Biosaintifika 15 (1) (2023): 26-35 p-ISSN 2085-191X | e-ISSN 2338-7610
Journal of Biology & Biology Education https://journal.unnes.ac.id/nju/index.php/biosaintifika

Analysis of Genetic Variations of *Ceiba pentandra* (L.) Gaertn. on Several Critical Lands in West Sumatra Using RAPD Molecular Markers

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Submitted: 2022-08-18. Revised: 2022-12-24. Accepted: 2023-02-25

Abstract. *Ceiba pentandra* (L.) Gaertn. is a plant whose fruit is used to produce fiber and seeds for biofuel, which has the potential to be developed in critical land because it is adaptive to grow in critical land that is less fertile and lacks water. In West Sumatra, several critical land locations are overgrown by *C. pentandra* plants. This study aims to determine the genetic variation of *C. pentandra* in five critical land populations in West Sumatra as the basis for selecting superior seeds for development in critical land. The research was conducted by the descriptive method using molecular data with the molecular marker RAPD (random amplified polymorphic DNA). The results showed that the primers OPA-01, OPA-02, and OPB-10 could detect polymorphisms. Pangkalan Koto Baru in the regency Lima Puluh Kota (H = 0.1212) was the population with the highest intrapopulation genetic variation value. Interpopulation genetic variation (D_{ST} = 0.0321) was lower than intrapopulation genetic variation (H_S = 0.1021), with a low genetic differentiation value (G_{ST} = 0.2392) and a high gene flow value (Nm = 1.5894). The genetic variation of *C. pentandra can* be used to select the parent in plant breeding programs.

Keywords: Ceiba pentandra, critical land, genetic variation, kapuk randu, RAPD.

How to Cite: Hefzi, F., Mansyurdin, M., & Maideliza, T. (2023). Analysis of Genetic Variations of *Ceiba pentandra* (L.) Gaertn. on Several Critical Lands in West Sumatra Using RAPD Molecular Markers. *Biosaintifika: Journal of Biology & Biology Education*, *15*(1), 26-35.

DOI: http://dx.doi.org/10.15294/biosaintifika.v15i1.40871

INTRODUCTION

Kapuk randu (*C. pentandra*) is a fiber-producing plant for textile materials, pillow fillers, and mattresses. The world's largest cotton-producing country is India, with production reaching 6.2 million tons per year, while Indonesia is in 68th place with a production of only 987 tons per year (Atlas Big, 2020), even though the land is sufficiently available. The low production of *C. pentandra* in Indonesia is due to the underdevelopment of the textile industry in Indonesia, which uses cotton fiber as raw material. In addition, synthetic fibers such as foam have replaced cotton as a filling for pillows and mattresses. Although *C. pentandra* is not significantly developed in industry, it can be used as a prospective plant for biodiesel fuel sources because *C. pentandra* contain 20-40% oil by dry weight (Ong et al., 2013).

By paying attention to prospects, it is necessary to develop *C. pentandra*, especially in critical lands, because *C. pentandra* has a high adaptation to growing on infertile land and lack of water. This is possible because in Indonesia, the critical land area reaches 14.01 million ha (PDASHL, 2021). West Sumatra is a province with a critical land area reaching 651,970 thousand ha, including the regencies of Lima Puluh Kota, Sijunjung, Solok, Tanah Datar, and Sawahlunto (BPS, 2022).

Genetic variation is a crucial feature that allows species to evolve and adapt to changing environments (Pakull et al., 2021). Furthermore, information on genetic variation is also valuable for supporting conservation and breeding programs, particularly in developing new superior seeds (Mursyidin et al., 2021). Therefore, the establishment of *C. pentandra* in critical land must be supported by the availability of seeds that were produced by a high-quality source. However, *C. pentandra* is still limited, and most are not superior seed sources. For this reason, *C. pentandra* plantations require seeds sourced from populations with a high level of genetic variation because it supports the adaptability and sustainability of the species (Ingvarson & Dahlberg, 2019). An accurate analysis of genetic variation can be performed using molecular markers such as RAPD (random amplified polymorphic DNA) (Zulfahmi, 2013). RAPD is a PCR-based method produced by PCR machines using genomic DNA and random primers. It is

quick, simple, affordable, and requires only a small amount of DNA (Uslan & Pharmawati, 2020). RAPD is also effective for identifying genetic polymorphism in plants (Wang et al., 2016), estimating of genetic variation (Hapsoro et al., 2015), and plant breeding (Fei et al., 2014). Research using the RAPD marker on 36 genotypes of *Ceiba pentandra* in Ghana showed a high genetic variation (Abengmeneng et al., 2016).

Information on the genetic variation of *C. pentandra* on critical land is used as the basis for selecting superior seeds. Therefore, an analysis of the genetic variation of *C. pentandra* in several populations of critical land in West Sumatra will be carried out using the RAPD marker. This study aims to analyze the genetic variation of *C. pentandra* in five populations of critical land in West Sumatra, using the RAPD marker as the basis for its selection and development in critical land.

METHODS

Sample collection

Twenty-five individuals of *C. pentandra* were obtained from five populations in West Sumatra: Pangkalan Koto Baru (50 Kota), Kupitan (Sijunjung), X Koto Singkarak (Solok), Lintau Buo Utara (Tanah Datar), and Talawi (Sawahlunto), each population consisting of five individual plants. Samples were stored in a plastic ziplock containing silica gel until DNA was extracted in the laboratory.

DNA plant extraction

DNA was extracted from young leaves using the CTAB (Cetyl Trimethyl Ammonium Bromide) method, according to Doyle and Doyle (1987). The DNA extraction was carried out with the following steps: 15 mg of young leaf tissue was frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. The pulverized materials were transferred to a microtube, and 750 mL of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM Na2EDTA, 1.4 M NaCl, 2% (w/v) CTAB, and 0.2% (v/v) mercaptoethanol) solution was added. The tubes were vortexed for a few seconds and incubated at 65 °C for 45 minutes (every 10 minutes, the tube is vortexed). Samples were centrifuged for 10 minutes at 27 °C and 12.000 rpm. The supernatant was transferred to a new sterile tube and chloroform: isoamyl alcohol (24:1) was added equal to the volume of the supernatant and vortexed. The tube was centrifuged at 27 °C and 12.000 rpm for 10 minutes. The supernatant was transferred to a sterile microtube, and isopropanol equal to the volume of the supernatant was added. The tube was centrifuged at 4 °C and 12.000 rpm for 10 minutes. The supernatant was removed, added 200 mL of cold ethanol 70% was. The tube was centrifuged at 4°C and 12.000 rpm for 5 minutes. The supernatant was removed and rinsed again with 200 mL cold ethanol 70%. The tube was centrifuged at 4°C and 12.000 rpm for 2 minutes. The DNA pellet was dried over the tissue for 1-2 hours by inverting the tube. Then, added 50 mL TE buffer to dissolve the DNA pellet. Furthermore, it was stored as a stock at -20 °C.

Primer selection and DNA amplification

A total of 10 RAPD primers were used for selection, as shown in Table 1. The selected primers that produced polymorphic bands were used for PCR amplification. The PCR was carried out at a total volume of 25 μL containing a mixture of 12.5 μL My Taq TM Red Mix Bioline as PCR reagent (10 mM dNTPs, 50 mM $MgCl_2$, 1 unit of Taq DNA Polymerase), 2 μL primer, 6.5 μL nuclease-free water, and 4 μL DNA isolate. Amplification was carried out in a SensoQuest thermocycler with a cycle programmed for 45 cycles of each of the following conditions: initial denaturation at a temperature of 94°C for 2 minutes, followed by denaturation at 94°C for 1 minute, annealing at 34°C for 1 minute, elongation at 72°C for 2 minutes 20 seconds, and the final condition was elongation at 72°C for 10 minutes.

Table 1. RAPD primer name and sequence

Primer name	Sequence (5' - 3')
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-10	GTGATCGCAG
OPA-13	CAGCACCCAC
OPA-16	AGCCAGCGAA
OPB-08	GTCCACACGG
OPB-10	CTGCTGGGAC

Electrophoresis

The PCR results were electrophoresed on a 2% agarose gel with 60 Volt, 150 mA, and 20 Watts for 2 hours. To determine the size of the DNA band, a 100-bp DNA ladder was inserted into the gel well in as much as 5 μL. Staining was done by soaking the gel in SYBR™ Safe DNA Gel Stain

for 30 minutes. The resulting amplified bands were observed using a GelDoc UV Transilluminator (Sambrook & Russell, 2001).

Data analysis

Each known size DNA band was scored. The DNA band was given a score of one (1) if present and a score of zero (0) if absent. The results of the binary data matrix were analyzed using the software POPGENE32 (Yeh et al., 1999). The obtained matrix data were then subjected to cluster analysis using the unweighted pair group method with the arithmetic mean (UPGMA) method based on the genetic distance matrix introduced by Nei (1978). A principal component analysis (PCoA) subjected to separate accessions to determine the relationship among accessions was conducted by the software MVSP (Multi-variate Statistical Package) 3.2 (Bousba et al., 2020).

RESULTS AND DISCUSSION

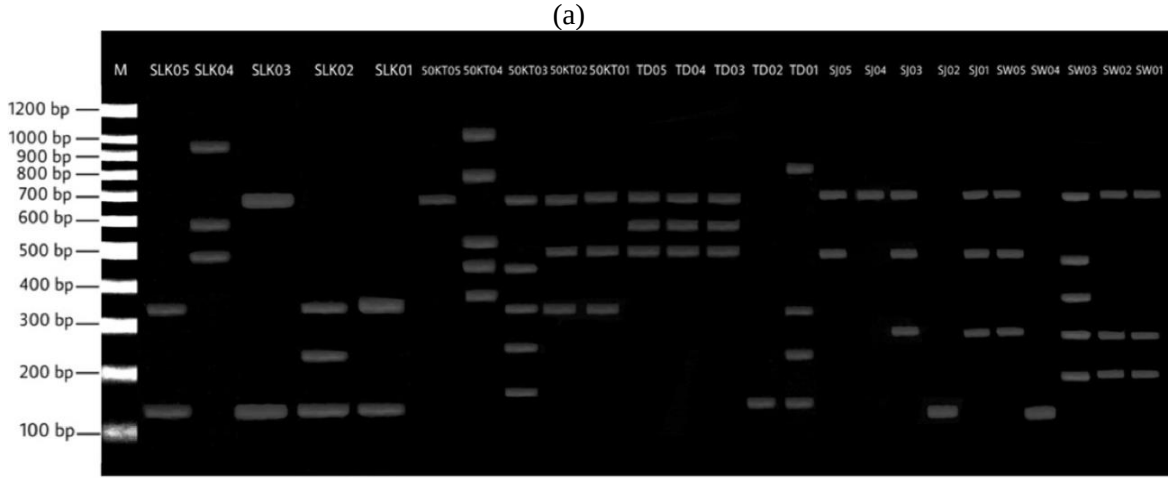
Polymorphism analysis of *C. pentandra*

Three useful primers (OPA-01, OPA-02, and OPB-10) were produced from ten primers that were screened. PCR amplification was carried out using these three primers. The three primers produced 44 bands, averaging 14.67 bands per primer. Primer OPA-01 produced the highest bands (20), whereas primer OPB-10 produced the lowest bands (10) (Table 2). DNA bands result from pairing nucleotides from primers and nucleotides from plant genomes (Lorenz, 2012).

The size of scorable and reproducible bands produced by three RAPD primers ranged from 133 to 1138 bp, and the polymorphic band percentage was 100% (Table 2). The different DNA band sizes are caused by differences in the length of DNA sites in plants, which primers can extend. The greater the distance between the primer site and the DNA template, the more extended and higher molecular-weight DNA fragments will be produced (Wahyudi et al., 2020). High polymorphic bands on the PCR amplification results indicated the high genetic variation of the species examined (Probojati et al., 2019).

The band profiles produced from three primers were clear, polymorphic, and reproducible (Figure 1). These results indicated that those three primers are suitable to be used as markers for studying the genetic variation within and among populations of *C. pentandra.*

(a)



(b)

(c)

Figure 1. RAPD-PCR profile from *C. pentandra* in five populations (a) Primer OPA-01, (b) Primer OPA-02, (c) Primer OPB-10. M = 100 bp DNA Ladder, SW01-05 = Sawahlunto, SJ01-05 = Sijunjung, TD01-05 = Tanah Datar, 50KT01-05 = Lima Puluh Kota, SLK01-05 = Solok.

Table 2. Polymorphism analysis results of three RAPD primers amplification of *C. pentandra*

No.	Primer name	Sequence (5'-3')	Number of bands	Number of polymorphic bands	Polymorphic bands (%)	Band size range (bp)
1	OPA-01	CAGGCCCTTC	20	20	100	133-972
2	OPA-02	TGCCGAGCTG	14	14	100	143-1041
3	OPB-10	CTGCTGGGAC	10	10	100	357-1138
Total			44	44	300	
Average			14.67	14.67	100	

Genetic variation of *C. pentandra*

Statistical analysis in five populations showed the value of the intrapopulation (within populations) and interpopulation (among populations) genetic variation of *C. pentandra*. Table 3 showed that the population of Pangkalan Koto Baru in Lima Puluh Kota regency had the highest values for alleles observed (Na), effective alleles (Ne), heterozygosity (H), and index Shannon (I), while the population of Lintau Buo Utara in Tanah Datar regency had the lowest values for Na, Ne, H, and I.

The heterozygosity value is used to assess genetic variation within a population. The heterozygosity values of the populations Talawi, Kupitan, Lintau Buo Utara, Pangkalan Koto Baru, and X Koto Singkarak, respectively, are 0.0859, 0.1071, 0.0802, 0.1212, and 0.1163 (Table 3). Based on these numbers, the five populations have low genetic variation values. This is based on the determination of the heterozygosity value by Nei (1978), according to which the heterozygosity value ranges from 0 (zero) to 1 (one); if the H value ranges from 0.1–0.4, it means low genetic variation; the value of 0.5–0.7 means moderate genetic variation; and a value of 0.8–1.0 means high genetic variation.

Based on the heterozygosity value, the Pangkalan Koto Baru population has the highest genetic variation compared to the other four populations. This is related to differences in habitat conditions within each population. In the Pangkalan Koto Baru population, the *C. pentandra* habitat is thought to be maintained and not disturbed. Meanwhile, in the Lintau Buo Utara, *C. pentandra* habitat has generally been disturbed due to community activities. Increased habitat fragmentation may contribute to low genetic variation (Plenk et al., 2019). Furthermore, habitat fragmentation may affect pollination- and animal-based seed dispersal mechanisms, especially at local level (Ony et al., 2020).

Table 3. Results of intrapopulation genetic variation estimations of *C. pentandra* in five populations

No.	Population	Total samples	N_a	N_e	*H*	*I*	*N*	*PLP*
1	Sawahlunto (Talawi)	5	1.2727	1.1426	0.0859	0.1321	12	27.27%
2	Sijunjung (Kupitan)	5	1.3636	1.1675	0.1071	0.1681	16	36.36%
3	Tanah Datar (Lintau Buo Utara)	5	1.2727	1.1282	0.0802	0.1256	12	27.27%
4	Lima Puluh Kota (Pangkalan Koto Baru)	5	1.4091	1.1901	0.1212	0.1901	18	40.91%
5	Solok (X Koto Singkarak)	5	1.4545	1.1724	0.1163	0.1892	20	45.45%

Note: N_a = The average number of alleles observed, N_e = The average number of effective alleles, *H* = Heterozygosity, *I* = Index Shannon, *N* = Number of polymorphic loci, *PLP* = Percentage of polymorphic loci

The total population heterozygosity (H_T) was calculated to be 0.1343 (13.43%), which means the genetic variation of *C. pentandra* was 23.93% among populations and 76.07% within the populations. The value of heterozygosity among population (D_{ST} = 0.0321) was lower than the value of heterozygosity within population (H_S = 0.1021) (Table 4). A lower D_{ST} value than H_S indicates that intrapopulation genetic variation is higher than interpopulation genetic variation. This is due to *C. pentandra* having an outrossing mating system. Fu et al. (2020) state that outcrossing species would be expected to have higher intrapopulation genetic variation and lower population differentiation values than selfing species.

The value of genetic differentiation (G_{ST}) on *C. pentandra* was equal to 0.2393, categorized as low. G_{ST} is calculated from the total genetic diversity in the pooled populations (H_T) and the mean diversity within each population (H_S) (Nei, 1973). G_{ST} values range from zero (0) to one (1), with low values indicating little variation among populations and high values indicating a lot of variation among populations (Hamrick & Godt, 1989). Nybom & Bartish (2000) state that the standard genetic differentiation number for an outcrossing species is equal to 0.23. Furthermore, Loveless & Hamrick (1984) state that outcrossing species have a G_{ST} less than 10%.

Low genetic differentiation among populations indicates that gene flow has occurred. This is evidenced by the high gene flow value (*Nm*) of 1.5894. High gene flow is due to high outcrossing, so plants tend to have the same genetic variation. Wright (1978) categorized the value of *Nm* into three categories: low ($0.0 < Nm < 0.249$), moderate ($0.250 < Nm < 0.99$), and high ($Nm \geq 1$). If the value of gene flow is greater than one ($Nm > 1$), there will be low genetic differentiation (Li et al., 2018).

Outcrossing of *C. pentandra* is helped by bats (Chiroptera) as pollinators. Singaravelan & Marimuthu (2004) reported that three types of bats (Pteropodidae) frequently visit *C. pentandra* trees: *Cynopterus sphinx* and *Pteropus giganteus*, which visit trees all night, and *Rousettus leschenaulti*, which visit trees in the afternoon. These pollinators' behavior is one factor that can determine the success of reproduction and the mating system in *C. pentandra* (Lobo et al., 2013).

The movement of pollinators also affects the genetic variation among populations because pollinators can move pollen relatively long distances and encourage outcrossing. Singaravelan et al. (2008) reported that the pollinator activities of *C. pentandra* in India, such as *C. sphinx* and *R. leschenaulti*, can fly up to 35 km, while *P. giganteus* can fly farther, up to more than 80 km every night, in search of food. The movement of these bats can move seeds to farther distances and broader areas, thus encouraging the outcrossing of plants.

Table 4. Results of interpopulation genetic variation estimations of *C. pentandra* in five populations

Total samples	H_T	H_S	D_{ST}	G_{ST}	*Nm*
25	0.1343	0.1021	0.0321	0.2393	1.5894

Note: H_T = The total population heterozygosity, H_S = Heterozygosity within population, D_{ST} = Heterozygosity among population, G_{ST} = Genetic differentiation, *Nm* = Gene flow value

Genetic distance and cluster analysis of *C. pentandra*

Based on the analysis of genetic distance, the highest genetic distance value was 0.0640 between the Lintau Buo Utara (Tanah Datar) population and the X Koto Singkarak (Solok) population. In contrast, the lowest genetic value was 0.0093 between the Talawi (Sawahlunto)

population and the Kupitan (Sijunjung) population (Table 5). A high genetic distance indicates a large relationship between the populations, and a small genetic distance indicates a close relationship between the two populations. The smaller the genetic distance, the more similar the populations (Muhajirah et al., 2021).

Table 5. Genetic distance matrix of five populations *C. pentandra* based on Nei 1978

Population	Sawahlunto	Sijunjung	Tanah Datar	Lima Puluh Kota	Solok
Sawahlunto	-	-	-	-	-
Sijunjung	0.0093	-	-	-	-
Tanah Datar	0.0591	0.0431	-	-	-
Lima Puluh Kota	0.0252	0.0114	0.0482	-	-
Solok	0.0273	0.0220	0.0640	0.0195	-

Cluster analysis was used to construct a UPGMA dendrogram of five populations of *C. pentandra* in West Sumatera (Figure 2). The dendrogram shows the population grouping according to their genetic distance. Based on the dendrogram, the Talawi (Sawahlunto) population and the Kupitan (Sijunjung) population are in the same cluster. This is due to the close geographic distance between populations. The closer two populations are to each other geographically, the more likely the two populations will have genetic similarities (Uslan & Pharmawati, 2020).

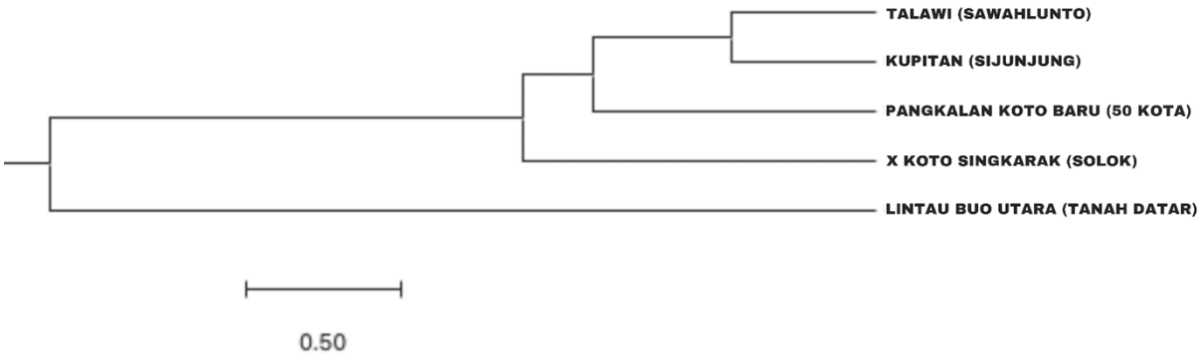


Figure 2. Dendrogram of five populations *C. pentandra* based on Nei 1978 genetic distance using the UPGMA method

The results of the cluster analysis on the 25 accessions of *C. pentandra* showed that the similarity coefficient ranges from 0.240-0.923 (minimal similarity > 0.2) (Figure 3). A high similarity coefficient indicates that the genetic relationship among accessions has low genetic diversity and vice versa. The highest similarity coefficient (0.923) was measured between accession TD05 and TD03. They have the same genome because they were collected from the same populations, i.e., Lintau Buo Utara in Tanah Datar Regency. Probojati et al. (2019) reported that if two cultivars have high similarity coefficients, they both will have the same genome and similar morphological characteristics.

The dendrogram showed that accessions tend to cluster based on their population. However, some accessions join other populations. All accessions were divided into two main clusters at a genetic similarity of 0.288. Cluster 1 consists of two sub-clusters from four populations: X Koto Singkarak (Solok), Lintau Buo Utara (Tanah Datar), Kupitan (Sijunjung), and Talawi (Sawahlunto). Cluster 2 consists of five sub-clusters from five populations: Pangkalan Koto Baru (Lima Puluh Kota), X Koto Singkarak (Solok), Kupitan (Sijunjung), Lintau Buo Utara (Tanah Datar), and Talawi (Sijunjung). Not all accessions from one population are in one cluster. It means that clustering based on genetic distance does not always show a real relationship with the geographical distribution of *C. pentandra* in the same habitat. It occurs due to environmental factors or because the accessions are from the same parent (Bhandari et al., 2017). Another possibility is due to the spread of seeds assisted by pollinators or there is a human factor (Syamsuardi et al., 2017). However, the tendency of such clustering is also shown in the results of research on plants that do outcross (Louwaars, 2018).

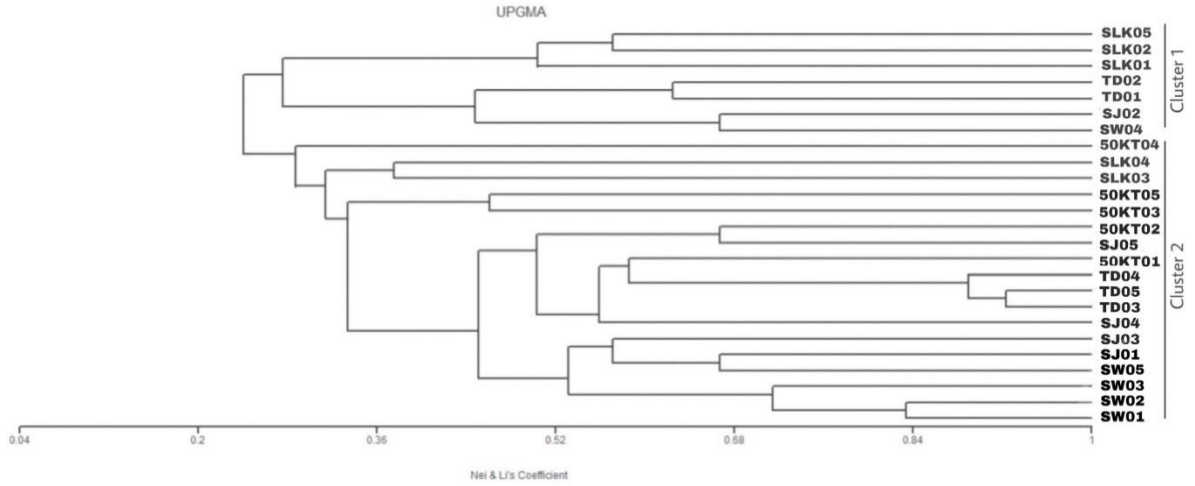


Figure 3. UPGMA dendogram of 25 accesions of *C. pentandra* based on Nei & Li similarity coefficient. SW01-05: Sawahlunto, SJ01-05: Sijunjung, TD01-05: Tanah Datar, 50KT01-05: Lima Puluh Kota, SLK01-05: Solok

The grouping pattern of 25 accessions of *C. pentandra* based on clustering analysis was confirmed using principal component analysis (PCoA). The results of the PCoA scatter plot diagram were also grouped into two main clusters (Figure 4). Cluster 1 consists of 7 accessions of *C. pentandra* from four populations, and cluster 2 consists of 18 accessions of *C. pentandra* from five populations. Hapsoro et al. (2015) reported that genotypes could be genetically related even though they have different geographic origins.

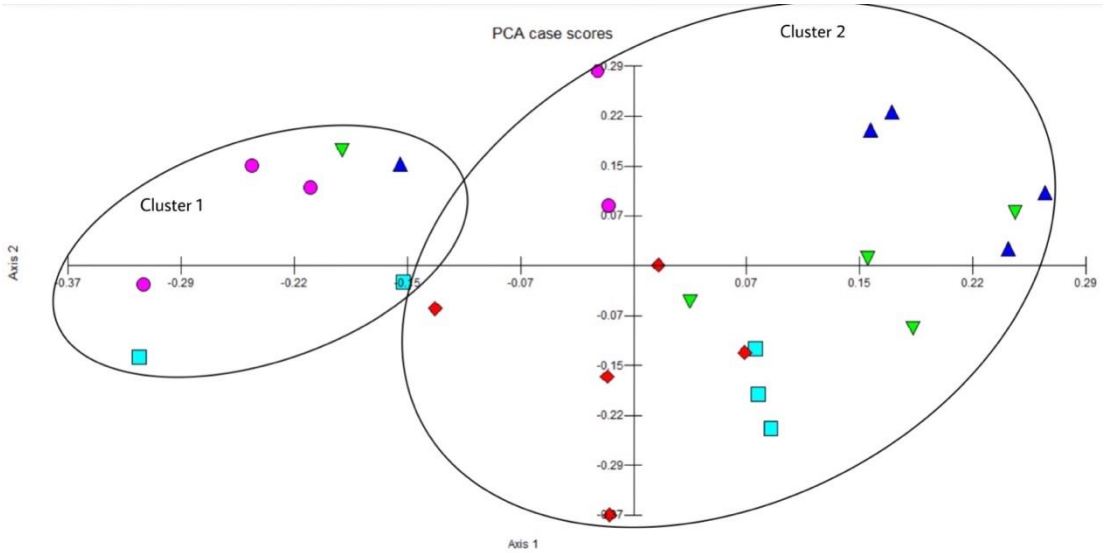


Figure 4. Principal Component Analysis (PCoA) scatter plot of 25 accessions of *C. pentandra* based on RAPD data

Note: ▲= Sawahlunto (Talawi), ▼= Sijunjung (Kupitan), ●= Tanah Datar (Lintau Buo Utara), ◆= Lima Puluh Kota (Pangkalan Koto Baru), ■= Solok (X Koto Singkarak)

This study reveals important information about the genetic variation of kapuk randu (*C. pentandra*). According to the results, RAPD markers can be used to study genetic variation within and among populations of *C. pentandra* in five populations at West Sumatra. This information can be used as a reference for developing the *C. pentandra* genetic resource area and supporting the conservation of this species. For breeding strategies, genetic variation within and among populations needs to be maintained (Larekeng et al., 2018). These efforts can be carried out by establishing seed orchards from various populations. The source is selected trees or seeds (superior seeds) to produce high-quality, genetically superior plants capable of adapting to

environmental change so biodiversity can be maintained (Kuswantoro et al., 2020).

The cluster analysis can be used to select diverse parents for hybridization programs to maximize heterosis. Diverse parents are preferred because hybridization involving divergent parents has increased the probability of obtaining desirable segregants in segregating generations. Based on the dendrogram (Figure 2), to maximize heterosis, the accessions from Lintau Buo Utara (Tanah Datar) with X Koto Singkarak (Solok) population were good candidates for parents to be hybridized, which have the farthest genetic distance. When specific allelic combinations from each parent are combined in a hybrid combination, they complete each other and yield heterosis expressions (Bingham et al., 1994). Pandey et al. (2015) reported that when heterosis is compared based on inter-cluster distances, it is noticed that the chance of obtaining highly heterotic hybrid results from the parental combination of low diversity and high diversity groups, or it may be possible between groups that have a high genetic distance. These findings can be used to aid in the development of better genotypes of *C. pentandra* in breeding programs to achieve a production population capable of producing quality seeds, as well as to inform conservation strategies for this species.

CONCLUSION

RAPD analysis of five populations *of C. pentandra* in West Sumatra showed that three RAPD primers (OPA-01, OPA-02, and OPB-10) successfully produced polymorphic bands. The heterozygosity of the Pangkalan Koto Baru (Lima Puluh Kota) population (H = 0.1212) was higher than other populations. The heterozygosity within populations (H_S = 0.1021) was higher than among populations (D_{ST} = 0.0321) with low genetic differentiation and high gene flow. Cluster analysis of five populations showed that Lintau Buo Utara (Tanah Datar) and X Koto Singkarak (Solok) have the farthest genetic distance. The 25 accessions of *C. pentandra* were grouped into two main clusters with a genetic similarity coefficient of 0.288. The genetic variation of *C. pentandra* is an important genetic resource that can be used in the future for a breeding program and the conservation of this species. For future research, the findings of this study are therefore recommendable to all stakeholders in the forestry industry, especially those using *C. pentandra* as a major species in their plantations.

ACKNOWLEDGEMENT

We extend our thanks to the Directorate of Research, Technology, and Community Service (DRTPM) under the National Competitive Research, Master Thesis Research No. 033/E5/PG.02.00/2022 for the management and funding support of the research to Andalas University, Padang, Indonesia. We also thank the Head of the Biology Department and the Dean of the Faculty of Mathematics and Natural Sciences, Andalas University, for the field and Laboratory work permit.

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