



MORPHOLOGICAL AND MOLECULAR PHYLOGENY OF CLOVE (*SYZYGIUM AROMATICUM* L.) FROM HAYA VILLAGE, SERAM ISLAND, INDONESIA

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SUMMARY

Clove is an economically valuable spice plant with numerous health and food-related benefits. The abundance of clove with higher diversity was prevalent in the Haya Village, Seram Island, renowned as the largest clove-producing center in Maluku, Indonesia. The presented research sought to quantitatively and qualitatively analyze the morphological diversity and genetic makeup of five clove accessions using seven RAPD primers. The results of coefficient variation showed that the leaf area holds the highest value (62.7%) compared with the petiole length (18.67%). Hierarchical cluster analysis based on 11 morphological traits, including crown shape, trunk type, bark type, branching direction, leaf shape, leaf apex shape, leaf base shape, leaf margin, leaf character, leaf venation, and leaves aroma exhibited a distant relationship between the clove accessions obtained from tuni and hutan. These accessions showed a high dissimilarity between the red and the white Zanzibar. The RAPD profile showed 92 bands ranging from 800 to 2700 bp, with a polymorphism of 66.3%. Based on genetic distance analysis, red Zanzibar and hutan have the highest value (0.58%), while red Zanzibar and white Zanzibar have the lowest (0.16%). These results were consistent with phylogenetic tree reconstruction and PCoA analysis that groups hutan at a long distance from red Zanzibar and tuni. According to morphological and molecular analysis, clove accessions tuni, red Zanzibar, and hutan were most suitable as promising parental genotypes for further improvement through plant breeding and conservation programs.

Keywords: Clove, genetic makeup, morphological trait, polymorphism, genetic distance, RAPD markers

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Key findings: Clove (*S. aromaticum* L.) showed variations in vegetative characters and a moderate polymorphism based on seven RAPD primers. The markers classified tuni and red Zanzibar clove accessions into the same group, while the hutan clove accession remained distinct. The results suggested that these three accessions have the potential to serve as parental genotypes in crossbreeding for further improvement in clove plant quality.

INTRODUCTION

Clove (*Syzygium aromaticum* [L.] Merrill & L.M. Perry) is an ancient spice plant native to Indonesia, particularly in the Maluku Islands (Batiha *et al.*, 2020). Belonging to the family Myrtaceae and genus *Syzygium*, it has been a recognized high-value spice. The clove flower extract is rich in secondary metabolites, such as flavonoids, hydroxybenzoic acid, hydroxycinnamic acid, hydroxyphenyl propene, eugenol, eugenol acetate, and gallic acid (Tomas *et al.*, 2022).

Extensive exotic spices cultivation is a practice by the local community in Maluku province and has become one of Indonesia's largest plantation commodities. Maluku Province is a prime producer of clove in Indonesia, with an area of 44,917 hectares and a production of 21,512 tons in 2022 (Ditjenbun, 2022). Seram, the largest island in Maluku Province, Indonesia, showed the highest clove productivity across various villages. The Haya Village hosts a diverse population of five distinct clove accessions. However, the authenticated scientific data based on the morphological and genetic aspects of the clove in Haya Village have unreleased reports. According to Mahulette *et al.* (2022), the Maluku clove population showed the highest morphological variations. It comprised nine accessions, i.e., tuni, hutan, raja, Bogor, Zanzibar, boiselang, jinten, tae, and damar. They have a wide distribution in several regions of Maluku. Haya Village only has five accessions—tuni, red Zanzibar, white Zanzibar, raja, and hutan. Hutan clove accessions are wild type, with the other accessions as cultivated.

Based on morphological and molecular parameters, genetic diversity plays a crucial role in selecting parental genotypes for

breeding programs. Morphological variables observed by their physical appearance in the crop plants are time-consuming field evaluations (Fatimah *et al.*, 2018). Additionally, morphological traits seemed to have many weaknesses because these quantitative traits gain influences from environmental factors, necessitating molecular analysis to complement them. According to Perdani *et al.* (2018), combining the morphological observations and the molecular markers offers significant opportunities for evaluating the different plant characteristics.

Molecular markers are the DNA sequences that mark differences at the genomic level, which can be associated with phenotypic characters (Agrawal and Srivastava, 2013). As an alternative, molecular markers have better potential in plant identification since numerous advantages are available. These include unlimited numbers, independence from environmental factors and growth conditions, ease of interpretation, and repeatable results (Datta *et al.*, 2011).

RAPD is one of the molecular markers widely used for plant identification to detect locus polymorphism rapidly. From Kumar *et al.* (2009), the PCR-based marker uses short oligonucleotide primers of 10 bases. The benefits of this method include the minimal DNA requirement, generation of a substantial number of fragments, independence from the need for plant genome information, and the capacity to analyze interspecific and intraspecific genetic diversity (Sasongko *et al.*, 2021). Based on the above discussion, the presented research will examine the morphological and genetic diversity of clove plants in Seram Island, Maluku Province, Indonesia, using RAPD markers as a reference for selecting parental genotypes for developing superior varieties.

MATERIALS AND METHODS

Plant materials

Five clove accessions selected for vegetative and genetic analysis came from the Haya Village, Seram Island, Maluku Province, Indonesia, belonging to phenotypically distinct tuni (C1), red Zanzibar (C2), white Zanzibar (C3), raja (C4), and hutan (C5) populations.

Morphological characterization

Five individuals at reproductive age (>20 years) were specimens from each of the five accessions, with their following vegetative characteristics measured: crown shape (CS), trunk type (TT), bark type (BT), branching direction (BD), leaf shape (LS), leaf apex shape (LAS), leaf base shape (LBS), leaf margin (LM), leaf venation (LV), leaf character (LC), leaf length (LL), leaf width (LW), and leaf area (LAR) (Mahulette *et al.* 2022). Five leaves were measured for leaf morphology, while five trees scored for tree characteristics. The leaf aroma determination from five leaves used the sense of smell. Only 11 qualitative characters served to perform hierarchical cluster analysis (HCA).

Molecular genetic analysis

The molecular analysis commenced at the Biochemistry and Molecular Biology Laboratory, Indonesian Research Institute for Biotechnology and Bioindustry, Bogor, Indonesia. DNA isolation from young leaves used the CTAB protocol (Orozco-Castillo *et al.*, 1994), starting with 200 mg of tissue ground to a fine powder with mortar and pestle under liquid nitrogen. DNA quantification employed a Nanodrop Spectrophotometer (Thermo Fischer Scientific). DNA fragment amplification with seven RAPD primers (Table 1) in 25 µl utilized a standard RAPD protocol (Williams *et al.*, 1990) with 12.5 µL MyTaq 2x PCR Mix [0.5%] (Bioline), 2 µL DNA sample, 1 µL primer [10 pmol], and 9.5 µL ddH₂O. DNA amplification ensued on a Veriti Thermal Cycler (Thermo Fisher Scientific) for 35 cycles with an initial

denaturation at 94 °C for 1 min, denaturation at 94 °C for 1 min, annealing at 50 °C–52 °C for 1 min, and an extension at 72 °C for 1 min. Amplification products sustained electrophoresis on a 2% agarose gel (Invitrogen).

Data analysis

The morphological data analysis used the R version 4.3.2 package Hierarchical Cluster Analysis (HCA) (Ihaka and Gentleman, 1996). The coefficient of variation (CV) calculation relied on a formula of Sokal and Braumann (1980), and the single factor analysis of variance performed pairwise comparison among the sample accessions' mean with t-test ($\alpha = 5\%$) (Mattjik and Sumertajaya, 2000). RAPD banding pattern scoring was 1 for the presence and 0 for the absence of a band of a particular size. Genetic distances and Principal Coordinates Analysis (PCoA) computations applied the GenAlEx 6.5 (Peakall and Smouse, 2012). Phylogenetic tree was constructed depending on the distance matrices obtained from the vegetative and genetic data using MEGA 11 (Tamura *et al.*, 2021) with the Neighbor-Joining method (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Morphological characters

According to quantitative observations of leaf morphology in the five accessions (Table 2), the hutan clove had larger leaves than all other accessions (Figure 1), which was consistent with the findings of Mahulette *et al.* (2019). Kamsurya *et al.* (2023) also stated that hutan clove leaves could be two to seven times larger than other accessions. Hutan cloves have a broader leaf size, which may be due to environmental and genetic factors; hence, hutan cloves may be genetically different from the other four accessions. Raja cloves have a smaller leaf area and length than the other accessions but have longer petioles. Red and white Zanzibar tended to have similar leaf

Table 1. The primers used in this study.

Primer	Base Sequence (5'-3')	Annealing (°C)
OPA-02	TGCCGAGCTG	51
OPA-04	AATCGGGCTG	50
OPA-11	CCATCGCCGT	51
OPA-12	TCGGCGATAG	50
OPA-13	CAGCACCCAC	51,5
OPA-17	GAAACGGGTG	50
OPA-19	CCAACGTCGG	52

Table 2. Summary of the observations based on clove characters.

Characters	C1 (Tuni)			C2 (Red Zanzibar)			C3 (White Zanzibar)			C4 (Raja)			C5 (Hutan)		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Quantitative															
Leaf Length (cm)	11	11.7	11.5±0.29 ^{bc}	10.7	11.5	11.1±0.33 ^{bc}	10.5	11.4	11±0.32 ^c	11.3	12.3	11.8±0.38 ^b	15.7	18.3	17±0.99 ^a
Leaf Width (cm)	3.5	4.2	3.7±0.29 ^c	3.9	4.5	4.3±0.244 ^b	4.1	4.3	4.2±0.10 ^b	2.7	4.2	3.4±0.60 ^c	6.7	7.4	7.1±0.26 ^a
Leaf Area (cm ²)	55.8	56.3	56.1±0.21 ^c	61.7	63	62.4±0.51 ^b	53.5	61	57.1±3.12 ^c	46.2	47.6	46.9±0.62 ^d	162.3	163.5	163±0.48 ^a
Petiole Length (cm)	1.6	2.3	1.8±0.29 ^a	2	3.1	2.4±0.43 ^a	1.6	2.8	2±0.53 ^a	2.4	2.6	2.5±0.10 ^a	1.4	2.1	1.6±0.29 ^a
Qualitative															
Crown shape	Slightly Rounded			Cylindrical			Cylindrical			Slightly Rounded			Conical		
Trunk type	Unsplit			Unsplit			Unsplit			Unsplit			Unsplit		
Bark type	Peeled			Peeled			Peeled			Peeled			Unpeeled		
Branching direction	Straight			Straight			Straight			Straight			Straight		
Leaf shape	Lanceolate			Lanceolate			Lanceolate			Lanceolate			Oblanceolate		
Leaf apex shape	Attenuate			Obtuse			Obtuse			Attenuate			Obtuse		
Leaf base shape	Attenuate			Attenuate			Attenuate			Attenuate			Attenuate		
Leaf margin	Wavy			Wavy			Wavy			Wavy			Flat		
Leaf character	Thin			Thick			Thick			Thick			Thin		
Leaf venation	Protrude			Unprotruded			Unprotruded			Protrude			Protrude		
Leaf aroma	Scented			Scented			Scented			Scented			Unscented		

Notes: Different letters for the mean values within the same parameters in the same row indicate a significant difference, while the same letters indicate a nonsignificant difference.



Figure 1. Leaf morphology of five clove accessions.

sizes and showed no significant difference in size. It may indicate that the two accessions have genetic similarities.

All five clove accessions showed greater diversity based on the four quantitative attributes, with varying coefficients of variation (CV). The CV metric represents variation, obtained by dividing the standard deviation by the mean (Prayoga *et al.*, 2022). The CV expression in percentage has a comparative value for inter-population character variability used in systematic research to measure differences in varying characteristics within a population. It is applicable for analyzing character similarities among different or within the same population with trait variations. CV has widely helped differentiate the morphological character variations in crop plants (Kozak *et al.*, 2013).

Gomes (2009) also classified CV values into several categories, i.e., low (<10%), moderate (10%–20%), high (>20%–30%), and very high (>30%). Based on the classification, the pertinent results revealed moderate variations in leaf length (CV: 20.4%) and very high variations in leaf width (CV: 32.54%) and leaf area (CV: 62.7%), but low variation in petiole length (CV: 18.67%). Therefore, leaf area and petiole showed the highest and lowest CV, respectively. In plant breeding programs, the classification of CV values is highly beneficial in providing

information about the quality of intermediate and final tests on crop plants (Carvalho *et al.*, 2003). The evaluation has also transpired on various plant species as a precise measure (Couto *et al.*, 2013) and served to measure the variations within a genetic population, determine phenotypic stability, and gauge individual variability (Bowman, 2001).

In five clove accessions, the 11 qualitative characters showed considerable variations (Table 2). The accessions especially indicated the variations in crown shape, including slightly rounded, cylindrical, and conical. Observations on leaf shape variations in lanceolate and oblanceolate shapes across the five accessions were also notable. The tuni clove has a slightly rounded crown and a lanceolate leaf shape with attenuate leaf apices and bases. Its leaf was thin with a wavy margin, prominent veins, and a fragrant aroma. Tuni has a pleasant aroma due to its high essential oil content (78%), contributing to its market value and widespread cultivation (Hariyadi *et al.*, 2020ab).

Red and white Zanzibar also shared characteristics, such as cylindrical crown shape, lanceolate leaf shape, obtuse leaf apices, attenuate leaf bases, and thick leaves. Additionally, both were aromatic due to their fragrant leaves, similar to the accession tuni. A known characteristic of Zanzibar was the abundant flower production compared with

other accessions (Alfikri *et al.*, 2020). Raja clove classification was a non-aromatic wild-type clove because the leaf has a lower aromatic level than the tuni and Zanzibar accessions (Mahulette *et al.*, 2022). Furthermore, qualitative observations found that raja shares similar morphological characteristics as tuni, such as lanceolate leaf shape, attenuated leaf apices, bases, and a slightly rounded crown. Alfian *et al.* (2019) stated that the raja clove shared many similar characteristics with tuni, specifically in leaf shape, tree structure, and aromatic properties resembling the hutan clove. It leads to the conclusion that raja comes from cross-pollination between the tuni and hutan clove accessions (Wattimena *et al.*, 2023).

Although categorized as a non-aromatic clove with no scent and low eugenol content in the previous study (Mahulette *et al.*, 2022), raja possesses a pleasant aroma in this study. Therefore, clove has the highest eugenol content (77.24%), as observed in past studies (Wael *et al.*, 2018). It occurs because environmental factors influence the secondary metabolites in plants, such as eugenol (Ncube *et al.*, 2012; Rakhmawati *et al.*, 2022). Hutan clove accessions showed numerous morphological trait differences from other accessions. These differences were visible in the crown shape (conical), bark texture (unpeeled), leaf shape (oblanceolate), and leaf margin (flat). Hariyadi *et al.* (2019) also reported that the hutan clove is an endemic

wild clove in the Maluku region with a very weak aroma (unscented) due to the significantly low eugenol content, categorized as a non-aromatic wild-type clove. The eugenol content was about 22.82% (Mahulette *et al.*, 2021), and hutan clove showed rapid growth, larger flower sizes, and resistance to various pests (Mahulette *et al.*, 2019).

Hierarchical cluster analysis (HCA) based on 11 distinct morphological characters in five clove accessions formed two main clades (Figure 2). The first clade consisted of four clove accessions, tuni, raja, red, and white Zanzibar, while the second clade contained only the hutan accession. The first clade further split into two sub-clades, with the first occupied by clove accessions raja and tuni and the second by the red and white Zanzibar. Based on the results, the clove accessions hutan and tuni showed significant dissimilarity, while the red and white Zanzibar accessions indicated substantial similarity. Mahulette *et al.* (2022) reported that clove accessions red and white Zanzibar received the same clove type classification, with a similarity value of 93%.

Additionally, the differences only came from the color of leaf apices, where red and white Zanzibar are slightly reddish and orange. The observed clove accessions appear to form clusters based on the similarity level in morphological traits. The plant accessions with similar characteristics seemed to cluster (Rosmaina *et al.*, 2021).

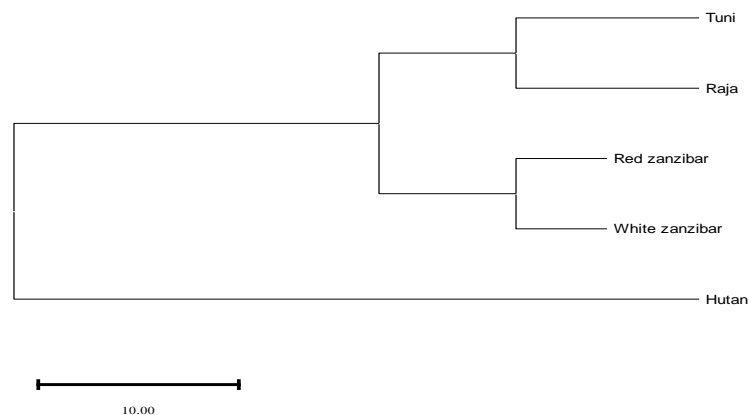


Figure 2. Dendrogram of clove accessions by 11 morphological characters.

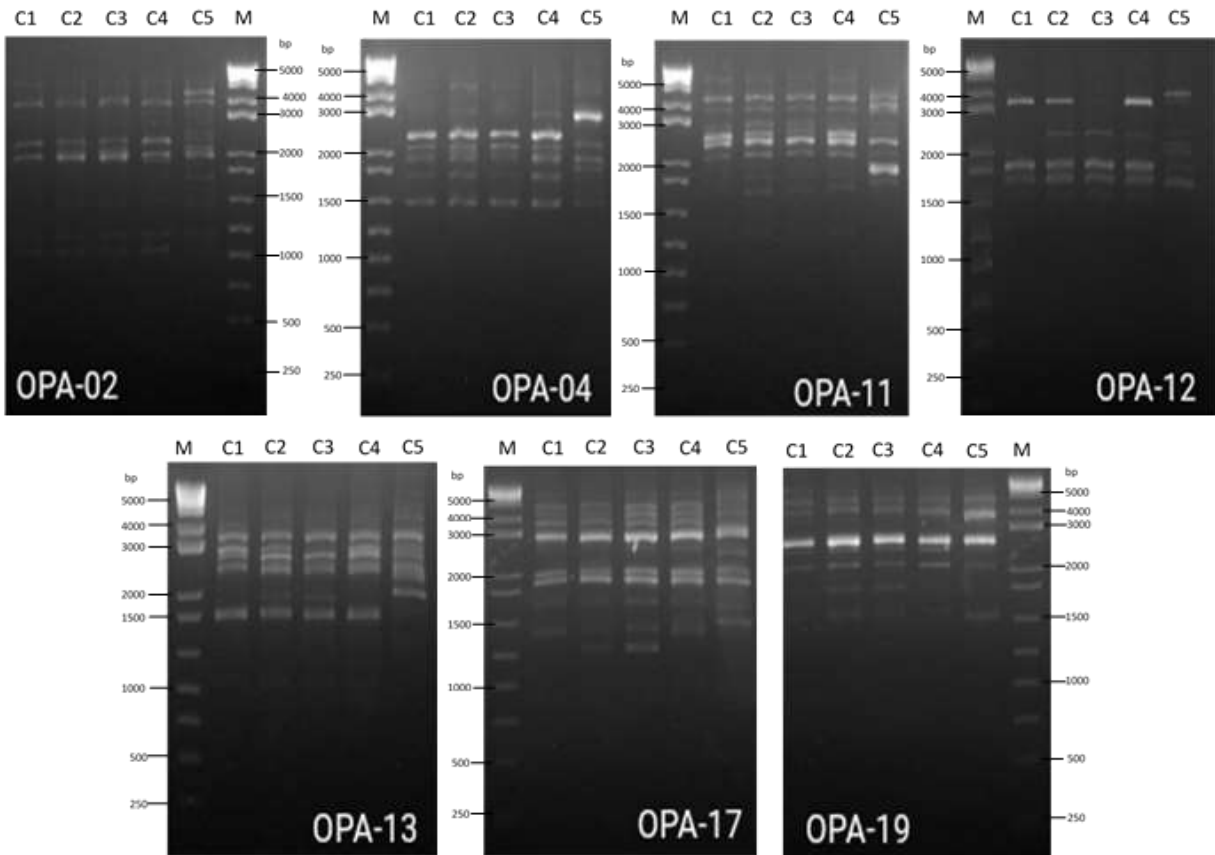


Figure 3. Amplification product of seven primers (OPA-02, OPA-04, OPA-11, OPA-12, OPA-13, OPA-17, OPA-19). M= marker 250bp; C1 = tuni; C2 = red Zanzibar; C3 = white Zanzibar; C4 = raja; and C5 = hutan.

Molecular genetic analysis

DNA amplification using seven RAPD primers obtained 92 bands with a size of 800 to 2700 bp (Figure 3). Total band of 61 were polymorphic, while 31 were monomorphic bands. The DNA bands with polymorphism revealed genetic diversity among the studied clove accessions. In the latest research, the primers with the highest and lowest polymorphism were OPA-02 (90%) and OPA-19 (40%), with 18 and 4 polymorphic bands, respectively. Polymorphism in DNA bands occurs due to differences in base sequences in each amplified DNA. The variation in size between primer binding sites and the target DNA sequence leads to polymorphism (Amiteye, 2021). DNA bands appear because the primer nucleotides bind to the plant

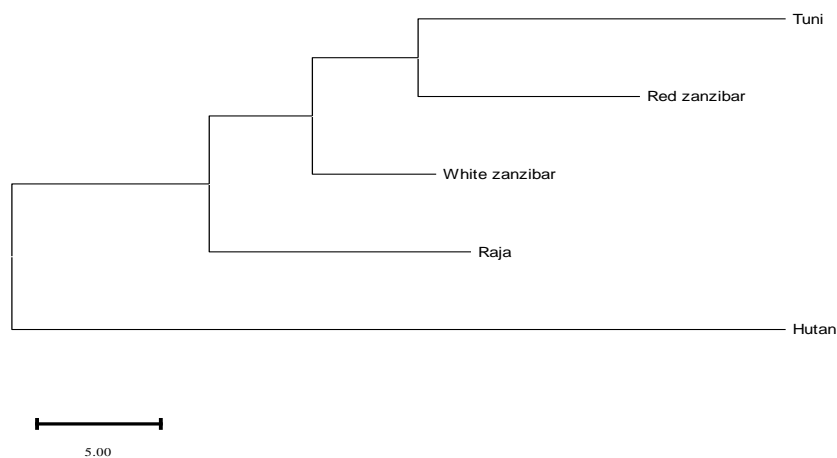
genome. The development of these bands varies in number and intensity due to the primer ability to recognize DNA sequences (Uslan and Pharmawati, 2020).

The analysis revealed that the clove accessions, red Zanzibar, and hutan have the highest genetic distance (0.58) (Table 3). However, the lowest genetic distance (0.16) was evident in red and white Zanzibar accessions. Nei (1972) classified genetic distance values into three levels: low (0.010–0.099), moderate (0.1–0.99), and high (1.00–2.00). This study showed that the five clove accessions have a moderate level of genetic diversity. Information on genetic distance is crucial to understand species relationships; this can reconstruct a phylogenetic tree (Takezaki and Nei, 1996). A high and low genetic distance between the two accessions

Table 3. Genetic distance among the five clove accessions using seven RAPD markers.

Accessions	C1	C2	C3	C4	C5
C1	0.00				
C2	0.24	0.00			
C3	0.27	0.16	0.00		
C4	0.38	0.30	0.17	0.00	
C5	0.57	0.58	0.51	0.50	0.00

Notes: C1 (tuni), C2 (red zanzibar), C3 (white zanzibar), C4 (raja), and C5 (hutan).

**Figure 4.** Dendrogram of the five clove accessions based on seven RAPD markers.

showed a distant or close relationship. Nugroho *et al.* (2017) stated that a low genetic distance was unsuitable for use as a parent in breeding, leading to inbreeding. According to Wicaksono *et al.* (2017), selecting parental genotypes with a high genetic distance greatly influences success in breeding due to the better chance of enhanced heterosis in the resulting progeny.

The phylogenetic tree reconstruction relied on the genetic distance using the Neighbor-Joining method, which divided the five clove accessions into two clades (Figure 4). The first clade occupants were tuni, red Zanzibar, white Zanzibar, and raja, while the second clade only had hutan. The first clade had two sub-clades formed, and the tuni, red, and white Zanzibar accessions were in the first sub-clade, while raja was in the second. The phylogenetic tree results showed that the hutan with red Zanzibar and tuni has a very distant relationship, while the red and white Zanzibar has a very close relationship. Additionally, the phylogenetic tree

reconstruction showed that accessions tuni and red Zanzibar were in the same clade despite having different genetic distance values. The reason is the values between the clove accession hutan and both other accessions were maximal. Based on morphological and molecular phylogenetic analysis, tuni and forest had a distant relationship, while red and white Zanzibar are close.

Information on the phylogenetic relationship among plant accessions has always been crucial for breeders and conservation practitioners (Mursyidin and Ahyar, 2022). According to Gouta *et al.* (2008), determining the similarity level among the accessions can assist breeders in selecting the best parents to produce new cross combinations. In the presented research, clove accessions used as parental genotypes in the crosses were tuni and red Zanzibar, with hutan having the furthest relationship. These relationship distinctions resulted from phylogenetic analysis, which refers to the

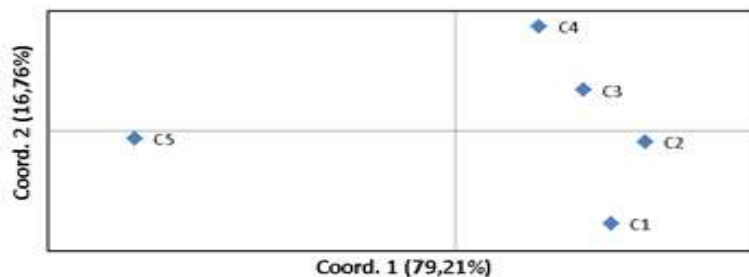


Figure 5. PCoA analysis of five clove accessions in a two-dimensional plot based on seven RAPD markers.

similarity of characters represented by the branching system (Rangkuti *et al.*, 2022). The clade length reflects the relationship and similarity levels among the accessions based on genetic distance (Tamura *et al.*, 2021).

According to the PCoA analysis, as outlined by Lu *et al.* (2021), the genetic distance information appears on a two-dimensional plot. This graphical representation distinctly categorizes the five clove accessions into three quadrants (Figure 5). Clove accession hutan forms a separate group in the third quadrant; the accessions tuni and red Zanzibar were in the fourth quadrant, while the white Zanzibar and raja were in the second quadrant. The position of the hutan clove in a different quadrant with a considerable distance from the two accessions, red Zanzibar and tuni, was in line with the phylogenetic tree reconstruction results. PCoA analysis aims to analyze plant diversity to determine the individual's relationship levels based on the similarity of characters with dimension simplification (Terryana *et al.*, 2018). The population of five clove accessions on the PCoA showed the highest contribution of diversity on coordinate axis 1 (Coord. 1) at 79.21%. Meanwhile, coordinate axis 2 (Coord. 2) has a low contribution of diversity at 16.76%, and the cumulative value on both axes was 95.97%.

CONCLUSIONS

Clove accessions tuni and hutan have significant morphological differences and

revealed the highest discrepancy. The molecular analysis showed that the clove accessions red Zanzibar and tuni were relatively distant and classified into moderate genetic diversity related to the clove accession hutan. These three clove accessions revealed the greater potential as parental genotypes for developing improved clove cultivars.

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