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Genetic diversity and population structure of *Glochidion* subgenus *Phyllanthodendron* (Phyllanthaceae) in Thailand and Laos revealed by SCoT markers

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Abstract. *Ilmi ZL, Chantaranothai P, Balslev H, Duanyai J, Pornpongrungrueng P. 2025. Genetic diversity and population structure of* Glochidion *subgenus* Phyllanthodendron *(Phyllanthaceae) in Thailand and Laos revealed by SCoT Markers. Biodiversitas 26: 5534-5543.* Two species of *Glochidion* subgenus *Phyllanthodendron* (Phyllanthaceae) were previously recorded in Thailand and Laos: *Glochidion mirabile*, which occurs throughout Thailand and adjacent Laos, and *Glochidion kaweesakii*, which is a local endemic in northeastern Thailand. During field work, we encountered three populations that were difficult to assign to either of the two known species on morphological grounds. In this study, we used SCoT markers to assess genetic diversity and relationships among 101 individuals from 22 populations to clarify their taxonomic status. Seven SCoT primers that provided 100% polymorphic bands (mean Polymorphic Information Content (PIC) = 0.26) were selected for the analysis. We found low genetic variation for *G. kaweesakii* (Nei's genetic diversity (H) = 0.05), unidentified populations 1 (H = 0.05), and unidentified populations 2 (H = 0.06). Both *G. mirabile* and the third unidentified population exhibited low genetic variation within populations but high variation between populations, which was associated with a high genetic differentiation coefficient among populations (Gst = 0.67 and 0.66, respectively). Clustering analysis grouped all individuals into three clusters. Cluster I included *G. mirabile* and the third unidentified population, Cluster II included the two remaining unidentified populations, and Cluster III consisted of *G. kaweesakii*. Population structure analysis identified two groups that are significantly different from clustering analysis. Genetic relationships were consistent with geographic distribution. The results support the possibility that the third unidentified population should be considered part of *G. mirabile*, whereas the two remaining unidentified populations represent potentially distinct lineages that require an integrative taxonomic approach to confirm. Given the low genetic diversity and limited gene flow, a comprehensive conservation strategy focusing on improving habitat connectivity and facilitating gene flow should be considered to enhance the long-term viability of these species.

Keywords: Genetic variation, *Glochidion kaweesakii*, *Glochidion mirabile*, molecular marker, taxonomy

INTRODUCTION

Glochidion J.R.Forst. & G.Forst. belongs to the family Phyllanthaceae (APG 2016), comprising approximately 300 species. The genus is divided into three subgenera: *Glochidion*, *Phyllanthodendron,* and *Pseudoactephila*. *Glochidion* subgenus *Phyllanthodendron* contains five species, which are all found in Asia: *G. anthopotamicum*, *G. dongmoense*, *G. dunnianum*, *G. mirabile*, and *G. kaweesakii* (Bouman et al. 2022)

Glochidion mirabile is restricted to limestone hill habitats in Thailand and Laos (Chantaranothai 2007). Another species in Thailand, *G. kaweesakii,* is a narrow endemic restricted to limestone hills in Loei Province (Pornpongrungrueng et al. 2017). Recently, three new populations with morphological characteristics of *Glochidion* subg. *Phyllanthodendron* was discovered in similar limestone habitats in both countries. They could not easily be assigned to any known *Glochidion* species. Thus, their taxonomic status requires clarification. These populations share some morphological characteristics with *G. kaweesakii* and *G. mirabile*, such as 5 sepals and 3 stamens in staminate flowers; 6 sepals and 3 stigmas in pistillate flowers, the sepals with long acuminate or aristate apex, the disc gland in flowers has a clavate shape, and a globose, 3-lobed capsule. However, they exhibit differences in several morphological characters, including branching type, inflorescence position, stem base, and leaf. *G. mirabile* and *Glochidion* unidentified population 3 (*Glochidion* upop 3) are similar in that they have a swollen stem base, two types of branches: plagiotropic (vegetative branches) and orthotropic (floriferous branches). However, the leaf of *G. mirabile* is oblong and subcoriaceous with an acute apex, while the leaves of *Glochidion* upop 3 are orbicular or ovate shaped and coriaceous with a rounded apex. In contrast, *G. kaweesakii*, *Glochidion* unidentified population 1 (*Glochidion* upop 1), and *Glochidion* unidentified population 2 (*Glochidion* upop 2) are similar in that they have a non-swollen stem base, only plagiotropic branches. They differ mostly in leaf characters. The leaves of *G. kaweesakii* are elliptic or ovate in shape, 2.3-8 × 1-4 cm in size, glabrous or glabrescent on the lower surface, while

Glochidion upop 1 and *Glochidion* upop 2 have oblong leaves with 2-4 × 0.5-1.5 cm in size, pubescent on the lower surface. The *Glochidion* upop 1 is distinct by the rounded or obtuse leaf apex and green branches, and *Glochidion* upop 2 is different in having an acute leaf apex and reddish branches.

Plants in isolated habitats face high extinction risks due to genetic variety loss caused by genetic drift and inbreeding (Kang et al. 2005). Maintaining the genetic diversity of endemic plants is crucial for their survival and adaptability in changing environments (Salgotra and Chauhan 2023). Thus, this information is essential for biodiversity conservation and management (Teixeira and Nazareno 2021; Guo 2022). DNA-based molecular markers have proven effective in assessing genetic diversity in endemic and endangered plants (Rameshkumar et al. 2019; Bagheri et al. 2020; Rahali et al. 2022; Wang et al 2023; Mahdavikia et al. 2024; Haque 2025). Start Codon Targeted (SCoT) markers are one such marker that is widely used for species identification and genetic diversity analysis in several endemic species (Thakur et al. 2016; Nitiworakarn et al. 2023; Bidyananda et al. 2024). Compared to the other markers, such as RAPD and ISSR, SCoT markers are favored for their high annealing temperature, enhancing amplification reliability (Abouseadaa et al. 2020). Targeting coding regions with single primers, SCoT markers offer higher polymorphism and reproducibility, providing more precise genetic information to improve endemic plant conservation (Rai 2023; Rizk et al. 2024). Despite their widespread application for endemic species (Hashim et al. 2021; Yu et al. 2021; Trieu et al. 2022), SCoT markers have not yet been used to study members of Phyllanthaceae.

This study is the first attempt to assess the genetic diversity of *Glochidion* subgenus *Phyllanthodendron* using SCoT markers based on samples from limestone hill habitats across Thailand and Laos. The primary objectives were to evaluate genetic diversity, genetic relationships, and population structure. It is hypothesized that the fragmented nature of limestone habitats may lead to genetic differentiation among populations through geographic isolation. The results aim to provide valuable information to support conservation strategies, especially for endemic species within the genus *Glochidion*.

MATERIALS AND METHODS

Plant materials

A total of 101 individuals were collected, comprising 5 individuals of *Glochidion kaweesakii*, 78 individuals of *Glochidion mirabile,* and 18 individuals from three populations of unidentified individuals of *Glochidion* (upop 1 to 3) from localities at various limestone hill habitats across Thailand and adjacent Laos (Figure 1). Geographical regions were defined based on floristic provinces in Thailand (Welzen et al. 2011). In the case of Laos, given the lack of a standard published floristic province division, this study adopts a division into three regions (North, Central and South) (Epprecht et al. 2008). Fresh leaf samples were dried in silica gel immediately after collection. For each population, three to five individuals were sampled. Voucher specimens for each population were prepared and kept in the KKU herbarium (Table 1).

DNA extraction

Fresh young leaf samples from all 101 individuals were dried and stored in silica gel. Total genomic DNA was extracted from 20 mg dry leaf tissue using the 2X CTAB (Cetyltrimethylammonium bromide) method (Cota-Sánchez et al. 2006). The genomic DNA quality was checked using 1% agarose gel electrophoresis, then visualized under ultraviolet (UV) light. DNA quantity was determined with a NanoDrop spectrophotometer by measuring absorbance at 260-280 nm and allowed DNA concentrations to range from 20 to 200 ng/µL. Extracted DNA samples were stored until further use at -20°C.

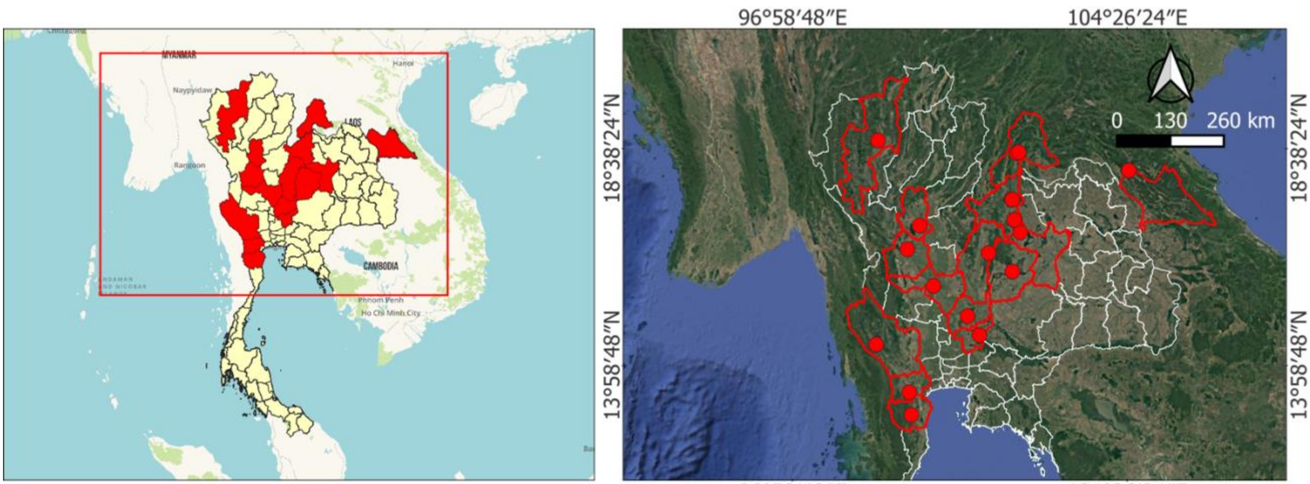

Figure 1. Location of the 22 populations of *Glochidion* subg. *Phyllanthodendron* that were sampled in Thailand and Laos. The map was generated using QGIS

Table 1. Genetic polymorphism of 22 populations of *Glochidion mirabile* and *G. kaweesakii* and unidentified *Glochidion* populations in Thailand and adjacent Laos, as revealed by seven SCoT primers

Primers ID	Sequences (5' to 3')	% GC	Amplicon size (bp)	TNL	NPL	PPL (%)	PIC
SCoT 2	CAACAATGGCTACCACCC	56	320-3000	30	30	100	0.28
SCoT 14	ACGACATGGGCACACGC	67	150-1750	35	35	100	0.21
SCoT 21	ACGACATGGCGACCCCACA	61	150-2000	29	29	100	0.30
SCoT 23	CACCATGGCTACCACCAG	61	150-1300	26	26	100	0.25
SCoT 30	CCATGGCTACCACCGGCG	72	200-2000	33	33	100	0.27
SCoT 34	ACCATGGCTACCACCGCA	61	150-3000	39	39	100	0.24
SCoT 36	GCAACAATGGCTACCACC	56	150-2000	23	23	100	0.29
Mean				31	31	100	0.26
Total				215		-	-

Note: TNL: Total number of loci, NPL: Number of polymorphic loci, PPL: Percentage of polymorphic loci, and PIC: Polymorphic information content

PCR amplification

Eight SCoT primers developed by Collard and Mackill (2009) were initially selected for PCR amplification based on the previous study by Duanyai et al. (2021) (Table 1). However, only seven primers were used for further analysis due to their consistent reproducibility across all samples used in this study and the clarity of the band visualization. Each 10 µL PCR reaction contained 50-60 ng (1 µL) of template DNA, 5 µL of 2XTaq Master Mix [Taq DNA Polymerase (0.05 U/ µL), 2X ViBufferA (100 mM KCl, 20 mM Tris HCl (pH 9.1 at 20°C) and 0.02% Triton™MX-100), 0.4 mM dNTPs and 3.0 mM $MgCl_2$, Vivantis], 0.1 µL of SCOT primer (25 µM, Vivantis), and nuclease-free water to a final quantity of 10 µL. The thermal cycling conditions included initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, which was applied for all primers, and extension at 72°C for 2 min with a final extension at 72°C for 10 min. Amplified PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV illumination. All PCR amplifications were performed in two independent reactions for each sample to ensure the accuracy and reproducibility of the results.

Data analysis

SCoT primer profiles were converted into binary data matrices, where bands were scored as either present (1) or absent (0). Only clear and reproducible bands were considered. The resulting binary data were used for calculating the total number of loci (TNL), Polymorphic Information Content (PIC), percentage of polymorphic loci (PPL), and genetic diversity parameters, including the number of polymorphic loci (NPL), effective number of alleles (Ne), observed number of alleles (Na), Nei's gene diversity (H), Shannon's information index (I) (Kanaka et al. 2023) using POPGENE version 1.32 (Yeh et al. 1999). Nei's expected heterozygosity (He) was calculated using GenAlEx 6.51b2 (Peakall and Smouse 2006). For species with more than one population (17 populations of *G. mirabile* and 2 populations of *Glochidion* upop 3), analysis of molecular variance (AMOVA) was performed to examine genetic differentiation within and among populations. The AMOVA was performed based on a binary distance matrix using GenAlEx 6.51b2, with 999 permutations. Additional estimates such as mean within population genetic diversity (Hs), genetic differentiation coefficient among different populations (Gst), and gene flow (Nm = 0.5(1-Gst)/Gst) were computed using POPGENE version 1.32 (Yeh et al. 1999). The Mantel test was performed to test the correlation between geographic and genetic distance using GenAlEx 6.51b2, with 999 permutations (Mantel 1967; Peakall and Smouse 2006). Clustering analysis was carried out using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method in PAST (Paleontological Statistics) version 4.02. (Hammer et al. 2001). Principal coordinate analysis (PCoA) was conducted to visualize the distribution of individuals based on Euclidean distance and was performed using PAST (Paleontological Statistics) version 4.02. (Hammer et al. 2001). Population structure was inferred using the admixture model implemented in STRUCTURE (version 2.3.4) (50,000 burn-in, 50,000 Markov Chain Monte Carlo (MCMC) iterations) with correlated allele frequencies and sampling location as a prior (LOCPRIOR). It performed 10 repetitions for each hypothesized number of genetic clusters (K), which ranged from 1 to 6. STRUCTURE HARVESTER was utilized to determine the optimal K using the approach outlined in Evanno et al. (2005).

RESULTS AND DISCUSSION

SCoT polymorphism

Seven SCoT primers were used to distinguish species and analyze genetic diversity. A total of 215 fragments were amplified, all of which were polymorphic. The amplified DNA fragment size ranged from 150 bp to 3000 bp, depending on the primer used (Table 1). The number of polymorphic fragments per primer ranged from 23 (SCoT 36) to 39 (SCoT 34), with an average of 31 fragments per primer. Each primer showed 100% polymorphism. The average polymorphic information content (PIC) was 0.26, with values ranging from 0.21 to 0.30.

Genetic diversity and genetic differentiation

The genetic diversity parameters of the 22 populations are summarized in Table 2. The SCoT analysis revealed that the number of observed alleles (Na) ranged between 1.03 and 1.29. The percentage of polymorphic loci (PPL) varied from 2.79% (pop 11) to 28.84% (pop 2). Shannon's information index (I) ranged from 0.02 to 0.17, and the expected heterozygosity ranged from 0.01 to 0.12, which is also confirmed by Nei's gene diversity (H) values ranged from 0.01 (Pop 11) to 0.12 (Pop 2). Population 11 exhibited the lowest genetic diversity across all parameters, while Population 2 exhibited the highest genetic diversity. However, overall genetic diversity parameters across *Glochidion* subg. *Phyllanthodendron* in this study remains low (Table 2).

Analysis of molecular variance (AMOVA) was conducted on 17 populations of *G. mirabile* and two populations of the *Glochidion* upop 3 but could not be conducted for *G. kaweesakii*, *Glochidion* upop 1, and *Glochidion* upop 2 because we had only been able to sample a single population of each. In *G. mirabile,* there was a higher proportion of genetic variation among populations (59%) than within populations (41%). This result was supported by a low mean within-population diversity (Hs = 0.07) and a high genetic differentiation index among populations (Gst = 0.67). Similarly, *Glochidion* upop 3 exhibited 63% variation among populations and 37% within populations, also corresponding to low Hs (0.03) and high Gst (0.66) values (Table 3). The Mantel test resulted in a significant positive correlation between the genetic and geographic distance (r = 0.471 and P = 0.001) (Figure 2).

Clustering analysis and principal coordinate analysis (PCoA)

The clustering analysis and the PCoA revealed that the 101 individuals are grouped into three main clusters corresponding to species (Figures 3, Figure 4). Group I included 78 individuals of *G. mirabile* and eight individuals of the *Glochidion* upop 3. Group II consisted of five individuals, each from the *Glochidion* upop 1 and the *Glochidion* upop 2. Group III comprised five individuals of *G. kaweesakii*.

Population structure

The population structure of the 101 individuals from 22 populations was assessed using STRUCTURE V2.3.4. Evanno's method identified a definite peak in ΔK at K = 2 (Figure 5.A). Thus, K = 2 is the most likely number of genetic clusters within the entire dataset and separates 22 populations into two clusters. The mean likelihood, LnP(K), and standard deviation for each value of K have been shown in Figure 5.B. Cluster I included one population of *G. kaweesakii* (Pop 1), two populations of *G. mirabile* from northeastern Thailand (Pop 2 and Pop 3), three populations of *G. mirabile* from Laos (Pop 18, Pop 19, and Pop 21), *Glochidion* upop 1 (Pop 21), and *Glochidion* upop 2 (Pop 22). Cluster II consisted of 12 populations of *G. mirabile* from East, North, Central, and Southwestern parts of Thailand (Pops 4-9 and 12-17) and two populations of *Glochidion* upop 3 (Pops 10 and 11) (Figure 5.C).

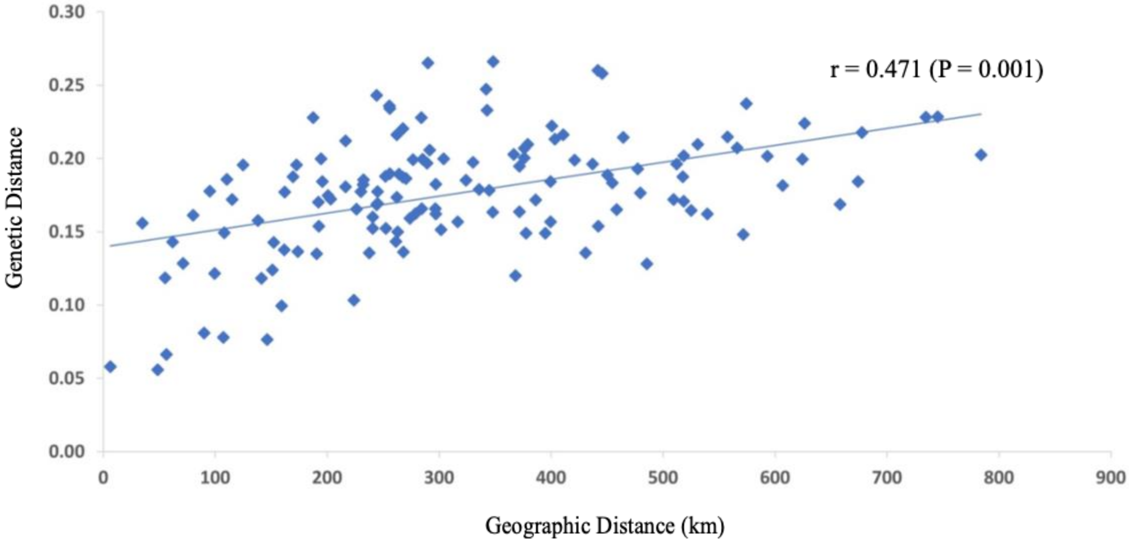

Figure 2. Mantel tests of genetic distance and geographical distance for populations of *Glochidion mirabile* performed using GenAlEx 6.51b2 (Peakall and Smouse 2006). Each point represents Nei's genetic distance plotted against geographic distance

Table 2. Genetic diversity parameter for 22 populations of *Glochidion* subg. *Phyllanthodendron* from Thailand and adjacent Laos by POPGENE and GenAlEx

Species	**Pop ID**	**Location**	**Voucher materials**	**N**	**Na**	**Ne**	**I**	**H**	**He**	**PPL (%)**
Glochidion kaweesakii	Pop 1	Pha Khao District, Loei Province, Thailand	ZL. Ilmi et al. 2 (KKU)	5	1.13±0.34	1.09±0.25	0.08±0.20	0.05±1.39	0.05	13.49
Glochidion mirabile	Pop 2	Pha Khao District, Loei Province, Thailand	ZL. Ilmi et al. 3 (KKU)	5	1.29±0.45	1.21±0.35	0.17±0.27	0.12±0.19	0.12	28.84
Glochidion mirabile	Pop 3	Muang District, Loei Province, Thailand	ZL. Ilmi et al. 4 (KKU)	5	1.27±0.44	1.19±0.34	0.15±0.26	0.11±0.18	0.11	26.98
Glochidion mirabile	Pop 4	Sri Chomphu District, Khon Kaen Province, Thailand	ZL. Ilmi et al. 5 (KKU)	5	1.21±0.40	1.16±0.34	0.13±0.25	0.09±0.18	0.09	20.93
Glochidion mirabile	Pop 5	Chon Daen District, Phetchabun Province, Thailand	J. Duanyai, T. Srisuk 93 (KKU)	5	1.25±0.43	1.16±0.31	0.14±0.25	0.09±0.17	0.09	25.12
Glochidion mirabile	Pop 6	Chai Badan District, Lopburi Province, Thailand	J. Duanyai et al. 118 (KKU)	5	1.14±0.35	1.10±0.28	0.08±0.21	0.06±0.15	0.06	13.95
Glochidion mirabile	Pop7	Khon San District, Chaiyaphum Province, Thailand	J. Duanyai, T. Srisuk 115 (KKU)	3	1.08±0.27	1.05±0.20	0.05±0.16	0.03±0.10	0.03	7.91
Glochidion mirabile	Pop 8	Chiang Dao District, Chiang Mai Province, Thailand	J. Duanyai, T. Srisuk 83 (KKU)	5	1.16±0.37	1.11±0.28	0.09±0.21	0.06±0.15	0.06	16.28
Glochidion mirabile	Pop 9	Sai Yok District, Kanchanaburi Province, Thailand	K. Keeratikiat P1 (KKU)	3	1.14±0.35	1.12±0.30	0.09±0.22	0.06±0.16	0.06	13.95
Glochidion upop 3	Pop 10	Sai Yok District, Kanchanaburi Province, Thailand	K. Keeratikiat P2 (KKU)	5	1.13±0.34	1.08±0.23	0.07±0.19	0.05±0.13	0.05	13.49
Glochidion upop 3	Pop 11	Phou Pha Marn, Kham Mouane Province, Laos	ZL. Ilmi et al. 10 (KKU)	3	1.03±0.16	1.02±0.11	0.02±0.19	0.01±0.06	0.01	2.79
Glochidion mirabile	Pop 12	Thung Saliam District, Sukhothai Province, Thailand	J. Duanyai et al. 146 (KKU)	5	1.19±0.39	1.13±0.30	0.11±0.23	0.07±0.16	0.07	19.07
Glochidion mirabile	Pop 13	Muak Lek District, Saraburi Province, Thailand	ZL. Ilmi et al. 1 (KKU)	5	1.18±0.38	1.12±0.29	0.10±0.22	0.07±0.15	0.07	17.67
Glochidion mirabile	Pop 14	Banphot Phisai District, Nakhon Sawan Province, Thailand	J. Duanyai et al. 131 (KKU)	5	1.23±0.42	1.15±0.30	0.13±0.24	0.08±0.16	0.08	22.79
Glochidion mirabile	Pop 15	Phran Kratai District, Kamphaeng Phet Province, Thailand	J. Duanyai et al. 144 (KKU)	2	1.07±0.25	1.05±0.17	0.04±0.15	0.03±0.10	0.03	6.51
Glochidion mirabile	Pop 16	Cha-am District, Phetchaburi Province, Thailand	J. Duanyai et al. 2 (KKU)	5	1.21±0.40	1.13±0.28	0.11±0.23	0.08±0.16	0.08	20.93
Glochidion mirabile	Pop 17	Pak Tho District, Ratchaburi Province, Thailand	J. Duanyai et al. 21 (KKU)	5	1.16±0.36	1.11±0.28	0.09±0.22	0.06±0.15	0.06	15.81
Glochidion mirabile	Pop 18	Na Yang village, Muang Feuang, Vientiane Province, Laos	ZL. Ilmi et al. 6 (KKU)	5	1.06±0.23	1.05±0.20	0.04±0.15	0.03±0.11	0.03	6.05
Glochidion mirabile	Pop 19	Xinxai Yaram Temple, Muang Feuang, Vientiane Province, Laos	ZL. Ilmi et al. 7 (KKU)	5	1.16±0.37	1.10±0.24	0.09±0.20	0.06±0.14	0.06	16.28
Glochidion mirabile	Pop 20	Phou Pha Marn, Kham Mouane Province, Laos	ZL. Ilmi et al. 8 (KKU)	5	1.21±0.40	1.14±0.30	0.12±0.24	0.08±0.17	0.08	20.93
Glochidion upop 1	Pop 21	Muang District, Loei Province, Thailand	ZL. Ilmi et al. 11 (KKU)	5	1.14±0.35	1.09±0.23	0.08±0.19	0.05±0.13	0.05	14.42
Glochidion upop 2	Pop 22	Phou Pha Marn, Kham Mouane Province, Laos	ZL. Ilmi et al. 9 (KKU)	5	1.17±0.37	1.10±0.24	0.09±0.20	0.06±0.14	0.06	16.74

Note: N: Sample size, Na: Observed number of alleles; Ne: Effective number of alleles, I: Shannon's information index, H: Nei's gene diversity, He: Expected heterozygosity, PPL (%): Percentage of polymorphic loci

Table 3. Molecular variance (AMOVA) for 17 populations of *Glochidion mirabile* and two populations of *Glochidion* upop 3

Species	**Source of variation**	**df**	**SS**	**MS**	**Est. Var.**	**%**	**Phi$_{pt}$**	**P value**	**H$_S$**	**G$_{ST}$**	**Nm**
Glochidion mirabile	Among population	16	1286.74	80.42	15.27	59	0.05	0.001	0.07	0.67	0.24
	Within population	61	640.33	10.49	10.49	41					
	Total	77	1927.08		25.77	100					
Glochidion upop 3	Among population	1	43.20	43.20	9.97	63	0.63	0.017	0.03	0.66	0.25
	Within population	6	34.80	5.80	5.80	37					
	Total	7	78.00		15.77	100					

Note: df: degrees of freedom, SS: sum of squares, MS: mean squares, Est. Var.: estimated variance components; %: percentage of total variance contributed by each component, H$_S$: the mean within-population genetic diversity, G$_{ST}$: genetic differentiation coefficient among populations, Nm: estimated gene flow among populations, calculated as Nm = 0.5 (1-GST)/GST

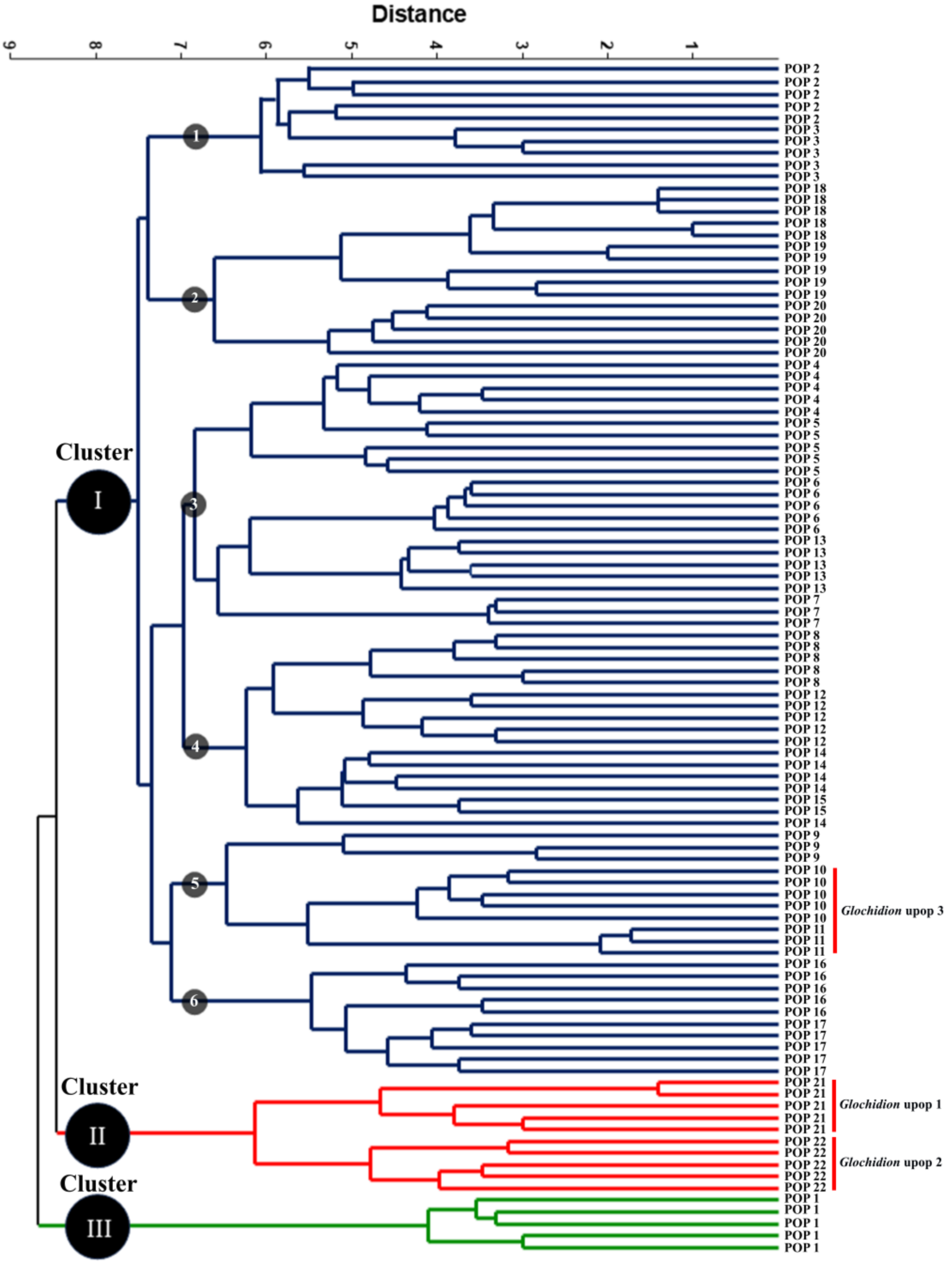

Figure 3. Dendrogram based on UPGMA analysis using Nei's genetic distance showing the genetic relationships among 101 individuals of *Glochidion* subg. *Phyllanthodendron* sampled in Thailand and adjacent Laos. Group I corresponds to *Glochidion mirabile* and the *Glochidion* upop 3. Group II corresponds to five individuals of the *Glochidion* upop 1 and five individuals of the *Glochidion* upop 2. Group III corresponds to five individuals of *G. kaweesakii*. ❶-❻ indicate subgroups 1-6

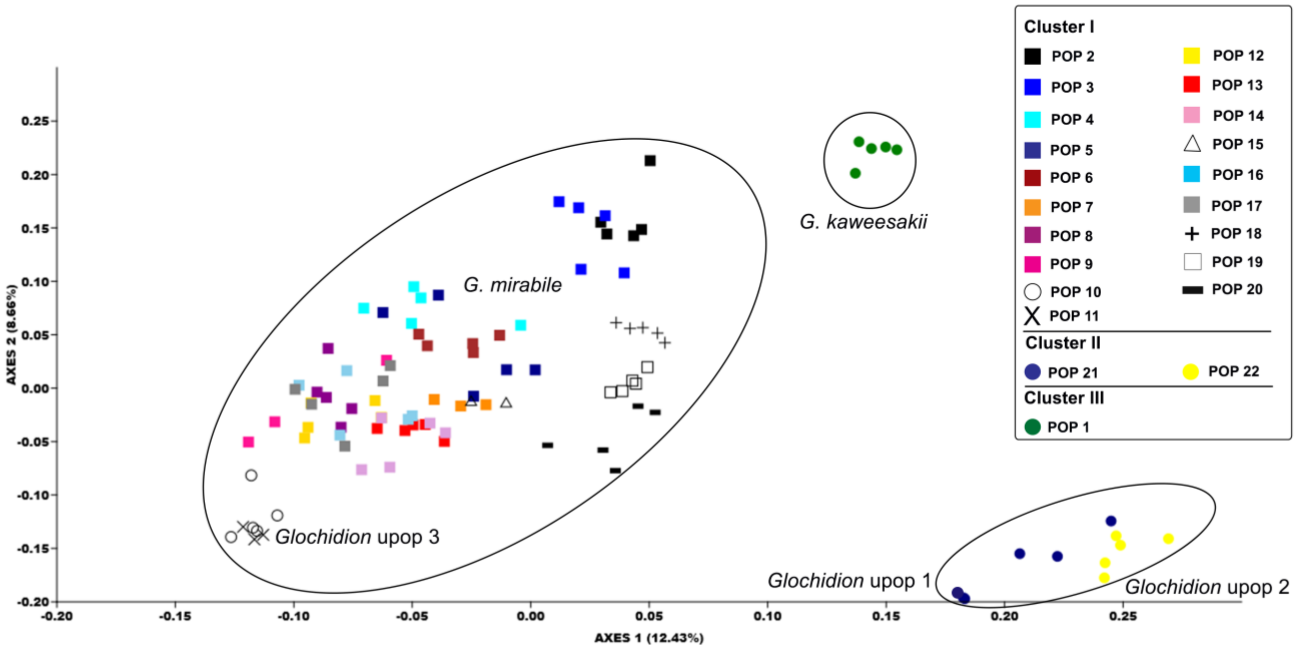

Figure 4. Principal Coordinate Analysis (PCoA) of 22 *Glochidion* subg. *Phyllanthodendron* populations, based on Euclidean distance and were performed using PAST (PAleontological STatistics) version 4.02. (Hammer et al. 2001). The analysis reveals genetic relationships among populations based on coordinate distribution. The plane of the first three axes of the PCoA explained a total of 29.05% of the genetic variation, with Axis 1 = 12.43%, Axis 2 = 8.66% and Axis 3 = 7.96%. All accessions correspond to the sample list in Table 2

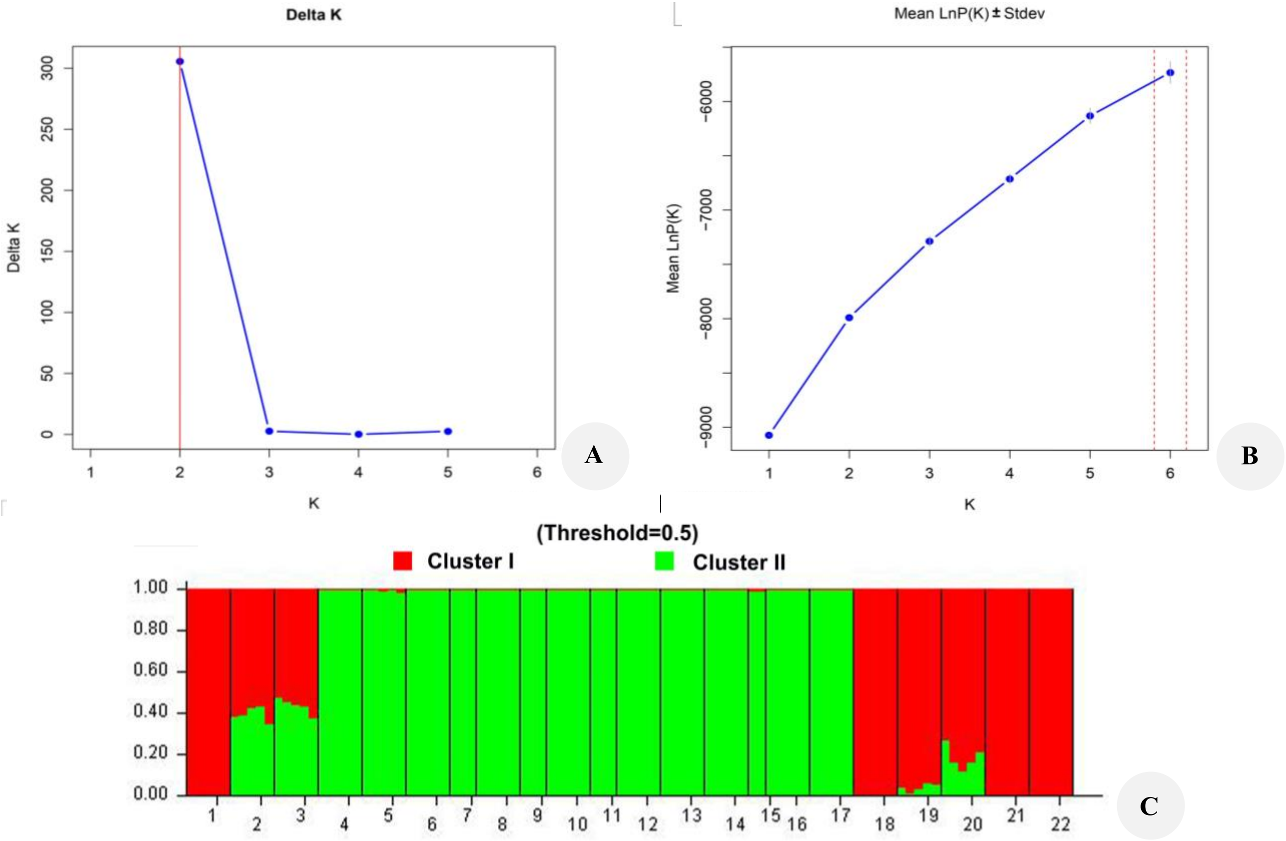

Figure 5. Structure analysis of 22 populations of *Glochidion* subg. *Phyllanthodendron* from Thailand and adjacent Laos as inferred by STRUCTURE. A. *ΔK* value for different number of assumed populations based on Evanno's method ran by STRUCTURE HARVESTER with the maximum of measure ΔK was found to be K = 2, B. The mean likelihood, LnP(K), and standard deviation for each value of K, C. Bar plot representation for K = 2 with population IDs (1-22) correspond to Table 2. The different colors in the bar plot represent different genetic clusters (Cluster I and Cluster II)

Discussion

The effectiveness of SCoT primers

There is no previous research on the use of the SCoT markers in *Glochidion* subg. *Phyllanthodendron*. Here, all seven primers used produced 100% polymorphic bands. These results indicate a higher-level polymorphism than reported by El-Hawary et al. (2021), who observed only 48% polymorphic bands in several species of *Euphorbia* (Euphorbiaceae) using SCoT markers. The high number of polymorphic bands suggests that the SCoT markers are effective in detecting genetic variation within *Glochidion* subg. *Phyllanthodendron*. The average polymorphic information content (PIC) was 0.26, which is comparable to the findings of Yang et al. (2019), who reported a PIC value of 0.26 in the Chinese grass *Miscanthus lutarioriparius* using SCoT markers. According to Kanaka et al. (2023), markers with PIC values above 0.5 are highly informative, those between 0.25 and 0.50 are moderately informative, and those below 0.25 are less informative. Based on this classification, the SCoT markers used in this study are moderately informative, and thus appropriate for preliminary assessments of genetic diversity.

Genetic diversity and differentiation

Species of *Glochidion* subg. *Phyllanthodendron* typically grows in limestone habitats (Chantaranothai 2007; Pornpongrungrueng et al. 2017). Geographic isolation of smaller populations affects not only species biodiversity but also genetic diversity (Qi et al. 2023). In this study, the lowest level of genetic diversity was found in the *Glochidion* upop 3 (Pop 11). This low diversity may be attributed to the small sample size of three individuals and the species' restricted distribution, found only in limestone habitats in Khammouanne Province in Laos and Kanchanaburi Province in Thailand. However, small sample sizes often lead to significant errors in estimating allelic richness, a key indicator of genetic diversity (Bashalkhanov et al. 2023); thus, increasing the sample size could improve the robustness of the analyses. The highest genetic diversity was observed in *G. mirabile* population 2, though overall genetic diversity across *G. mirabile* remains low (Table 2). Similarly, *G. kaweesakii*, *Glochidion* upop 1, and *Glochidion* upop 2 also exhibited low levels of genetic variations based on all parameters. The expected heterozygosity (He) ranged from 0.01 to 0.12, lower than that reported for the endemic *Camellia dilinhensis* using SCoT markers (He = 0.21) (Trieu et al. 2022). Low expected heterozygosity indicates a reduced number of alleles with equal frequency in the population (Kanaka et al. 2023).

Low genetic diversity at the species level was also revealed in the AMOVA results. Genetic differentiation based on Gst values is typically categorized as low (<0.05), moderate (0.05-0.15), or high (>0.15) (Li et al. 2018). Here, *G. mirabile* had a high level of differentiation among populations (Gst = 0.67, Nm = 0.24) and *Glochidion* upop 3 also had a similar level (Gst = 0.66, Nm = 0.25). In *G. mirabile*, 59% of the total genetic variation was among populations, while in *Glochidion* upop 3, it was 63%. This finding differs from *Phyllanthus emblica*, widely distributed species that occupies diverse habitats, which exhibit higher genetic variation within populations, but lower genetic differentiation among populations (Liu et al. 2020). The difference may be attributed to the fact that the species investigated in this study are geographically restricted and occur in isolated limestone habitats, which might limit gene flow among populations. In general, plant species with geographically restricted distribution often possess reduced genetic diversity and higher genetic variation among populations compared to widespread species. This case is also found in *Camellia huana*, the endangered species with a narrow distribution in limestone hills of northern Guangxi and southern Guizhou Provinces, China, that exhibits a small distribution range and low level of genetic diversity (Li et al. 2020). Additionally, the Mantel test indicated a positive correlation between geographic and genetic distance among populations, suggesting that geographic isolation may have contributed to the observed genetic structure of *G. mirabile*. This pattern is similar to that found in another isolated limestone species, *Ceiba pubiflora* (Brandão et al. 2024). Although inbreeding was not directly assessed in this study, the observed low gene flow and geographic isolation among populations, as well as small population size, may increase the potential risk of inbreeding, which could lead to reduced within-population diversity and increased inter-population differentiation. The observed low genetic diversity in *Glochidion* species might reflect natural adaptation to their unique environments. Similarly, Yan et al. (2019) suggested that limited gene flow caused by habitat fragmentation and ecological specialization in *Mallotus oblongifolius* facilitated local adaptation and contributed to genetic divergence among populations.

Genetic relationships and population structure

The UPGMA analysis grouped 78 individuals of *G. mirabile* and *Glochidion* upop 3 together, and 6 subgroups of *G. mirabile* were identified: Subgroup 1 consists of samples from northeastern Thailand. This subgroup clustered in the same clade as Subgroup 2. Subgroup 2 consists of populations from Laos, located in the northern and central parts of Laos. This suggests a linear distribution of geographical connections between *G. mirabile* habitats in northeastern Thailand and northern Laos. Subgroup 3 includes several populations from northeastern Thailand, eastern Thailand, and central Thailand. Subgroup 4 consists of populations from northern Thailand. Subgroup 5 comprises one population from southwestern Thailand and two populations of *Glochidion* upop 3. Subgroup 6 includes two populations from southwestern Thailand. However, the populations from southwestern Thailand clustered together in the same clade at a distance >7 (Figure 3). This result indicated that *Glochidion* upop 3 shares genetic similarity with *G. mirabile* and is temporarily suggested to represent a variation of *G. mirabile*. *Glochidion* upop 1 and *Glochidion* upop 2 were clearly confirmed as members of the subgenus *Phyllanthodendron* due to their significant genetic similarities. High genetic similarity was also discovered between *Glochidion* upop 1 and *Glochidion* upop 2. Thus, because they differ in morphology, the two taxa should be separated. However, additional formal taxonomic work is required to confirm this status.

SCoT markers produced clear results for categorizing the population structure of *Glochidion* subg. *Phyllanthodendron* in Thailand and Laos. The 101 analyzed individuals of *Glochidion* subg. *Phyllanthodendron* were differentiated according to populations and geographical distribution, except for *Glochidion* upop 3 (Pop 11), which was found in Khammouanne, Laos, and grouped with *G. mirabile* and *Glochidion* upop 3 from southwestern Thailand. *Glochidion kaweesakii*, *G. mirabile* (northeastern Thailand and Laos), along with *Glochidion* upop 1 and *Glochidion* upop 2 clustered together in Cluster I. This suggests that the habitats of those populations in Cluster I are connected by a linear distribution, which has directly influenced the genetic structure of these populations and is related to their geographical distribution. *Glochidion* upop 1 and *Glochidion* upop 2 were included in this study due to their similar morphological characteristics with *G. mirabile* and *G. kaweesakii*, and their occurrence in limestone hill habitats. This statement is supported by the result of the bar plot in population structure analysis, which grouped *Glochidion* upop 1 and *Glochidion* upop 2 together with G. *kaweesakii* and several populations of *G. mirabile*.

The UPGMA and STRUCTURE analyses yielded different numbers of genetic clusters (three and two clusters, respectively). This discrepancy stems from the fundamental methodological differences: UPGMA is a distance-based, hierarchical clustering method, while STRUCTURE uses a Bayesian approach with strict assumptions, including Hardy-Weinberg and linkage equilibrium. These differing mathematical models often lead to varied cluster inferences, especially in populations experiencing complex or recent admixture. Moreover, the limitation of the current sample size may further contribute to this inconsistency between the analyses. To establish robust species boundaries and confirm the taxonomic status of these populations, further integrative studies, including morphological and DNA sequence data, are required. Therefore, the current taxonomic conclusions derived solely from these analyses are subject to further verification.

In conclusion, the genetic analyses revealed clear population structuring and low genetic diversity among *Glochidion* subg. *Phyllanthodendron* populations in Thailand and Laos. The distinct genetic structure of *Glochidion* upop 1 and 2 supports the hypothesis that these may represent potentially distinct lineages, but extensive morphological and molecular studies are required to confirm their taxonomic status. *Glochidion* upop 3 clusters within *G. mirabile* in all our genetic analysis, and we recommend that the morphologically based taxonomic circumscription of that species should be amended to include the variation found in *Glochidion* upop 3. We found low genetic diversity at the species level in *Glochidion* subg. *Phyllanthodendron* populations in Thailand and Laos. This may reduce the species' ability to adapt to changing environmental conditions and withstand various stressors, ultimately threatening their long-term survival as endemic species. However, the study was constrained by limited sampling for some populations and a sole reliance on SCoT markers. Future work should integrate additional genomic and morphological analyses to clarify taxonomic relationships and assess adaptive diversity. Moreover, genetic variation between populations was higher than within populations, which is likely a consequence of low gene flow and the geographic isolation of populations. Based on these findings, a comprehensive conservation strategy focusing on improving habitat connectivity, protection of remaining limestone ecosystems, and strategies that facilitate genetic exchange to sustain long-term population viability is needed. However, the environmental factors were not evaluated in this study, which limit ecological interpretation. More detailed landscape or habitat-fragmentation analyses are necessary to support these conservation recommendations.

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