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Genetic Diversity and Phylogenetic Relationships of Mountain Papaya (*Vasconcellea pubescens*) in Dieng Plateau Based on Internal Transcribed Spacer Sequence

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ABSTRACT

The Dieng Plateau is an area used to cultivate Mountain Papaya (*Vasconcellea pubescens* A.DC.) as a food commodity in Indonesia. Research on diversity and relationships is vital as a first step in Mountain Papaya conservation in the Dieng Plateau. The study aims to determine the genetic diversity and relationship between Mountain Papaya accessions using Internal Transcribed Spacer (ITS) rDNA sequences. Fourteen accessions of Mountain Papaya with different sex distributions and altitudes are amplified using ITS1 and ITS4 primers. The genetic diversity is analyzed using the DnaSP 5.10.1 program. The Maximum Likelihood (ML) approach in MEGA 11 is utilized for assessing phylogenetic tree data based on ITS-rDNA regional sequences. With high haplotype diversity (Hd) values of 1.000 ± 0.027 and high nucleotide diversity (π) values of 0.09674 ± 0.00978, Mountain Papaya exhibits a high level of genetic diversity. Three main clades were identified in the phylogram tree based on the 14 ITS-rDNA sequences of Mountain Papaya. The results of this diversity data can support breeding programs intended to boost Mountain Papaya variety production.

INTRODUCTION

Mountain Papaya (*Vasconcellea pubescens*, A.DC.) is a member of the *Vasconcellea* genus originating from South America. Mountain Papaya can be grown in subtropical and warm climates such as North America, Europe, Japan, and Australia. However, they require an environment free from frost and excessive heat. These plants are susceptible to certain pests and diseases, especially nematodes, mites, root rot, and viruses. Little is known about the climatic requirements and cultural practices used to produce maximum production and the best flavor of Mountain Papaya (National Research Council, 1989). This plant thrives on 1400–2400 meters above sea level (asl), with low temperatures and high rainfall. In Indonesia, Mountain Papayas spread in the Dieng Plateau area, Central Java, and in the Bromo and Cangar areas, East Java. This plant was introduced by the Dutch colonial government to Indonesia in the period before the Second World War (Laily et al., 2021). Mountain Papaya has a variety of tastes and qualities. Some Mountain Papaya specimens have a bland taste and must be cooked with sugar to be delicious to eat (National Research Council, 1989). People often call Mountain Papaya “Carica Dieng” in the Dieng Plateau. The locals also process the fruit of the Mountain Papaya to create candied Carica fruit in small-scale domestic industries (Sarno & Wahyudi, 2018).

A study on Mountain Papaya’s genetic diversity, relationships, and environmental adaptation is urgently needed for future conservation (Scheldeman et al., 2007). Low genetic diversity in Mountain Papaya can be interpreted as an indicator of inbreeding depression and increased genetic drift. In addition, low genetic diversity is associated with reduced life span and health in individuals and reduced population growth capacity. In contrast, high

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levels of gene diversity in Mountain Papaya can be vital in improving population viability and ensuring the adaptability of natural populations to rapidly changing environmental pressures (Teixeira & Huber, 2021). Various aspects can affect genetic diversity in a population, especially in Mountain Papaya populations, including breeding systems, distribution of germplasm and pollen, plant age, farming practices, and variation in plant distribution among and within populations (Hamrick & Godt, 1996). One type of conservation that can be done on Mountain Papaya is germplasm conservation. Germplasm conservation can help preserve extinct, wild, and living plant species. Ex-situ and in-situ conservation can be used in conservation efforts (Priyanka et al., 2021). At least three benefits can be obtained from germplasm conservation efforts, such as new plant varieties, samples in plant hybridization, and genetic variant alleles in plant breeding programs (Quazi et al., 2021).

The molecular marker used in this Mountain Papaya study is the Internal Transcribed Spacer (ITS) sequence. Studies on Mountain Papaya using ITS sequences have been carried out previously by Kyndt et al. (2005). ITS sequences are widely used to determine genetic diversity and study phylogenetics at the genera and below genera level in plants (Álvarez & Wendel, 2003). Studies on nuclear genes usually involve ribosomal DNA. The ribosomal genes are arranged collectively, up to some thousands of copies. Every set-in ribosomal gene has a small subunit (18S) and a large subunit (26S), separated by more minor genes (5.8S). The 18S and 26S gene sequences were utilized in phylogenetic research, as they have some very conservative regions that assist in alignment and most variable areas that help differentiate phylogenetic groups (Singh, 2019). According to Brinegar (2009), ITS1 and ITS2 of ribosomal DNA are non-structural and have relatively higher mutation rates than the 18S, 5.8S, and 26S gene regions of ribosomal DNA. These regions are generally conserved within species but show considerable variation among species and genera when used in phylogenetic studies. Hence, selecting ITS sequences is suitable for studying intrapopulation in Mountain Papaya.

Studies on the genetic diversity and relationship of Mountain Papaya growing in Indonesia, especially in the Dieng Plateau area, are still limited. Previous research by Laily et al. (2012) discussed the characterization of Mountain Papaya based on morphological characteristics, antioxidant capacity, and protein banding patterns. Most other studies on Mountain Papaya in the Dieng Plateau area discuss its benefits and health potential as a medicinal plant. Novalina (2013) researched the antibacterial activity of *C. pubescens* leaf extract from the Dieng Plateau against bacteria that cause diarrhea. The two studies did not study the genetic diversity and relationships of the Dieng Mountain Papaya. Hence, this study aims to determine Mountain Papaya's genetic diversity and phylogenetic relationships from the Dieng Plateau based on Internal Transcribed Spacer sequences. Data from this research can support the development of gene diversity in Mountain Papaya, which will become a food and medicinal commodity in the future.

MATERIALS AND METHODS

Study Area

The study was conducted from August to October 2022. Fresh leaf samples of Mountain Papaya were collected from the Dieng Plateau in the Banjarnegara and Wonosobo regions of Central Java, Indonesia (Fig. 1). DNA extraction, PCR amplification, and data analysis were conducted at the Genetics Laboratory, Integrated Laboratory of UIN Sunan Kalijaga, Yogyakarta. DNA sequencing was performed at 1st BASE Malaysia.

Sample Preparation

Sampling was conducted from August 7 to 22, 2022. The sampling method for Mountain Papaya was purposive sampling using the Explore Method (Rugayah et al., 2004). Mountain papaya samples were obtained based on three locations that have different altitudes. These locations are Kejajar (1400 m asl) and Sembungan (2200 m asl), located in Wonosobo district, and Kepakisan (1800 m asl), located in Banjarnegara district. Each location sampled Mountain Papaya with a different sex distribution (Table 1). Observations of molecular characters used young leaves in the order of the fifth stalk from the shoot. Previously, the leaves were cleaned using technical alcohol, then stored in a small Ziplock plastic bag containing silica gel and put in an ice box to maintain the freshness of the leaves while traveling. Sample storage was carried out by crushing with liquid nitrogen and storing at -20ºC.

Reagent and Chemical

An extraction buffer consisting of 100 mM tris-HCl (pH 8), 20 mM EDTA (pH 8), 1,4 M NaCl, 3% CTAB (w/v), and 2% PVP was prepared. In addition, Proteinase K (10 mg/ml), RNAse (10 mg/ml), β-Mercaptoethanol, phenol:chloroform:isoamyl alcohol (25:24:1), 5 M NaCl, Wash solution [70% (v/v) ethanol] and TE buffer containing 10 mM tris-HCl (pH 8) and 1 mM EDTA (pH 8) were prepared and applied.

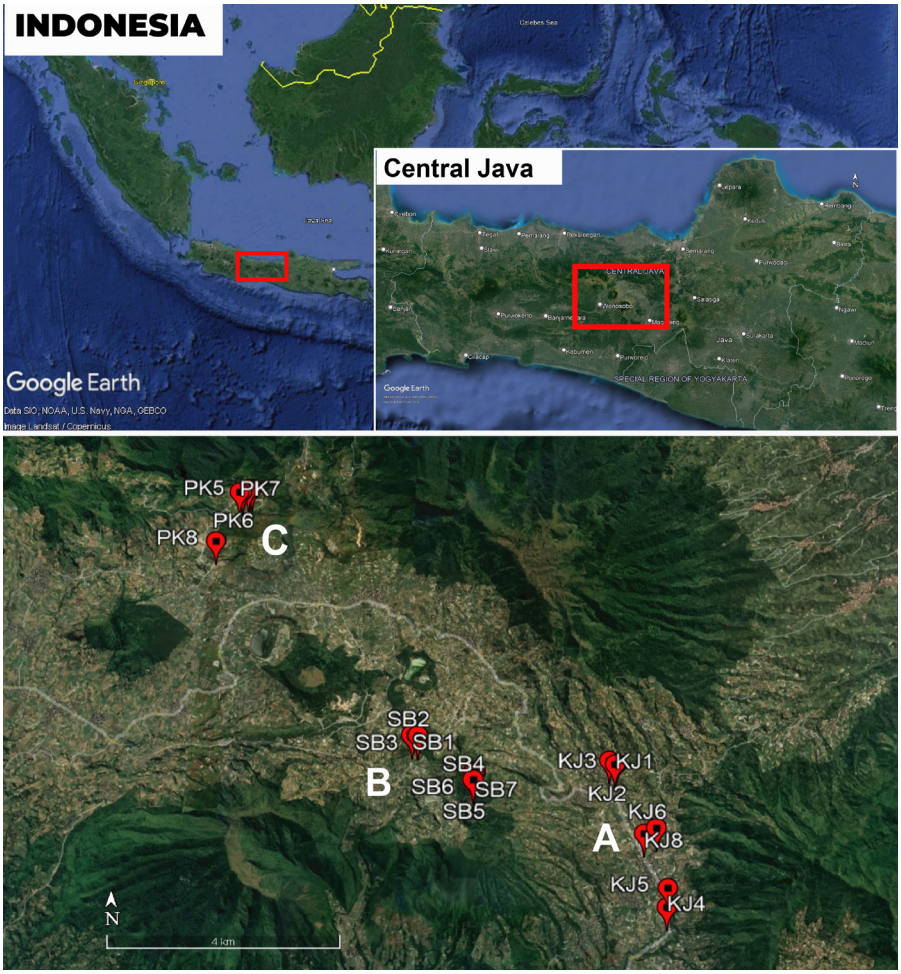


Fig. 1. Mountain Papaya sampling location in the Dieng Plateau: **A**. Kejajar, Kejajar, Wonosobo. **B**. Sembungan, Kejajar, Wonosobo. **C**. Kepakisan, Batur, Banjarnegara

Table 1. Mountain Papaya accessions

Code	Collection Site			Altitude (m asl)	Sex Distribution
	Sub-District	Regency	Province		
KJ1	Kejajar	Wonosobo	Central Java	1579	Female
KJ2	Kejajar	Wonosobo	Central Java	1567	Female
KJ4	Kejajar	Wonosobo	Central Java	1376	Hermaphrodite
KJ6	Kejajar	Wonosobo	Central Java	1473	Male
KJ7	Kejajar	Wonosobo	Central Java	1579	Hermaphrodite
SB1	Sembungan	Wonosobo	Central Java	2227	Female
SB2	Sembungan	Wonosobo	Central Java	2223	Female
SB5	Sembungan	Wonosobo	Central Java	2184	Male
SB6	Sembungan	Wonosobo	Central Java	2185	Hermaphrodite
SB7	Sembungan	Wonosobo	Central Java	2224	Hermaphrodite
PK1	Kepakisan	Banjarnegara	Central Java	1887	Hermaphrodite
PK2	Kepakisan	Banjarnegara	Central Java	1887	Male
PK4	Kepakisan	Banjarnegara	Central Java	1921	Female
PK6	Kepakisan	Banjarnegara	Central Java	1921	Hermaphrodite

DNA Extraction

DNA extraction was performed by modifying the CTAB (Cetyltrimethylammonium Bromide) method of Conlon et al. (2022). Dry leaf tissue (50–100 mg) was ground using a mortar and pestle with liquid nitrogen. Leaf samples were transferred into 1.5 ml microtubes and added to 1 ml of extraction buffer, 10 µl proteinase K, 10 µl RNAse A, and 10 µl β-Mercaptoethanol. Incubate the sample at 65°C for 45 minutes (vortex sample every 15 minutes, for 1 minute). Centrifuge the sample at 13000 rpm for 15 minutes. Transfer 700 µl of the supernatant to a new tube. Add phenol:chloroform:isoamyl alcohol (25:24:1) as much as the supernatant and mix by gentle inversion 50 times and centrifuge at 13000 rpm for 20 minutes.

Transfer the supernatant obtained to a new tube. Add the chloroform:isoamyl alcohol (24:1) solution as much as the supernatant and centrifuge at 13000 rpm for 15 minutes. Transfer the upper phase to a new tube and add 1/3 of the supernatant volume of 5 M NaCl and 2/3 of the supernatant volume of ice-cold isopropanol to the tube. Incubate the tube overnight at -20°C. Centrifuge the tube at 13000 rpm for 15 minutes. Discard the supernatant and wash the pellet by adding 500 µl 70% ethanol. Centrifuge at 13000 rpm for 5 minutes; wash twice to increase purity. Discard the supernatant and air-dry the pellet. Dissolve the pellet by adding 40–100 µl of TE buffer. DNA quantity was confirmed using a spectrophotometer with a 260 nm/280 nm wavelength. The results of DNA extraction were stored at -20ºC.

Polymerase Chain Reaction (PCR) Amplification

PCR amplification of the ITS region, including the 5.8 S rDNA region, was carried out using the primers ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) as forward and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) as reverse (White et al., 1990). The PCR reaction was performed in a 50 µl volume, consisting of a 5 µl DNA template (10 ng/µl), 5 µl forward primer (10 pmol/µl), 5 µl reverse primer (10 pmol/µl), 10 µl nuclease-free water, 25 µl PCR mix (Bioline MyTaq HS Red MixTM).

PCR amplification followed the protocol: 4 minutes of initial denaturation at 94°C, 30 cycles of 30 second denaturation at 94°C, 30 seconds of annealing at 55°C, and 1 minute of extension at 72°C. The amplification ends with a final extension at 72°C for 4 minutes. PCR products were visualized using electrophoresis on a 1% agarose gel stained with EtBr and 1X TBE buffer. Electrophoresis was performed at 90 V for 55 minutes. The gel was observed using a UV transilluminator to visualize DNA bands and compared with a 100 bp DNA ladder. PCR products with positive target bands were sent for sequencing at 1st Base Malaysia using the Sanger sequencing method.

Data Analysis

ITS-rDNA data analysis verified the nucleotide sequences of the ITS-rDNA and 5.8 S regions using the BLAST (Basic Local Alignment Search Tool) program on the NCBI website. Multiple sequence alignments were performed with accessions of ITS-rDNA sequences from *Cylicomorpha parviflora*, *Jarilla chocola*, and *Jarilla heterophylla* (available in the NCBI database) used as outgroups. The sample sequence data and outgroup sequences were processed using the OPAL program from MESQUITE (Maddison & Maddison, 2019) to align and convert sequence data from Notepad into Fasta format. Phylogenetic tree construction used the Maximum Likelihood (ML) algorithm, while genetic distance was analyzed using the Kimmura-2 parameter model with MEGA 11 software. The bootstrap method with 1000 replications was used to analyze branch formation on the phylogenetic tree. Genetic variation between clades in the ITS rDNA gene in the form of the number of haplotypes, number of polymorphic sites, number of parsimony sites, haplotype diversity, and nucleotide diversity was analyzed using the DnaSP 5.10.01 program (Librado & Rozas, 2009).

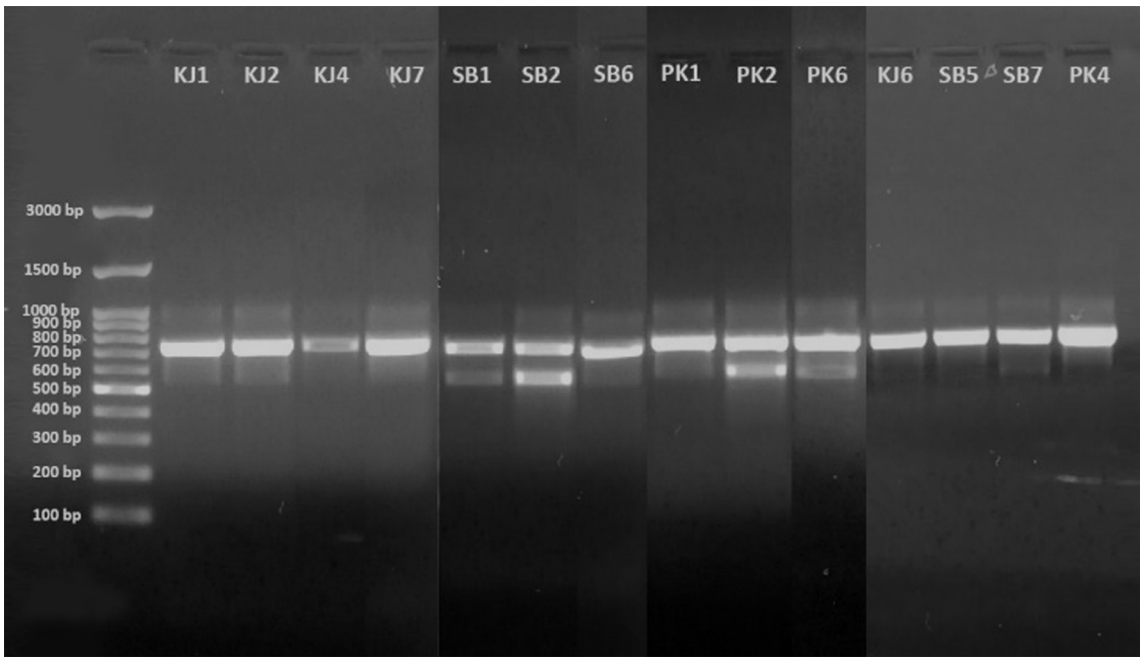
RESULTS AND DISCUSSION

Phylogenetic Relationship of Mountain Papaya

The total Mountain Papaya samples are successfully amplified using ITS1 and ITS4 primers, as indicated by the appearance of the target band on 1% agarose gel visualization. The amplification results show that the Mountain Papaya target band appeared at 737 bp (Fig. 2). Previous research by Kyndt et al. (2005) stated that ITS-1 and ITS-4 primers failed to produce PCR products in some taxa, so they used the ITS-VASF and ITS-VASR primer designs in their research.

The results of aligning Mountain Papaya sequences with sequences from the database using the BLAST program from NCBI are shown in Table 2. BLAST analysis showed varying percent identity values of 99.5%–81.02%. The sequence alignment of Mountain Papaya accessions showed two distinct species. Accessions KJ1, KJ4, KJ7,

SB1, SB5, PK1, PK2, PK6, and SB6 show that the sequences are similar to the species *Vaconcellea sprucei*. However, of the 9 accessions, there are 4 with a percent identity value below 90% (PK1, PK2, PK6, and SB6).



Remarks: KJ = accession from Kejajar sub-district, Wonosobo regency; SB = accession from Sembungan sub-district, Wonosobo regency; PK = accession from Kepakisan sub-district, Banjarnegara regency

Fig. 2. Agarose gel electrophoresis of the ITS1 and ITS4 regions of Mountain Papaya

Table 2. ITS sequence homology analysis of Mountain Papaya

No	Accession Code	Species	Query Cover (%)	Per Ident (%)	E value	Accession Number
1	PG_KJ1_ITS	*Vasconcellea sprucei*	90	98.34	0	AY461549.1
2	PG_KJ4_ITS1	*Vasconcellea sprucei*	84	93.30	0	AY461549.1
3	PG_KJ7_ITS1	*Vasconcellea sprucei*	86	93.50	0	AY461549.1
4	PG_SB1_ITS1	*Vasconcellea sprucei*	86	95.17	0	AY461549.1
5	PG_SB2_ITS1	*Vasconcellea cundinamarcensis*	91	88.41	0	AY461550.1
6	PG_SB5_ITS	*Vasconcellea sprucei*	80	99.5	0	AY461549.1
7	PG_PK1_ITS1	*Vasconcellea sprucei*	99	87.33	0	AY461549.1
8	PG_PK2_ITS1	*Vasconcellea sprucei*	99	89.86	0	AY461549.1
9	PG_PK6_ITS1	*Vasconcellea sprucei*	97	87.84	0	AY461549.1
10	PG_SB6_ITS1	*Vasconcellea sprucei*	99	87.85	0	AY461549.1
11	PG_PK4_ITS1	*Vasconcellea cundinamarcensis*	86	92.2	0	AY461550.1
12	PG_KJ2_ITS1	*Vasconcellea cundinamarcensis*	85	85.91	3.00E-149	AY461550.1
13	PG_KJ6_ITS1	*Vasconcellea cundinamarcensis*	86	81.02	2.00E-107	AY461550.1
14	PG_SB7_ITS1	*Vasconcellea cundinamarcensis*	85	86.36	2.00E-151	AY461550.1

Remarks: *V. cundinamarcensis* is a synonym of *V. pubescens*; KJ = Accession from Kejajar sub-district, Wonosobo regency; SB = Accession from Sembungan sub-district, Wonosobo regency; PK = Accession from Kepakisan sub-district, Banjarnegara regency

Five accessions (SB2, PK4, KJ2, KJ6, and SB7) have sequence similarity with the species *V. cundinamarcensis*, a synonym of *Vasconcellea pubescens*. However, only two accessions have percent identity values above 90%, including accessions SB2 and PK4. Accessions KJ2, KJ6, and SB7 have an identity value of 81–86% with an E-value between 0.01 and 10. E-values between 0.01 and 10 indicate that the similarity of sequence pairs is less significant but may suggest homology (Aprilyanto & Sembiring, 2016).

A phylogenetic tree reconstruction was performed by aligning sample and outgroup sequences. The outgroups selected were *Cylicomorpha parviflora*, *Jarilla chocola*, and *Jarilla heterophylla*, species in the Caricaceae family. Huelsenbeck (2002) mentioned that good outgroup selection requires taxa outside the in-group but still has a close relationship with the in-group. Phylogenetic tree reconstruction is based on the Maximum Likelihood algorithm using the Kimura 2-parameter evolutionary model and a 1000-time bootstrap reliability test. The maximum likelihood method has the main principle of determining the tree topology, branch length, and evolutionary model that maximizes the probability of observing the range of sequences possessed (Aprilyanto & Sembiring, 2016). Hence, finding phylogenetic trees involving topology and branch length searches will provide the most significant possibility of observing DNA sequences in the acquired data (Cho, 2012). The results of the phylogenetic tree analysis of Mountain Papaya based on ITS sequences formed 3 main clades, which are clades I, II, and III, with ML value of 99 (Fig. 3). Clade groupings of 14 Mountain Papaya accessions in the Dieng Plateau based on ITS sequences showed no correlation based on altitude or sex distribution.

Clade I form two subclades with an ML value of 80. Subclade A consists of eight Mountain Papaya accessions and two comparison species from the database, which are *V. sprucei* and *V. pubescens* (syn. *V. cundinamarcensis*). Accessions included in subclade A are accessions identified as *V. sprucei* and *V. pubescens* with identity values above 88%, indicating that the eight accessions are closely related. Following phylogenetic analysis conducted by Kyndt et al. (2005) using ITS sequences and by Antunes Carvalho & Renner (2012) based on nuclear and chloroplast sequences, *V. sprucei* and *V. pubescens* species are closely related by forming the same clade. Meanwhile, a multilocus phylogenetic study by Tineo et al. (2020) states that *V. pubescens* and *V. sprucei* are conspecific species. Subclade B consists of three accessions (PK1, PK6, and SB6), identified species of *V. sprucei*, but the identity value is only 87%. Identity values below 90% indicate they do not belong to the Mountain Papaya species *V*. *sprucei*. Clade II consists of one accession (KJ2), while Clade III consists of two (KJ6 and SB7) of Mountain Papaya.

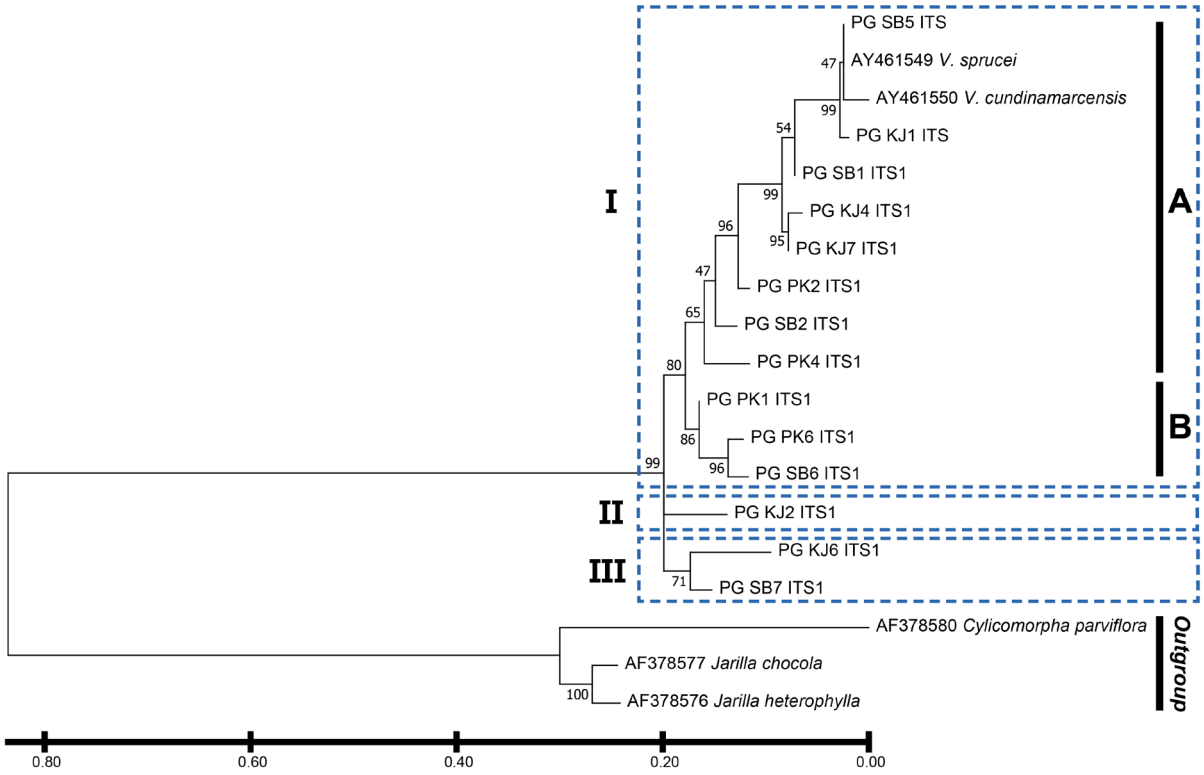
Previous research on Mountain Papaya found in Chile in structure and Bayesian analysis of population structure clustered into 8 different genetic groups: 3 in the southern region and 5 in the northern region. This difference is because farmers in the south are more active in exchanging plant material than farmers in the north, who rarely or never do so. The genetic diversity of Mountain Papaya in Chile can also be explained by founder events and extended selection (Carrasco et al., 2009). Research conducted by Warnakula et al. (2017) based on the analysis of morphological markers found that Mountain Papaya and *Carica papaya* in Sri Lanka are clustered in two main clusters. Mountain Papaya is clustered separately from the Carica papaya species sub-cluster. Meanwhile, molecular markers of SSR and ISSR show that Mountain Papaya is clustered separately in one central cluster from the other two main clusters. This proves that Mountain Papaya and *C. papaya* are genetically distant relatives.

The evolutionary difference between homologous gene sequences of the same gene from a common ancestor is known as genetic distance. Genetic distance determines the genetic differences between individuals within a species (intra-species) or between species (inter-species) (Warseno et al., 2022). Genetic distance analysis on Mountain Papaya accessions and sequences from the GenBank using the Kimura-2 parameter method showed a genetic distance value of 0.000–2.182 (0.0%–218.2%) (Table 3). According to Qin et al. (2017), dicotyledon plants have an average interspecific threshold of genetic distance based on the ITS2 gene of 7.69%. Genetic distance values of Mountain Papaya accessions compared to the species *V. pubescens* showed 11 accessions (KJ4, KJ7, PK1, PK2, PK4, PK6, SB2, SB6, KJ2, KJ6, and SB7) had genetic distance values above the interspecific threshold (8.0%–27.8%). While the genetic distance of accessions compared to

V. sprucei shows, 9 (PK1, PK2, PK4, PK6, SB2, SB6, KJ2, KJ6, and SB7) accessions have genetic distance values higher than the interspecific threshold (10.6%–27.1%). Genetic distance values above the interspecific threshold indicate that accessions are different species.

Genetic Diversity of Mountain Papaya

Nucleotide composition is a simple way of characterizing genomes. However, nucleotide composition is a major determining factor, among other features, including dinucleotide frequency, repetitive short DNA sequences, and codon and amino acid usage (Simón et al. 2021). The significance of nucleotide composition (GC content) in the evolution of genomes has been well recognized from genomic, ecological, and biological viewpoints (Castillo & Almeida, 2021). The GC Content value of Mountain Papaya is 0.575 (57.5%). According to Lynch (2007), eukaryotes have an average GC content variation of ~20 to 60%. The nucleotide composition of Mountain Papaya accessions showed variations in the percentage differences in thymine (T) or uracil (U), cytosine (C), adenine (A), and guanine (G) (Table 4). The average percentage of nucleotide frequency of the ITS sequence of Mountain Papaya is 22.05% (T), 31.71% (C), 21.03% (A), and 25.22% (G). The difference in the percentage of nucleotide composition in Mountain Papaya indicates genetic variation in the population.



Remarks: KJ = Accession from Kejajar sub-district, Wonosobo regency; SB = Accession from Sembungan sub-district, Wonosobo regency; PK = Accession from Kepakisan sub-district, Banjarnegara regency

Fig. 3. The Maximum Likelihood tree of Dieng Mountain Papaya

Table 3. Genetic distance ITS sequence of Mountain Papaya using *Kimura-2 Parameter* method

	KJ1	KJ4	KJ7	PK1	PK2	PK4	PK6	SB1	SB2	SB5	SB6	KJ2	KJ6	SB7	VP	VS	JC	JH	CP
KJ1																			
KJ4	0.075																		
KJ7	0.068	0.014																	
PK1	0.140	0.110	0.110																
PK2	0.112	0.062	0.058	0.060															
PK4	0.136	0.102	0.104	0.075	0.073														
PK6	0.134	0.112	0.107	0.042	0.068	0.076													
SB1	0.051	0.028	0.017	0.100	0.066	0.104	0.106												
SB2	0.131	0.092	0.085	0.057	0.053	0.073	0.070	0.088											
SB5	0.012	0.077	0.066	0.136	0.108	0.132	0.134	0.047	0.127										
SB6	0.140	0.141	0.132	0.048	0.096	0.097	0.035	0.124	0.091	0.136									
KJ2	0.193	0.161	0.150	0.098	0.106	0.108	0.111	0.151	0.104	0.189	0.124								
KJ6	0.278	0.225	0.218	0.128	0.168	0.149	0.141	0.228	0.126	0.273	0.145	0.138							
SB7	0.192	0.143	0.145	0.070	0.097	0.109	0.074	0.152	0.089	0.192	0.085	0.103	0.097						
VP	0.037	0.091	0.080	0.154	0.125	0.149	0.156	0.066	0.139	0.024	0.156	0.210	0.278	0.209					
VS	0.012	0.075	0.064	0.134	0.106	0.130	0.133	0.046	0.126	0.000	0.135	0.187	0.271	0.191	0.024				
JC	1.558	1.505	1.494	1.429	1.431	1.429	1.435	1.494	1.373	1.515	1.520	1.368	1.394	1.350	1.454	1.509			
JH	1.562	1.472	1.462	1.412	1.398	1.430	1.436	1.457	1.358	1.497	1.544	1.338	1.424	1.387	1.415	1.494	0.050		
CP	2.081	2.081	2.182	1.841	1.942	1.667	1.981	2.048	1.701	1.922	2.022	1.685	1.574	1.698	1.951	1.919	0.361	0.370	

Remarks: PK = Mountain Papaya from Kepakisan, SB = Mountain Papaya from Sembungan, KJ = Mountain Papaya from Kejajar, VP = *V. pubescens*, VS = *V. sprucei*, JC = *J. chocola*, JH = *J. heterophylla*, CP = *C. parviflora*

Analysis of polymorphism sites showed that the 14 accessions of Mountain Papaya in the Dieng Plateau were divided into 14 haplotypes with 169 polymorphic sites. According to Pandin (2010), mutation mechanisms such as deletions, insertions, transitions, and transversions can happen naturally or induced, and this causes changes in nucleotide bases in the DNA sequence. Mutation analysis was performed separately on single-base substitutions and multiple-base substitutions. Transitions and transversions occur in single-base substitutions, which are then recorded separately. Four patterns arise in the transition: A→G, G→A, T→C, and C→T. In transversion, there are eight patterns, such as A→T, T→A, A→C, C→A, G→T, T→G, G→C, and C→G. The number of multiple base substitutions, deletions, and insertions is recorded and analyzed separately (Luo et al., 2016). Mutations in Mountain Papaya accessions show transitions at 49 sites, transversions at 97 sites, multiple substitutions at 16 sites, and insertions-deletions at 11 sites (Table 5).

Table 4. Nucleotide composition of ITS sequence data in Mountain Papaya

Accession	T(U)	C	A	G	Total
KJ1	20.61	29.90	20.44	29.05	592
KJ4	20.71	29.71	22.24	27.33	589
KJ7	20.37	30.73	21.73	27.16	589
PK1	22.96	33.16	21.09	22.79	588
PK2	22.24	31.75	21.39	24.62	589
PK4	22.28	32.14	22.11	23.47	588
PK6	24.40	32.42	20.14	23.04	586
SB1	20.71	30.73	21.56	26.99	589
SB2	20.47	31.64	23.01	24.87	591
SB5	20.27	30.24	20.44	29.05	592
SB6	24.70	33.05	19.76	22.49	587
KJ2	21.60	33.33	20.58	24.49	588
KJ6	23.47	32.99	19.90	23.64	588
SB7	23.93	32.14	20.00	23.93	585
Average	22.05	31.71	21.03	25.22	588.64

Remarks: KJ = Accession from Kejajar sub-district, Wonosobo regency; SB = Accession from Sembungan sub-district, Wonosobo regency; PK = Accession from Kepakisan sub-district, Banjarnegara regency

Table 5. Mutation type of the ITS sequence of Mountain Papaya

No	Type of mutation	Nucleotide Positions	Total
1	Transition	11, 23, 90, 95, 114, 151, 167, 172, 183, 234, 258, 261, 268, 271, 274, 283, 290, 294, 310, 319, 327, 331, 335, 342, 359, 364, 366, 376, 380, 397, 412, 424, 457, 501, 507, 510, 511, 512, 516, 524, 529, 550, 555, 559, 560, 561, 570, 573, 574	49
2	Transversion	5, 78, 80, 82, 84, 99, 107, 110, 118, 121, 130, 138, 147, 157, 169, 171, 175, 182, 188, 195, 197, 206, 209, 211, 219, 225, 232, 245, 249, 251, 263, 265, 269, 278, 281, 286, 296, 299, 301, 304, 312, 317, 321, 324, 329, 339, 348, 358, 370, 372, 374, 377, 385, 389, 391, 393, 396, 399, 409, 422, 430, 431, 438, 446, 453, 462, 465, 466, 468, 472, 473, 476, 487, 489, 497, 499, 505, 508, 513, 519, 523, 528, 533, 534, 536, 538, 541, 544, 551, 557, 562, 565, 567, 571, 572, 591, 592	97
3	Multiple Substitution	435, 484, 515, 518, 522, 531, 549, 554, 564, 568, 582, 583, 584, 585, 589, 590	16
4	Insertion-Deletion	6, 7, 13, 23, 24, 531, 547, 567, 568, 569, 588	11

The variation in the 14 accessions of Mountain Papaya in the Dieng plateau is known after alignment. Sequence alignment resulted in a total of 592 sites of sequence variation. Among the 592 sites compared, there were 423 monomorphic sites, 156 polymorphic sites, and 112 parsimony-informative sites (Table 6). A parsimony-informative site is one where there are at least two distinct character states in the sequences at that site. The site includes at least two types of nucleotides (or amino acids), and at least two appear with the minimal frequency necessary for the most minor evolutionary changes in the genome. This informative position helps inform the evolutionary relationships between the genomes under study (Saha et al., 2020).

The results of haplotype diversity analysis (Hd) on Mountain Papaya accessions show a high diversity value of 1,000 ± 0.027 and a nucleotide diversity value (π) of 0.09674 ± 0.00978 (Table 6). The Hd value can be used to identify whether a sample has high or low diversity. Hd values ≥ 0 < 0.5 indicate low haplotype diversity, while Hd values > 0.5 ≤ 1 indicate samples have high haplotype diversity (Hobbs et al., 2013). The high haplotype diversity in this study demonstrates that increased mutation rates occur in the rDNA gene.

Mountain Papaya's haplotype diversity (Hd) from 14 accessions is high (1,000). Genetic diversity is essential for the population's long-term viability, as it enables it to be adaptable to changes in the environment, making it less vulnerable to extinction. The existence of high genetic diversity in a population is favorable for its survival, adaptability, and evolution (Nonić & Šijačić-Nikolić, 2019). Nucleotide diversity (π), according to Nei & Kumar (2000), has a range of values between 0.001-0.01 divided into three categories. Nucleotide diversity in the range of 0.08–0.1 shows a high category; the range of 0.05–0.07 is in the medium category, and the range of 0.01–0.44 is in the low category. Mountain Papaya accessions showed high nucleotide diversity (0.09674).

In contrast, the genetic diversity study of Mountain Papaya conducted by Carrasco et al. (2009) in Chile using ISSR sequence markers showed that the genetic diversity of Mountain Papaya was very low compared to the average variety reported in plants when using dominant markers. At the same time, the percentage of polymorphic loci showed similar levels to other crop studies. This reflects an interesting situation where high polymorphism does not necessarily result in high gene diversity. Rifqi & Chasani (2023), in their study, mentioned that the diversity of Mountain Papaya in Dieng Plateau based on morphological markers is classified as medium categories.

Table 6. Pholymorphic analysis of Mountain Papaya accessions

Accession	bp	Number of haplotypes	Monomorphic sites	Polymorphic sites	Parsimony informative sites	Haplotype diversity (Hd)	Nucleotide diversity (π)
KJ1	592	14	423	156	112	1.000 ±0.027	0.09674 ±0.00978
KJ4	589						
KJ7	589						
PK1	588						
PK2	589						
PK4	588						
PK6	586						
SB1	589						
SB2	591						
SB5	592						
SB6	587						
KJ2	588						
KJ6	588						
SB7	585						

Remarks: KJ = Accession from Kejajar sub-district, Wonosobo regency; SB = Accession from Sembungan sub-district, Wonosobo regency; PK = Accession from Kepakisan sub-district, Banjarnegara regency

The high haplotype diversity (Hd) and nucleotide diversity (π) in Mountain Papaya accessions indicate that Mountain Papaya in the Dieng plateau area has high genetic diversity. The high genetic diversity in the intrapopulation of Mountain Papaya on the Dieng plateau is caused by mutations and gene flow (migration). Eriksson & Ekberg (2001) mentioned that gene flow is the presence of individuals from a population participating in producing a new generation in the recipient population, where the donor and recipient populations have different allele frequencies. In plants, for example, gene flow occurs through pollen, seeds, or fruit dispersal. The high diversity of Mountain Papaya on the Dieng Plateau can provide benefits in supporting resilience to adverse environmental changes, integrity, community structure, and ecosystem function. In addition, high diversity can help plant breeding with the availability of genetically diverse parents, increasing varietal productivity in Mountain Papaya (Salgotra & Chauhan, 2023).

One of the strategies in plant conservation mentioned in the Convention on Biological Diversity (2012) is that plant diversity can be well understood, documented, and recognized. This can be done by including data on Mountain Papaya in the Dieng Plateau in the Online World Flora data, the first step in conservation. In addition, it is also important to conserve germplasm in Mountain Papaya by conducting in-situ and ex-situ conservation. In-situ conservation of Mountain Papaya is carried out by maintaining genetic resources in their natural habitat and original place. With in-situ conservation, plants can evolve naturally with minimal intervention from humans. In addition, conservation can also be carried out outside the original habitat or ex-situ conservation, such as with seed gene banks, plant tissue culture, and others. In its development, the biotechnological approach can also be used in conservation strategies for Mountain Papaya without using biological materials (such as seeds or organs), including in vitro culture, cryopreservation, and molecular biology (Salgotra & Chauhan, 2023).

CONCLUSION

The phylogenetic relationships of Mountain Papaya in the Dieng plateau form three clades and two subclades. At the same time, the analysis of the genetic diversity of Mountain Papaya shows high diversity, supported by high haplotype diversity values (Hd) and nucleotide diversity values (π). The results can help breeding programs increase the productivity of Mountain Papaya varieties. Further studies using other markers need to be carried out so that the diagnostic characters required to study the diversity and relationship between Mountain Papaya accessions can be complete and more accurate.

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