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NUCLEOTIDE VARIATIONS OF KAPOK (*CEIBA PENTANDRA*) BASED ON INTERNAL TRANSCRIBED SPACER (ITS) SEQUENCE DATA IN WEST SUMATRA, INDONESIA

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SUMMARY

Kapok (*Ceiba pentandra* L. Gaertn) is native to the tropics of Central and South America, the Caribbean, and West Africa. This plant earned intentional introduction and cultivation in numerous tropical regions, including Indonesia. However, West Sumatra has experienced a decline in its population. The promising study aimed to assess the genetic variability based on nucleotide variation in several accessions of *C. pentandra* belonging to two different populations in West Sumatra through the internal transcribed spacer (ITS) sequences. In the presented study, eight sequences of *C. pentandra* from West Sumatra and 12 from GenBank - NCBI gained usage. The analyzed *C. pentandra* sequences identified 683 conserved sites and three polymorphic sites, with AT (34.8%) and GC (65.2%) contents. Nucleotide diversity was evident in the 64th, 149th, and 224th sequences. Also, recognizing four haplotypes showed a haplotype diversity (Hd) value of 0.4953. Overall, the genetic diversity was low (Pi = 0.00130). It is because of the *C. pentandra* plant's introduction to several areas by humans (anthropogenic). In addition, the outcrossing mating system, seed dispersal through wind or water, and propagation techniques further contribute to its distribution and low genetic diversity. The prevailing results can add genetic information about the *C. pentandra* DNA sequence data based on ITS markers in West Sumatra, Indonesia. The obtained genetic data can serve as a basis for germplasm conservation strategies that have the potential for development in the future breeding program.

Keywords: Kapok (*Ceiba pentandra* L.), genetic variability, polymorphic sites, haplotype, ITS sequence data, nucleotide diversity, tropical regions

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Key findings: The analyzed *C. pentandra* sequences identified three polymorphic sites and four haplotypes, indicating low genetic variability. The pertinent data can add genetic information about *C. pentandra* DNA sequences based on ITS markers in West Sumatra, Indonesia. This information can serve as a basis for germplasm conservation strategies and a source of genetic data that has the potential to develop in the breeding program.

INTRODUCTION

Kapok (*Ceiba pentandra* L. Gaertn) is a tropical plant of the order Malvales and family Malvaceae, native to tropical regions in South and Central America, the Caribbean, and West Africa (Alvarado *et al.,* 2002). This plant gained introduction and cultivation in all the tropics, including Indonesia (Abdullah *et al.,* 2010), extending to various regions, such as Sumatra, Java, Kalimantan, Madura, Bali, Lombok, Nusa Tenggara, Sulawesi, Maluku, Ambon, and Irian Jaya (Agoes, 2010). *C. pentandra* has attained broad utilization by the community for medicinal and non-medicinal purposes (Chan *et al*., 2023). Given its several benefits and uses, *C. pentandra* became one of the potential plants used by humans (Nkoum *et al.,* 2017). However, due to a lack of conservation efforts, the population of *C. pentandra* L. has shown a current decline in the West Sumatra province, Indonesia (BPS, 2020; Hidayati, 2020).

Population decline in Kapok (*C. pentandra* L.) can lead to reduced levels of genetic diversity (Furlan *et al.,* 2011), providing fewer opportunities for selecting potential accessions within the population (Hedrick and Fredrickson, 2010). Hefzi *et al.* (2023) conducted a study in West Sumatra to examine the genetic diversity of *C. pentandra* using RAPD markers, and their findings revealed a low genetic diversity in *C. pentandra* populations. Furthermore, they also identified two populations of *C. pentandra* L., i.e., Pesisir Selatan and Tanah Datar, which further revealed the most significant genetic distance. This considerable genetic divergence seemed to result from variations in nucleotide sequences among the various *C. pentandra* accessions.

In *C. Pentandra,* a low genetic diversity indicates the obligation of conserving the populations (Abengmeneng *et al.,* 2016). The study of genetic diversity in germplasm is one of the vital efforts to preserve the *C. pentandra* population as a potential source. Information on genetic diversity can help optimize germplasm conservation in specific areas (Abbas *et al*., 2020) and be a basis for selection through plant breeding programs (Enaberuel *et al.,* 2014). Measuring the levels of genetic diversity based on the nucleotide variations can proceed through DNA sequencing techniques using molecular markers (Grattapaglia, 2007), such as the internal transcribed spacer (ITS) in the angiosperms (Baldwin *et al*., 1995).

The ITS is a repetitive region consisting of non-coding DNA sequences located between regions flanked by coding segments of ribosomal RNA molecules (Badotti *et al.,* 2017). In plant research, these ITS markers gain broad use in phylogenetic analysis, genetic diversity investigation, and DNA barcoding due to their smaller size, usually ranging from 600 to 700 base pairs, highly conserved, large copy number, high-mutation rate, and rapid evolution (Fitmawati *et al.,* 2018; Zhou *et al.,* 2018). Therefore, based on the above premise, the apposite study aims to assess the genetic variability based on nucleotide variations in several accessions of Kapok (*C. pentandra* L.) in two populations (Pesisir Selatan and Tanah Datar) through the internal transcribed spacer (ITS) sequences in West Sumatra, Indonesia. These results could serve as a basis for germplasm conservation strategies. Additionally, it can provide genetic information that may benefit breeding programs.

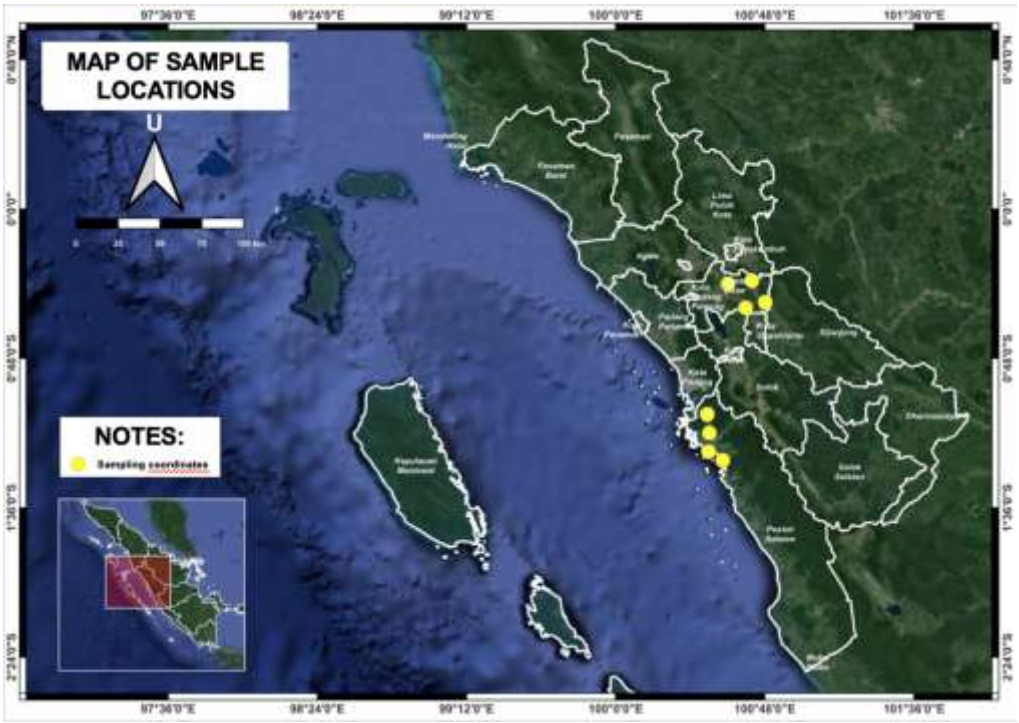

Figure 1. Sampling locations of eight accessions in two populations of *C. pentandra* L. in West Sumatra, Indonesia.

MATERIALS AND METHODS

Plant material and study area

In Kapok (*C. pentandra* L.), a collection of eight accessions came from two different populations (Pesisir Selatan and Tanah Datar) in West Sumatra, Indonesia (Figure 1). The ITS spacer region of eight *C. pentandra* accessions attained sequencing in this study, with an additional 12 sequences of *C. pentandra* from the GenBank NCBI (Table 1).

DNA isolation and PCR amplification

The CTAB (Cetyl Trimethyl Ammonium Bromide) method proceeded to extract the DNA from young leaves, following the procedure outlined by Doyle and Doyle (1987). The PCR amplification used a SensoQuest PCR Thermal Cycler. This study employed two specific primer sets: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as the forward primer, and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as the reverse primer (White *et al*., 1990). The PCR reactions progressed in 25 µl volumes composed of 12.5 µl of Bioline My Taq HS Red Mix reagent, 3.5 µl of ddH2O, 2 µl of the forward primer, 2 µl of the reverse primer, and 5 µl of DNA template. The amplification process followed these conditions: an initial pre-denaturation cycle at 95 °C for four minutes, followed by 30 amplification cycles. Each amplification cycle included denaturation at 95 °C for 45 seconds, annealing at 54 °C for 45 seconds, and elongation at 72 °C for a minute. A final elongation step at 72 °C took two minutes. The PCR products then received electrophoresis for 55 minutes at 100 V, 200 mA, and 20 watts on a 2% agarose gel. Gel visualization ensued on a UV transluminator for documentation. Subsequent dispatch of the PCR products to the PT Genetics Science followed for further analysis at the 1st BASE DNA Sequencing Service Malaysia, employing the Sanger sequencing method.

Table 1. The *C. pentandra* sequences used in this study.

No.	Species	Accessions	Locations	Authors
1	*Ceiba pentandra*	TD01	West Sumatra	Present study
2	*Ceiba pentandra*	TD02	West Sumatra	Present study
3	*Ceiba pentandra*	TD03	West Sumatra	Present study
4	*Ceiba pentandra*	TD04	West Sumatra	Present study
5	*Ceiba pentandra*	PSL01	West Sumatra	Present study
6	*Ceiba pentandra*	PSL02	West Sumatra	Present study
7	*Ceiba pentandra*	PSL03	West Sumatra	Present study
8	*Ceiba pentandra*	PSL04	West Sumatra	Present study
9	*Ceiba pentandra**	MF629751.1	North Maluku	Sundari *et al.* (2017) Unpublished
10	*Ceiba pentandra**	ON908445.1	Vietnam	Vu *et al.* (2022) Unpublished
11	*Ceiba pentandra**	HQ658386.1	Brazil	Duarte *et al.* (2011)
12	*Ceiba pentandra**	KM453169.1	Brazil	Carvalho-Sobrinho (2014) Unpublished
13	*Ceiba pentandra**	EF432374.1	Africa	Dick *et al.* (2007)
14	*Ceiba pentandra**	EF432375.1	Africa	Dick *et al.* (2007)
15	*Ceiba pentandra**	DQ284820.1	Africa	Dick *et al.* (2007)
16	*Ceiba pentandra**	DQ284827.1	Africa	Dick *et al.* (2007)
17	*Ceiba pentandra**	DQ284829.1	Africa	Dick *et al.* (2007)
18	*Ceiba pentandra**	DQ284830.1	Africa	Dick *et al.* (2007)
19	*Ceiba pentandra**	DQ284847.1	Africa	Dick *et al.* (2007)
20	*Ceiba pentandra**	DQ284848.1	Africa	Dick *et al.* (2007)

Note: *sequence from GenBank.

Data analysis

Sequences were contig using the SeqMan DNA STAR program (Burland, 2000). Obtaining alignment of the sequence analysis using the ClustalX version 2.0 program (Larkin *et al.*, 2007) and editing by the Bioedit program (Hall, 1999). The study used the MEGA 11 program for sequence characteristics (Tamura *et al.,* 2021). The analysis of the number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (Pi) utilized the DnaSP 6 program (Rozas *et al.*, 2017). Haplotype network creation ran the NETWORK version 10.2.0.0 based on median-joining (Bandelt *et al.,* 1999).

RESULTS AND DISCUSSION

Profile of ITS sequence in *C. Pentandra*

The CTAB method can help extract the *C. pentandra* DNA. The fresh leaves of *C. pentandra* contain high amounts of polysaccharides, polyphenols, and mucilage (Kuruvilla and Anilkumar, 2018). Past studies reported that the CTAB method was most suitable for isolating plant DNA containing high polysaccharides and polyphenols (Turaki *et al.*, 2017). DNA isolation using the CTAB method has also succeeded in other species of the family Malvaceae (Hafizah *et al.,* 2018). Eight accessions of the *C. pentandra* from West Sumatra incur successful amplifications using ITS1 and ITS4 primers at 54 °C annealing temperatures. The primers amplified regions were ITS1, 5.8S, and ITS2, with a total length of about 770 bp (Figure 2).

The ITS1 and ITS4 primers are universal primers that can amplify ITS1 and ITS2 regions, including 5.8S rDNA (White *et al.,* 1990). Past studies authenticated that amplification using ITS1 and ITS4 primers can produce amplicons with a size of about 600–780 bp (Chen *et al.*, 2010). Baldwin *et al*. (1995) reported that the ITS1 and ITS2 regions in plants have a length of about 300 bp each, and the 5.8S subunit in most of the angiosperms has a length of about 163–164 bp, with the length of the entire ITS region and 5.8S rDNA ranged from 565 to 700 bp. Dick *et al.* (2007) also obtained the same results and reported an average ITS sequence length of about 750 bp from 51 individuals of *C. pentandra* in Africa and Neotropics. Tate *et al.*

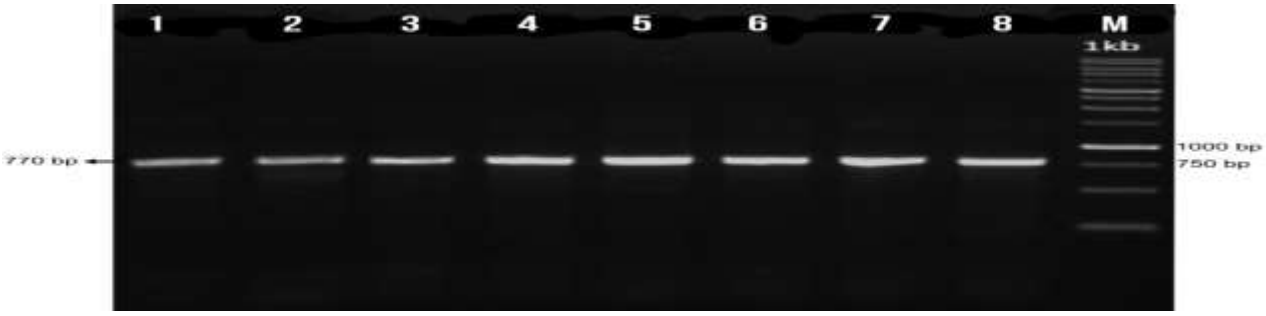

Figure 2. Visualization of PCR amplification of the ITS region on agarose gel 2% (1-4 = Tanah Datar, 5-8 = Pesisir Selatan, M = 1 kb DNA ladder).

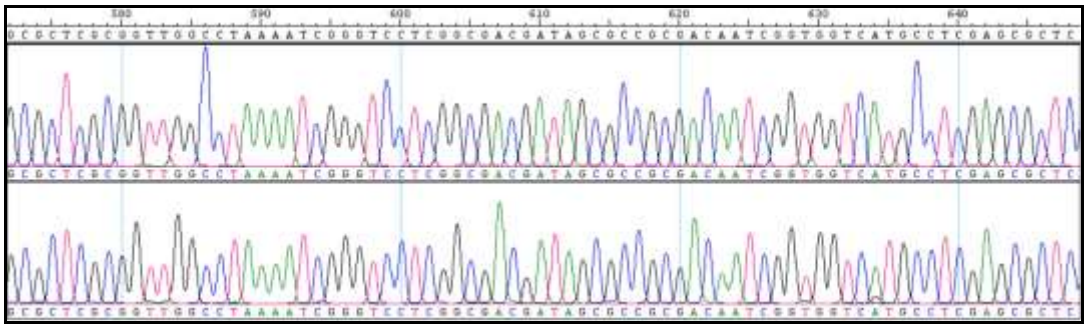

Figure 3. Performance of electropherogram sequencing results of the *C. pentandra* accessions from West Sumatra based on the ITS region.

(2005) reported that the length of ITS1 in several species of the family Malvaceae ranged from 253 to 297 bp, and the length of ITS2 ranged from 207 to 231 bp. Furthermore, Mukhamedov *et al.* (1994) also mentioned the length of ITS1 in *Gossypium* sp. (Malvaceae), which was around 287 bp, while the length of ITS2 was around 229 bp.

The sequencing results of *C. pentandra* sequences' visualization appear as an electropherogram (Figure 3). The electropherogram showed a peak consisting of four colors, each representing one nucleotide base—green for adenine (A), red for thymine (T), blue for cytosine (C), and black for guanine (G). The best sequencing results were characteristics of non-overlapping peaks and uniform space between the peaks (Wibawa, 2022).

In *C. pentandra* L., the alignment of 20 sequences obtaining 686 bp incurs analysis. Among 686 sites, 683 were conserved sites (monomorphic), three were variable sites (polymorphic), two parsimony sites, one singleton site, and two gaps (Table 2). Parsimony sites occurred at the nucleotides 149 and 224, singleton sites showed at the nucleotide 64, with gaps identified on the nucleotides 9 and 68 (Table 3). Dick *et al.* (2007) also reported similar results on 51 aligned sequences of *C. pentandra* L., obtaining five variable sites, with two singleton sites at the nucleotides 139 and 141 and three parsimony sites at the nucleotides 224, 299, and 413.

The exact nucleotides appear throughout all sequences in conserved sites. However, this site has no mutation in the nucleotide bases. Less genetic diversity was noticeable by having more conserved sites in the sequence (Warseno *et al.,* 2022). Liu and Schardl (1994) reported that the high level of conservation of ITS sequences seemed involved with its function in processing rRNA

Table 2. Characteristics of the ITS sequence of *C. pentandra.*

Variables	Values
Total sequence	20
Length of the analyzed sequence (bp)	686
Conserved (monomorphic) sites	683 (99.56%)
Variable (polymorphic) sites	3 (0.43%)
Parsimony-informative sites	2 (0.29%)
Singleton sites	1 (0.14%)
Gaps	2 (0.29%)
A+T content (%)	34.8
G+C content (%)	65.2

Table 3. Variation in *C. pentandra* sequences.

Accessions code	Base nucleotide position				
	9	64	66	149	224
C. pentandra TD01	A	C	C	G	T
C. pentandra TD02	.	.	.	.	.
C. pentandra TD03	.	.	.	.	.
C. pentandra TD04	.	.	.	.	.
C. pentandra PSL01	.	.	.	.	.
C. pentandra PSL02	.	.	.	T	.
C. pentandra PSL03	.	.	.	.	.
C. pentandra PSL04	.	.	.	.	.
C. pentandra MF629751.1	–	.	.	.	.
C. pentandra ON908445.1	.	.	.	T	.
C. pentandra HQ658386.1	.	.	.	.	.
C. pentandra KM453169.1	.	.	.		.
C. pentandra EF432374.1	.	A	–	T	.
C. pentandra EF432375.1	.	.	.	T	.
C. pentandra DQ284820.1	.	.	.	.	C
C. pentandra DQ284827.1	.	.	.	.	C
C. pentandra DQ284829.1	.	.	.	.	C
C. pentandra DQ284830.1	.	.	.	.	C
C. pentandra DQ284847.1	.	.	.	.	C
C. pentandra DQ284848.1	.	.	.	T	.

gene transcripts. In addition, the gene 5.8S presence with a length of 163–165 bp plays a vital role in the ribosomal complex and makes the ITS region highly conserved (Hershkovitz and Lewis, 1996; Jobes and Thien, 1997).

Polymorphic sites contain at least two different types of nucleotides. These variable sites may be singleton or parsimony-informative (Tamura *et al.,* 2021). Parsimony sites also became beneficial for reconstructing phylogenetic trees, while singleton sites are unable to provide information about parsimony trees (Ubaidillah and Sutrisno, 2009). The variation in nucleotide bases might be due to the substitutions, such as transitions and transversions, while the existing gaps were due to insertions and deletions (Mir *et al.,* 2010).

In *C. pentandra* L., the A+T content in all the sequences ranged from 34.7% to 35.0%, with an average of 34.8%, while the G+C content ranged from 64.8% to 65.2%, with an average of 65.2%. The G+C content was higher than the A+T. These results were analogous to past findings reported that on 51 sequences of *C. pentandra* in Africa based on ITS markers, the average A+T content was 35%, and the average G+C content was 65% (Dick *et al.*, 2007). Hershkovitz and Zimmer (1996) also reported that sequences on ITS1 and ITS2 appeared rich in G+C bases because

this part occurred highly conserved in angiosperms.

Nucleotide diversity of *C. Pentandra*

Based on the analyzed 20 sequences of *C. pentandra* L., the obtained four haplotypes (H = 4) had a haplotype diversity (Hd) value of 0.4953 and nucleotide diversity (Pi) of 0.00130 (Table 4). A visualization of the relationship between haplotypes (haplotype network) is available in Figure 4.

The Hap_1 has 12 accessions from West Sumatra, North Maluku, Central America, and Brazil. The Hap_2 comprises three accessions from West Sumatra, Vietnam, and Africa. The Hap_3 contains four accessions from America and Africa, while Hap_4 includes a single accession from Africa. The grouping of haplotypes depended on the variations in the ITS sequence bases of all the accessions of *C. pentandra* analyzed due to point mutations, such as transitions and transversions. Accessions occupying the same haplotype have the exact position of a point mutation and identical sequences (Deng *et al.,* 2020).

Table 4. Haplotypes of 20 *C. pentandra* sequences using 2-parameter Kimura model analysis.

No	Haplotype	n	Accessions code	Hd	Pi
1	Hap_1	10	*C. pentandra*_TD01*, *C. pentandra*_TD02*, *C. pentandra*_TD03*, *C. pentandra*_TD04*, *C. pentandra*_PSL01*, *C. pentandra*_PSL03*, *C. pentandra*_PSL04*, *C. pentandra*_MF629751.1_North Maluku, *C. pentandra*_HQ658386.1_Brazil, *C. pentandra*_KM453169.1_Brazil		
2	Hap_2	4	*C. pentandra*_PSL02*, *C. pentandra*_ON908445.1_Vietnam, *C. pentandra*_EF432375.1_Africa, *C. pentandra*_DQ284848.1_Africa	0.4953	0.00130
3	Hap_3	5	*C. pentandra*_DQ284827.1_Africa, *C. pentandra*_DQ284820.1_Africa, *C. pentandra*_DQ284829.1_Africa, *C. pentandra*_DQ284830.1_Africa, *C. pentandra*_DQ284847.1_Africa		
4	Hap_4	1	*C. pentandra*_EF432374.1_Africa		

Note: *accessions from West Sumatra, n = total accession, Hd = haplotype diversity, Pi = nucleotide diversity.

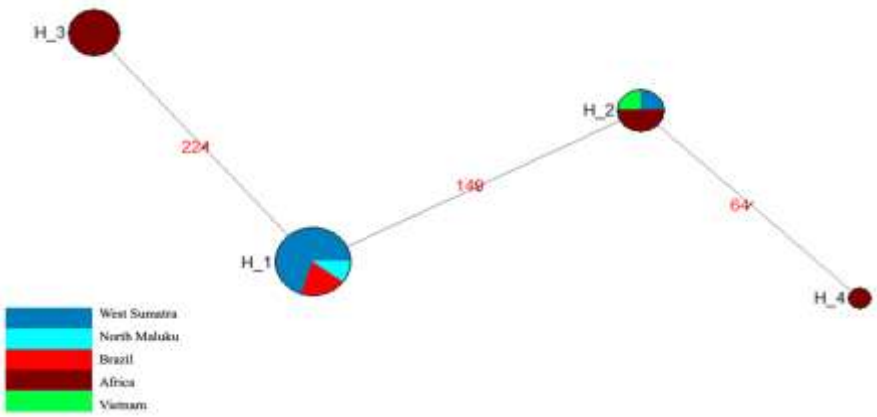

Figure 4. Median-joining haplotype network of the ITS region of *C. pentandra.*

The *C. pentandra* accessions from West Sumatra were generally visible on Hap_1. However, one accession, PSL02, was on Hap_2. It is because of a difference in one nucleotide base in the PSL02 accession compared with other West Sumatra accessions. Based on field surveys, no differences in the habitat among all accessions existed during sample collection. The difference in nucleotide bases occurs due to a point mutation in the form of a transition that causes a change from base C to T (Table 2). Point mutation usually results from errors in DNA replication during cell division. However, such mutation also occurs due to deamination, exposure to mutagens (ecological factors that cause mutations), and viral contamination (Shoaie, 2022). Nucleotide variation due to mutation implies that further understanding can aid in their use in crop improvement. Using haplotype graphs to present variability can add genomic data for plant breeding programs.

Based on the Hd and Pi values, it can be a low category. According to Nei and Kumar (2000), the Hd value ranges from 0 to 1; if $\geq 0 < 0.5$, it is low, and if $>0.5 \leq 1$, it is high. The value of Pi ranges from 0.001 to 0.01; if 0.08–0.1, it is high; if 0.05–0.07, it is medium; and if 0.01–0.04, it is low. The value of haplotype diversity (Hd) has linkages to the level of genetic diversity. The higher the value of haplotype diversity, the higher the level of genetic diversity, and vice versa (Wulandari *et al.,* 2021). However, the low nucleotide diversity value (Pi) indicates a minimal difference between haplotypes. Adriansyah *et al.* (2021) reported that low nucleotide diversity can be due to highly conserved sequences and accessions' low substitution rates. Dick *et al.* (2007) also found comparable findings in their study of 54 accessions of *C. pentandra* from the Neotropics and Africa. They identified five polymorphic sites among all ITS sequences, classifying them as low. The low levels of nucleotide divergence, in this case, are attributive to extreme long-distance dispersal of *C. pentandra* through wind or marine currents.

In *C. pentandra* L., the low genetic diversity was due to the outcrossing mating system and existing pollinator agents (bats). Hamrick and Godt (1996) reported that outcrossing plants tend to be more genetically homogenous and have low genetic differentiation among the different populations due to gene flow that causes chromosomal mixing among populations. Furthermore, generative (seeds) and vegetative (cuttings) propagation of *C. pentandra* L. also allows low variation between accessions (PROSEA, 2003).

CONCLUSIONS

In Kapok (*C. pentandra* L.), the sequences of several accessions in two populations of West Sumatra and accessions in other populations obtained 683 conserved sites and three polymorphic sites, one singleton site, and two parsimony sites, as well as AT (34.8%) and GC (65.2%) content. The haplotype diversity of *C. pentandra* accessions has two haplotypes with low genetic diversity. The presented results can add genetic information about *C. pentandra* DNA sequence data based on ITS markers in West Sumatra, Indonesia, which can serve as a basis for the germplasm conservation strategies and genetic data as a potential source for development in the future breeding program.

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