

SABRAO Journal of Breeding and Genetics
 57 (5) 2024-2035, 2025
<http://doi.org/10.54910/sabrao2025.57.5.23>
<http://sabraojournal.org/>
 pISSN 1029-7073; eISSN 2224-8978



HAPLOTYPE DIVERSITY OF *UNCARIA GAMBIR* (W. HUNTER) ROXB. LANDRACES IN WEST SUMATRA, INDONESIA, BASED ON INTERNAL TRANSCRIBED SPACER (ITS) MARKERS

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SUMMARY

Indonesia is the leading global producer of *Uncaria gambir* (W. Hunter) Roxb., with West Sumatra having the largest plantation. The assessment of haplotype diversity is crucial for the production and quality of gambir in West Sumatra, Indonesia. The following study utilized ITS markers to ascertain the haplotype diversity in 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*) from four populations in main plantation areas located in West Sumatra, Indonesia. The research isolated, amplified, and sequenced the DNA. The assessment of genetic differentiation utilized various bioinformatics tools, including BioEdit for sequence alignment, DnaSP for detecting polymorphisms and analyzing haplotype diversity, and MEGA 11. The analysis of 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*) among four examined populations, identified seven distinct haplotypes (H1–H7). Haplotype diversity emerged to be moderate among all the groups ($H_d = 0.633$). The population of Taratak Tempatih demonstrated the greatest haplotype variety ($H_d = 0.90$), while the population of Simpang Kapuak exhibited the lowest diversity ($H_d = 0$), while all populations possessed the identical haplotype (H1). The genetic identity of Udang, Riau Besar, Riau Kecil, and Cubadak accessions will support seedling selection and breeding programs.

Keywords: *Uncaria gambir* (W. Hunter) Roxb., landraces, genetic uniformity, haplotype diversity, ITS, phylogenetic relationship

Key findings: The presented study identified seven distinct haplotypes in *U. gambir* (W. Hunter) Roxb., demonstrating the highest genetic diversity, using ITS markers. The highest haplotype diversity indicates low genetic uniformity, affecting gambir production and quality. This genetic diversity is crucial for the conservation of gambir landraces in West Sumatra, Indonesia.

Communicating Editor: Dr. Kamile Ulukapi

Manuscript received: February 13, 2025; Accepted: May 28, 2025.

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Citation: Audina Z, Syamsuardi, Mildawati, Nurainas, Christy P (2025). Haplotype diversity of *Uncaria gambir* (W. Hunter) Roxb. landraces in West Sumatra, Indonesia, based on internal transcribed spacer (ITS) markers. *SABRAO J. Breed. Genet.* 57(5): 2024-2035. <http://doi.org/10.54910/sabrao2025.57.5.23>.

INTRODUCTION

Indonesia, particularly the Sumatra region, is the primary home of *Uncaria gambir* (W. Hunter) Roxb., a species of the genus *Uncaria* (Wardi *et al.*, 2022). The medicinal plant is well-known for its several pharmacological qualities, which include antihypertensive, sedative, depressive, and antithrombotic effects (Dai *et al.*, 2023). Furthermore, *U. gambir* plays a remarkable role in Indonesian traditional medicines, which contribute to betel mixes and cure burns, headaches, diarrhea, dysentery, ulcers, and skin problems. It also works as a mouthwash, diabetic treatment, and a natural textile dye (Armenia *et al.*, 2024).

Gambir production is mainly prevalent in West and South Sumatra, with the West Sumatra region being the largest producer in Indonesia (Rauf *et al.*, 2015). Traditionally, gambir cultivation has been occurring in community gardens using seeds derived from diverse landraces native to West Sumatra, such as Udang, Cubadak, Mancik (Riau Kecil), and Riau (Riau Besar). Each gambir type has a considerable impact on the quality of the finished products, particularly its catechin level (Wardi *et al.*, 2022). In gambir, catechin is the significant bioactive ingredient and a reliable quality indicator. Among the several types, the Udang has the highest catechin concentration (14% to 45%), followed by three other types of gambir—Mancik (3% to 33%), Riau (9% to 25%), and Cubadak (9% to 17%). The landraces' genetic diversity and the catechin content variations can better help in genotype selection for the development of promising gambir cultivars (Kristina *et al.*, 2016).

The diversity of gambir plant types used in cultivation significantly contributes to variations in production, both in quantity and quality. It is essential to establish an obvious genetic identity for the gambir plant types used in each cultivation area to ensure sustainable and consistent production. This allows for accurate identification of the plant types. However, in practice, the seedlings used in many cultivation sites often lack authenticated information about their type and origin (Wardi

et al., 2020). This uncertainty can affect the cultivation efficiency and hinder the development of improved gambir cultivars. Therefore, identification and evaluation of the genetic diversity in gambir types is crucial. The knowledge about the genetic variations in these valuable resources can help in developing a sustainable and thriving gambir industry.

A landrace refers to a cultivated plant variety with a distinct historical origin, often characterized by relevant genetic diversity and local adaptation. Traditional agricultural systems have close links to it (Villa *et al.*, 2005), and these landraces hold greater genetic potential for their further development through future breeding programs (Cristobal and Herrero, 2016). Gambir landraces represent a crucial genetic resource for the sustainable utilization of Indonesia's medicinal plants. It is vital to look at the levels of genetic variation and divergence to understand how evolution might work and protect the genetic diversity of tropical medicinal plants. Molecular analysis by using ITS markers is a promising way to identify the genetic diversity and divergence in these gambir accessions. Therefore, the applicable study aimed to ascertain the haplotype diversity in 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*) from four populations by using ITS markers in West Sumatra, Indonesia. These results can better help make decisions on the protection and further use of gambir species.

Wardi *et al.* (2020) have tried to identify genetic differences among the four types of gambir plants using molecular analysis based on *matK* and *rbcl* sequences. However, the results showed no genetic differentiation among the types. Furthermore, Wardi *et al.* (2021) analyzed the four types of gambir using ITS sequences. However, based on the results of this study, only the Cubadak type showed significant genetic differences compared with the other types. Hence, to overcome the limitations in genetic identification between *U. gambir* types, a more precise molecular authentication approach is essential. This approach includes increasing the number of

representative samples and the use of molecular markers, such as ITS, to likely find genetic differentiation between gambir plant types.

MATERIALS AND METHODS

Plant material

For this study, the collection of 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*) occurred in two districts in West Sumatra—Pesisir Selatan (has the Taratak Tempatih and Siguntur locality) and the Lima Puluh Kota (has the Ampalu and Simpang Kapuak

Kapuak locality) (Figure 1). These gambir accessions represented various morphological types and one wild species of *Uncaria*. The dataset included four *U. gambir* accessions from Taratak Tempatih, as well as four accessions each from the Cubadak, Riau Besar, Riau Kecil, and Udang types, collected from Siguntur. Additionally, the research obtained four *U. gambir* accessions from the Ampalu and Simpang Kapuak localities. For comparative purposes, the study also included a wild species (*U. homomalla*), collected from the Taratak Tempatih locality. The detailed information about the locations and characteristics of the gambir accessions is available in Table 1.

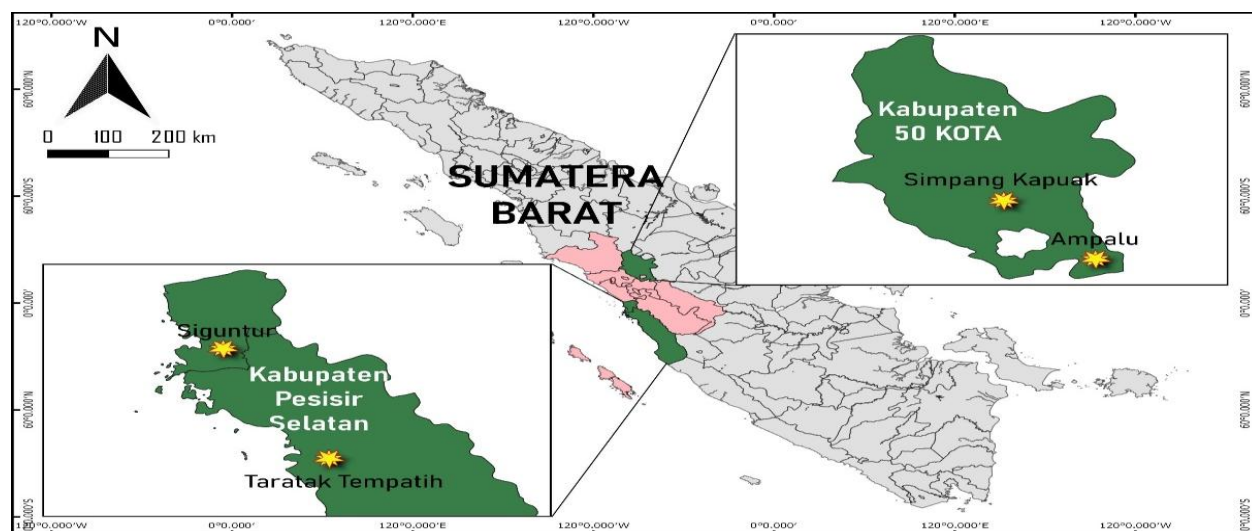


Figure 1. Sampling sites of four populations of *U. gambir* in West Sumatra, Indonesia.

Table 1. Distribution of *U. gambir* accessions used in this study.

Accession Codes	Number of Accessions (n)	Locations	Description
PG1, PG2, PG3, PG4	4	Taratak Tempatih	Gambir cultivation
C1, C2, C3, C4	4	Siguntur	Gambir cultivation
RB1, RB2, RB3, RB4	4	Siguntur	Gambir cultivation
RK2, RK3, RK4, RK5	4	Siguntur	Gambir cultivation
U1, U2, U3, U4	4	Siguntur	Gambir cultivation
A1, A2, A3, A4	4	Ampalu	Gambir cultivation
S1, S2, S3, S4	4	Simpang Kapuak	Gambir cultivation
PL5 (<i>Uncaria homomalla</i>)	1	Taratak Tempatih	Wild Gambir

DNA extraction, amplification, and sequencing

DNA extraction commenced by using the CTAB method on 28 accessions of *U. gambir* and one wild species (*U. homomalla*) (Table 1) (Doyle and Doyle, 1987). DNA amplification progressed by using ITS primers (ITS4 and ITS5) (White *et al.*, 1990). PCR reactions succeeded in using Bioline MyTaq Red Mix at 95 °C conditions for one minute for pre-denaturation, followed by 15 seconds of denaturation at 95 °C, 15 seconds of annealing at 48.8 °C, and 10 seconds of extension at 72 °C. The PCR process ended with a final extension at 72 °C for 10 minutes. The PCR products entailed examination using the electrophoresis method and documenting with Gel Doc (Biometra). Performing sequencing on the PCR product used the single-pass DNA sequencing method at Apical Scientific Laboratory, Malaysia.

Data analysis

Data analysis involved processing the forward and reverse sequences derived from the sequencing results. First, the forward and reverse sequences underwent merging, or contigs, to generate consensus sequences. Afterward, alignment continued using BioEdit to make sure the nucleotide comparisons for further analysis were correct (Ohied and Al-Badran, 2020). The DnaSP version 5.10.01 aided in viewing genetic variations or sequence polymorphism. Using MEGA version 11 helped measure genetic distance and the construction of phylogenetic trees (Nuratika *et al.*, 2020). This approach provided valuable insights into the evolutionary relationships and genetic diversity of the gambir populations studied.

RESULTS AND DISCUSSION

The ITS sequence alignment from 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*) revealed initial lengths ranging from 722 to 956 bp. The sequence lengths underwent standardization to 699 bp following alignment. Baldwin *et al.* (1995)

stated that the size of its sequence was around 700 bp. The successful alignment achieved uniform sequence lengths for subsequent analyses. The G-C content of the aligned sequences varied between 57.7% and 61.3%, with a mean value of 60.77%. All the gambir accessions exhibited a G-C content exceeding 50%, indicating the highest prevalence of guanine and cytosine in the ITS region. The thermodynamic stability of DNA structures attained enhancement due to the stronger hydrogen bonds formed between guanine and cytosine nucleotides (Smarda *et al.*, 2014).

Accession U2 demonstrated the lowest G-C content (57.7%), positioning it as an outlier relative to other accessions and possibly reflecting genetic variation. In contrast, accessions P1 and P2 exhibited the highest G-C content (61.3%). High G-C indicates that the accession resists various environmental stresses and reflects evolutionary adaptability to certain environmental conditions. Smarda *et al.* (2014) reported that these accessions displayed a higher level of genetic conservation. The uniform G-C content across most accessions implies genetic similarity among *U. gambir* populations. The small differences in G-C content might be due to genetic diversity within the same species, which could be biologically important, with the same also pointed out (Smarda *et al.*, 2014; White *et al.*, 1990). The results indicated that ITS regions were effective for examining genetic diversity and stability in *U. gambir* accessions. Further investigation into the relationship between the variations in G-C content and ecological adaptations may enhance the understanding of genetic composition in the species.

Based on the ITS sequence characteristic analysis, the 29 accessions had differences, and seven haplotypes (H1–H7) appeared. The seven haplotypes varied in the four populations of *U. gambir*. In these accessions, 59% had Haplotype H1, 17% had Haplotype H4, 10% had Haplotype H2, and 3% had each of Haplotypes H3, H5, H6, and H7. The findings further showed many haplotype variations in *U. gambir* existed, and the gambir accessions used for growing were not the same. However, based on the evolution and

conservation, 5–10 haplotype differences were enough to show sufficient genetic variation occurred. The more haplotypes identified in a population, the higher the haplotype diversity (Ellestad *et al.*, 2022). Nucleotide variation at the ITS locus is an important factor in the formation of haplotypes. Nucleotide variations in the ITS sequence of *U. gambir* include seven different nucleotide positions. Some nucleotide positions indicating haplotypes include positions 42, 57, 92, 214, 412, 482, and 579. For example, position 92 distinguishes the second haplotype from the first haplotype (H1); position 482 characterizes the third haplotype (H3), position 42 for the fourth haplotype (H4), position 57 for the fifth haplotype (H5), position 214 for the sixth haplotype (H6), and positions 412 and 579 mark the seventh haplotype (H7). These nucleotide markers provide an overview of the pattern of genetic variation in the *U. gambir* population.

This haplotype analysis describes the combination of alleles or genetic variants found at certain loci in the genome, which are inherited together as a unit (Huda *et al.*, 2019). The pattern of genetic variation reflected in haplotypes provides an overview of genetic diversity among individuals or varieties within the same species, making it a crucial tool in the study of genetic diversity. In the field of plant breeding, haplotype analysis is valuable as a tool for variety identification, allowing clear differentiation between varieties based on their genetic characteristics. Haplotype information also supports the selection of optimal hybrid combinations, thus facilitating the development of improved varieties with desirable agronomic traits. Moreover, haplotype analysis plays an essential role in the reconstruction of domestication history and species evolution (Hu *et al.*, 2022).

Studies of haplotypes help scientists understand how evolution works, how genes change over time, and how genetic variation can link to specific haplotypes (Bhat *et al.*, 2021). Using genetic traits to tell the distinctions among the different types of plants is possible with haplotypic analysis. As such, plant breeders need to know about haplotypes

to choose the best hybrid combinations leading to better cultivars with desirable agronomic traits. Haplotypic analysis is essential for understanding species evolution and domestication processes. This also provides insights into the origins of genetic diversity within a species and the influence of selection on its development (Hu *et al.*, 2022).

The identification of the 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*) used in this research was also successful. Four accessions of the Cubadak type came from Siguntur, with four additional accessions obtained from Simpang Kapuak. Four accessions of the Riau Besar type, four accessions of the Riau Kecil type, and four accessions of the Udang type reached sourcing from Siguntur. Eight accessions of an unidentified type attained inclusion, with four sourced from Taratak Tempatih and four from Ampalu. The study detected a haplotype similarity relationship with the *U. gambir* type used. The research indicated the Cubadak type from Siguntur and Simpang Kapuak shared the same haplotype, signifying that all this type of accessions possessed identical nucleotide sequences. The Riau Besar type exhibited two distinct haplotypes. The RB2 accession displayed a nucleotide variation at position 92, with cytosine (C) substituting thymine (T). The Riau Kecil type showed two haplotypes. The accession code RK3 provided a nucleotide alteration at position 482, where guanine (G) sustained an adenine (A) replacement. The Udang type gave two distinct haplotypes.

The U1 accession substituted adenine (A) with cytosine (C) at position 42 (Table 2). Additionally, the unidentified type comprised five distinct haplotypes. Haplotype 1 consisted of accessions A1, A4, and P3 and displayed identical nucleotide sequences. Haplotype 2 included the accession designated as P1, which exhibited a nucleotide alteration at position 92 (T to C). P2 and P4 accessions, comprising Haplotype 3, provided nucleotide alterations at position 42 (C to A). Haplotype 4 included accessions designated A2, exhibiting a nucleotide substitution at position 57 (T to C). The accession labeled A3 in Haplotype 5 exhibited a nucleotide alteration at position

Table 2. Seven haplotypes of 29 accessions of *U. gambir* based on ITS marker.

No.	Accession Code	Types	Locations	ITS							Haplotype	
				42	57	92	214	412	482	579		
				A	T	T	G	G	G	G		
1	C1	Cubadak	Siguntur									H1
2	C2	Cubadak	Siguntur									H1
3	C3	Cubadak	Siguntur									H1
4	C4	Cubadak	Siguntur									H1
5	RB3	Riau Besar	Siguntur									H1
6	RB4	Riau Besar	Siguntur									H1
7	RB5	Riau Besar	Siguntur									H1
8	RK1	Riau Kecil	Siguntur									H1
9	RK2	Riau Kecil	Siguntur									H1
10	RK4	Riau Kecil	Siguntur									H1
11	A1	Unidentified	Ampalu									H1
12	A4	Unidentified	Ampalu									H1
13	S1	Cubadak	Simpang Kapuak									H1
14	S2	Cubadak	Simpang Kapuak									H1
15	S3	Cubadak	Simpang Kapuak									H1
16	S4	Cubadak	Simpang Kapuak									H1
17	P3	Unidentified	Taratak Tempatih									H1
18	RB2	Riau Besar	Siguntur			C						H2
19	U1	Udang	Siguntur			C						H2
20	P1	Cf Udang	Taratak Tempatih			C						H2
21	RK3	Riau Kecil	Siguntur						A			H3
22	U2	Udang	Siguntur	C								H4
23	U3	Udang	Siguntur	C								H4
24	U4	Udang	Siguntur	C								H4
25	P2	Unidentified	Taratak Tempatih	C								H4
26	P4	Unidentified	Taratak Tempatih	C								H4
27	A2	Unidentified	Ampalu		C							H5
28	A3	Unidentified	Ampalu				C					H6
29	PL5	Uncaria homomalla	Taratak Tempatih					A		C		H7

214 (G to C). This comprehensive haplotypic analysis provides considerable insights into the genetic identity and potential evolutionary relationships among *U. gambir* populations (Ellestad *et al.*, 2022). Haplotypes also indicated the genetic identity of two landraces of pine from Kerinci, Aceh, and Tapanuli (Rinaldi *et al.*, 2023).

The haplotype grouping specifies that *U. gambir* exhibited significant genetic diversity, evidenced by the variations found in the number of haplotypes across different types. Haplotypes are also a set of alleles and DNA sequences inherited simultaneously in a genome and can serve as genetic markers to analyze the differences among the accessions (Huda *et al.*, 2019). Genetic variation in the

specific types results from nucleotide substitution mutations at designated positions (Table 2). The Cubadak type demonstrated genetic homogeneity, indicating all accessions within this group possess identical nucleotide sequences. The genetic homogeneity is crucial for obtaining better product quality (Syamsuardi *et al.*, 2018), as demonstrated by a single haplotype. The observed similarity suggested that the Cubadak type may have originated from a population exhibiting minimal gene flow, resulting in limited genetic variation among the individuals (Romdhane *et al.*, 2017). The unidentified types displayed the greatest genetic diversity, consisting of five distinct haplotypes. The diverse traits of this group revealed a complex evolutionary history.

Diverse selection pressures may have led to increased genetic differentiation. Genetic diversity varies among the types, indicating that ecological and evolutionary factors may influence the genetic diversity in *U. gambir* populations (Chung *et al.*, 2023).

This study emphasizes the importance of using a representative number of individual replicates in genetic analysis, especially in haplotype studies in *U. gambir*. The results obtained indicate the existence of haplotype variation among individuals previously considered homogeneous based on their morphological characters. Utilizing a single sample for each type or population can overlook genetic variation within the same population, which is often invisible when relying on a single sample (Wardi *et al.*, 2021).

Gambir haplotype distribution

Based on ITS sequences, the haplotype distribution in various *U. gambir* populations in Siguntur, Taratak Tempatih, Ampalu, and Simpang Kapuak exhibited distinct genetic variations. Siguntur showed four haplotypes: the first haplotype accounts for 62%, the second for 13%, the third for 6%, and the fourth for 19%. Figure 2 illustrates the distribution of haplotypes derived from ITS sequences. Among the 10 individuals, the primary haplotype identified was the dominant variant, while the remaining haplotypes resulted in lesser frequencies. Taratak Tempatih displayed four haplotypes: the first haplotype accounts for 20%, the second for 20%, the fourth for 40%, and the seventh haplotype constitutes 20%.

The fourth haplotype had two individuals representing it, whereas the first, second, and seventh haplotypes each have a single individual. Ampalu exhibited three haplotypes: the first haplotype at 50%, the fifth haplotype at 25%, and the sixth haplotype at 25%. The predominant haplotype in this region was the first, represented by two individuals, while the fifth and sixth haplotypes each comprise a single individual. Simpang Kapuak provided a pronounced genetic homogeneity, evidenced by the presence of a single haplotype, specifically the first haplotype

(100%), identified among the four individuals. All sampling sites exhibited the distribution of the predominant haplotype. Syamsuardi *et al.* (2018) emphasized that haplotype diversity within a population serves as an indicator of the highest genetic variation. Therefore, maintaining haplotype-based genetic diversity is crucial for the conservation of genetic resources.

Haplotype diversity

Genetic diversity within a population can undergo evaluation through parameters including the number of haplotypes (H_n), haplotype diversity (H_d), and nucleotide diversity (P_i). The analysis employing ITS markers revealed varying degrees of genetic variation across the populations (Table 3). The Taratak Tempatih population exhibited the highest haplotype diversity ($H_d = 0.9$), indicating substantial genetic variation within this group. The Simpang Kapuak population gave an H_d of 0 and a P_i of 0, signifying genetic homogeneity within this group. The Taratak Tempatih population enunciated the ultimate nucleotide diversity ($P_i = 0.00296$), suggesting significant variation at the nucleotide level. The pooled populations exhibited moderate overall genetic diversity, characterized by an H_d of 0.633 and a P_i of 0.00137. The results indicated that various populations owned both high and low levels of genetic diversity. This authenticated that varying degrees of evolutionary and ecological pressures influence genetic differentiation.

The distribution of genetic diversity among the populations revealed that groups like the Ampalu and Taratak Tempatih display considerable genetic variation. This is likely due to larger populations and a significant genetic flow. Nonetheless, the Simpang Kapuak population exhibited genetic homogeneity ($H_d = 0$, $P_i = 0$), suggesting a smaller population size and limited genetic exchange. Genetic homogeneity can yield plants exhibiting identical advantageous traits. Populations, such as Simpang Kapuak, may serve as a relevant genetic resource for breeding programs targeting specific traits in *U. gambir*. Nei (1987) categorizes genetic

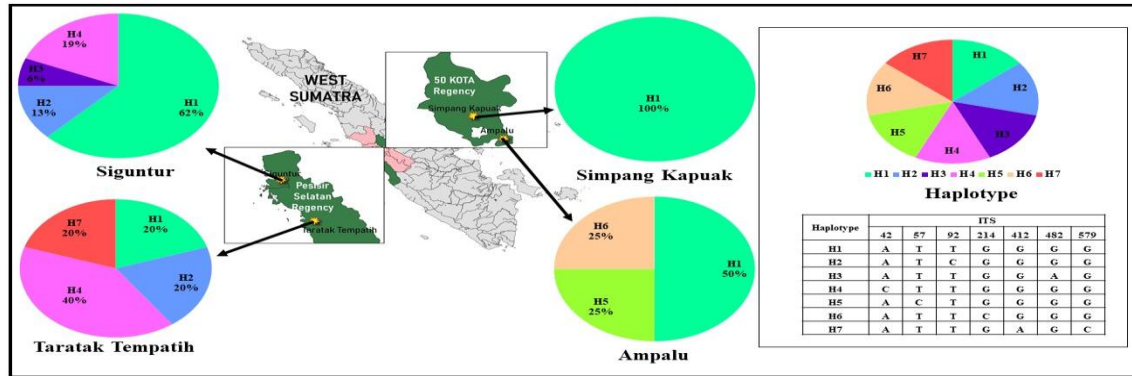


Figure 2. Haplotype distribution of *U. gambir* using ITS marker.

Table 3.-Analysis of haplotype diversity using ITS.

Populations	n	Hn	Hd	Pi
Siguntur	16	4	0.591	0.00112
Taratak Tempatih	5	4	0.9	0.00296
Population of Pesisir Selatan	21	5	0.676	0.00152
Ampalu	4	3	0.833	0.00164
Simpang Kapuak	4	1	0	0
Population of Lima Puluh Kota	4	3	0.464	0.00082
All Populations	29	7	0.633	0.00137

Description: n = Number of Samples, Hn = Number of Haplotypes, Hd = Haplotype Diversity, and Pi = Nucleotide Diversity.

diversity values as follows: values ranging from 0.8 to 1.0 were classified as high, 0.5 to 0.7 as medium, and values between 0.1 and 0.4 as low. The observed haplotype and nucleotide diversity values reflected the extent of genetic variation within the species. Grant and Bowen (1998) reported that haplotype diversity values span from 0 to 1, with a value of 0 indicating the absence of genetic differences among the analyzed accessions and higher values reflecting increased genetic variation within the population.

Nucleotide diversity values ranging from 0% to 10% exhibited a relatively low level of genetic diversity within the species. The haplotype analysis can provide significant insights into population structure and evolutionary relationships among various crop varieties. Haplotype network mapping serves as an effective method for identifying the distinct genetic groups within a population, which enables the identification of substantial genetic variations. This approach was crucial

for comprehending the development of crop diversity over time and can assist in identifying adaptive traits across various genetic lineages (Lopez and Bonasora, 2017). The various haplotypes observed across the populations indicated the presence of distinct genetic lineages. This signifies that these species possess diverse evolutionary histories that have incurred influences from their present genetic composition (Table 3).

The results further revealed that *U. gambir* populations displayed moderate genetic diversity. Populations exhibiting substantial genetic diversity, such as those from Ampalu and Taratak Tempatih, showed considerable potential for adaptation and enduring genetic sustainability. These populations possess the ability to adapt to varied environmental conditions, hence ensuring their long-term resilience. Conversely, populations demonstrating little genetic variation, such as the Simpang Kapuak, may be particularly advantageous for breeding efforts focused on

developing genotypes with consistent characteristics. With genetic uniformity, these populations can serve as dependable sources for commercial agricultural initiatives that prioritize stability in desired agronomic features.

Phylogenetic relationship

The analysis of phylogenetic relationship was successful on 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*) as an out-group. Figure 3 presents the resulting phylogenetic tree, constructed based on ITS sequences. The application of a bootstrap resampling method with 1000 replicates enhanced the reliability of the phylogenetic inference. This approach also ensures statistical robustness and confidence in the inferred phylogenetic relationship. Bootstrap values serve as indicators of the reliability of branching patterns within the phylogenetic tree. A higher bootstrap value reflects greater confidence in the accuracy and stability of the inferred relationship. When bootstrap values between 70 and 100 were so high, Rosidiani *et al.* (2013) mentioned a lesser chance for the branch topology to change. However, bootstrap values below 70 mean less confidence, which revealed there could be

instability in the formation of clades and a higher chance that the topology of the phylogenetic tree will change. According to Kress *et al.* (2009), bootstrap values' categorization depended on their level of reliability, namely, strong (>85%), medium (70%–85%), weak (50%–69%), and low (<50%). The phylogenetic tree constructed from ITS sequences disclosed two primary clades. The outgroup *Uncaria homomalla* clearly showed separation from the *U. gambir* accessions observed in the first clade. The second clade comprised multiple subclades, indicating genetic differentiation between the wild gambir and 28 *U. gambir* accessions.

The study detected identical genetic relationships among the 17 accessions: S4, P3, S3, S2, S1, A4, A1, RK4, C4, C3, C1, C2, RB3, RB4, RB5, R1, and RK2. These showed relatedness to the haplotype of the 17 accessions, namely, haplotype H1. The tree diagram also revealed the separation of the accessions of the Haplotype H6 group (accession A3), Haplotype H5 (accession A2), Haplotype H3 (accession RK3), Haplotype H4 group (accessions U2, U3, U4, P2, and P4), and Haplotype group 2 (accessions RB2, U1, and P1) from Group 17 accessions with Haplotype 1. Based on the branching pattern of the phylogenetic tree with the wild gambir out-

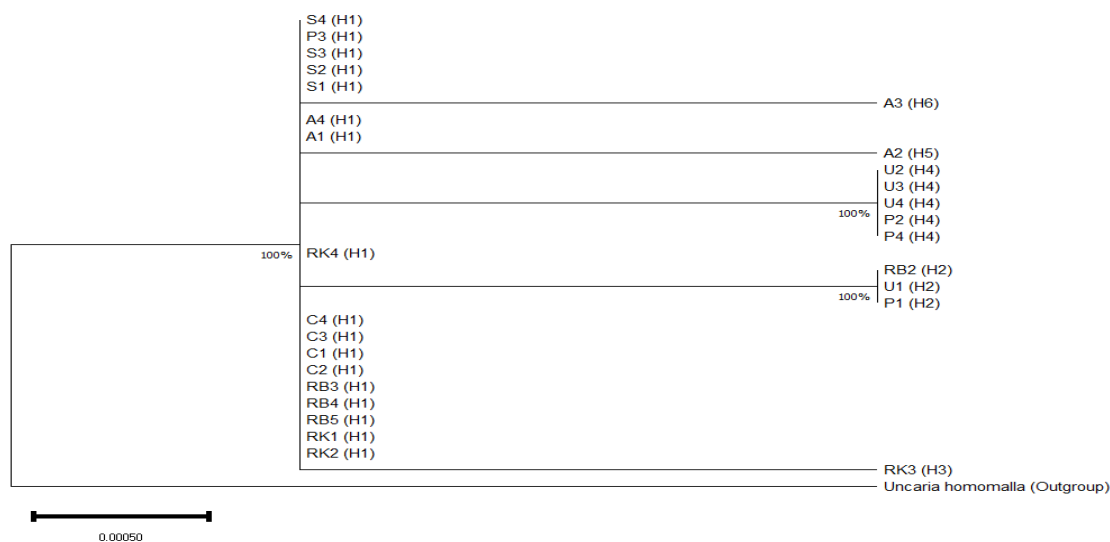


Figure 3. The genetic relationship tree of *U. gambir* based on ITS sequences using the neighbor-joining method.

group, one can suspect that Haplotype 1 was an ancestral genetic character while the other haplotypes were derivatives of Haplotype H1. Additionally, in more detail, one can estimate that accession A3 was a derivative from A4, with A2, U2, U3, U4, P2, and P4 derived from RK4. Then RB2, U1, and P1 were originating from Group C2, C1, C3, and C4. However, if one compares the molecular characters with ITS markers, one can conclude that Haplotype 1 is a plesiomorphic character, while the other haplotypes (H2-H6) were the apomorphic characters.

In the phylogenetic tree, the branch lengths provide insight into the evolutionary trajectories of these gambir accessions. Longer branches indicate more nucleotide substitutions, suggesting a longer evolutionary history and increasing the genetic divergence (Tabatabaee *et al.*, 2023). The examiners observed this pattern in the accession *Uncaria homomalla* having the highest branch length, which suggested a longer evolutionary distance and a possible more ancestral lineage within *U. gambir*. The shorter branches showed fewer nucleotide substitutions, which revealed the accessions were genetically more similar, and the divergence happened recently (Figure 3) (Liu *et al.*, 2022; Tabatabaee *et al.*, 2023).

CONCLUSIONS

The ITS sequence analysis revealed seven distinct haplotypes identified in 28 accessions of *U. gambir* and one wild gambir species (*U. homomalla*), distributed across four studied populations. Haplotype diversity appeared to be moderate ($H_d = 0.633$) across all the populations. The Taratak Tempatih population exhibited the highest haplotype diversity ($H_d = 0.90$), whereas the Simpang Kapuak population displayed the lowest level of diversity ($H_d = 0$), and all gambir accessions shared the same haplotype (H1).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the Institute for Research and Community Service (LPPM), Andalas

University, Indonesia, for their financial support for this research (322/UN16.19/PT.01.03/PTM/2024). Likewise, they thank the Balai Pengujian Standar Instrumen Tanaman Buah Tropika (BSIP) in Solok for providing the necessary facilities and technical assistance in conducting the molecular testing of the accessions.

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