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GENETIC VARIATION OF SIAM ORANGE (*CITRUS NOBILIS* LOUR.) IN SEVERAL SUMATRAN PRODUCTION CENTERS BASED ON ITS MARKERS

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SUMMARY

Siam orange (*Citrus nobilis* Lour.) is a major horticultural commodity with wide distribution and the largest share in *Citrus* production in Indonesia. The information on genetic relationships among *C. nobilis* populations from major production centers remains limited in Indonesia. This study evaluated the genetic diversity, haplotype structure, and phylogenetic relationships of *C. nobilis* populations from four major production centers in Sumatra (Gunung Omeh, Pasaman, Bangkinang, and Berastagi) using ITS markers. Forty DNA samples were amplified using ITS1 and ITS4 primers, generating ITS (internal transcribed spacer) fragments of 603–666 bp. The resulting sequences incurred analysis for nucleotide composition, genetic diversity, haplotype structure, and phylogenetic relationships. The results revealed eight polymorphic nucleotide sites and a high GC content (65.24%). Eight haplotypes identified had the Hap_1, representing 65% of all samples and occurring across all populations. Haplotype diversity was the highest in Gunung Omeh and lowest in Berastagi. The haplotype network positioned Hap_1 as the central node, while other haplotypes differed by one to three nucleotide substitutions. This study provides the first ITS-based assessment of genetic relationships among major *C. nobilis* populations in Sumatra and complements previous random amplified polymorphic DNA (RAPD)-based findings with sequence-level evidence. The genetic information obtained may support germplasm selection, conservation efforts, and future breeding programs for the Siam orange in Indonesia.

Keywords: Siam orange (*Citrus nobilis*), genetic variation, ITS marker, haplotype diversity, phylogenetic analysis, Sumatra

Key findings: The analysis of *C. nobilis* from four production centers using ITS markers revealed eight haplotypes with low nucleotide diversity. The shared and region-specific haplotypes disclosed an extensive exchange of planting materials among the production centers. Phylogenetic analysis placed all accessions within a single monophyletic clade, reflecting a close genetic relationship.

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INTRODUCTION

Citrus nobilis Lour. is one of the most important citrus crops in Indonesia with a wide cultivation throughout the archipelago. Although the evolutionary origin of *Citrus* has an association generally with Southern China and Mainland Southeast Asia, the cultivation and dissemination of *C. nobilis* in Indonesia have a historical linkage to several essential development and selection centers. This includes Tlekung (Batu, East Java), Purworejo (Central Java), and Tulungagung (East Java) (Agisimanto *et al*., 2007). Its extensive distribution has the successful facilitation of its tolerance to a broad elevational range, from sea level to approximately 1,500 masl. Nevertheless, yield performance and fruit quality traits remain strongly influenced by local climatic and edaphic conditions, contributing to phenotypic variation among cultivated populations (Ashari *et al*., 2014).

The long cultivation history of Siam orange has resulted in numerous local cultivars across Indonesia. Reports stated releases of approximately 20 cultivars, with previous morphological and RAPD analyses classifying them into four major cultivar groups (Martasari *et al*., 2012). In Sumatra, *C. nobilis* relatively exhibited the highest genetic and morphological diversity. Morphological variations were especially visible in fruit size, canopy structure, and postharvest fruit characteristics (Devy and Hardiyanto, 2017). Genetic studies showed that *C. nobilis* populations from Gunung Omeh exhibited low diversity during the early intensification period. However, genetic variation increased in subsequently established populations, likely due to the inconsistent use of certified planting materials and the introduction of propagules from multiple sources (Mulyani *et al*., 2017; Devy and Hardiyanto, 2017).

RAPD-based analyses of populations from four different locations (Gunung Omeh, Pasaman, Bangkinang, and Berastagi) uncovered substantial genetic differentiation among populations. Genetic distances ranged from 0.0614 between Gunung Omeh and Bangkinang to 0.1622 between Gunung Omeh and Berastagi, indicating relatively close relationships among former populations and greater divergence of the Berastagi population. Accordingly, cluster analysis grouped populations into two major clusters, namely (a) Gunung Omeh, Pasaman, and Bangkinang, and (b) Berastagi (Setriani *et al*., 2025). However, because RAPD is a dominant marker system that generates fragment-size polymorphisms without providing nucleotide sequence information, the resulting clustering patterns may not fully reflect underlying genetic relationships. In addition, homoplasy and the limited phylogenetic resolution of RAPD markers can obscure patterns of genetic divergence among closely related populations. Consequently, the evolutionary origin, genetic divergence, and potential gene flow among Sumatran *C. nobilis* populations remain insufficiently resolved and require further evaluation using sequence-based molecular markers.

Since RAPD markers provide only fragment-size information without sequence data, their phylogenetic resolution is inherently limited. A more informative approach involves sequence-based markers, such as the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, which has been a widely used approach in phylogenetic and taxonomic studies of *Citrus* (Baldwin *et al*., 1995; Amar *et al*., 2014; Sun *et al*., 2015). The ITS region has had wide usage for species identification, phylogenetic reconstruction, hybrid origin assessment, and genetic diversity analyses in *Citrus* (Li *et al*., 2010; Kumar *et al*., 2012; Pessina *et al*., 2011; Hynniewta *et al*., 2014; Amar *et al*., 2014; Sun *et al*., 2015; Kim *et al*., 2021; Trang *et al*., 2021). Despite its utility, recent phylogenomic studies have revealed that evolutionary processes, such as incomplete lineage sorting and introgression, contribute to phylogenetic discordance within *Citrus*. This highlights both the advantages and limitations of single-locus markers like ITS in resolving the genus's complex evolutionary history (Witharana *et al*., 2025).

Previous studies have demonstrated that ITS markers provide sufficient sequence variation to resolve genetic relationships among closely related *Citrus* taxa (Amar *et al*., 2014; Sun *et al*., 2015). ITS sequence data

provide locus-specific information that can complement RAPD markers and help reassess genetic relationships among Sumatran *C. nobilis* populations using sequence-level evidence.

Based on previous findings, this study aimed to analyze the nucleotide variation and genetic divergence of *C. nobilis* populations from four major production centers in Sumatra, Indonesia, using ITS markers. This study provides the first ITS-based assessment of genetic relationships among major Sumatran populations and generates information relevant to germplasm conservation and breeding programs.

MATERIALS AND METHODS

Plant material and sampling locations

Fresh leaf samples of *C. nobilis* totaled 40, collected from four major production centers in Sumatra, Indonesia, comprising 10 samples from each population. These are Gunung Omeh (West Sumatra), Pasaman (West Sumatra), Bangkinang (Riau), and Berastagi (North Sumatra) (Figure 1). The conduct of sampling was purposive from trees owned by different farmers to represent genetic variations across the locations. The collection of healthy young leaves came from plants representing the cultivars commonly cultivated in each production center. Sampled plants, grown under conventional orchard management, entailed selection based on normal growth and the absence of visible pest or disease symptoms. Fresh leaf samples reached immediate placement in Ziploc bags containing silica gel before storage at room temperature until DNA extraction.

DNA extraction and quantification

DNA isolation, as carried out from dried leaves, used a modified CTAB method following Doyle and Doyle (1987). Leaf tissues sustained grinding in liquid nitrogen, followed by chloroform alcohol purification, isopropanol precipitation, and ethanol washing. DNA quality and concentration measurement used a NanoDrop spectrophotometer (Thermo Scientific NanoDrop™ 2000, USA). Samples with an A260/280 ratio between 1.8 and 2.0 and concentrations above 100 ng/μL entailed selection for further analysis.

PCR amplification and sequencing

Amplification of the ITS region succeeded in using primers ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990), targeting the ITS1/2 and 5.8S rDNA. The PCR mixture consisted of 12.5 μL MyTaq™ Red Mix (Bioline), 3.5 μL nuclease-free water,

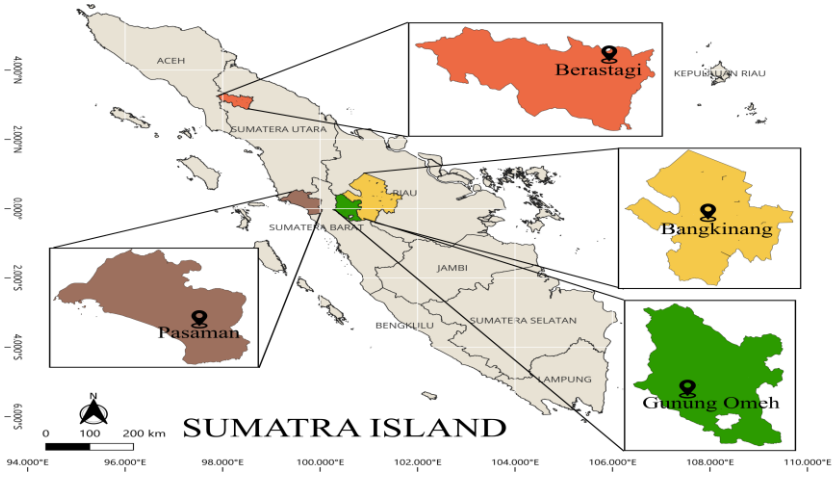

Figure 1. Sampling sites of four populations of *C. nobilis* in Sumatra, Indonesia.

2 µL of each primer, and 5 µL DNA template in a total volume of 25 µL. The PCR conditions included an initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 2 min. The PCR products' visualization continued on a 2% agarose gel stained with SYBR Safe (Invitrogen). DNA fragments of the expected size (600–750 bp) gained subsequent purification using the MEGAquick-spin™ Agarose Gel DNA Extraction Kit (iNtRON Biotechnology, Korea). Bidirectional sequencing proceeded at the 1st BASE Sequencing Services.

Sequence editing and alignment

Forward and reverse sequence reads reached assembling into contigs and editing using DNASTAR Lasergene 7.1 software (DNASTAR Inc., USA). Sequence alignment performance used ClustalX version 2.0, while the manual inspection was in BioEdit version 7.2.5. Ambiguous bases, if present, attained verification against sequence chromatograms and correction before downstream analyses (Hall, 1999; Larkin *et al*., 2007).

Data analysis

Nucleotide composition, conserved and variable sites, as well as GC content, underwent analysis using MEGA version 11 (Tamura *et al*., 2021). Haplotype diversity (Hd) and nucleotide diversity (π) calculations engaged DnaSP version 6.12.03 (Rozas *et al*., 2017). Performing haplotype network visualization was successful using PopART version 1.7 (Leigh and Bryant, 2015). Phylogenetic analysis continued in MEGA version 11 using the neighbor-joining (NJ) method with the Kimura 2-parameter (K2P) substitution model and 1,000 bootstrap replicates. Two *C. nobilis* sequences from GenBank (FJ641927.1 and AB456087.1) and two outgroup species, *Citrus hystrix* (MN638988.1) and *Murraya paniculata* (MZ824494.1), entailed inclusion in the phylogenetic reconstruction. The selected outgroups sought to represent both a closely related *Citrus* species and a more distantly related member of the Rutaceae family, thereby providing an appropriate framework for rooting the phylogenetic tree.

RESULTS AND DISCUSSION

ITS sequence characteristics of *C. nobilis*

DNA amplification of *C. nobilis* using universal primers ITS1 (forward) and ITS4 (reverse) with an annealing temperature of 54 °C produced a single and distinct band of approximately 600–750 bp. These primers target the ITS1, 5.8S rDNA, and ITS2 regions (White *et al*., 1990), and the obtained fragment size falls within the range commonly reported for *Citrus* species and other angiosperms (Baldwin *et al*., 1995; Amar *et al*., 2014; Hynniewta *et al*., 2014). The successful amplification confirmed the suitability of the ITS region for subsequent analyses of genetic variation among studied populations.

According to BLAST analysis, the ITS sequences showed 97.32%–97.97% similarity to *C. nobilis* (FJ641927.1) in the GenBank, with 100% query coverage and an E-value of 0.0, confirming their close genetic relationship and species identity. Alignment of 42 ITS sequences, including two outgroups (*C. hystrix* and *M. paniculata*), produced a consensus length of 508 bp. No insertions or deletions (indels) appeared in the aligned sequences. The alignment comprised 500 conserved sites (98.43%) and eight variable sites (1.57%), including three parsimony-informative sites and five singleton sites (Table 1). Despite the high level of sequence conservation, the detected nucleotide substitutions were sufficient to distinguish eight haplotypes and reveal genetic differentiation among populations from different production centers. The highest ITS sequence similarity has reached wide employment to confirm the species identity and explore the close genetic relationship in *Citrus* (Warseno *et al*., 2022). Previous studies also reported a predominance

Table 1. Sequence characteristics of *C. nobilis* ITS regions.

Variables	Values
Total sequence	42
Length of the analyzed sequence (bp)	508
Conserved (monomorphic) sites	500 (98.43%)
Variable (polymorphic) sites	8 (1.57%)
Parsimony-informative sites	3 (0.50%)
Singleton sites	5 (0.98%)
Thymine (T)	15.45%
Cytosine (C)	34.89%
Adenine (A)	19.31%
Guanine (G)	30.35%
A+T content	34.76%
G+C content	65.24%

Table 2. Haplotype distribution and GenBank accession numbers of 40 *C. nobilis* sequences from production centers in Sumatra.

No.	Haplotype	N	Sample code
1	Hap_1	26	GO1_PZ528304, GO2_PZ528305, GO3_PZ528306, GO6_PZ528309, GO7_PZ528310, PSM1_PZ528314, PSM2_PZ528315, PSM5_PZ528318, PSM6_PZ528319, PSM7_PZ528320, PSM9_PZ528322, PSM10_PZ528323, BK1_PZ528326, BK2_PZ528327, BK3_PZ528328, BK6_PZ528331, BK7_PZ528332, BK10_PZ528335, BTG1_PZ528336, BTG2_PZ528337, BTG3_PZ528338, BTG5_PZ528340, BTG6_PZ528341, BTG7_PZ528342, BTG9_PZ528344, BTG10_PZ528345
2	Hap_2	4	GO4_PZ528307, GO10_PZ528313, PSM4_PZ528317, BTG4_PZ528339
3	Hap_3	4	GO5_PZ528308, GO8_PZ528311, PSM8_PZ528321, BK4_PZ528329
4	Hap_4	1	GO9_PZ528312
5	Hap_5	1	PSM3_PZ528316
6	Hap_6	1	BK5_PZ528330
7	Hap_7	2	BK8_PZ528333, BK9_PZ528334
8	Hap_8	1	BTG8_PZ528343

Hap_1 – Hap_12 = Haplotypes, N = number of haplotypes, GO1–GO10 = Gunung Omeh, PSM1–PSM10 = Pasaman, BK1–BK10 = Bangkinang, BTG1–BTG10 = Berastagi.

of conserved ITS sites with limited variability among the cultivated *Citrus* accessions (Amar *et al*., 2014; Hynniewta *et al*., 2014).

Nucleotide composition analysis enunciated an average A+T content (34.76%) and G+C content (65.24%) across the studied *C. nobilis* samples, indicating a GC-rich ITS region with minor intraspecific variations. The predominance of conserved sites is consistent with the functional constraints of ribosomal DNA regions and has had numerous reports in *Citrus* species (Amar *et al*., 2014; Hynniewta *et al*., 2014). Reports stated the GC-rich ITS sequences have also emerged in various *Citrus* species, with those considered informative for evaluating the genetic variations despite overall sequence conservation (Amar *et al*., 2014; Hynniewta *et al*., 2014; Tamura *et al*., 2021).

Haplotype analysis of *C. nobilis* based on ITS markers

In the 40 *C. nobilis* samples collected from Gunung Omeh, Pasaman, Bangkinang, and Berastagi, ITS sequence analysis identified eight haplotypes (Table 2). Hap_1 was the dominant haplotype, accounting for 65% of all samples and occurring in all four production centers. The remaining haplotypes occurred as low-frequency variants or singletons, indicating limited sequence differentiation among

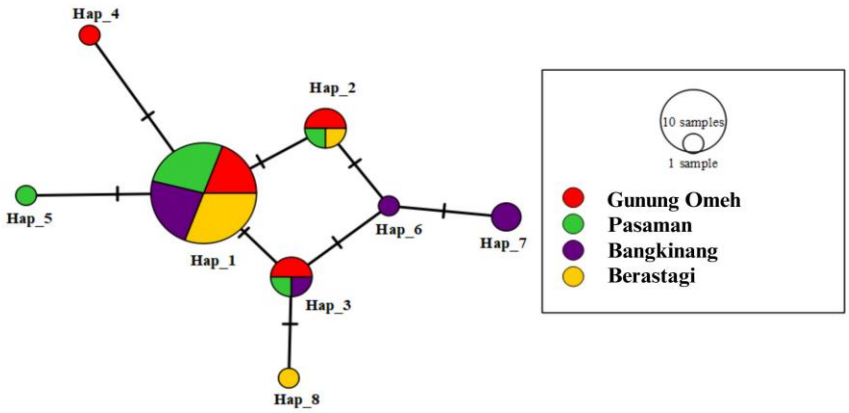

Figure 2. Haplotype network of 40 *C. nobilis* individuals based on ITS sequences.

Table 3. Haplotype diversity analysis of ITS sequences based on sampling locations of *C. nobilis.*

No.	Production center	n	Hn	Hd	π
1	Gunung Omeh	10	4	0.73333	0.00179
2	Pasaman	10	4	0.53333	0.00118
3	Bangkinang	10	4	0.64444	0.00267
4	Berastagi	10	3	0.37778	0.00118

n = number of samples, Hn = number of haplotypes detected, Hd = haplotype diversity, *π* = nucleotide diversity.

populations despite their geographic separation. Dominant haplotypes shared across multiple locations are commonly prevalent in clonally propagated crops and may reflect extensive exchange of planting materials among cultivation areas (Ellestad *et al*., 2022).

The results further revealed that nucleotide substitutions at specific ITS positions controlled the haplotype differentiation. Several variable ITS sites acted as haplotype markers, distinguishing individual haplotypes through one to three nucleotide substitutions. Within ITS regions, the point mutations, including transitions and transversions, typically play a primary role in haplotype formation, widely used to differentiate the closely related plant taxa (Syamsuardi *et al*., 2018).

The haplotype network analysis disclosed Hap_1 as the central node, while other haplotypes radiated through one to three mutational steps (Figure 2). This configuration represents a star-like network topology, characterized by a common central haplotype surrounded by low-frequency derivative haplotypes. Such a pattern showed a typical association with recent population expansion, extensive vegetative propagation, and limited recombination among cultivated lineages (Ollitrault *et al*., 2012). The widespread occurrence of Hap_1 across all production centers further suggests extensive exchange of planting materials among cultivation regions.

Genetic diversity analysis showed the location of Gunung Omeh had the highest haplotype diversity (Hd = 0.73333; π = 0.00179), while the Bangkinang location exhibited the highest nucleotide diversity (π = 0.00267) (Table 3). In contrast, the Berastagi location displayed the lowest level of genetic diversity. High Hd, coupled with low π, was a primary characteristic of populations with multiple closely related haplotypes and limited nucleotide divergence (Nei and Kumar, 2000).

Overall, ITS-based haplotype analysis revealed relatively low sequence variation among the studied *C. nobilis* populations, although Gunung Omeh and Bangkinang

locations exhibited slightly higher diversity than Pasaman and Berastagi locations. This pattern may reflect long-term clonal propagation and the exchange of planting materials among cultivation areas, which tend to homogenize genetic structure while maintaining limited haplotypic variation (Wu *et al*., 2018).

Phylogenetic analysis

Based on ITS sequences, the phylogenetic analysis used the neighbor-joining (NJ) method. The resulting phylogenetic tree resolved three major clusters (Figure 3). Cluster 1 comprised all the *C. nobilis* samples from Sumatra, Cluster 2 consisted of reference sequences from the GenBank, and Cluster 3 had representation from the species *Murraya paniculata* as the outgroup. Cluster 1 further underwent division into two subclusters, with Subcluster 1 including most samples from all production centers and Subcluster 2 containing a few divergent accessions.

Accessions in Subcluster 1 formed a compact and well-supported monophyletic group, indicating a high degree of sequence similarity within the ITS region (Alvarez and Wendel, 2003; Qin *et al*., 2017). Subcluster 2, represented by some *C. nobilis* samples (GO4, PSM4, and PSM10), showed an obvious separation from Subcluster 1. However, it gained placement within the same species clade, suggesting intraspecific genetic differentiation within *C. nobilis*.

Cluster 2 comprised reference sequences of *C. nobilis* and *C. hystrix* obtained from GenBank, which displayed phylogenetic separation from the main sample cluster but remained within the genus *Citrus*. The outgroup species *M. paniculata* (Cluster 3) gave the greatest genetic divergence from the studied *Citrus* accessions, supporting its suitability for rooting the phylogenetic tree (Liu *et al*., 2022; Tabatabaee *et al*., 2023).

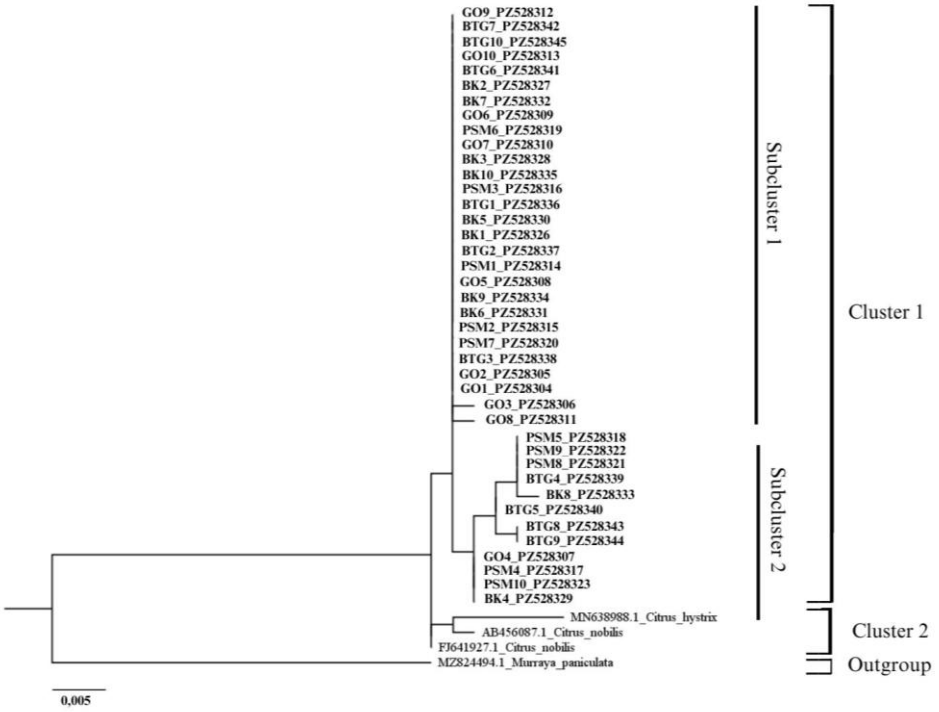

Figure 3. Phylogenetic tree of *C. nobilis* and reference species based on ITS sequences constructed using the neighbor-joining method (bootstrap 1000).

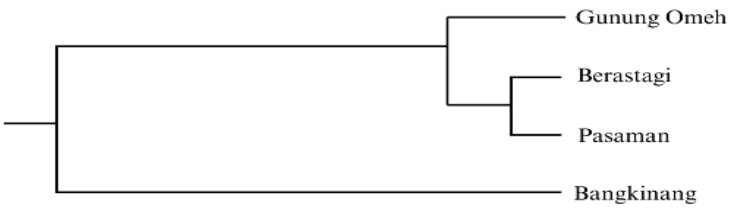

Figure 4. Dendrogram of four *C. nobilis* populations in Sumatra based on ITS sequences.

Table 4. Sequence divergence matrix among four *C. nobilis* production centers in Sumatra based on ITS markers.

Locations	Gunung Omeh	Pasaman	Bangkinang	Berastagi
Gunung Omeh	-	-	-	-
Pasaman	0.0053	-	-	-
Bangkinang	0.0152	0.0241	-	-
Berastagi	0.0053	0.0027	0.0255	-

Genetic distance analysis revealed a very low intraspecific divergence among the *C. nobilis* accessions, ranging from 0.0 to 0.008. Most accessions exhibited near-zero genetic distances, indicating a high degree of sequence similarity among the studied samples. This level of divergence is consistent with the low ITS variation commonly reported in angiosperm species, where intraspecific divergence typically remains below 1.20% (Qin *et al*., 2017).

Inter-population genetic distance values ranged from 0.0027 to 0.0255 (Figure 4, Table 4). The smallest genetic distance was evident between Pasaman and Berastagi (0.0027), whereas Bangkinang showed the highest genetic distance relative to Berastagi (0.0255) and Pasaman (0.0241). Gunung Omeh exhibited intermediate genetic distances with both Pasaman and Berastagi (0.0053), consistent with the dendrogram clustering pattern. According to Nei and Kumar (2000), these values indicate low to moderate population differentiation.

Based on the ITS dendrogram, *C. nobilis* populations from Gunung Omeh, Pasaman, and Berastagi clustered together, whereas Bangkinang formed a separate and more divergent cluster, revealing its distinct genetic position among the four production centers of *C. nobilis* (Figure 4). This pattern was consistent with the inter-population genetic distance matrix and further supported the distinct genetic position of the Bangkinang population. The distinct position of Bangkinang received further support from its relatively high nucleotide diversity (π = 0.00267), suggesting the presence of unique nucleotide variants within this population. Such differentiation could reflect differences in planting material sources, introduction history, or localized cultivation practices that contributed to the maintenance of a distinct genetic background. In contrast, the close clustering of Gunung Omeh, Pasaman, and Berastagi may indicate greater germplasm exchange or shared propagation history among these production centers.

The clustering pattern obtained in the potential study differs from that reported by Setriani *et al*. (2025) using RAPD markers. In the RAPD-based UPGMA (unweighted pair group method with arithmetic mean) dendrogram, Gunung Omeh and Bangkinang obtained grouping within the same subcluster; Pasaman formed a separate subcluster within the same major cluster, with Berastagi placed in a distinct cluster. In contrast, the ITS-based analysis grouped Gunung Omeh, Pasaman, and Berastagi together, while Bangkinang created a separate cluster. This discrepancy may be in relation to differences in the genomic regions

targeted by the two molecular markers. RAPD markers assess polymorphisms across multiple loci throughout the genome, whereas the ITS region represents a relatively conserved nuclear ribosomal sequence. Consequently, the two marker systems could capture different aspects of genetic variation and population structure. Despite the differences in clustering topology, both studies consistently demonstrate the existence of genetic variation among *C. nobilis* populations from major production centers in Sumatra.

Overall, ITS-based analysis revealed a low sequence divergence among the studied *C. nobilis* populations from Sumatra, suggesting limited genetic differentiation despite geographical separation. The conservative nature of the ITS region may restrict the detection of subtle population-level variation, particularly in domesticated taxa (Baldwin *et al*., 1995; Alvarez and Wendel, 2003). In vegetatively propagated species, such as *Citrus*, long-term clonal propagation could contribute to the maintenance of similar genetic backgrounds and reduce sequence variation, although additional molecular markers would be essential to assess genome-wide genetic diversity.

The predominance of Hap_1 across all production centers indicates that a substantial proportion of the sampled germplasm shares a common genetic background. While such uniformity may facilitate the maintenance of desirable horticultural traits, it could also limit the genetic resources available for future crop improvement. Contrastingly, the relatively high haplotype diversity observed in Gunung Omeh (Hd = 0.73333) suggests this population can represent an important reservoir of genetic variation for germplasm conservation and breeding programs. The utilization of genetically distinct accessions from diverse production centers could contribute to the development of improved Siam orange cultivars with broader genetic backgrounds.

CONCLUSIONS

The ITS sequence analysis of 40 *C. nobilis* samples from four major production centers in Sumatra revealed high nucleotide conservation, with only eight variable sites and a GC-rich composition (65.24%). The study identified eight haplotypes, with Hap_1 predominating across all production centers, while phylogenetic analysis disclosed a low genetic divergence among accessions. The observed genetic uniformity suggests a relatively narrow genetic base, likely associated with the widespread use and exchange of closely related planting materials. As this may support the maintenance of desirable horticultural traits, it could also limit adaptive potential to environmental changes, pests, and diseases. This study provides the first ITS-based assessment of genetic variation among major *C. nobilis* production centers in Sumatra and offers valuable genetic information for germplasm conservation, breeding, and sustainable cultivation.

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