E. pellita, E. urophylla,* simple sequence repeat

INTRODUCTION

Eucalypts is one of the most important industrial forest plantations due to their rapid growth capacity, wider adaptability, and versatile purposes such as pulp, paper, timber, and eucalypt oil production (Sumathi and Yasodha, 2014). Eucalypt belongs to the Myrtaceae family and comprises over 800 species, including 3 closely related genera, namely *Angophora*, *Corymbia,* and *Eucalyptus* (Nicolle 2019). The genus of *Angophora* consists of only one genus commonly present in New South Wales and Queensland, Australia (Nicolle 2019). Meanwhile, the 2 subgenera of *Corymbia* (bloodwoods) include *Corymbia citriodora* which belongs to the *Blakella* subgenus (Nicolle 2019). The natural distribution of *Corymbia* extended to northern or eastern Australia (Schuster et al. 2019; Goodine and Oelgemoller 2020). Out of the 9 subgenus of *Eucalyptus, Eucalyptus urophylla*, *E. globulus*, *E. deglupta,* and *E. pellita* belong to the same subgenus namely *Symphyomyrtus* (Nicolle, 2019). Similarly, the natural distribution of *Eucalyptus* species is mostly present in Australia and partly in Papua New Guinea, while *E. deglupta* Blume and *E. urophylla* S.T. Blake are the only

two species spread in Eastern Indonesia (Nicolle 2019).

Plant genetic resources (PGR) need to be continuously enriched, conserved and maintained. Meanwhile, the strategy for preserving of eucalypts germplasm was carried out for ex-situ conservation in Forest Area with the Special Purpose (*Kawasan Hutan Dengan Tujuan Khusus,* KHDTK) Bromo Forest of Sebelas Maret University, Central Java Province. In forest management, the main focus is for the benefit of forestry research, development, and education (Apriyanto and Kusnandar 2020; Wicaksono et al. 2020). Moreover, the advancement of molecular genetic techniques is becoming increasingly important for studying biodiversity and nature conservation. Molecular tools are also used to decipher distributions and affiliation of a population distribution to identify the populations with immediate conservation concerns (Mukherjee and Ramakrishnan 2018).

Microsatellite or simple sequence repeat (SSR) is a DNA marker with a simple sequence consisting of one to six repeated bases, it is commonly present in plant genomes (Brondani et al. 1998). Meanwhile, the high level of polymorphism makes the SSR markers be distributed genetically based on species and individuals (Burke and Long 2012). The characteristics of SSR include the

predominant distribution in genome, locus specificity, co-dominance, multi-allelic, high mutation rate, heterozygous, transferability across species, and associated with the gene expression and function (Sumathi and Yasodha 2014). However, the disadvantage of SSR markers in the genetic analysis is size homoplasy and a high polymorphism rate (Hodel et al. 2016). Therefore, phylogenetic studies need to be carried out with caution for distantly related species (Abdurakhmonov 2016). The quick examination of SSR with low cost and technical efficiency makes it preferable and attractive to be applied in the large practice of molecular analyses.

Previous studies on eucalypt using SSR markers include the development of SSR markers (Grattapaglia et al. 2015), genetic diversity (Liu et al. 2018b), population structure analyses (Costa et al. 2017), genetic linkage map (Sumathi et al. 2018), phenotypic and genotypic variation (Padovan et al. 2017; Zhou et al. 2020), and hybrid purity assessment (Subashini et al. 2014). However, in Indonesia, there are limited information and studies on the characterization of eucalypts germplasm based on SSR markers such as the genetic diversity of *E. urophylla* from 7 islands in eastern Indonesia (Payn et al. 2007) and genetic variation of *E. deglupta, E. urophylla, and E. pellita* from arboretum in Yogyakarta as well as seed garden in South Kalimantan (Nurtjahjaningsih et al. 2013).

Therefore, there is a need to have new insights about the distribution and genetic diversity of eucalypt in Indonesia for more comprehensive examination using the molecular technique. Meanwhile, this is the first study in KHDTK Bromo Forest to measure the genetic diversity of *Corymbia* and *Eucalyptus* as a preliminary study for the development of eucalypts germplasm conservation.

MATERIALS AND METHODS

Plant materials

The leaves of *Corymbia* and *Eucalyptus* used for DNA analysis were from a seedling at several locations in Central and West Java, Indonesia collected in 2021 (Figure 1, Table 1). These plants were planted and cultivated in a Forest Area with the Special Purpose (KHDTK) Bromo Forest for germplasm conservation. Subsequently, each leaf sample was collected, placed in a plastic bag, and stored in a deep freezer (-20°C) until DNA extraction.

Procedures

This molecular analysis was carried out in 2021 at the Molecular Biology Laboratory of Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor. The fresh leaf samples were weighed 100 mg each and crushed using liquid nitrogen, while the DNA extraction was conducted using a modified CTAB method (Yu 1994). Furthermore, the PCR reaction was carried out at 20 μL volume, which contained a PCR buffer mix, 0.5 μM primers, DNA (50 ng/μL), and 1 unit of Mytaq DNA polymerase. The PCR program used was initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 60 sec (denaturation), 55°C for 60 sec (annealing), and 72°C for 120 sec (extension), while the final extension was at 72°C for 7 min and soaked at 4⁰C. Subsequently, the PCR products were separated using 8% polyacrylamide gel electrophoresis and the DNA staining was conducted with ethidium bromide. The visualization of PCR products used a gene analyzer machine (BioRad). The 23 microsatellite markers used were developed from *E. grandis, E. urophylla, E. globulus, E. pellita*, and *C. citriodora* (Table 2). Meanwhile, out of 23 primers, 14 were used for further analysis.
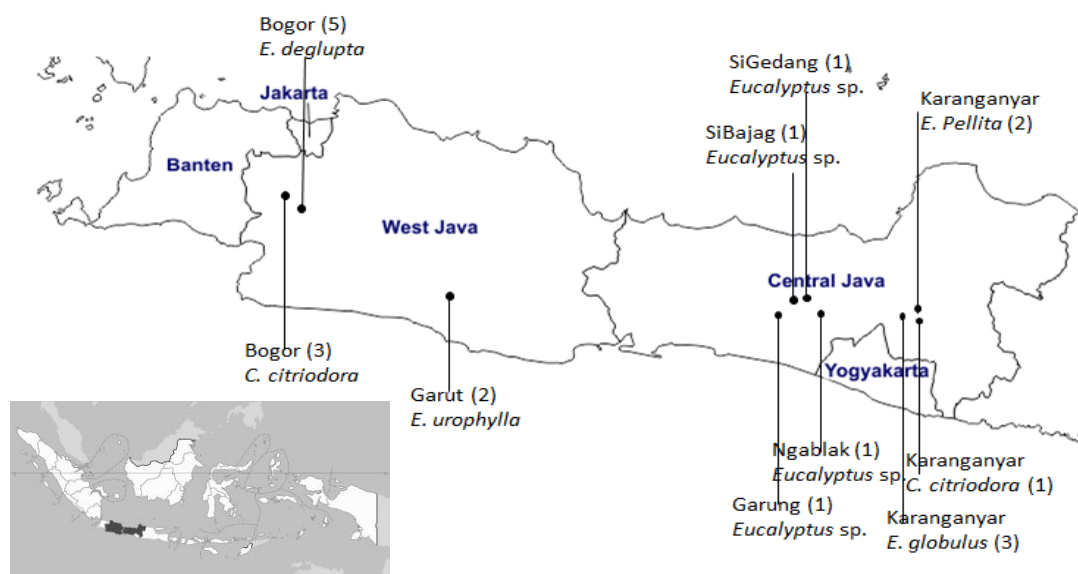

Figure 1. Map of West Java and Central Java, Indonesia, shows the location of eucalypt sampling sites collected from two provinces. The lines indicate the province and the number of eucalypts sampled from each location

Table 1. Data of collected *Corymbia* and *Eucalyptus* used in this study

Accession	Species	Local name	Natural distribution	Section	Source	Province
E16	*Corymbia citriodora*	Ekaliptus Lemon	Northern Australia	*Maculatae*	Karanganyar	Central Java
E2	*C. citriodora*	E. Lemon	Northern Australia	*Maculatae*	Bogor	West Java
E6	*C. citriodora*	E. Lemon	Northern Australia	*Maculatae*	Bogor	West Java
E10	*C. citriodora*	E. Lemon	Northern Australia	*Maculatae*	Bogor	West Java
E221	*Eucalyptus urophylla* S.T. Blake	Ampupu	Wetar Islands, Timor, Alor, Pantar, Lomblen, Adonara, and Flores	*Latoangulatae*	Garut	West Java
E222	*E. urophylla* S.T. Blake	Ampupu	Wetar Islands, Timor, Alor, Pantar, Lomblen, Adonara, and Flores	*Latoangulatae*	Garut	West Java
E18	*E. deglupta* Blume	E. Pelangi (Rainbow Gum)/Leda	West Papua, Seram, and Sulawesi	*Equatoria*	Bogor	West Java
E31	*E. deglupta* Blume	E. Pelangi (Rainbow Gum)/Leda	West Papua, Seram, and Sulawesi	*Equatoria*	Bogor	West Java
E21	*E. deglupta* Blume	E. Pelangi (Rainbow Gum)/Leda	West Papua, Seram, and Sulawesi	*Equatoria*	Bogor	West Java
E231	*E. deglupta* Blume	E. Pelangi (Rainbow Gum)/Leda	West Papua, Seram, and Sulawesi	*Equatoria*	Bogor	West Java
E232	*E. deglupta* Blume	E. Pelangi (Rainbow Gum)/Leda	West Papua, Seram, and Sulawesi	*Equatoria*	Bogor	West Java
E26	*Eucalyptus* sp.	Ekaliptus	-	-	SiBajag	Central Java
E25	*Eucalyptus* sp.	Ekaliptus	-	-	Ngablak	Central Java
E27	*Eucalyptus* sp.	Ekaliptus	-	-	SiGedang	Central Java
E24	*Eucalyptus* sp.	Ekaliptus	-	-	Garung	Central Java
E13	*E. globulus* subsp. *globulus* Labill.	*E. globulus* (Tasmanian Blue Gum)	Native to southeast Australia, originated in Victoria and Tasmania and the islands in the Bass Strait	*Maidenaria*	Karanganyar	Central Java
E14	*E. globulus* subsp. *globulus* Labill.	*E. globulus* (Tasmanian Blue Gum)	Native to southeast Australia, originated in Victoria and Tasmania and the islands in the Bass Strait	*Maidenaria*	Karanganyar	Central Java
E28	*E. globulus* subsp. *globulus* Labill.	*E. globulus* (Tasmanian Blue Gum)	Native to southeast Australia, originated in Victoria and Tasmania and the islands in the Bass Strait	*Maidenaria*	Karanganyar	Central Java
E20	*E. pellita* F. Muell	*E. pellita*	West Papua, Papua New Guinea, and northern Australia	*Latoangulatae*	Karanganyar	Central Java
E32	*E. pellita* F. Muell	*E. pellita*	West Papua, Papua New Guinea, and northern Australia	*Latoangulatae*	Karanganyar	Central Java

Data analysis

The amplified bands of the SSR data obtained were scored based on the allele sizes (bp) across multiple samples using PhotoCapMW. The species compatibility survey of SSR markers was classified into three categories, namely unamplified markers, amplified markers that produced 1 allele (monomorphic), and amplified markers with many alleles (polymorphic) (Nurtjahjaningsih et al. 2013). Meanwhile, the genetic parameters used to characterize microsatellite markers were genetic diversity per locus, the number of alleles detected (Na), observed heterozygosity (Ho), expected heterozygosity (He), and Principal Coordinate Analysis (PCoA). These parameters were calculated to measure the genetic diversity between eucalypts species using PowerMarker v3.25 (Liu and Muse 2005) and GenAlex v6.41 (Peakall and Smouse 2006). Furthermore, the dendrogram was compiled from allele frequency data to describe the genetic relationship between eucalypts species and was analyzed using the NTSys v2.1 program (Rohlf 2005). The population structure of eucalypts was also analyzed using Structure v2.1 (Pritchard et al. 2000). The posterior probabilities were assessed as *K* values between 1 and 10 using the Marcov Chain Monte Carlo method (MCMC). The parameter was used for the

length of burning period = 10,000, the iteration of MCMC after burning = 100,000, with 5 replications. Also, the output of Ln P (D) as the estimated probability of k and delta k was calculated to assess the k value, while the structure harvester program was used to estimate the main population (Earl and von Holdt 2012).

RESULTS AND DISCUSSION

SSR amplified product

Out of the 23 markers, the SSR markers amplification gave 4 unamplified (EMBRA1, EMBRA7, EMBRA19, and gSSR-GU023), 5 monomorphic, 3 e-SSR markers (eSSR-GR046, eSSR-GR124, eSSR-GR127, gSSR-CA013, EMCRC49), and 14 polymorphic markers (Table 2). The comparison of genomic SSRs with e-SSR markers showed that the 3 e-SSR markers used displayed monomorphic in 1 *Corymbia* and 5 *Eucalyptus* species. This indicated that the e-SSR markers used have low levels of polymorphism for genomic markers. Subsequently, the 14 polymorphic markers were continued for genotyping analysis on all *Corymbia* and *Eucalyptus* accession (Table 3, Figure 2). In the characterization of the 14 markers, the amplification of SSR markers in *Eucalyptus* sp. and *E. urophylla* have the highest polymorphic markers compared to *E. deglupta*, *E. globulus,* and *C. citriodora,* which have a similar range of polymorphic markers, while *E. pellita* had the lowest polymorphic marker. From the number of amplified markers, the highest polymorphism rate of species compatibility of SSR markers was *Eucalyptus* sp.

Genetic diversity of eucalypts

Based on table 4, the parameters of genetic diversity per locus of eucalypts species showed that 49 alleles were detected with 3 as the average number of alleles per marker. *C. citriodora* had the highest number of alleles detected (Na: 1−4 alleles), while *E. pellita* had the lowest (Na: 1−2 alleles). Furthermore, *E. urophylla, E. globulus*, *Eucalyptus* sp., and *E. deglupta* had a similar number of alleles (Na: 1−3 alleles). The gene diversity (He) value of *Corymbia* and 5 *Eucalyptus* species gave a low-level genetic diversity (He = 0.00–0.66, mean He = 0.26). Meanwhile, the He value in *E. urophylla* (He: 0.00–0.63, mean He = 0.35) and *Eucalyptus* sp. (He: 0.00–0.66, mean

He = 0.42) have the highest He value compared to *C. citriodora* (He: 0.00–0.66, mean He = 0.24), *E. deglupta* (He: 0.00–0.64, mean He = 0.23) and *E. globulus* (He: 0.00–0.61, mean He = 0.27) with similar range, while *E. pellita* has the lowest He value (He = 0.00–0.50, mean He = 0.04). The value of heterozygosity (Ho) per marker also has the same range in *E. urophylla, E. globulus, E. pellita*, and *C. citriodora*, (Ho: 0.00–1.00) compared to the Ho value of *Eucalyptus* sp. (Ho: 0–0.75) and *E. deglupta* (Ho: 0–0.4). In this study, the Ho parameter indicated a wide range of heterozygosity in *Corymbia* and *Eucalyptus* (0.0–1.0, mean Ho = 0.18) (Table 4).

The PIC values ranged from 0.35 on the EMCRC41 marker to 0.69 on the EMBRA2 marker with an average of 0.55. The 11 markers were very informative (PIC >0.5) with 4 markers having a PIC value >0.60, namely EMBRA13, EMBRA8, EMCRC11, and EMBRA2. Meanwhile, 7 out of 12 polymorphic markers in *Eucalyptus* sp. and 6 out of 8 polymorphic markers in *C. citriodora* have high inbreeding coefficient (Fis) values and significantly deviated from the Hardy-Weinberg equilibrium (HWE) law. Although a similar result was also shown in 3 out of the 7 polymorphic markers in *E. deglupta,* several polymorphic markers in *E. urophylla* did not significantly deviate from the HWE law (Table 4).

Genetic relationships among eucalypts species

The dendrogram of the UPGMA tree analysis showed that 20 *Corymbia* and *Eucalyptus* were grouped into two main clusters at a similarity coefficient of 0.59 (Figure 3). Cluster I consisted of 4 accessions of *Corymbia* (*C. citriodora*) and Cluster II consisted of 16 accessions from 5 *Eucalyptus* species. Meanwhile, Cluster II is divided into two sub-clusters, namely sub-cluster IIA and IIB. Also, sub-cluster IIA is divided into two sub-clusters, namely sub-cluster IIA (i) and sub-cluster IIA (ii). The sub-cluster IIA (i) corresponds to *E. globulus* in sub-cluster IIA (ia), together with *E. pellita, E. globulus,* and *Eucalyptus* sp. in sub-cluster IIA (ib), Furthermore, sub-cluster IIA (ii) consisted of 5 accessions of *E. deglupta*. Sub-cluster IIB is divided into two sub-clusters, namely sub-cluster IIB (i) and sub-cluster IIB (ii). Similarly, sub-cluster IIB (i) corresponds to *E. urophylla,* while sub-cluster IIB (ii) corresponds to *Eucalyptus* sp.
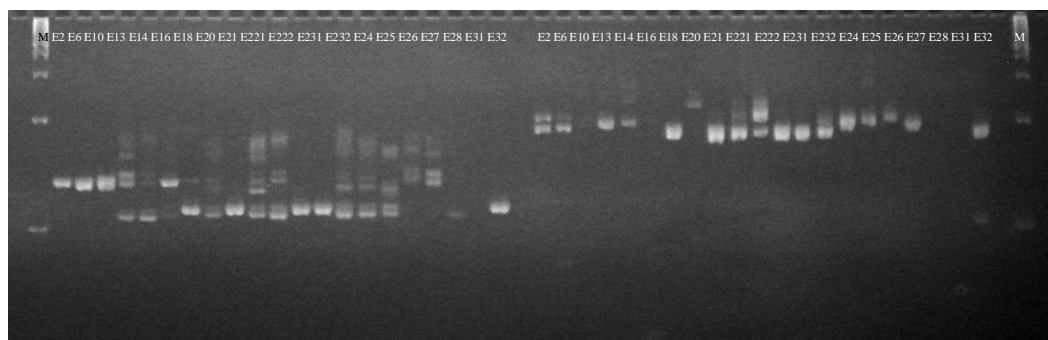

Figure 2. Electropherogram of eucalypts genotyping analysis with EMCRC3 (left) and EMCRC4 (right) markers on 8% gel electrophoresis. Lane 1: 100 bp Ladder, Lane 2−21: eucalypts accessions. M: marker

Table 2. Primer sequences used in this study

Primer	SSR motif	Primer sequences	Size (bp)	Source	Reference
EMBRA1	(AG)33	5-GATAGAACTTTCCTATTTGATCG-3 5-GTAGGATTTGATGTCTGCAA-3	127	*E. grandis × E. urophylla*	Brondani et al. (1998)
EMBRA2*	(AG)15	5-CGTGACACCAGGACATTAC-3 5-ACAAATGCAAATTCAAATGA-3	121	*E. grandis × E. urophylla*	Brondani et al. (1998)
EMBRA3*	(AG)19	5-GATCGGATTGGA GGAGAC-3 5-AATTCAATTCATCCAAAGC-3	123	*E. grandis ×E. urophylla*	Brondani et al. (1998)
EMBRA7	(AG)15	5-CACACCGTGTCAGTTAGC-3 5-AATAAG GAGGATTCCATGG-3	115	*E. grandis × E. urophylla*	Brondani et al. (1998)
EMBRA8*	(AG)21	5-CACAACTAAAAATCAAAACCC-3 5-AAAGAGCAGATTATTACAGAAGC-3	127	*E. grandis × E. urophylla*	Brondani et al. (1998)
EMBRA13*	(AG)27	5-ATTTCCCTAGGTTTGACATG-3 5-TCCAACATCTTACTCAACCA-3	130	*E. grandis × E. urophylla*	Brondani et al. (1998)
EMBRA17*	(AG)18	5-AGGATACTCGTG AGAGAAGC-3 5-GTAGATCTGTTCTGCATG TTG-3	184	*E. grandis × E. urophylla*	Brondani et al. (1998)
EMBRA19	(AG)23	5-GACGGTTGATTTCCTGAT T-3 5-GTGGTGCTCCTCTCCTCT-3	124	*E. grandis × E. urophylla*	Brondani et al. (1998)
EMCRC3*	(CA)10	5-AGATGGGGTTTCTCATGGTTT-3 5-ACCGTACTATGCAGCTGGAAC-3	109-145	*E. globulus*	Steane, et al. (2001)
EMCRC4*	(AC)17	5-GTAATCTTTCATTCTCCGACC-3 5-CTCGAGGACATGTTGAGTG-3	178-268	*E. globulus*	Steane, et al. (2001)
EMCRC6*	(CT)11(CA)23	5-CTTCAAGGTTCACAGATGG-3 5-TCTTCATAAGTCCCCTAATCA-3	151-193	*E. globulus*	Steane, et al. (2001)
EMCRC11*	(TC)10(AC)10	5-AACTGACTGTGGATTTAAGC-3 5-GTGAGTCATTATTTGGCAACC-3	221-255	*E. globulus*	Steane, et al. (2001)
EMCRC12*	(CT)8(CA)14	5-CTCCGACCTCCTCCACT-3 5-AATCGTCTTCATCGAATCAAG-3	70-128	*E. globulus*	Steane, et al. (2001)
EMCRC41*	(GA)24	5-GACGGTAGCATTCTGTTCTTTGGCA-3 5-TGGGTCGACTTTCAAGTCGCGT-3	114-150	*C. citriodora*	Shepherd et al. (2006)
EMCRC49	(GA)11(A)7(GA)2	5-ACCAAGAAACGGGGAAAGAG-3 5-GTTTTGGAGAGAGGGGCAAG-3	251-286	*C. citriodora*	Shepherd et al. (2006)
EMCRC51*	(GA)15	5-CTCGACCACCCGAGAGAA-3 5-CACAGAATTCACTCCCTCCTAAA-3	250-277	*C. citriodora*	Shepherd et al. (2006)
EMCRC55*	(GA)21	5-TGTAGATAAGGCGTGGAGAGG-3 5-GCATACAATTATCCGCCAGAG-3	270-297	*C. citriodora*	Shepherd et al. (2006)
EMCRC93*	(GA)16	5-GCAACTCCAACGACAACAAC-3 5-GGTCAATCTCCTCCACCAGTAA-3	131-166	*C. citriodora*	Shepherd et al. (2006)
eSSR-GR046	(GGACCG)4	5-AAACAACCCGAACGAAAGAA-3 5-GGAGGTCCTACTACACCCACAA-3	170	*E. pellita*	Liu et al. (2018a)
eSSR-GR124	(GTT)8	5-ATTGATTCACGGACACGG-3 5-GAACGCAATCCCACCTAA-3	279	*E. pellita*	Liu et al. (2018a)
eSSR-GR127	(GCAGCG)3	5-CGCTGTCCATCGTGTATTTG-3 5-TGACGCTGTCGGTGAAACT-3	371	*E. pellita*	Liu et al. (2018a)
gSSR-CA013	(CT)10	5-AGATGGGACGGACGAGGAT-3 5-GGTGGTGCTGGGAAGAAT-3	121	*E. pellita*	Liu et al. (2018a)
gSSR-GU023	(AGAAAA)3	5-GAGCCTGTTACAAATGGA-3 5-TCACAGCAGTCGGTCTTT -3	316	*E. pellita*	Liu et al. (2018a)

Note: *Fourteen selected primers were used for further analysis

In sub-cluster IIA (ib), the two closely related accessions of *E. pellita* are E20 and E32 from the same regions (Karanganyar, Central Java), with a genetic similarity value of 100%. The two closely related accessions of *E. deglupta* namely in sub-cluster IIA (ii) include E23-1 and E23-2 from the same regions (Bogor, West Java), with a genetic similarity value of 90%. In addition, there are also two distant related accessions namely E16 (*C. citriodora*) from Karanganyar, Central Java, while E22-2 (*E. urophylla*) is from Garut, West Java with a genetic similarity value of 43% (Table 5).

The Principal Coordinate Analysis (PCoA) in two spatial dimensions (XY coordinates) showed the total value of variance at each coordinate. Based on this result, the PCoA has identified two groups that clearly distinguish and each genotype is grouped based on its genus, namely *Corymbia* (right) and *Eucalyptus* (left) (Figure 4). The *Corymbia* in the blue rhombus type is grouped into *C. citriodora,* while within the *Eucalyptus* group, the individual trees with a triangular shape are grouped into *E. deglupta* was separated from other *Eucalyptus* species at the upper left coordinate. Although the other 4 *Eucalyptus* species (*E. urophylla, E. pellita, E. globulus,* and *Eucalyptus* sp) were not distinguished, there is a slight difference between each species such as in the shape of crosses grouped into *Eucalyptus* sp., in blue star grouped into *E. globulus,* in orange box grouped into the *E. urophylla,* in green circle grouped into *E. pellita*.

Population structure of eucalypts

There were 14 SSR markers used to determine the population structure of eucalypts species. The results showed that the peak of delta-K continued to decline after $k = 6$, which suggested that the existence of 6 populations (Figure 5). At $k = 6$, eucalypts species were distinguished into 6 populations and most of the populations were homogeneous with little admixture apart. This showed that Group 1 corresponded to *C. citriodora* (20%), Group 2 corresponded to *E. urophylla* (15%), Group 3 corresponded to *E. deglupta* (25%), Group 4 corresponded to *E. pellita* (10%), Group 5 corresponded to *Eucalyptus sp.* (10%), and Group 6 corresponded to *E. globulus* (20%). In addition, the *Eucalyptus* sp. consisted of E26 which corresponded to Group 2 or belongs to *E. urophylla,* E24 corresponded to Group 6 or belongs to *E. globulus,* while E25 and E27 corresponded to Group 5 or belongs to *Eucalyptus* sp.

Discussion

This study determined the genetic diversity and population structure of *Corymbia* and *Eucalyptus* species using 14 SSR markers. Although the success rate of genomic SSR marker in eucalypt from is transferable, it is relatively low (>50%) compared to cross-species transferability on other eucalypt species (Subashini et al. 2014; Kotrappa et al. 2017; Liu et al. 2018b). Many isolated SSR markers from *E. grandis* and *E. urophylla* (EMBRA code) were cross-amplified in different *Eucalyptus* species such as *E. globulus, E. pilularis, E. nitens, E. urophylla, E. pyrocarpa, E. camaldulensis,* and *E. tereticornis* (Bundock et al. 2000; Steane et al. 2001; Glaubitz et al. 2001; Agrama et al. 2002; Ottewell et al. 2005; Arumugasundaram et al. 2011; Subashini et al. 2014). Similarly, e-SSR markers were successfully cross-amplified in several species such as *E. dunnii, E. saligna, E. camaldulensis, E. viminalis, E. urophylla, and E. tereticornis* (Faria et al. 2010; Hudson et al. 2012; Acuna et al. 2012; He et al. 2012; He et al. 2015; Breed et al. 2012; Bradbury et al. 2013).

One of the determining factors for successful amplification of SSR markers is the genetic relationship of each taxonomic or phylogenetic distance (Chandra et al. 2011). The success of SSR amplification occurred with subgenus, genus, sub-family, even family (McCulloch and Stevens 2011). In this study, the SSR markers used are transferable in different genera (*Corymbia* and *Eucalyptus)* and the *Eucalyptus* species used belong to the same subgenus, namely *Symphyomyrtus* (Brondani et al. 1998). Moreover, the polymorphism rate of SSR amplification in *E. urophylla* and *Eucalyptus* sp. was higher than *C. citriodora*, *E. globulus,* and *E. deglupta,* while *E. pellita* was weakly transferred. This is in line with a study by Nurtjahjaningsih et al. (2013) which stated that the amplification of *E. urohpylla* was higher than *E. deglupta.*

The SSR markers were successfully amplified when carried out in the same genus with the source of the marker. However, when it was conducted against other species, the success rate decreased even within the same taxa (Ujino et al. 1998). Although the source of SSR marker was from *E. grandis X E. urophylla* (EMBRA) and *E. globulus* (EMCRC)*,* however, all the SSR markers did not amplify polymorphic allele on *E. urophylla* and *E. globulus* accession. This was similar to a study by Nurtjahjaningsih et al. (2013) which showed that the SSR amplification does not always work even on the same species. This indicated that in addition to proximity taxonomically, the size and complexity of DNA composition are also important in the polymorphism of the SSR screening. The size of the genetic disorders caused by the evolutionary process also affected successful SSR amplification, even the deviation varies between loci. In addition, the polymorphic allele is affected by perfect or the SSR repeat structure. Since the mutation rate interrupts the base arrangement in SSR sequences, therefore, the structure of the replication is not perfect. This affects the degree of polymorphism of allele amplification or causes inhibition. Moreover, the decreasing distance also influences the phylogenetic allelic polymorphism of the species (Jan et al. 2012).

Table 3. The number of amplified markers of eucalypt in this study

Primer	*C. citriodora*	*E. urophylla*	*E. deglupta*	*Eucalyptus* sp.	*E. globulus*	*E. pellita*
EMCRC11	M	P	P	P	M	M
EMCRC41	P	M	M	M	M	M
EMCRC51	P	P	M	P	P	M
EMCRC55	P	M	P	P	P	M
EMCRC93	P	P	M	P	P	M
EMBRA2	P	P	M	P	P	M
EMBRA3	M	P	P	P	M	P
EMBRA8	P	M	P	P	P	M
EMBRA17	M	P	P	P	M	M
EMCRC3	M	P	M	P	P	M
EMCRC4	P	M	M	P	P	M
EMBRA 13	P	P	P	P	P	M
EMCRC6	M	P	P	M	M	M
EMCRC12	M	P	M	P	M	M
Polymorphic (P)	8	10	7	12	8	1
Monomorphic (M)	6	4	7	2	6	13
Polymorphism rate (%)	57.1	71.4	50.0	85.7	57.1	7.1

Table 4. Genetic diversity of eucalypts generated by 14 SSR markers

Population		EMCRC11	EMCRC41	EMCRC51	EMCRC55	EMCRC93	EMBRA2	EMBRA3	EMBRA8	EMBRA17	EMCRC3	EMCRC4	EMBRA13	EMCRC6	EMCRC12	Mean
								SSR marker								
C. citriodora	N	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4.0
	Na	1	2	2	2	2	4	1	2	1	1	2	2	1	1	1.71
	Ho	0.00	0.25	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.14
	He	0.00	0.22	0.38	0.38	0.38	0.66	0.00	0.50	0.00	0.00	0.38	0.50	0.00	0.00	0.24
	Fis	~	-0.14ns	1.00*	1.00*	1.00*	-0.14ns	~	1.00*	~	~	1.00*	-1.00*	~	~	0.46
E. urophylla	N	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Na	2	1	2	1	2	2	3	1	2	2	1	3	3	2	1.93
	Ho	0.50	0.00	0.00	0.00	0.50	1.00	0.50	0.00	0.00	0.50	0.00	1.00	0.50	0.50	0.36
	He	0.38	0.00	0.50	0.00	0.38	0.50	0.63	0.00	0.50	0.38	0.00	0.63	0.63	0.38	0.35
	Fis	-0.33ns	~	1.00ns	~	-0.33ns	-1.00ns	0.20ns	~	1.00ns	-0.33ns	~	-0.60ns	0.20ns	-0.33ns	-0.05
E. deglupta	N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	Na	2	1	1	2	1	1	3	2	2	1	1	3	2	1	1.64
	Ho	0.00	0.00	0.00	0.20	0.00	0.00	0.40	0.00	0.00	0.00	0.00	0.40	0.60	0.00	0.11
	He	0.32	0.00	0.00	0.50	0.00	0.00	0.56	0.32	0.48	0.00	0.00	0.64	0.42	0.00	0.23
	Fis	1.00*	~	~	0.60ns	~	~	0.29ns	1.00*	1.00*	~	~	0.38ns	-0.43ns	~	0.55
Eucalyptus sp.	N	4	3	4	4	4	4	4	4	4	4	4	4	4	4	3.93
	Na	3	1	2	3	3	2	2	2	2	3	2	3	1	3	2.29
	Ho	0.00	0.00	0.00	0.50	0.25	0.25	0.00	0.00	0.00	0.75	0.00	0.75	0.00	0.75	0.23
	He	0.63	0.00	0.38	0.59	0.53	0.47	0.38	0.38	0.38	0.66	0.38	0.59	0.00	0.53	0.42
	Fis	1.00*	~	1.00*	0.16ns	0.53ns	0.47ns	1.00*	1.00*	1.00*	-0.14*	1.00*	-0.26ns	~	-0.41ns	0.53
E. globulus	N	3	2	3	3	3	3	3	3	3	3	3	3	3	3	2.93
	Na	1	1	2	2	2	2	1	2	1	3	2	2	1	1	1.64
	Ho	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.67	0.00	0.00	0.14
	He	0.00	0.00	0.44	0.50	0.44	0.44	0.00	0.44	0.00	0.61	0.44	0.44	0.00	0.00	0.27
	Fis	~	~	1.00ns	-1.00ns	1.00ns	1.00ns	~	1.00ns	~	0.45ns	1.00ns	-0.50ns	~	~	0.49
E. pellita	N	1	2	2	1	2	1	2	2	2	2	2	2	1	1	1.64
	Na	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1.07
	Ho	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.07
	He	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.04
	Fis	~	~	~	~	~	~	-1.0ns	~	~	~	~	~	~	~	-1.00
PIC		0.68	0.35	0.54	0.57	0.58	0.69	0.55	0.65	0.51	0.48	0.43	0.63	0.55	0.52	0.55

Note: N: Number of samples. Na: Number of the detected allele. Ho: Observed heterozygosity. He: Expected heterozygosity. Fis: Inbreeding coefficient. PIC: Polymorphic Information Content. ns: not significant. *$P < 0.05$; ~: allele monomorphic

Table 5. The genetic similarity matrix of eucalypts in this study was generated by 14 SSR markers

| Accession	E10	E13	E14	E16	E18	E2	E20	E21	E22_1	E22_2	E23_1	E23_2	E24	E25	E26	E27	E28	E31	E32	E6
E10	1.00																			
E13	0.55	1.00																		
E14	0.53	0.86	1.00																	
E16	0.73	0.61	0.59	1.00																
E18	0.65	0.65	0.67	0.67	1.00															
E2	0.84	0.55	0.61	0.73	0.69	1.00														
E20	0.63	0.67	0.73	0.65	0.65	0.63	1.00													
E21	0.57	0.65	0.71	0.67	0.80	0.65	0.73	1.00												
E22_1	0.57	0.57	0.55	0.59	0.55	0.61	0.61	0.59	1.00											
E22_2	0.53	0.53	0.67	0.43	0.59	0.53	0.61	0.55	0.67	1.00										
E23_1	0.47	0.63	0.69	0.53	0.69	0.51	0.71	0.78	0.57	0.69	1.00									
E23_2	0.53	0.61	0.67	0.59	0.76	0.57	0.65	0.84	0.63	0.67	0.90	1.00								
E24	0.61	0.65	0.67	0.63	0.71	0.53	0.57	0.67	0.59	0.59	0.61	0.67	1.00							
E25	0.61	0.49	0.51	0.51	0.63	0.57	0.69	0.55	0.67	0.67	0.61	0.63	0.67	1.00						
E26	0.65	0.53	0.51	0.59	0.59	0.53	0.69	0.47	0.59	0.63	0.57	0.55	0.59	0.67	1.00					
E27	0.69	0.57	0.63	0.76	0.67	0.65	0.78	0.67	0.63	0.59	0.57	0.59	0.63	0.67	0.71	1.00				
E28	0.59	0.71	0.69	0.57	0.57	0.55	0.80	0.69	0.57	0.61	0.67	0.65	0.53	0.57	0.57	0.73	1.00			
E31	0.53	0.69	0.71	0.63	0.71	0.61	0.73	0.71	0.55	0.63	0.82	0.76	0.63	0.67	0.59	0.67	0.69	1.00		
E32	0.63	0.67	0.73	0.65	0.65	0.63	1.00	0.73	0.61	0.61	0.71	0.65	0.57	0.69	0.69	0.78	0.80	0.73	1.00	
E6	0.82	0.53	0.55	0.76	0.63	0.82	0.57	0.59	0.51	0.47	0.57	0.63	0.55	0.51	0.59	0.63	0.53	0.63	0.57	1.00
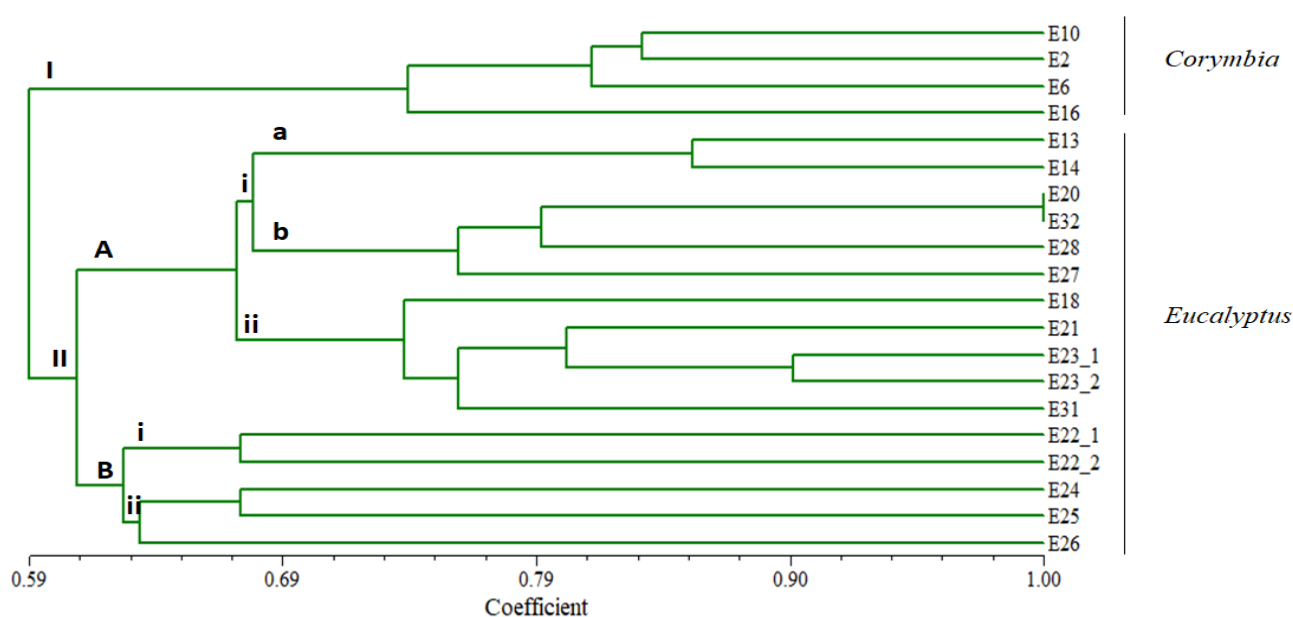
Figure 3. UPGMA dendrogram of *Corymbia* and *Eucalyptus* showing the genetic relationships among the 20 accessions generated by 14 SSR markers
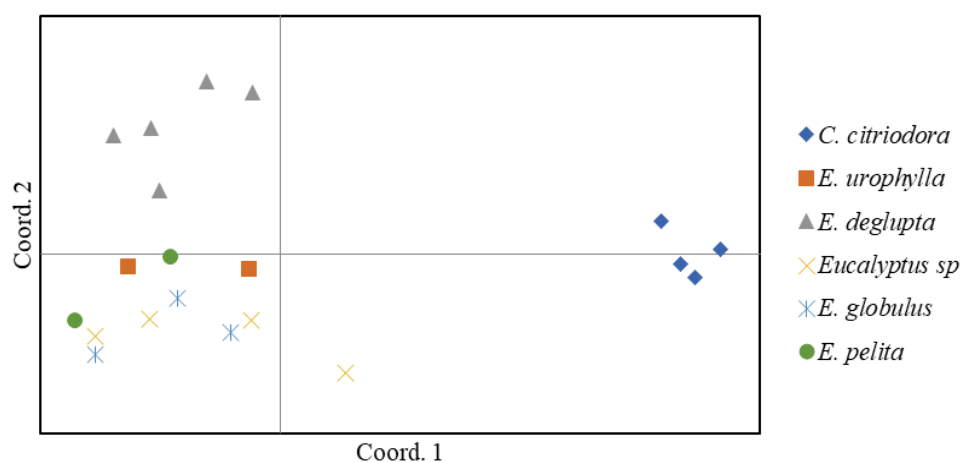

Figure 4. PCoA of inter-individual coordinates of Corymbia and Eucalyptus

In this study, the relatively small plant material coverage gave smaller allele numbers (average of 3 alleles per marker) and a low level of genetic diversity (mean H*e* = 0.26). This showed that *Eucalyptus* sp. and *E. urophylla* were the highest gene diversity, followed by *E. globulus*, *C. citriodora*, *E. deglupta*, and *E. pellita* compared to previous studies (Nurtjahjaningsih et al. 2013; Kotrappa et al. 2017; and Liu et al. 2018b). The genetic diversity of *E. globulus*, *C. citriodora*, *E. deglupta* was not significantly high compared to *Eucalyptus* sp. and *E. urophylla* with the continuous (connected) distribution. Also, fragmented distribution hindered the flow of genes or migration rate per generation, which affected the structure of genes and caused the low value of genetic diversity (Hu et al. 2010;

Karan et al. 2012). However, the low level of gene diversity value in *C. citriodora, E. deglupta,* and *Eucalyptus* sp. did not show a heterozygosity deficit because of the inbreeding coefficient value (F*is*) were 0.46, 0.55, and 0.53. respectively. This indicated that each of the three species used in this study came from a random mating system. Although the value of gene diversity in *E. deglupta* was lower than *Eucalyptus* sp. and *C. citriodora,* the inbreeding coefficient value of *E. deglupta* was higher. In addition, the low value of gene diversity in *E. globulus* did not significantly deviate from the HWE. However, a previous study by Costa et al. (2017) stated that the high value of gene diversity in *E. globulus* did not significantly deviate from the HWE. Since the mean observed

heterozygosity (H*o*) was 0.19 derived from *C. citriodora, E. urophylla, E. deglupta, Eucalyptus* sp*.,* and *E. globulus,* it indicated that most markers used detected more than one allele per accession, as expected for accessions directly derived from landraces. The mean H*o* in *E. pellita* was 0.07, which indicated that the accessions are highly inbred since the accessions of a largely self-pollinated species are maintained in the collections. Therefore, further studies are required by increasing the number of populations and samples with more diverse populations. In addition, the use of genetically related species also causes a high inbreeding coefficient.

The PIC value provided information about the polymorphism of a marker, meanwhile, a study by Botstein et al. (1980) stated that a PIC value >0.5 gave high information. Also, PIC values between 0.25–0.5 gave moderate information, while a PIC value of < 0.25 gave little information. In this study, the PIC value (PIC=0.55) was higher than Liu et al. (2017) which observed the PIC value of 0.49 from six species of *Eucalyptus*. In contrast, higher mean PIC value (>0.75) from 4 *Corymbia* and 36 *Eucalyptus* species (Liu et al. 2018b), *C. citriodora* (Liu et al. 2016), and 20 eucalypt genotypes (He et al. 2015). Therefore, the higher the PIC value of the SSR marker, the greater the potential of identifying genetic relationships, which are influenced by the characteristics of the marker and species differences. Among 14 SSR markers, 4 makers with high PIC values (>0.6), which indicated their ability to distinguish between and within the individuals in a population. However, the e-SSR markers used gave low levels of polymorphism in *Corymbia* and 5 *Eucalyptus* species compared to the genomic SSR marker. In the e-SSR markers, the relatively low level of polymorphism is due to the location of these markers in more conserved and expressed sequences compared to the genomic sequences which are spread throughout (Parthiban et al. 2018).

In this study, the cluster analysis illustrated by the UPGMA dendrogram showed the genetic relationship of eucalypt into two distinct groups, namely *Corymbia* species, *C. citriodora* which belongs to subgenus *Blakella,* and *Eucalyptus* species from the subgenus *Symphyomyrtus* as the largest (Júnior and Garcia 2021). This is in line with the *Eucalyptus* taxonomic classifications of Nicolle (2019) and chloroplast genome analysis (Bayly et al. 2013). Meanwhile, the Australian Plants Society (2016) stated the differences between *Corymbia* and *Eucalyptus* based on the type of bark, juvenile, and mature leaves, as well as fruit/capsules and buds. In *Corymbia,* the diverse and widespread bloodwoods, spotted, lemon-scented, and ghost gums as well as the fruit has many typical bloodwood shapes with no teeth and are not ribbed. Meanwhile, *Eucalyptus* is very diverse and widespread gum, while peppermint, ironbark, stringybark, mahogany, ash, blackbutt, box, tallowwood, and the fruit is a woody capsule, size variable, not ribbed or toothed, and variable

shape. In this study, *E. globulus* (section *Maidenaria), E. pellita* (section *Latoangulatae*), and *E. deglupta* (section *Equatoria*) were clustered together in group IIA, while *Eucalyptus* sp. was in the same cluster with *E. urophylla* (section *Latoangulatae*) in group IIB. Moreover, the clustering of group IIA supported the hypothesis of Bayly et al. (2013) that analyzed chloroplast genomes of 39 eucalypt species of *Eucalyptus*, *Corymbia,* and *Angophora* discovered that *E. globulus* (subgenus *Eucalyptus*) together with *E. deglupta* was placed with each other in close genetic proximity (at node 27). The clustering in group IIB, *E. urophylla* were clustered separately with *E. pellita* in group IIA. This is notably different from a study by Liu et al. (2018b) which clustered *E. urophylla* and *E. pellita* in the same cluster.

The PCoA analysis showed that one species of *Corymbia* and five species of Eucalyptus were distinguished by each genus. The genetic relationship among the species of *Eucalyptus* and *E. deglupta* was separated from the other 4 *Eucalyptus* species (*E. urophylla, E. globulus, E. pellita,* and *Eucalyptus* sp.), which showed that the genetic purity of each species is still maintained. Moreover, the PCoA analysis usually indicated the geographic location with the genetic analysis of a species. In this study, *E. deglupta* originated from Bogor, West Java which is closer to *E. urophylla* from Garut, West Java, however, it was separated in the different ordinate side with *E. urophylla*. The *E. urophyllla* was clustered together with *E. pellita, E. globulus,* and *Eucalyptus* sp. from Karanganyar, Central Java. This is not in line with a study by Nurtjahjaningsih et al. (2013) which stated that the PCoA gave the *E. urophylla* and *E. deglupta* from Yogyakarta, clustered together on the same ordinate side (right side of form the Y ordinate), while *E. pellita* on the other side of the ordinate (to the left of Y-ordinate).

Based on the peak of delta-K, the population structure was divided into six, where Group 1 corresponded to *C. citriodora),* Group 2 corresponded to *E. urophylla*, Group 3 corresponded to *E. deglupta*, Group 4 corresponded to *E. pellita*, Group 5 corresponded to *Eucalyptus* sp., and Group 6 corresponded to *E. globulus*. Based on the geographical location, the first three groups (*C. citriodora*, *E. urophylla*, and *E. deglupta*) were cultivated from West Java, and the second three groups (*E. pellita*, *Eucalyptus* sp., and *E. globulus*) from Central Java. In this study, it was assumed that the geographic distribution of West and Central Java promotes the adaptation to the same environment (tropical environment) caused similarities in gene structure. Meanwhile, the genetic variation within a species generally has a geographic basis, since the processes of adaptation, gene flow, and genetic drift which act differently in heterogeneous landscapes and are strongly influenced by the demographics and spatial distribution of populations (Eckert et al. 2008; Junior and Garcia 2021).
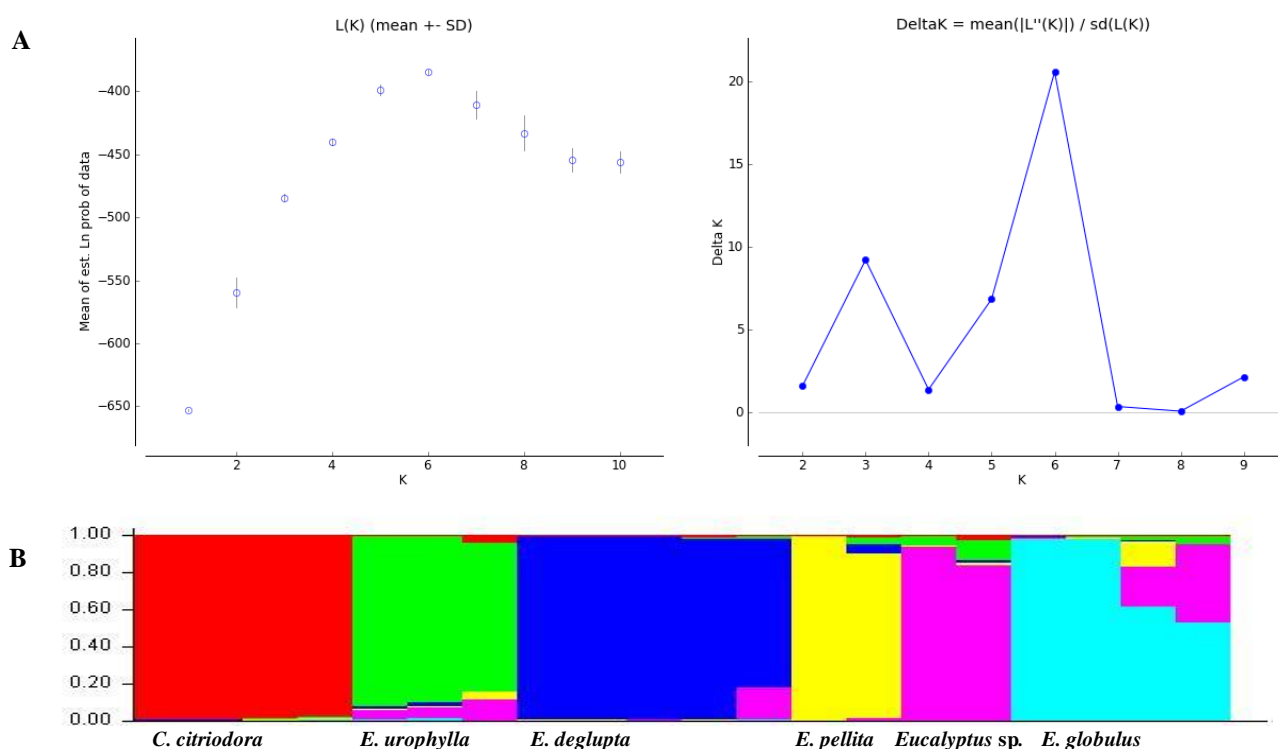
Figure 5. Population structure analysis based on 14 SSR markers. A) Left: The probability of the data Ln P(D) (± SD) (y-axis) against the number of K clusters (x-axis), and Right: ΔK values from the mean log-likelihood probabilities (y-axis) from STRUCTURE runs where inferred clusters (K) ranged from 1 to 10 (x-axis). B) Clusters were from 20 accessions of *Corymbia* and *Eucalyptus* in a bar plot. The proportion of each individual (y-axis) indicate by colors measured the genetic clusters correspond to six main populations (x-axis).

Implications to conservation management

The availability of eucalypt cultivated in the KHDTK Bromo Forest can contribute as a reference set that provides marker-based germplasm classification. Moreover, the new accession can easily be compared to this reference set. The 14 SSR markers are potentially used as a marker set for easy application, such as its use for local studies in Sebelas Maret University. This can be very useful for characterizing eucalypt germplasm and enriching eucalypts collections, especially for uncovered novel accession. Furthermore, the 14 SSR markers provided a considerable backup for the mining of germplasm diversity. The molecular data were used for complementing the eucalypt reference set with new additional eucalypt accession and morphological or phenotypic information to characterize the genetic resource and substantial economic benefits, which can also be accommodated. This combination enriches and captures most of the genetic variation within the species of both new and eucalypt accession from other sources to be incorporated in ex-situ conservation in KHDTK Bromo Forest to generate a genetic diversity database for germplasm management and conservation. In the long term, this will contribute to the global eucalypt community to focus on biological investigations by accumulating and compiling data to develop a better biological understanding of eucalypt species, especially species from Indonesia. In addition, the analysis of genetic relationships and distance among *Eucalyptus* species can be used as a tool for predicting the potential compatibility of new interspecific combinations to create viable F$_1$ hybrids. This showed that the two species of *Eucalyptus* can be hybridized easily when they belong to the same subgenus/section/series compared to the species from different sections/series and/or subgenera (Potts and Dungey 2004).

In conclusion, the molecular analysis on *Corymbia* and *Eucalyptus* detected a low number of alleles per locus and a low level of genetic diversity. Furthermore, the genetic relationships among eucalypts species and the population structure of eucalypts showed the grouping of individuals based on its genus *Corymbia* and *Eucalyptus*. This showed that the genetic purity of eucalypts is still maintained.

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