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Genetic diversity of *Sterculia quadrifida* from Kupang based on ISSR profiles, stomatal density, and chlorophyll content

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Abstract. *Jannah N, Pharmawati M, Uslan. 2022. Genetic diversity of* Sterculia quadrifida *from Kupang based on ISSR profiles, stomatal density, and chlorophyll content. Biodiversitas 23: 2690-2698.* This study investigated the genetic diversity of *Sterculia quadrifida* R.Br. or *faloak* based on Inter Simple Sequence Repeats (ISSR) molecular markers and determined stomatal density and chlorophyll content from several localities in Kupang District, East Nusa Tenggara, Indonesia. For genetic diversity analysis, samples were collected from the Oelsonbai forest area of Maulafa Sub-district, Kupang City, whereas for stomatal density and chlorophyll content analysis, samples were collected from areas with various degrees of anthropogenic activity from Kupang city. DNA was isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) method and amplified by three ISSR primers (UBC 811, UBC 812, and UBC 815), whereas stomatal density was observed using a microscope and chlorophyll content was determined using a spectrophotometer. Genetic data were analyzed using the unweighted pair group method with arithmetic mean (UPGMA) method and Nei & Li coefficient similarity in the MVSP software. A total of 78 ISSR bands ranging from 320-3200 bp were obtained, where populations were divided into four main clusters and six sub-clusters, similarly having moderate heterozygosity (He=0.50) not clustered based on their geographical distribution. There was a difference in stomatal density and chlorophyll content, probably influenced by anthropogenic disturbances. A conservation action plan is required to ensure the *S. quadrifida*'s survival in the future.

Keywords: Genetic conservation, Indonesia, medicinal plant, plant anatomy, plant conservation

INTRODUCTION

Sterculia quadrifida R.Br. or locally known as *faloak* is a medicinal plant in the Sterculiaceae family that is widely distributed in Nusa Tenggara Timur (NTT) province. *S. quadrifida* is found across the NTT island including Belu district, Timor Tengah Utara District, Timor Tengah Selatan district, Kupang district, and Kupang city (Siswadi et al. 2021). *S. quadrifida* is a unique species of dryland plant that grows on degraded soil typical of lesser Sunda islands with short rainy season (3-4 months) and a dry season (8-9 months) (BPS NTT 2015).

Sterculia quadrifida has been consumed as traditional medicine by the local community for a long time. A previous study has revealed that the bark of *S. quadrifida* contains flavonoids and phenolic acids (Dillak et al. 2019), which yields a high antioxidant activity that acts as a scavenger of free radicals (Olajuyigbe and Afolayan 2011), stimulates a non-specific immune response (Winanta and Hertiani 2019), stimulates macrophage phagocytosis activity *in-vitro* and acts as a cancer chemopreventive (Hertiani et al. 2019), inhibits hepatitis C virus JFH1 (Dean et al. 2019), and promoting antiplasmodial activity (Tenda et al. 2019). Since *S. quadrifida* in Kupang city was subjected to intensive peeling harvested for their bark, this medicinal plant is highly vulnerable to excessive harvesting (Saragih and Siswadi 2019). Given that the plant is expected to become one of the future commodity products, efforts should be made immediately to establish fundamental information on *S. quadrifida*. Several steps that need to be taken to provide genetic material for crop improvement include exploration, conservation and evaluation of the character of the plants so that the knowledge is widely known. Therefore, studies on breeding conservation and anatomical characteristics associated with growth and survival are crucial for maintaining *S. quadrifida* existence.

Conserving genetic diversity is important to understand and reduce the risk of species extinction (Plenk et al. 2019), where low genetic diversity and inbreeding may negatively affect the population's fitness (Jiménez et al. 2014). Numerous studies indicate that species extinction is mainly caused by low genetic diversity, which results in low survival rates and reduced population growth (Jiménez et al. 2014; Plenk et al. 2019). Additionally, extrinsic factors (e.g., environmental stressors, drought response, atmospheric CO_2 concentration, anthropogenic disturbance) influenced the stomatal density (Dunn et al. 2019) and chlorophyll content (Spinedi et al. 2019) as an adaptive response of plants. Thus, it is important to evaluate the genetic variation and anatomical characteristics associated with the growth and survival of *S. quadrifida* (e.g., stomatal density, chlorophyll content) in an effort for breeding conservation.

In identifying plants, the molecular markers technique was known to be more accurate than the morphological approach. Molecular markers are classified into three

groups based on their detection methods: hybridization-based, PCR-based (Wahyudi et al. 2020b), and DNA sequence-based (Nikmah et al. 2016). Several DNA molecular methods are often used to understand genetic characteristics (Govindaraj et al. 2015), e.g., Restriction Fragment Length Polymorphism (RFLP) (Hapsari et al. 2015), Random Amplified Polymorphic DNA (RAPD) (Uslan and Pharmawati 2020), Variable Number Tandem Repeats (VNTR) (Hermenegildo et al. 2021), Sequence-Related Amplified Polymorphism (SRAP) (Li et al. 2015), and Inter Simple Sequence Repeats (ISSR) (Wahyudi and Rifliyah 2020). ISSR is considered more reliable than others and produces more polymorphism bands than RAPD (Das et al. 2018; Poerba et al. 2018).

Previous studies have investigated the genetic diversity of *S. quadrifida* from Kupang based on the morphological approach of the leaves (Uslan et al. 2020). Still, information on *S. quadrifida*'s genetic variety is scarce; only one study examined *S. quadrifida*'s genetic diversity using Random Amplified Polymorphic DNA (RAPD), a PCR-based method (Uslan and Pharmawati 2020). Since *S. quadrifida* is a type of dryland plant that may adapt to various environmental factors, there may also be a variation in stomatal density and chlorophyll content in response to environmental stress and pressure. Thus, this study aimed to investigate the genetic diversity of *S. quadrifida* in Kupang, East Nusa Tenggara, Indonesia, as well as the leaf anatomy, stomatal density, and chlorophyll content, all of which are critical for breeding conservation efforts.

MATERIALS AND METHODS

Study time and sample collection

Our studies were conducted from May 2017 to February 2018, by collecting several *S. quadrifida*'s leaves from various localities in Kupang, East Nusa Tenggara, Indonesia. For genetic diversity analysis, using the purposive sampling method, samples were collected from four different site encompassing the east, west, north, and east side of the Oelsonbai forest area of Maulafa Sub-district, i.e., Talaka, Sokon, Naioni, and Sikumana (Table 1). Two *S. quadrifida*'s leaves from the same trees were collected on each site, only fresh young leaves were collected for further DNA extraction. For leaf anatomy, stomatal density, and chlorophyll content analysis, using a random sampling method, samples were collected from areas with various degrees of anthropogenic activity in Kupang city, i.e., Oebobo, Kelapa Lima, Kota Raja, and Alak (Table 1). Nine leaves from two different trees were collected on each site, only fresh young leaves were collected for further examination.

Both analyses were then conducted in the Biology Education laboratory of Universitas Muhammadiyah Kupang, Bio-science laboratory of Universitas Nusa Cendana Kupang, and Animal Physiology laboratory of Department Biology, Universitas Brawijaya Malang.

Genetic diversity analysis

DNA isolation

DNA was extracted from young leaves using Doyle and Doyle's (1990) CTAB method, with Pharmawati's (2009) modifications. A total of 0.1 g of young *S. quadrifida* leaf samples were homogenized in a mortar and pestle, followed by the addition of 1 µL of extraction buffer [2% CTAB, 0.2% β-mercaptoethanol, 100 mM Tris-HCL (pH 8.0), 1.4 M NaCl, 50 mM Na2EDTA (pH 8.0)]. The sample was then incubated for 60 min at 65°C in a water bath, inverting every 10 min. Samples were added with 700 µL of chloroform: isoamyl alcohol (24:1), vortexed, and then centrifuged at 12,000 rpm for 5 min. The supernatant was transferred into a clean tube, and cold ethanol was added in the amounts given in the previous tube. The tube was gently mixed and incubated for 1 hour in a freezer at -20°C. Additionally, the tubes were centrifuged for 3 min at 12,000 rpm, the supernatant was carefully removed, and the pellets were washed with 400 µL of 70% alcohol and centrifuged for 3 min at 12,000 rpm. After removing the supernatant, the pellet was dried over the tissue till all the alcohol was evaporated. After drying, the pellet in the tube was dissolved in 100 µL of sterile H_2O. It was stored at -20 °C as a stock for further analysis.

Table 1. Sampling locations and distributions of *Sterculia quadrifida* in Kupang, East Nusa Tenggara, Indonesia, were used for analysis of genetic diversity, leaf anatomy, stomatal density, and chlorophyll content in this study

Analysis	Site	Number of samples	Coordinates	Altitude (m asl.)
Genetic diversity	Talaka (East side)	2	123º60.8522'E, 10º23.4843' S	299
			123º60.9183'E, 10º23.5934' S	313
	Sokon (South side)	2	123º60.9577'E, 10º23.6942' S	305
			123º60.9610'E, 10º23.6953' S	286
	Naioni (West side)	2	123º61.0028'E, 10º23.7445' S	298
			123º61.2972'E, 10º24.0616' S	333
	Sikumana (North side)	2	123º61.3231'E, 10º24.0939' S	309
			123º61.3380'E, 10º24.1985' S	306
Stomatal density and chlorophyll content	Oebobo	18	-	-
	Kelapa Lima	18	-	-
	Kota Raja	18	-	-
	Alak	18	-	-

Note: Talaka, Sokon, Naioni, and Sikuman were located in the Oelsonbai forest area of Maulafa Sub-district, whereas Oebobo, Kelapa Lima, Kota Raja, and Alak were Sub-district located in Kupang city

DNA quantification

The DNA concentration was measured by comparing it to lambda DNA (50 ng/µL). Electrophoresis of isolated DNA was conducted using a 1.8 % of agarose gel in 1 x TAE buffer [40 mM Tris-acetate (pH 7.9), 2 mM Na_2EDTA]. On parafilm paper, a total of 3 µL of the sample was mixed with loading dye. Each sample was placed in a gel well. Lambda DNA (50 ng/µL) as much as 2, 4, and 6 µL were put into the gel well to estimate DNA concentration. For 30 min, electrophoresis was performed at 100 volts. After 30 min of soaking in ethidium bromide (EtBr), the gel was examined in the GelDoc UV Transilluminator

ISSR (Inter Simple Sequence Repeats)

Three selected primers proven to produce amplicon based on previous research (Pharmawati et al. 2004) were utilized to assess the genetic diversity of *S. quadrifida* (Table 2). PCR reacted was performed in total volume 20 µL containing 12 µL master mix (composed of 2 µL dNTP mix containing dATP, dTTP, dGTP, dan dCTP, 2 µL Taq buffer polymerase, 1.5 µL $MgCl_2$, 0.2 µL Taq polymerase, 1 µL glycerol, 2.3 ddH_2O), 8 µL primer, and 3 µL DNA. DNA was amplified using PCR BioRad with one cycle of pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 4 s, annealing temperature at 40°C for 1.5 min, followed by 45 cycles of extension at 72°C for 2 min. The amplification was ended by 1 cycle of final extension at 72°C for 7 min.

Leaf anatomy and stomatal density determination

Leaf anatomy of *S. quadrifida* was observed and described quantitively following Uslan et al. (2020). The duplicate method was used to prepare the longitudinal cross-section for assessing leaf anatomy and stomatal density (Schuler et al. 2018), which involved cleaning the surface of fresh and young *S. quadrifida* leaves with tissue. Young *S. quadrifida* leaves were coated with nail polish and left to dry for 5 min. Masking tape was used to hold the dry nail polish. The masking tape was gradually removed, and the attached leaf's structure was placed into a glass slide and labeled. The stomata were observed using a Zeiss Primo Star digital microscope at a magnification of 10 x 40.

Chlorophyll content determination

Fresh and young *S. quadrifida* leaves weight up to 2 g were cut and macerated in the dark conditions for 48 h before being extracted with 20 mL of 85% acetone. The absorbance of the extracts was determined using spectrophotometry at 642.5 and 660 nm (Spectronic 20D) to estimate the chlorophyll-a, chlorophyll-b, and total chlorophyll content.

Table 2. List of ISSR primers used in this study

Primer	The primer nucleotide sequence (5'→ 3')	Annealing temperature (°C)
UBC 811	GAG AGA GAG AGA GAG AC	53
UBC 812	GAG AGA GAG AGA GAG AA	52
UBC 815	CTC TCT CTC TCT CTC TG	55

Data analysis

PyElph ver.1.4 software was used to evaluate the PCR data (polymorphism band). The ISSR band was assigned the value of 1 if it was present and 0 if it was not. Clustering analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm based on the Nei & Li coefficient similarity, implemented in the MVSP ver.3.2 software. The polymorphism information content (PIC) was used to determine the primer's discriminating potential (Probojati et al. 2019) using the following equation (1):

$$PIC = 1 - \sum_{j=1}^{n} Pij^2 \quad [1]$$

Where PIC denotes the polymorphism information content, and Pij indicates the presence of a primer fragment. The genetic diversity parameter was determined by Na (observed number of alleles), Ne (effective number of alleles), and He (expected number of heterozygosity), using the following equations (2-4):

$$Na = \frac{1}{\sum_i pi^2} \quad [2]$$

$$Ne = \frac{\sum Allele}{\sum Locus} \quad [3]$$

$$He = 1 - \sum_i Pi^2 \quad [4]$$

Where pi is the genetic frequency. The criterion for genetic diversity levels is based on Nurdianawati et al. (2016), who defined low genetic diversity as less than 0.25, moderate genetic diversity as 0.25-0.50, and high genetic diversity as greater than 0.50. Stomatal density (µM) was determined using the following equation (5):

$$\text{Stomatal density} = \frac{\text{Field of view area}}{\text{Total stomata}} \quad [5]$$

The chlorophyll content (mg/g) was determined using the following equation (6):

$$\text{Chlorophyll content} = (7{,}12A_{660} + 16{,}8A_{642{,}5})\ (V/W) \quad [6]$$

Where A is the absorbance, V is the volume (L), and W is the sample mass (g).

RESULTS AND DISCUSSION

ISSR profile and genetic diversity of *Sterculia quadrifida* from Oelsonbai forest area, Kupang

A total of 78 ISSR bands with a range of 320-2300 bp were obtained using three ISSR primers (UBC-811, UBC-812, and UBC-815), and they were all polymorphic (100%) (Figure 1; Table 3). UBC-815 generated the most bands (28 bands), whereas UBC-812 generated the fewest (24 bands). The polymorphism information contents (PICs) of the primer range between 0.87 and 0.88, indicating that it can detect polymorphism in a population of 87-88 % in all *S. quadrifida* leaves samples.

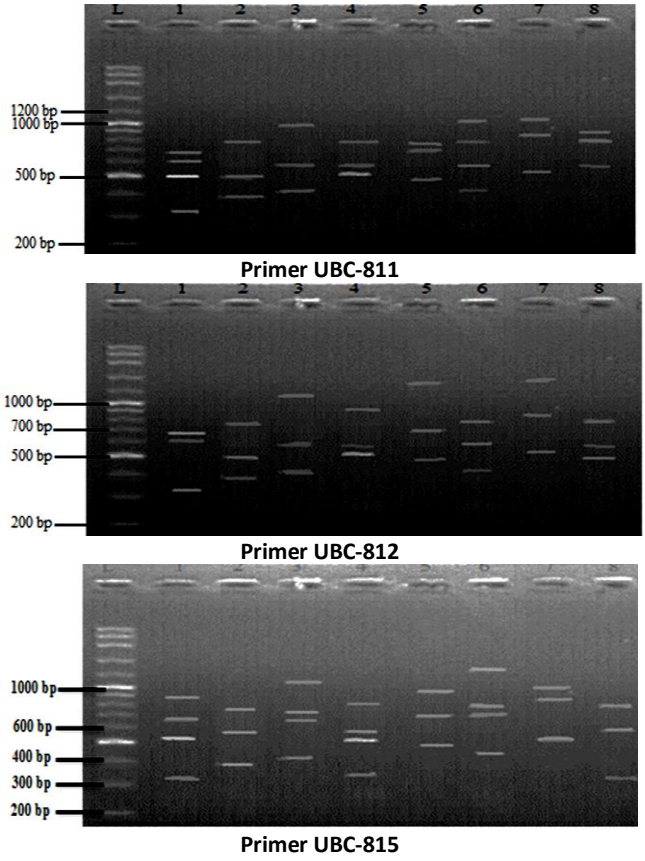

Figure 1. ISSR profile of each primer (UBC-811, UBC-812, and UBC 815) on *Sterculia quadrifida* from Oelsonbai forest area, Kupang. Note: 1-2: Talaka, 3-4: Sokon, 5-6: Naioni, 7-8: Sikumana. DNA ladder VC 100bp Plus (L)

Table 3. Number of polymorphism band and the polymorphism information content (PIC) of each ISSR primer on *Sterculia quadrifida* from Oelsonbai forest area, Kupang

Primer	Range of band (bp)	Number of bands	Number of polymorphic bands	Polymorphism percentage (%)	PIC
UBC-811	320-1200	26	26	100	0.87
UBC-812	330-1700	24	24	100	0.88
UBC-815	320-2300	28	28	100	0.87
Total		78	78	100	

Table 4. Genetic diversity of *Sterculia quadrifida* from Oelsonbai forest area, Kupang using ISSR markers

Site	No. of samples	Na	Ne	He
Talaka (East side)	2	2.00	6.67	0.50
Sokon (South side)	2	2.00	6.67	0.50
Naioni (West side)	2	2.00	6.67	0.50
Sikumana (North side)	2	2.00	6.00	0.50
Total	8	8.00	26.00	2.00
Mean average		2.00	6.50	0.50

Note: Na (number of allele), Ne (number of effective allele), and He (expected heterozygosity)

DNA bands are formed when nucleotides from primers are paired with nucleotides from the plant genome. According to Wahyudi et al. (2020a), the amount and strength of generated DNA bands greatly depend on the primer's capacity to recognize the complementary DNA sequence in the DNA template. Additionally, DNA amplification is dependent on the primer's compatibility with the *S. quadrifida* plant DNA sequence. The difference in the size of the DNA bands is related to the length of DNA sites in plants, which can be extended using primers. The greater the distance between the primer site and the remaining bases in the DNA template, the longer the new DNA will be and the higher its molecular weight will be (Wahyudi et al. 2020b). DNA polymorphisms are differences in DNA sequences among individuals, groups, or populations. Polymorphism occurs when two or more alleles at the same locus have a substantial enough frequency in the same population, where the minimum frequency is generally 1% (Sukhumsirichart 2018).

Earlier studies employing six RAPD primers on *S. quadrifida* plants from 8 different sub-districts in Kupang identified 131 RAPD bands ranging in size from 250-1400 bp, all of which were polymorphic (100%) with a higher PIC value (0.95) (Uslan and Pharmawati 2020). The primer's high PIC value is suitable for use as a molecular marker (Graebner et al. 2016). One could suggest that ISSR are capable of analyzing *S. quadrifida*'s genetic diversity. Additionally, ISSR may be an effective molecular marker due to its low cost (Poerba et al. 2018) and rapid analysis time (Kiran et al. 2015).

These are all the parameters used to assess genetic diversity in a population: observed number of alleles (Na), the effective number of the alleles (Ne) and expected heterozygosity (He) (Yulita and Rahmat 2019). The average observed number of an allele (Na) in *S. quadrifida* populations from the Oelsonbai forest area, Kupang was 2.00, the average effective number of the alleles (Ne) was 6.50, and the average value of expected heterozygosity (He) was 0.50 (Table 4). This indicated that the genetic diversity of *S. quadrifida* from the Oelsonbai forest area in Kupang was moderate.

Prior studies using 6 RAPD primers on *S. quadrifida* from 8 different sub-districts in Kupang revealed low genetic diversity (<0.25) (Uslan and Pharmawati 2020). *S. quadrifida*'s high genetic diversity could be a result of natural mutations. The low value of genetic diversity may be caused by population isolation and habitat fragmentation, which may result in low genetic variation; on the other hand, the high value of genetic diversity may be caused by interbreeding between populations, aided by pollinators and herbivores as seed dispersers, or wind as a means of gene transfer via pollen displacement (Siswadi et al. 2021). Additionally, widely spread species frequently exhibit a higher degree of genetic diversity (Hahn et al. 2017).

Genetic distance and clustering of *Sterculia quadrifida* from Oelsonbai forest area, Kupang

According to the similarity matrix, Naioni 2 and Sokon 1 have the greatest genetic distance (Table 5), although these two populations are geographically separated (Table

1). Additionally, Sikumana 1 and Sokon 1 have the smallest genetic distance (Table 5). The low similarity of populations demonstrates the genetic diversity generated by isolation, which drives the development of a certain trait. Furthermore, a diversified habitat or geographical condition will lead to additional adaptation patterns and genetic features (Poerba et al. 2018).

Sterculia quadrifida from the Oelsonbai forest region in Kupang was divided into four major clusters and six sub-clusters based on Nei and Li's coefficient similarity of >0.50. Sikumana 1 is included in the first cluster; Naioni 1 is included in the second cluster; Sokon 2, Sikumana 2, and Talak 2 are included in the third cluster; and Naioni 2, Sokon 1, and Talak 2 are included in the fourth cluster. These clustering analyses revealed that *S. quadrifida* samples from the Oelsonbai forest area in Kupang were not clustered geographically, and both adjacent and isolated *S. quadrifida* populations in Kupang's Oelsonbai forest area tend to cluster together. This suggests that clustering based on genetic distance does not always correspond to geographical distribution, which could be due to environmental factors, differing plant ages (which results in increased genetic diversity) or plant crossbreeding (Carvalho et al. 2019).

Leaf anatomy and stomatal density of *Sterculia quadrifida* from Kupang city

Stomata were found on both the upper and lower sides of *S. quadrifida* leaves; however, the lower side had the most stomata. Thereby, this study examined only the lower side of the leaf for stomatal density observation in *S. quadrifida* from Kupang city. The leaf anatomy of *S. quadrifida* from Kupang city mainly comprises trichome, epidermis, stomata, and guard cells (Figure 3). Based on the observations, the stomata on the leaves of *S. quadrifida* plants have a spherical form. The stomatal type of *S. quadrifida* in Kupang city was categorized as xerophyte with anomocytic type, which implies that the stomata are protected by several uniform cells of the same size and shape. Because xerophyte plants thrive in low humidity and dry conditions, they require low transpiration rates (Xi et al. 2018). They also exhibit characteristics such as small cell size, thick cell wall, palisade tissue that is thicker than sponge tissue and is protected by trichomes, as observed in this current study.

Table 5. Similarity matrix based on UPGMA method clustering using MVSP software of 8 samples of *Sterculia quadrifida* from Oelsonbai forest area, Kupang based on Nei & Li's coefficient similarity

	1	2	3	4	5	6	7	8
Talaka 1 (East side)	1.000							
Talaka 2 (East side)	0.100	1.000						
Sokon 1 (South side)	0.286	0.005	1.000					
Sokon 2 (South side)	0.381	0.316	0.200	1.000				
Naioni 1 (West side)	0.100	0.222	0.105	0.105	1.000			
Naioni 2 (West side)	0.182	0.300	0.571	0.381	0.200	1.000		
Sikumana 1 (North side)	0.200	0.006	0.004	0.316	0.111	0.005	1.000	
Sikumana 2 (North side)	0.200	0.556	0.105	0.526	0.222	0.400	0.007	1.000

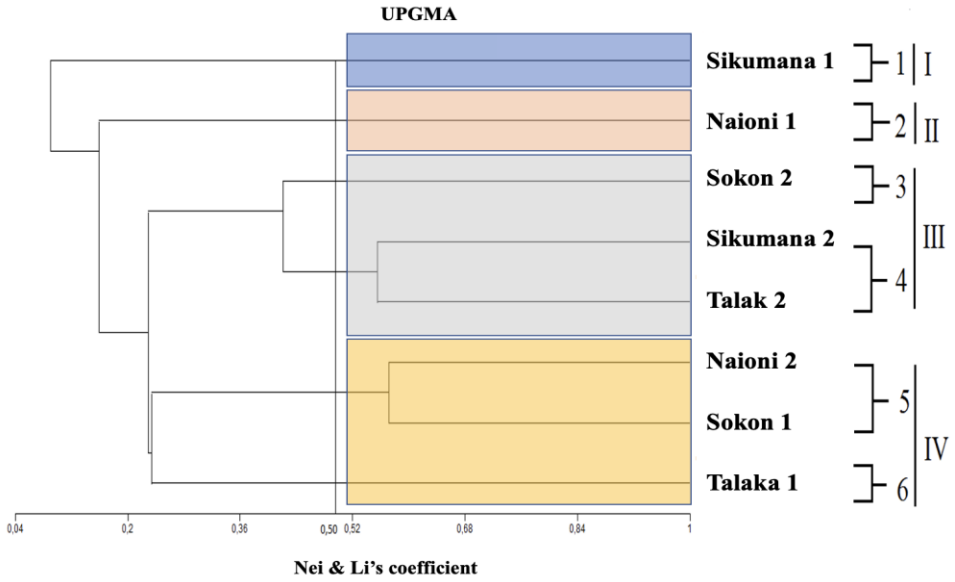

Figure 2. Dendrogram of 8 samples of *Sterculia quadrifida* from Oelsonbai forest area, Kupang based on Nei & Li's coefficient similarity with UPGMA method in MVSP software. Note: Arabic number denoted sub-cluster, Roman number denoted cluster

Our results were in line with Uslan et al. (2020), investigating the genetic relationship of *S. quadrifida* from Kupang District (with different localities, i.e., Kampung Barat, Taebenu) based on morphological and anatomical parameters. The type of stomata showed the genetic and evolutionary history of plants (Augstein and Carlsbecker 2018). Ultimately, the information on a cell's structure is important in plant systematics (Silva et al. 2014). Plant species in the same family often have the same forms of stomata, while there are notable exceptions in plants that have differences in the type of stomata in one type.

Trichomes on *S. quadrifida* plant leaves were non-glandular trichomes with a stellate shape exhibiting multiple branches (4-8 branches) that did not secrete any substances. It resembled the tomato trichome structure, having needle-like structures. According to Watts and Kariyat (2021), tomato, *Lycopersicon pimpilellifolium*, has a trichome morphology comparable to non-glandular trichomes observed in *Solanum tuberosum* with pointy ends. Trichome also serves a unique purpose because it comes in various forms. Each trichome has a distinct purpose; for example, non-glandular trichomes act as a pathogen barrier through the stomata, whereas glandular trichomes release secondary compounds. The form, size, and density of the shape and type of trichome also impact the trichome's function in protecting a plant's leaf organs (Barthlott et al. 2009).

Based on our observation, the *S. quadrifida* samples from Kota Raja showed the highest stomatal density (5.8 stomata/µM) while the population from Kelapa Lima showed the lowest stomatal density (4.4 stomata/µM) (Figure 4). Stomatal density developed due to the plant's adaptation to its environment (Bertolino et al. 2019). Numerous factors influence stomatal density, including light intensity (which may result in temperature changes), the availability of water resources, CO_2 concentration, and pollution.

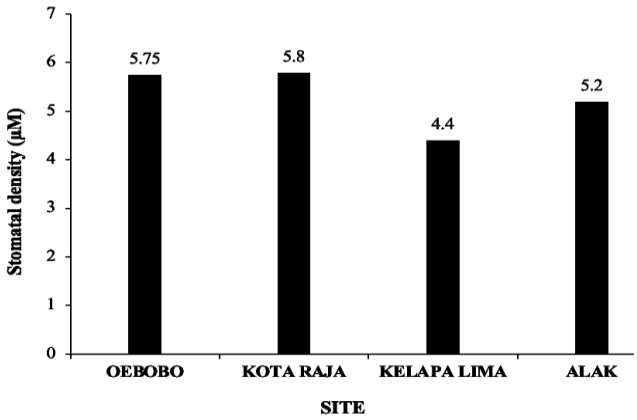

Figure 4. Stomatal density of 72 samples of *Sterculia quadrifida* from Kupang city

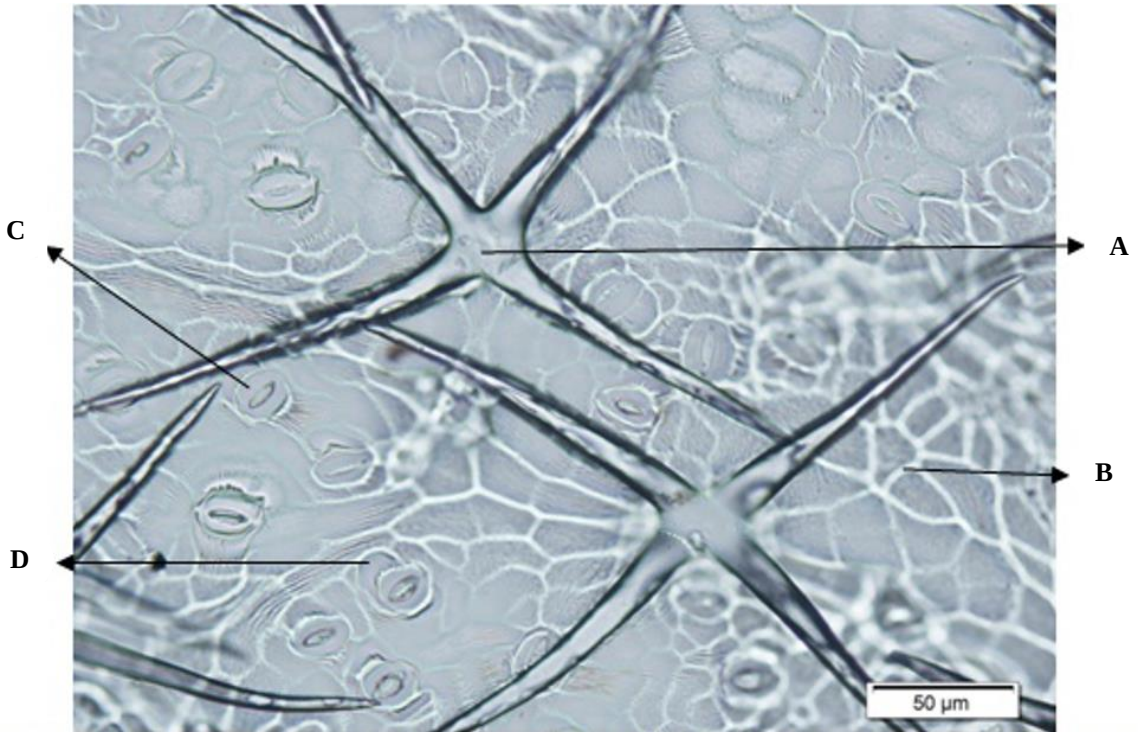

Figure 3. Leaf anatomy of representative samples of *Sterculia quadrifida* from Kupang city. A. Trichome. B. Epidermis. C. Stomata. D. Guard cell

Our results indicated that pollution might affect the stomatal density of each *S. quadrifida* population in Kupang city. In our observations, environmental contamination (e.g., vehicle exhaust fumes) was more severe in Kelapa Lima than in other sites, resulting in a low stomatal density; yet, other sites, such as Kota Raja, were more pristine than Kelapa Lima, resulting in a higher stomatal density (Figure 4). This was consistent with previous studies on the stomatal density of *S. quadrifida* from Kupang by Uslan and Pharmawati (2020), which found that the locality of Kupang Barat 3 (a population thought to be heavily polluted by vehicle exhaust fumes) had a lower stomatal density value than the locality of Kupang Barat 1 (population thought to be more pristine). In addition, Gostin (2009) stated that the level of air pollution affected the structure and functionality of the stomata. Stomatal density may strongly affect the rate of basic physiological processes such as photosynthesis transpiration besides determining the rate and type of respiration (Bertolino et al. 2019). This may also indicate that environmental stressors have threatened the *S. quadrifida* from Kupang city.

Chlorophyll content of *Sterculia quadrifida* from Kupang city

Based on our analysis, Alak has the highest total chlorophyll content (1.12 mg/g), followed by Kelapa Lima (1.01 mg/g), Oebobo (0.99 mg/g), and Kota Raja (0.95 mg/g). Oebobo had the highest chlorophyll-a content (4 mg/g), followed by Kota Raja, Kelapa Lima, and Alak (3.9 mg/g); also, Oebobo and Alak had the highest chlorophyll-b content (4.1 mg/g), followed by Kota Raja and Kelapa Lima (3.9 mg/g) (Figure 5). Tanaka and Tanaka (2006) stated that chlorophyll-a is the primary pigment found in all species of plants that undertake photosynthesis. Because it is not required for photosynthesis to occur, chlorophyll-b is categorized as an accessory pigment where not all organisms synthesize chlorophyll-b. Chlorophyll-a primary function in the electron transport chain is as a primary electron donor. The sun's energy is converted to chemical energy that the organism may employ for cellular processes. The primary function of chlorophyll-b is to expand the absorption spectrum of organisms. One of the primary differences between chlorophyll-a and chlorophyll-b is the wavelength of light they absorb; chlorophyll-b absorbs blue light. Thus, organisms may absorb greater energy from the blue light spectrum's higher frequencies.

According to our observations, *S. quadrifida* from Kupang city had higher chlorophyll-b than chlorophyll-a. One explanation is that the *S. quadrifida* plants receive less sunshine and more chlorophyll-b in cell chloroplasts is adaptive (Tanaka and Tanaka 2006). Plants that receive less sunlight have more chlorophyll-b in their chloroplasts; hence, an increase in chlorophyll-b is an adaptation to the shadow since it helps the plant absorb a wider spectrum of light wavelengths (Lakitan 1993; Samanhudi et al. 2017).

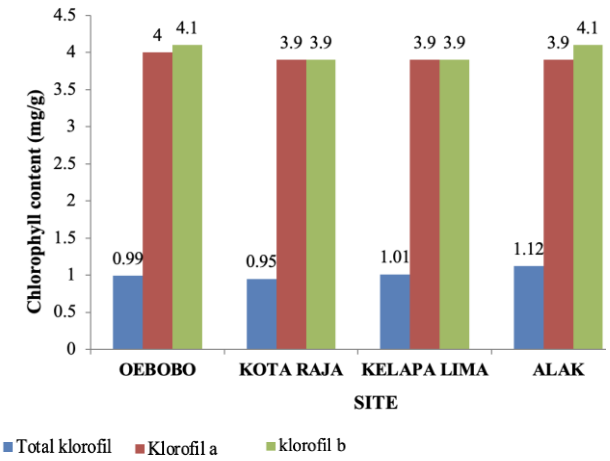

Figure 5. Mean average of total chlorophyll, chlorophyll-a, and chlorophyll-b contents of 72 samples of *Sterculia quadrifida* from Kupang city

The differences in total chlorophyll contents could also be attributed to differences in soil habitat, with some *S. quadrifida* inhabiting shallow solums above the rock and others on a sandy substrate. This is characteristic of the climatic and geographical conditions of Kupang city, which include a semi-arctic soil structure with rocky sand, allowing *S. quadrifida* plants to thrive naturally (BPS NTT 2015). Additionally, the relatively high total chlorophyll content in Alak was likely due to the area's proximity to the beach, which had the highest light intensity and less disruption from pollution fumes than other sites. This was consistent with Schuler et al. (2018), who stated that light intensity is critical for plant growth.

This study agreed with Uslan and Pharmawati (2020), who stated that genetic studies might help breeders comprehend and define *S. quadrifida* identity in the future. The assessment of genetic diversity will be valuable for mapping the genetic identity necessary for breeding programs as well as knowing the anatomical characteristics associated with development and survival, which are critical for *S. quadrifida* conservation.

To conclude, it can be concluded that ISSR primers (UBC 811, UBC 812, and UBC 815) were discovered to be a valid marker for determining genetic diversity in *S. quadrifida* from Kupang, East Nusa Tenggara, Indonesia, where populations were divided into several clusters, similarly having moderate heterozygosity, but not clustered based on their geographical distribution. Based on stomatal density and chlorophyll content, *S. quadrifida* is likely threatened by anthropogenic disturbances. A conservation action plan is required to ensure the *S. quadrifida*'s survival in the future. Utilizing other DNA molecular markers and genomic analyses will be important in the *S. quadrifida* breeding effort in the future.

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