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Assessment of morphology, ISSR profile, and taxonomic relationship of rough lemon, nasnaran mandarin and kaffir lime

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Abstract

Rough lemon (*Citrus jambhiri* lush) is used widely as rootstock for commercial Citrus species. Its morphological similarity to *C. amblycarpa* and *C. hystrix* causes difficulty distinguishing between the three species. This study aimed to provide a scientific basis for confirming the taxonomic status of rough lemon as a species distinct from *C. amblycarpa* and *C. hystrix,* based on morphological and molecular characterization using Inter Simple Sequence Repeats (ISSR) markers. A total of 18 samples from three Citrus species were used. Morphological characterization was based on descriptors for Citrus. Observations of plant habit, leaf and fruit morphology generated 49 characters for morphological characterization. This data was subjected to cluster analysis to assess taxonomic relationships. ISSR markers were obtained from amplification of genomic Deoxyribonucleic acid (DNA) using five primers, which produced 43 DNA fragments with polymorphisms ranging from 33.3% to 62.5%. Results of cluster analysis based on morphological characters and ISSR markers showed clear differences between the species, indicating they have low phenotypic and genotypic variability. Some ISSR markers were found to be species-specific, with potential to be developed into molecular markers for species identification. of the three Citrus species.

Keywords: Molecular marker, Morphology, Phenetics, Species identification, Taxonomy

1. Introduction

Citrus is a genus of horticultural plants cultivated for its fruit either as table fruit or food additives Citrus also known as aromatic plants from which the essential oils is extracted from the peel or leaves for pharmaceutical and industrial needs due to the distinctive refreshing aroma [1,2]. Several Citrus species are cultivated to other purposes, such as rough lemon (*Citrus jambhiri* lush), which is used as rootstock for commercial Citrus species due to its adaptability [3], disease resistance [4], and positive effects on scion's performances and yield [5]. Molecular analysis of the origin of Citrus species confirmed that rough lemon was a hybrid between citron and mandarin [6]. Phylogenetic analysis revealed rough lemon's origin from *C. medica* and *C. reticulata* [7]. Evidence suggests Citrus's Southeast Asian origin [8]. It gained species diversity from admixture among progenitor species through interspecies hybridization followed by clonal propagation and cultivation [9]. Accurate species identification is very important for the exploration of biodiversity [10].

Most studies on rough lemon are related to its role as rootstock for commercial Citrus. These have focused on morphology [11], growth and physiology under salt stress [12], and rooting and budding performance as rootstock [13]. Research on rough lemon's potential for other functions, such as antibacterial [14] and insecticide [15], have been linked to its secondary metabolite compounds. The potential of rough lemon as nutritious food product in India showed that fruit juice and peel of rough lemon was processed into several value-added products such as squash, pickle, candy, and jelly [16]. Meanwhile, [17,18] reported the essential oils content of rough lemon. The economic importance of rough lemon is particularly its role as vigorous, highly tolerant, and highly adaptable rootstock for commercial plantations of other Citrus species [19]. The essential oils extracted from fruit rind of rough lemon was reported as having antimicrobial activity against Gram positive bacteria and yeast [20]. In this

case, the importance of Citrus species as source of ecologically friendly botanicals has been mentioned by [21]. Meanwhile, the medicinal importance of rough lemon has been recognized as having therapeutic properties in Ayuverda formulations as antidiarrhea and to improve digestion [22]. The medical benefit of rough species is high in crude fiber and minerals such as magnesium (Mg), iron (Fe), zinc (Zn) and manganese (Mn) relative to another Citrus [23]. It has been processed into value-added food products [16], its essential oils are reported to have antimicrobial effects [20], and it is used in Ayurvedic medicine [22]. Rough lemon is particularly popular as a food product in Indonesia's South Kalimantan and Central Kalimantan provinces, while in North Sumatra, it is sold as food flavoring [3]. Rough lemon has high intraspecific variation, especially in fruit morphology [4,24]. This causes misrecognition with morphologically similar species such as *C. hystrix* and *C. amblycarpa,* which can be overcome through studies on characterization, intraspecific variation, and taxonomic relationships of rough lemon with other species.

Studies reveal variation within a species plays an important role as an initial step in mapping the wealth of biological resources and serves as basis for formulating strategies for developing its potential. Such studies start with comprehensive characterization on species of interest. Characterization is needed to identify accessions with similar genotypes, taxonomic relationships, and effective germplasm management of germplasm [25]. Phenotypic characterization is generally based on morphology while genotypic characterization uses molecular markers. There have been no publications regarding rough lemon's taxonomic relationship with morphologically similar species, and the potential of *C. jambhiri* for food and other purposes is underexplored. This research was performed with the aim of producing comprehensive characterization of *C. jambhiri* based on morphological characters and Inter Simple Sequence Repeats (ISSR) markers, as well as revealing taxonomic relationships with *C. amblycarpa* and *C. hystrix*.

2. Materials and methods

2.1 Sample collection and morphological characterization

C. jambiri samples were collected from South Kalimantan and Central Kalimantan. Nasnaran mandarin (*C. amblycarpa*) and kaffir lime (*C. hystrix*) samples were obtained from Central Java and Yogyakarta: varied collection sites ensured control for environmental factors. 18 total samples were used (Table 1). Leaf samples for DNA isolation were preserved in silica gel. Morphological characterization was based on observation of 20 leaves and 5 mature fruits. Morphological characters examined referred to [26]. Morphological data used for characterization and analysis of taxonomic relationships consisted of 49 characters obtained from observations of plant habit, leaves, and fruit (Table 2). For each character, binary or multiple character states were determined according to sample conditions. For purposes of cluster analysis, each character state was converted into numerical data, referring to research on morphological characterization of *C. maxima* [27].

Table 1 Citrus samples used in this study.

No.	Species name	Common name	Sample code	Sample origin (Province)
1.	*C. jambhiri*	rough lemon	CJ-C1	Central Kalimantan
2.	*C. jambhiri*	rough lemon	CJ-C2	Central Kalimantan
3.	*C. jambhiri*	rough lemon	CJ-C3	Central Kalimantan
4.	*C. jambhiri*	rough lemon	CJ-C4	Central Kalimantan
5.	*C. jambhiri*	rough lemon	CJ-C5	Central Kalimantan
6.	*C. jambhiri*	rough lemon	CJ-C6	Central Kalimantan
7.	*C. jambhiri*	rough lemon	CJ-C7	Central Kalimantan
8.	*C. jambhiri*	rough lemon	CJ-S1	South Kalimantan
9.	*C. jambhiri*	rough lemon	CJ-S2	South Kalimantan
10.	*C. jambhiri*	rough lemon	CJ-S3	South Kalimantan
11.	*C. jambhiri*	rough lemon	CJ-S4	South Kalimantan
12.	*C. hystrix*	kaffir lime	CH-Y1	Yogyakarta
13,	*C. hystrix*	kaffir lime	CH-Y2	Yogyakarta
14.	*C. hystrix*	kaffir lime	CH-J	Central Java
15.	*C. amblycarpa*	nasnaran mandarin	CA-Y	Yogyakarta
16.	*C. amblycarpa*	nasnaran mandarin	CA-J1	Central Java
17.	*C. amblycarpa*	nasnaran mandarin	CA-J2	Central Java
18.	*C. amblycarpa*	nasnaran mandarin	CA-C	Central Kalimantan

2.2 DNA extraction and ISSR amplification

Genomic Deoxyribonucleic acid (DNA) was isolated from young, fully expanded leaves in the third and fourth position from the twig tip of leafy twigs. The use of leaves in the third or fourth positions from the tip was because they are young leaves that have been fully expanded, with the consideration that Citrus leaves are known to have high polysaccharide content which can cause problems in polymerase chain reaction (PCR) [28]. Leaves were cut into pieces. 50 mg of leaves were ground in liquid nitrogen. DNA isolation was performed using Geneaid™ Genomic DNA Mini Kit (Plant) according to manufacturer's protocol. Primers used were ISSR-A [(AC)8YA], ISSR-B [(AC)8YG], ISSR-C [HVH(TCC)5], ISSR-D [(TCC)5RY], and ISSR-E [(GT)8YC]. Amplification of ISSR markers was performed using MyTaq™ HS Red Mix (Bioline), in 25 µL volume consisting of 50 ng of DNA template, 10 pmol of primer, 12.5 µL of MyTaq™ HS Red Mix and 10 µL of Ultra-Pure Water (Bio Basic™). PCR was performed with initial denaturation at 94°C for 3 min, followed by 35 amplification cycles consisting of denaturation at 94°C for 50 sec, annealing at 50°C for 2 min, elongation at 72°C for 90 sec, and a final extension at 72°C for 6 min. Electrophoresis of PCR products was done using 2% agarose gel (GeneDireX™) in 1x TBE, stained with FloroSafe DNA Stain (1st Base™), performed at 100 volts for 45 min. Visualization of DNA fragments was done under UV-transilluminator.

2.3 Data analysis

Morphological data consisted of qualitative and quantitative characters converted into binary or multi-state numerical scores and subjected to cluster analysis to determine taxonomic relationships between three Citrus species. Cluster analysis to construct dendrogram was based on Euclidean distance and unweighted pair group method with arithmetic mean (UPGMA) clustering. Input data for cluster analysis consisted of two data sets. The first contained morphological data from 18 samples. 49 characters were used, covering plant habit, leaf, and fruit morphology. The second contained molecular information in ISSR fingerprinting profiles. ISSR data, amplified using five primers, were scored as binary data as either 1 or 0, representing either presence or absence of each DNA fragment. ISSR analysis was performed using the Jaccard coefficient and UPGMA clustering. Observation of plant habit, leaf, and fruit morphological characters produced data for cluster analysis to construct dendrogram. Forty-nine morphological characters were used (Table 2). Cluster analysis was performed using Paleontological Statistics Software Package for Education and Data Analysis (PAST) 2.01 [29].

Table 2 List of morphological characters.

No.	Character	Character states/unit	No.	Character	Character states/unit
1.	Tree shape	ellipsoid, obolid, spheroid	15.	Leaf base	acute, rounded
2.	Tree growth habit	erect, spreading, drooping	16.	Absence/presence of petiole wing	presence
3.	Branch density	sparse, medium, dense	17.	Petiole wing length (mm)	measurement
4.	Branch angle	narrow, medium, wide	18.	Petiole wing width (mm)	Measurement
5.	Adult tree spine density	low, medium, high	19.	Petiole wing shape	obcordate, obovate, linear
6.	Adult tree spine length (mm)	measurement	20.	Petiole and lamina junction	articulate
7.	Leaf division	bifoliate	21.	Leaf lamina/petiole wing ration	ratio
8.	Leaf lamina attachment	brevipetiolate, longipetiolate	22.	Petiole wing length/width ratio	ratio
9.	Leaf lamina length (mm)	measurement	23.	Fruit weight (g)	weight
10.	Leaf lamina width (mm)	measurement	24.	Fruit diameter (mm)	measurement
11.	Ratio leaf lamina length/width	ratio	25.	Fruit shape	spheroid, pyriform
12.	Leaf lamina shape	elliptic, ovate, orbicular	26.	Shape of fruit base	necked, convex, truncate
13.	Leaf lamina margin	crenate	27	Fruit apex shape	truncate
14.	Leaf apex	acuminate, acute	28.	Fruit surface texture	rough, grooved

Table 2 (Continued) List of morphological characters.

No.	Character	Character states/unit	No.	Character	Character states/unit
29.	Epicarp color	green	40.	Diameter of fruit axis (mm)	measurement
30.	Equatorial epicarp width (mm)	measurement	41.	Pulp color	green, yellowish-white, yellow
31.	Mesocarp to endocarp adherence	weak, medium, strong	42.	Pulp firmness	soft, intermediate, firm
32.	Fruit surface oil gland conspicuousness	conspicuous, strongly conspicuous	43.	Vesicle length	short, medium, long
33.	Fruit surface oil glands	intermediate, high	44.	Vesicle thickness	thin, medium
34.	Mesocarp color	white	45.	Average seeds per fruit	count
35.	Mesocarp thickness (mm)	measurement	46.	Seed shape	spheroid, ovoid
36.	Segments per fruit	count	47.	Seed surface	smooth, wrinkled
37.	Segment wall adherence	weak, medium	48.	Seed color	cream
38.	Fruit axis	solid, semi-hollow	49.	Cotyledon color	green, white, and green
39.	Fruit axis cross-section shape	round			

3. Results and discussion

3.1 Morphological characterization of C. jambhiri, C. amblycarpa and C. hystrix

Eleven rough lemon samples in fruiting condition were obtained from South and Central Kalimantan. Kaffir lime samples were collected from Central Java and Yogyakarta. Nasnaran mandarin samples were collected from South Kalimantan, Central Java, and Yogyakarta. Varied collection locations ensured control for environmental factors [30]. Rough lemon samples are shown in (Figure 1). Comparative fruit and leaf morphology of species studied is presented in (Figure 2).

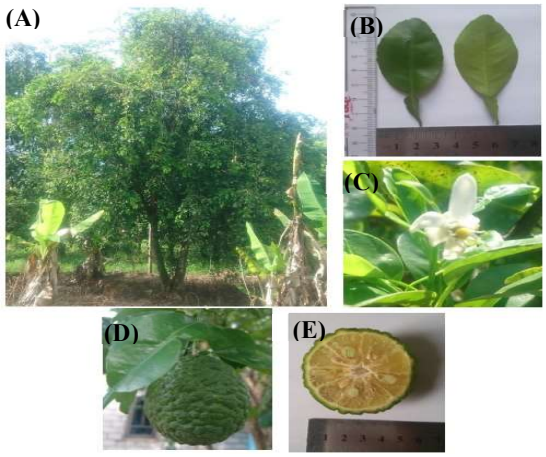

Figure 1 Morphology of *C. jambhiri*: (A) habit, (B) leaves, (C) flower, (D) whole fruit, and (E) half fruit.

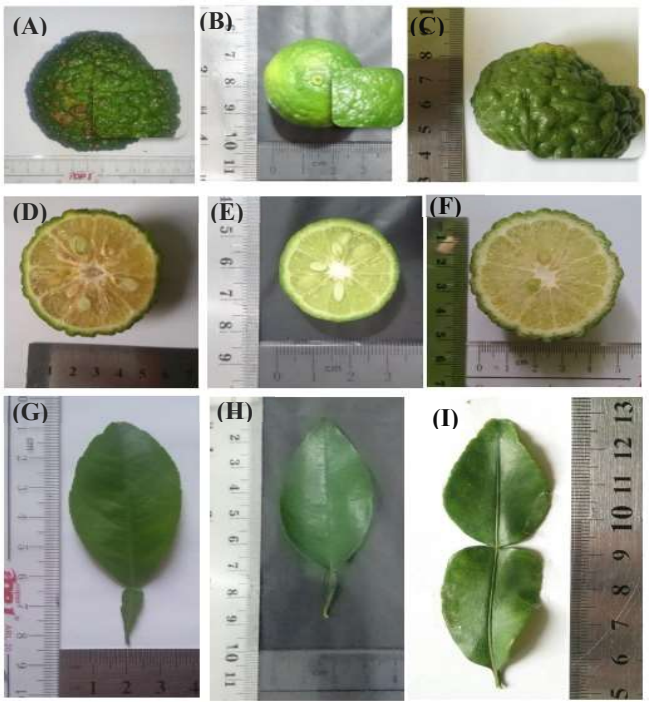

Figure 2 Comparisons of three Citrus species on prominent distinguishing morphological characters: (A-C) whole fruit *of C. jambhiri, C. amblycarpa, C. hystrix*, (D-F) half fruit of *C. jambhiri, C. amblycarpa, C. hystrix*, and (G-I) leaf shape of *C. jambhiri, C. amblycarpa, C. hystrix*.

Morphological observations recorded 49 characters (Table 2). as bases for determining taxonomic relationships using cluster analysis. These characters distinguished *C. jambhiri* from other species. This result supported the recognition of morphological characters as reliable taxonomic evidence for species identification and classification. This result supported the recognition of morphological characters as reliable taxonomic evidence for species identification and classification. In this regard [31] notes morphology's significance to plant systematics. Morphology remains relevant for recognizing Citrus genotypes and species, although molecular markers have now been used in plant systematic research [25].

3.2 Molecular characterization of C. jambhiri, C. amblycarpa and C. hystrix using ISSR markers

Amplifying genomic DNA using primers generated 43 DNA fragments representing ISSR markers (Table 3). Number of markers produced ranged from 5 to 12, with an average 8.6 per primer. Among the 43 markers, 21 were polymorphic. Polymorphism ranged from 33.3% to 62.5%. In a previous study ISSR markers produced 80.72% polymorphism for *C. jambhiri* [25]. A study of *C. aurantifolia* using ISSR markers reported 87.18% polymorphism [26]. A study of *C. aurantifolia, C. limetta, C. medica, C. limon* and *C. jambhiri* reported average polymorphism of 66.2% for ISSR markers generated from 13 primers [32]. ISSR fingerprinting profiles are presented in (Figure 3).

Table 3 Size and number of ISSR markers obtained from five primers.

Primer name	Sequence	DNA fragments of ISSR markers				Polymorphism (%)
		Size (bp)	Total number	Monomorphic	Polymorphic	
ISSR-A	HVH(TCC)5	500-2000	9	5	4	44.4
ISSR-B	(TCC)5RY	480-2800	9	6	3	33.3
ISSR-C	(TCC)5RY	400-1000	5	3	2	40.0
ISSR-D	(GA)8YG	290-1500	12	5	7	58.3
ISSR-E	(GA)8YG	450-1400	8	3	5	62.5
Total number			43	22	21	
Average			8.6	4.4	4.2	

ISSR profiles in agarose gel (Figure 3). showed four of the five producing species-specific markers. Only the ISSR-C primer did not produce any. ISSR-C produced the lowest number of amplification products. The ISSR-A primer had one marker each for *C. jambhiri* (DNA fragment 1700 bp), and *C. hystrix*, (DNA fragment 2000 bp). The ISSR-B primer produced three fragments for *C. jambhiri*, (550 bp, 1000 bp, and 1100 bp). The ISSR-D primer introduced the highest number of markers, with four unique DNA fragments, namely two each for *C. hystrix* (400 bp and 500 bp), and *C. amblycarpa* (1400 bp and 1500 bp). The ISSR-E primer had two markers for *C. amblycarpa* (450 bp and 1400 bp), and one for *C. jambhiri* (500 bp). The presence of species-specific DNA fragments suggested that ISSR is appropriate molecular marker for genotypic characterization in Citrus.

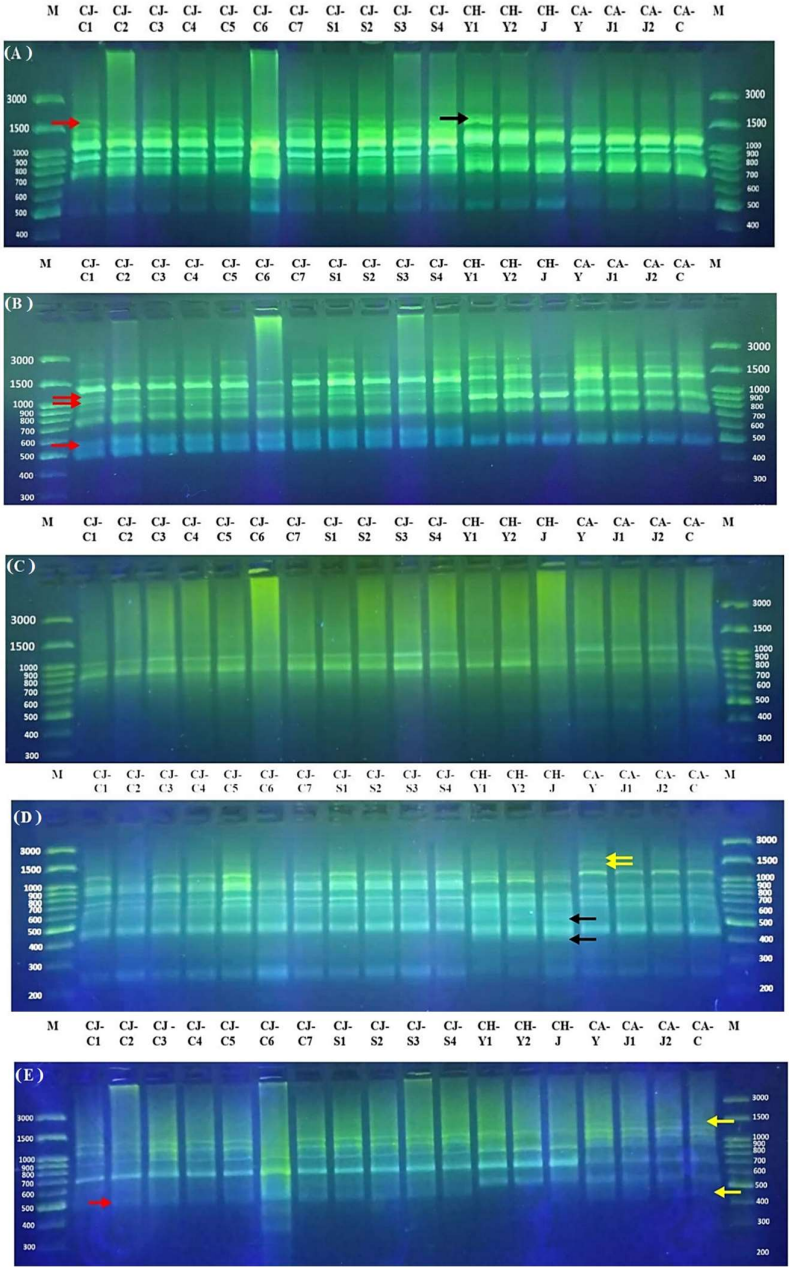

Figure 3 Profiles of ISSR markers generated by five primers: (A) Primer ISSR-A, (B) Primer ISSR-B, (C) Primer ISSR-C, (D) Primer ISSR-D, and (E) Primer ISSR-E. M: standard DNA molecular weight. Sample codes (CJ-, H-, CA-) referred to Table 1. Red arrow: unique marker for *C. jambhiri*, black arrow: unique marker for *C. hystrix*, yellow arrow: unique marker for *C. amblycarpa*.

3.3 Comparison of taxonomic relationships based on morphology and ISSR markers

The dendrogram generated from cluster analysis (Figure 4). shows samples grouped in three clusters according to species. Dendrogram topology and the taxonomic distance in each cluster shows low intraspecies diversity. This low variability in morphological characters might be caused by vegetative propagation practices that promote phenotypical uniformity [33]. Another explanation for the near-identical phenotypes is a nucellar embryo with special characteristics of polyembryony [34,35]. In this case asserts that the low genetic diversity of *C. jambhiri* results from its high polyembryony [25]. Most Citrus species have polyembryony resulting from sporophytic apomixis which generates nucellar embryos [35]. Regarding *C. jambhiri's* use as rootstock, the nucellar embryo is beneficial in producing uniform offspring with similar morphology and identical genetic properties to mother plants [34].

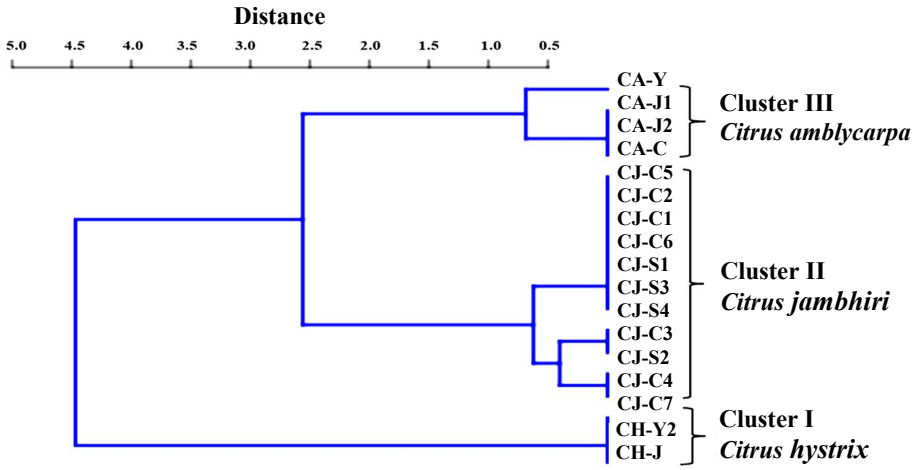

Figure 4 Dendrogram showing taxonomic relationship of samples from three Citrus species based on morphological characters.

The dendrogram showed that *C. jambhiri* is related more closely to *C. amblycarpa* than to *C. hystrix*. This result might explain why some publications mischaracterize rough lemon as *C. amblycarpa*. The misrecognition was found in some publications regarding rough lemon, which is indicated by the mention of different scientific names [36,37]. *C. jambhiri* resembles *C. amblycarpa* in leaf and fruit morphology and *C. hystrix* in fruit size and peel texture. Confusion regarding taxonomy can be resolved by cluster analysis. Many studies have used cluster analysis to assess taxonomic relationships, phenotypic diversity, and characterization in Citrus species like *C. aurantifolia* [38], and *C. latifolia* [39]. Moreover, cluster analysis for taxonomic identity and species delimitation has been reported in genus *Nicaea* [40], and *Legousia* [41].

Results of cluster analysis for determining taxonomic relationships between three Citrus species based on ISSR markers are presented in a dendrogram (Figure 5). Three clusters formed, representing three distinct species. A vertical line in each cluster, indicating identical ISSR profiles, showed low within-species genetic diversity. This result indicated that ISSR markers had important role in the analysis of genetic diversity and relationship in genus Citrus. This finding was in line with those reported in *C. jambhiri* [42], *C. aurantifolia*, [43] and *C. reticulata* [44].

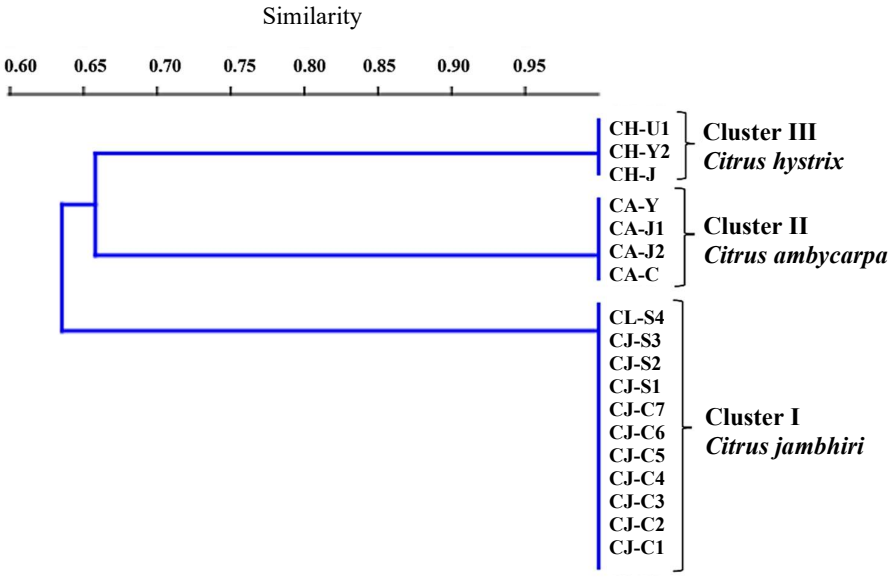

Figure 5 Dendrogram of taxonomic relationships of three Citrus species based on ISSR markers.

The dendrogram based on ISSR markers resembled the dendrogram generated from morphological data, in which three clusters corresponded to three species. Cluster analysis on both data sets suggests the three species had low phenotypic variability due to a narrow genetic base. Analysis of variability through morphological characterization aids in estimating genetic diversity [25,45], which is linked to species crop development and conservation potential [46,47].

4. Conclusion

This study is the first to report ISSR-based morphological and molecular characterization, and taxonomic relationships, between *C. jambhiri, C. amblycarpa,* and *C. hystrix.* Cluster analysis on morphological and ISSR markers, showed clear differences between the species and illuminated phenotypic and genotypic variability. This study thus showed ISSR's potential as a molecular identification tool for Citrus. Results therefore provided a scientific basis for recognizing differences in morphological and molecular profiles of *C. jambhiri, C. amblycarpa*, and *C. hystrix,* despite their morphological resemblances. These species show low genetic diversity based on ISSR markers, meaning conservation of all three Citrus species is recommended.

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