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# Development of Microsatellite Markers to Assess Genetic Diversity and Population Structure in Mitragyna Parvifolia (Roxb.) Korth

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***Abstract:*** ***Mitragyna parvifolia (Roxb.) Korth., Rubiaceae, is an economically important timber tree species. To meet the increasing market demand for M. parvifolia, it is necessary to assess genetic diversity within individuals to accelerate genetic improvement. Microsatellites, or simple sequence repeats (SSRs), are the most widely used molecular markers in population genetic studies. The present study estimated genetic variation in M. parvifolia among 20 individuals collected from wild populations using 10 polymorphic SSR markers. Allelic data were used to calculate genetic diversity parameters, including genotype distance, the Shannon index, and pairwise relatedness. The Analysis of molecular variance (AMOVA) was also performed to assess the distribution of genetic variation within and among the individuals. Further genotypic distance analysis revealed a wide range of divergence (6–30), indicating both close kinship and distinct lineages among individuals. The Principal Coordinates Analysis (PCoA) explained 45.97% of the total variation across the first three axes, with clear clustering patterns among populations. Another observation from the Analysis of Molecular Variance (AMOVA) showed that most genetic diversity resides within individuals (72%), followed by among individuals within populations (16%) and among populations (12%). Further, the analysis indicated moderate genetic differentiation (FST = 0.124, p < 0.001), with gene flow estimated at Nm = 1.768, suggesting substantial interpopulation connectivity. Shannon's diversity index further supported high within-population diversity (sH = 0.559) compared to among-population variation (sH = 0.155). Pairwise relatedness estimates indicated that most individuals were genetically unrelated, confirming a broad and heterogeneous genetic base. The presence of population-specific alleles and moderate structuring highlights the importance of conserving diverse populations. The genetic diversity of M. parvifolia provides valuable insight for conservation strategies and future genetic improvement programs.***

***Keywords:*** ***Mitragyna* Parvifolia, *SSR Markers, Genetic Diversity, Genetic Improvement***

***Nomenclature:***
STR: Sathyamangalam Tiger Reserve
KL: Kerala Region
QTL: Quantitative Traits
AMOVA: Analysis of Molecular Variance

## I. INTRODUCTION

*Mitragyna parvifolia* (Roxb.) Korth., commonly known as Kadam/Kaim/Kalamb, is an indigenous, fast-growing tree species in the family Rubiaceae, native to South Asia, and is considered highly endangered. The genus *Mitragyna* comprises 10 species, primarily distributed in tropical and arid/semi-arid regions of Africa, India, China, Bangladesh, Myanmar, Sri Lanka, and Southeast Asia. Among them, seven species are found in Asia, and in India, 4 species, viz., M. parvifolia, M. tubulosa, M. rotundifolia, and M. diversifolia, are distributed. The chromosome number is n=22. *M. parvifolia* grows in semi-evergreen to moist deciduous forests up to 1200 m altitude and in ravine areas from plains. The growing demand for wood-based panels, driven by global population growth, which adversely affects the sustainable utilisation of forest resources, has led to ongoing efforts to identify alternative materials [1]. With the growing demand for woody raw materials, the need for alternatives has become inevitable. Alternative raw materials, such as underutilised species, fast-growing species, crops, and other plant fibres, will play an important role in balancing supply and demand [3]. The advantages of biomaterials over synthetic counterparts include low cost, high toughness, low density, high specific strength, good processability, and biodegradability [4].

*M. parvifolia* wood is pinkish-brown in colour, even-grained, moderately hard and durable if not exposed to wet conditions. It is used in construction, furniture making, agricultural implements, carved articles, and the paper industry. The wood bark yields cordage fibres [8]. It is credited with numerous medicinal properties and is widely used by tribal communities and other Ayurvedic practitioners [17]. The phytochemical studies reveal active compounds, including alkaloids, flavonoids, tannins, saponins, and glycosides, with anti-inflammatory, antimicrobial,

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antioxidant, and hepatoprotective effects [18]. Most forest tree species remain largely undomesticated and exhibit high levels of heterozygosity because of their predominantly outcrossing breeding systems [6], which results in a substantial amount of genetic diversity[15]. Genetic diversity acts as a crucial resource for improvement programmes aimed at developing well-adapted tree species or varieties. It forms the foundation of biological diversity, enabling species to adjust to changing environments and persist through natural selection. Populations with limited genetic variation are more susceptible to pests, diseases, pollution, climate change, and habitat destruction caused by human activities [14] [5]. The level and distribution of genetic diversity are greatly influenced by the mating system and gene flow among dispersed populations of the same species, which determine the extent of genetic isolation among individual tree populations [16].

Molecular markers have proven effective tools for evaluating genetic diversity across diverse species and populations. DNA markers are used in tree improvement programmes to quantify genetic diversity. DNA markers, *viz.* RAPD (Randomly Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeats), SSR (Simple Sequence Repeats) and AFLP (Amplified Fragment Length Polymorphism) are commonly used for studying the genetic diversity in various tree species. Among molecular markers, microsatellites (SSRs; Simple Sequence Repeats), which are codominant and multi-allelic, are widely distributed across genomes and highly polymorphic [10]. SSR markers have been used to determine genetic diversity in several species, including maize, rice, common beans and rubber trees [7]. For the past 20 years, SSRs have been the predominant markers for plant genotyping due to their high informativeness, codominant inheritance, multi-allelic variation, and strong experimental reproducibility, and their transferability among related species [9]. SSRs are useful in studies of diversity measured by genetic distance, in estimating gene flow and recombination rates, in constructing linkage maps, in mapping loci involved in quantitative traits (QTL), and in using marker-assisted selection [9]. Currently, RNA sequencing has become a popular high-throughput sequencing technology that enables the development of SSR markers due to its wide dynamic range, high accuracy, and strong sensitivity [2]. In addition, compared with genomic-derived SSRs, transcriptome-derived SSRs are characterised by high efficiency, strong transferability, and correlation with potential genes [21].

In this study, the aim was to determine the genetic diversity of *M. parvifolia.* This study highlights the extent of genetic variation in M. parvifolia, as detected by SSR markers. This study would help conserve M. parvifolia populations with high genetic diversity for the genetic improvement programme.

## II. MATERIALS AND METHOD

### A. Sample Collection

The genetic diversity of M. parvifolia was assessed using natural populations from four locations: Sirumalai (SM), Sathyamangalam Tiger Reserve (STR), Anamalai Tiger Reserve (ATR), and the Kerala Region (KL). A total of 20 M. parvifolia samples were collected from 4 natural populations (SM, STR, ATR, and KL). 5 individuals were selected from each population. Leaf samples were collected from each tree and were kept in cold storage, transported to the laboratory and stored at -20°C. (Table 1).

**Table I: Details of Sample Collection of *M. Parvifolia* in Different Locations**

| S. No | Sample Id | Latitiude | Longitude |
|---|---|---|---|
| 1 | SM 1 | 10°57'08.43 | 76°42'08.43 |
| 2 | SM2 | 10°56'23.36 | 76°41'48.87 |
| 3 | SM3 | 10°56'20.25 | 76°43'51.02 |
| 4 | SM4 | 10°22'41.82 | 78°04'01.27 |
| 5 | SM5 | 10°22'48.81 | 78°03'94.03 |
| 6 | KL43-6 | 11°16'31.0 | 76°19'51.5 |
| 7 | KL37-7 | 11°16'22.3 | 76°22'08.8 |
| 8 | KL34-8 | 11°16'24.7 | 76°22'07.1 |
| 9 | KL75-9 | 10°11'47.9 | 76°34'26.8 |
| 10 | KL58-10 | 10°14'27.0 | 76°4'01.5 |
| 11 | STR11 | 11◦36'29.14 | 77◦01'29.34 |
| 12 | STR12 | 11◦36'50.60 | 77◦01'19.84 |
| 13 | STR13 | 11◦41'02.12 | 77◦07'27.13 |
| 14 | STR14 | 11◦42'08.20 | 77◦07'20.48 |
| 15 | STR15 | 11◦43'58.06 | 77◦06'45.37 |
| 16 | ATR16 | 8◦41'31.32 | 77◦10'31.16 |
| 17 | ATR17 | 10◦27'03.32 | 76◦50'04.96 |
| 18 | ATR18 | 10◦26'47.35 | 76◦50'31.22 |
| 19 | ATR19 | 10◦26'42.92 | 76◦50'30.66 |
| 20 | ATR20 | 10◦26'40.43 | 76◦51'05.49 |

### B. DNA Extraction and Primer Optimization in Mitragyna

The total genomic DNA was extracted from the leaf samples (150 mg) of *M. parvifolia* using the ARBOREASY DNA isolation kit, ICFRE-IFGTB patent. The extracted DNA was treated with 2 µl of 50 mg/ml RNase and incubated at 37°C for 30 min. The presence of the DNA was confirmed through electrophoresis in ethidium-bromide-stained 1% agarose gels. The DNA was quantified using a spectrophotometer (Nanodrop 2000 UV-VIS spectrometer (Thermo Scientific, USA)) at absorbance A260nm/A280nm. All 20 samples were diluted in nuclease-free $H_2O$ to a final concentration of 25 ng/µl and stored at -20°C before amplification. A total of 20 primers were developed from the transcriptome sequences of Mitragyna, and 10 primers were selected for the genetic diversity study. (Table 2)

**Table II: List of Selected SSR Primers for *M Parvifolia*. F: Forward; R: Reverse Primers**

| S.No. | Oligo Name | Sequence Forward and Reverse | Annealing Temperature | Expected Product Size | Amplified Product Size |
|---|---|---|---|---|---|
| 1 | MP21(P1) | F- CCACACAAGAGAGCGACCTT | 59.35 | 129bp | 100- 150bp |
| | | R-TTGCCATCGGAGGTAGAGGA | | | |
| 2 | MP22(P2) | F- CTCTCCTCCTCTTCCTGCCT | 60.3 | 131bp | 100- 150bp |
| | | R- AAGATGATGCGGTCCTCCTG | | | |
| 3 | MP23(P3) | F- CAGCCCCAATCCTCCTCTTC | 60.3 | 227bp | 200 – 250bp |
| | | R – GCTCAACTGCAAATCCCCCT | | | |
| 4 | MP24(P4) | F – AAACCCCCAACTCCCGTCATC | 59.35 | 156bp | 150 – 200bp |
| | | R- CCCTGTTTCTACCACCACCCA | | | |
| 5 | MP25(P5) | F – AAGTTGGCAAAGCTCTCGGA | 59.35 | 121bp | 100- 150bp |
| | | R – CATCTCCCGACTGACTGACG | | | |
| 6 | MP26(P6) | F-TGTCTCCTGCACACCACTTG | 59.6 | 114bp | 100-150bp |
| | | R-GCCTTCTTTGGTCGATCCA | | | |
| 7 | MP29(P7) | F-AGTTGCCTCTTAGTTGCTCA | 58.6 | 106bp | 100-150bp |
| | | R-CCAAAGCCTTCGAACAAGCC | | | |
| 8 | MP9(P8) | F-ACGGAGCCTTCCCACTTTT | 56.9 | 242bp | 200 – 250bp |
| | | R-GGCATCACCCAGAATACGAA | | | |
| 9 | MP11(P9) | F- CACGGATGGGCTGATATGCT | 58.3 | 212bp | 200-250bp |
| | | R- TGCTTCTTCACAACAGGGCT | | | |
| 10 | MP 3(P10) | F-TCGATGCAGTTTGTCCATTG | 55.25 | 196bp | 150-200bp |
| | | R-CGCCCATTTCACCATACACAT | | | |

### C. PCR (Polymerase Chain Reaction) Optimization and Amplification of Mitragyna

The PCR amplification was carried out in a 10 µL reaction containing 1 µL of template DNA, 1 µL of Taq buffer, 0.4 µL of dNTP, 0.4 µL of $MgCl_2$, 0.4 µL of diluted forward and reverse primers each, 0.3 µL of Taq polymerase and 6.1 µL of sterile water. The final volume was made up to 10 µL. A master cycler gradient (Qiagen) was used with the following conditions: Initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 0.45 sec, 58.1°C – 60.3°C (primer-specific annealing temperature) for 40 sec, and elongation at 72°C for 55 sec and final extension at 72°C for 8 min.

### D. Separation and Detection of Amplified PCR Products

The amplification products were separated by gel electrophoresis on a 7% PAGE gel in 1× TAE buffer at a constant 200 V for 3 hours, then stained with silver nitrate solution. The gel was observed under white light provided by a transilluminator (Syngene G: Box). The size of the amplification products was estimated using a 50-bp molecular weight ladder (Thermo Fisher) and depended on the product size.

### E. Microsatellite Marker Data Analysis

Due to the tetraploid nature of M. parvifolia, allele information for 10 loci from selected individuals was assembled as co-dominant data. The samples were categorised on a sample-wise basis across all populations. The software GenAlex v 6.5[12] was used to estimate the following genetic diversity parameters at the individual level and population level: Number of alleles (Na), Effective number of alleles (Ne), Shannon information index (I), F statistics (FIS, FIT & FST), and Nie's genetic distance (1978) were estimated at the population level. Gene flow was calculated by using the formula Nm = 0.25 (1 − FST)/FST. Analysis of molecular variance (AMOVA) was used to determine genetic variation between and within populations.

## III. RESULTS AND DISCUSSION

Based on the tree's geographical origin, the individuals were grouped into 4 populations, namely Sirumalai, Sathyamangalam Tiger Reserve, Anamalai Tiger Reserve, and Kerala, using GenAlEx 6.502 [13]. Analysis of molecular variance (AMOVA) was also conducted to assess genetic variance among groups, among individual trees, and within each tree. Python was used to generate a dendrogram illustrating the hierarchical clustering of individuals.

### A. Genotypic Distance Analysis

Codominant genotypic distance matrices were generated among the 20 *Mitragyna parvifolia* individuals from four natural populations. The results revealed a broad range of genetic distances, reflecting both close kinship and considerable divergence. The minimum observed genetic distance was 6, while the maximum reached 30, indicating substantial variation among individuals (Table 3). Several pairs showed relatively low genetic distances (e.g., SM vs KL individuals), suggesting close genetic similarity. In contrast, other pairs, such as KL7 and ATR18, showed the greatest divergence, indicating the presence of genetically distinct lineages within the species. This wide distribution of genetic distances underscores the heterogeneous genetic background of *M. parvifolia* populations.

Overall, the distance matrix indicates that while some populations maintain genetically cohesive groups, others exhibit clear divergence, contributing to the broad genetic base of *M. parvifolia*. This variation supports the AMOVA results, confirming that a substantial portion of genetic diversity is within individuals and populations and that population structure is detectable.

**Table III: Genotypic Distance of *M. Parvifolia***

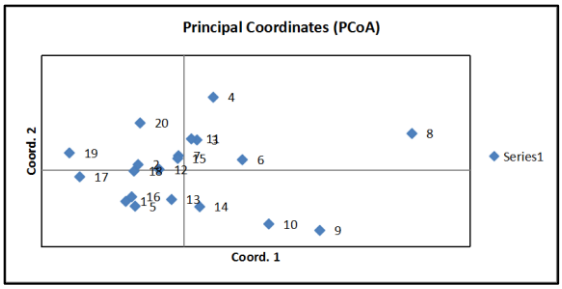
| 1 | 20 | 4 | 5 | 5 | 5 | 5 | 1 | 20 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PGD | GD | SM | KL | STR | ATR | | | | | | | | | | | | | | |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | |
| 0 | | | | | | | | | | | | | | | | | | | | 1 |
| 11 | 0 | | | | | | | | | | | | | | | | | | | 2 |
| 8 | 11 | 0 | | | | | | | | | | | | | | | | | | 3 |
| 16 | 11 | 6 | 0 | | | | | | | | | | | | | | | | | 4 |
| 10 | 7 | 15 | 20 | 0 | | | | | | | | | | | | | | | | 5 |
| 18 | 13 | 15 | 10 | 14 | 0 | | | | | | | | | | | | | | | 6 |
| 14 | 12 | 15 | 11 | 19 | 11 | 0 | | | | | | | | | | | | | | 7 |
| 30 | 25 | 22 | 22 | 30 | 24 | 25 | 0 | | | | | | | | | | | | | 8 |
| 21 | 15 | 19 | 21 | 17 | 15 | 18 | 17 | 0 | | | | | | | | | | | | 9 |
| 11 | 13 | 14 | 16 | 13 | 13 | 14 | 20 | 9 | 0 | | | | | | | | | | | 10 |
| 15 | 6 | 15 | 11 | 13 | 13 | 12 | 21 | 19 | 15 | 0 | | | | | | | | | | 11 |
| 10 | 5 | 11 | 10 | 12 | 12 | 5 | 22 | 13 | 11 | 7 | 0 | | | | | | | | | 12 |
| 7 | 9 | 10 | 12 | 13 | 12 | 8 | 23 | 12 | 8 | 10 | 4 | 0 | | | | | | | | 13 |
| 12 | 12 | 11 | 14 | 18 | 14 | 13 | 24 | 11 | 9 | 15 | 9 | 3 | 0 | | | | | | | 14 |
| 10 | 7 | 11 | 12 | 14 | 14 | 10 | 18 | 17 | 14 | 11 | 6 | 7 | 9 | 0 | | | | | | 15 |
| 11 | 13 | 14 | 17 | 16 | 14 | 11 | 27 | 18 | 15 | 18 | 8 | 5 | 7 | 10 | 0 | | | | | 16 |
| 12 | 10 | 17 | 18 | 12 | 18 | 13 | 28 | 19 | 17 | 15 | 8 | 9 | 13 | 10 | 8 | 0 | | | | 17 |
| 13 | 7 | 14 | 15 | 13 | 19 | 8 | 25 | 16 | 16 | 12 | 7 | 10 | 12 | 9 | 13 | 9 | 0 | | | 18 |
| 14 | 6 | 14 | 14 | 12 | 18 | 15 | 30 | 21 | 18 | 11 | 8 | 10 | 12 | 10 | 11 | 4 | 9 | 0 | | 19 |
| 15 | 12 | 10 | 8 | 16 | 14 | 13 | 22 | 21 | 17 | 15 | 10 | 10 | 12 | 11 | 10 | 6 | 13 | 6 | 0 | 20 |

## B. Principal Coordinates Analysis (PCoA)

The PCoA scatter plot visualizes the separation among the samples, with each point representing an individual sample. The samples are numbered from 1 to 20 on the plot (Fig 1). The coordinates represent the principal axes derived from the analysis: **Coord. 1** (x-axis): Represents the primary direction of variance (20.49%). **Coord. 2** (y-axis): Represents the secondary direction of variance (13.67%). This means that the first coordinate (principal component) explains 20.49% of the total variance, the second explains 13.67%, and the third axis contributes 11.81%. Together, the first three axes account for 45.97% of the total variation in the data (Table 4), indicating that these axes capture almost half of the structural information in the dataset. Samples that cluster together on the plot are more similar in genetic or measured traits, while those farther apart are more distinct. The plot helps to identify patterns of clustering, outliers, and relationships among the 20 samples across the 4 populations.

**Table IV: Percentage of Variation by Axes**

| Axis | 1 | 2 | 3 |
|---|---|---|---|
| Percentage (%) | 20.49 | 13.67 | 11.81 |
| Cumulative (%) | 20.49 | 34.16 | 45.97 |




**[Fig.1: Principal Coordinate Plot Representing Genetic Distance Among *M. parvifolia* Samples]**

## C. Analysis of Molecular Variance (AMOVA)

(Fig 2). The overall F-statistics (Table 6) further supported these observations: Fst = 0.124 (moderate genetic differentiation among populations; $p < 0.001$). Fis = 0.176 (indicating some degree of inbreeding within populations, $p = 0.003$). Fit = 0.278 (total differentiation across all hierarchical levels, $p < 0.001$). The number of migrants per generation (Nm) was estimated as 1.768, suggesting moderate gene flow among populations. The AMOVA results clearly demonstrate that the majority of genetic variation resides within individuals, which is typical for outcrossing tree species with sexual reproduction. The relatively low differentiation among populations (12%) indicates weak genetic structuring, likely maintained by ongoing gene flow.

**Table 5& 6: Analysis of Molecular Variance (AMOVA) of M. Parvifolia Accessions Based on Genotype with SSR Markers and F-Statistics**

| Source | Df | SS | Est. Var. | Total Var % |
|---|---|---|---|---|
| Among Pops | 3 | 18.625 | 0.339 | 12% |
| Among Indiv | 16 | 45.1 | 0.422 | 15% |
| Within Indiv | 20 | 39.5 | 1.975 | 72% |
| Total | 39 | 103.225 | 2.736 | 100% |

| F-Statistics | Value | P (Rand >= Data) |
|---|---|---|
| Fst | 0.124 | 0.001 |
| Fis | 0.176 | 0.003 |
| Fit | 0.278 | 0.001 |
| Fst max | 0.521 | |
| F'st | 0.238 | |
| Nm | 1.768 | |

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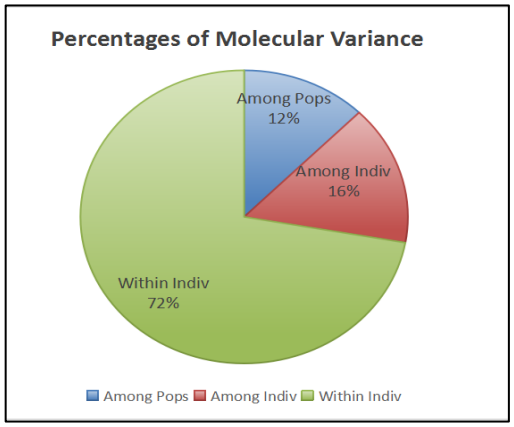



**[Fig 2: Distribution of Molecular Variance]**

### D. Population Genetic Structure

The estimated gene flow (Nm) among populations was 1.768, indicating moderate migration and gene exchange. The maximum FST observed was 0.521, indicating some loci show higher differentiation than average.

**Shannon's Diversity Index**

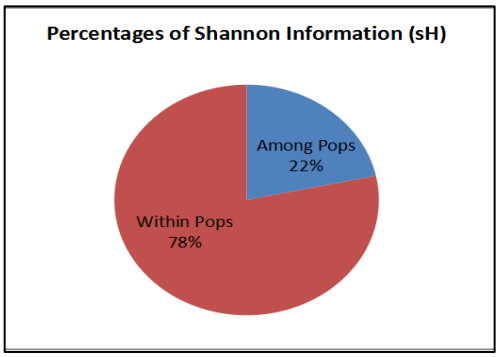
Shannon's informational diversity index (sH) was used to quantify genetic diversity within and among populations. (Fig 3)

Within populations: sH = 0.559 (\~78% of total variation).
Among populations: sH = 0.155 (\~22% of total variation).
Total Shannon Index (sH Tot) = 0.714.

The scaled Shannon diversity estimates also indicated substantial allele overlap among populations (O' = 0.476), indicating shared genetic variation across populations (Table 7). These results align with the AMOVA findings, highlighting that most of the genetic diversity in M. parvifolia resides within populations.

**Table VII: Summary Shannon Information**

| | DF | G-Test | Sh | Inform. | exp(sH) | D' | O'=1-D' | P (rand >= data) |
|---|---|---|---|---|---|---|---|---|
| Among Pops | 3 | 11.59 | 0.15 | 21.65 | 1.16 | 0.19 | 0.8 | 0.002 |
| Within Pops | 36 | 41.84 | 0.55 | 78.34 | 1.74 | 0.48 | 0.52 | 0.999 |
| Total | 39 | 53.43 | 0.71 | 100 | 2.04 | 0.52 | 0.47 | |



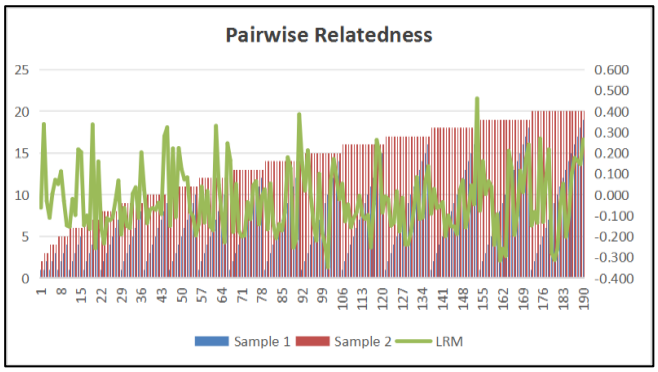

**[Fig.3: Scaled Diversity and Percentage of Shannon Information]**

### E. Pairwise Relatedness

Pairwise relatedness was estimated using the Lynch and Ritland (1999) method across the 190 possible pairwise comparisons (Fig 4). Mean relatedness (r) = -0.030, which is close to zero, indicating that most individuals are genetically unrelated. Median r = -0.063. Range of relatedness values: Minimum = -0.348 (strongly unrelated individuals). Maximum = 0.462 (moderately related individuals) (Table 8). The negative average relatedness suggests that the sampled individuals represent genetically diverse and largely independent genotypes. Only a few pairs showed moderate relatedness, reflecting localized kinship but not widespread inbreeding.

**Table VIII: Lynch & Ritland Estimator - Mean**

| | LRM |
|---|---|
| N | 190 |
| Sum | -5.765 |
| Mean | -0.03 |
| Median | -0.063 |
| SD | 0.157 |
| SE | 0.011 |
| Min | -0.348 |
| Max | 0.462 |




**[Fig.4: SSR-Based Pairwise Relatedness of M. Parvifolia Individuals]**

### F. Allele Frequencies

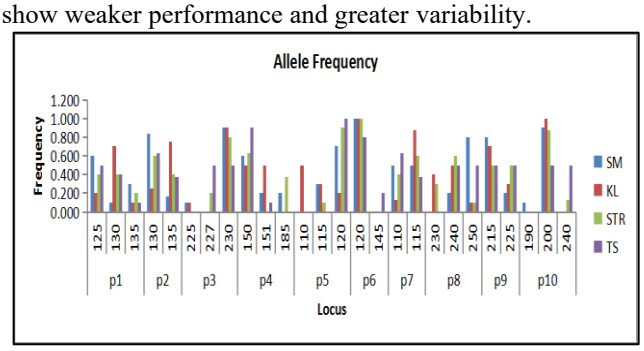
The allele frequency data across 10 loci (p1–p10) for the four populations (SM, KL, STR, and ATR) indicate that allele distributions vary across populations and loci (Table 9). The highest allele frequencies indicate loci with strong amplification, while variation among populations reflects polymorphism levels. Among all loci, locus p6 exhibits the highest and most consistent allele frequency across all populations — SM (1.000), KL (1.000), STR (1.000), and ATR (0.800). This indicates that the p6 primer is the most efficient, as it successfully amplified the target allele in all populations with minimal variation. Other loci, such as p3, p5, and p10, also exhibit high allele frequencies (0.8–1.0) in some populations but show greater variation among groups. Loci like p1, p8, and p9 present lower frequencies, suggesting weaker amplification or lower primer binding efficiency.

**Table IX: Allele Frequencies and Sample Size by Populations**

| Locus | Allele/n | SM | KL | STR | ATR |
|---|---|---|---|---|---|
| p1- MP21 | N | 5 | 5 | 5 | 5 |
| | 125 | 0.6 | 0.2 | 0.4 | 0.5 |
| | 130 | 0.1 | 0.7 | 0.4 | 0.4 |
| | 135 | 0.3 | 0.1 | 0.2 | 0.1 |
| p2- MP22 | N | 3 | 4 | 5 | 4 |
| | 130 | 0.833 | 0.25 | 0.6 | 0.625 |
| | 135 | 0.167 | 0.75 | 0.4 | 0.375 |
| p3-MP23 | N | 5 | 5 | 5 | 2 |
| | 225 | 0.1 | 0.1 | 0 | 0 |
| | 227 | 0 | 0 | 0.2 | 0.5 |
| | 230 | 0.9 | 0.9 | 0.8 | 0.5 |
| p4-MP24 | N | 5 | 4 | 4 | 5 |
| | 150 | 0.6 | 0.5 | 0.625 | 0.9 |
| | 151 | 0.2 | 0.5 | 0 | 0.1 |
| | 185 | 0.2 | 0 | 0.375 | 0 |
| p5-MP25 | N | 5 | 5 | 5 | 5 |
| | 110 | 0 | 0.5 | 0 | 0 |
| | 115 | 0.3 | 0.3 | 0.1 | 0 |
| | 120 | 0.7 | 0.2 | 0.9 | 1 |
| p6-MP26 | N | 5 | 3 | 5 | 5 |
| | 120 | 1 | 1 | 1 | 0.8 |
| | 145 | 0 | 0 | 0 | 0.2 |
| p7-MP29 | N | 5 | 4 | 5 | 4 |
| | 110 | 0.5 | 0.125 | 0.4 | 0.625 |
| | 115 | 0.5 | 0.875 | 0.6 | 0.375 |
| p8-MP9 | N | 5 | 5 | 5 | 5 |
| | 230 | 0 | 0.4 | 0.3 | 0 |
| | 240 | 0.2 | 0.5 | 0.6 | 0.5 |
| | 250 | 0.8 | 0.1 | 0.1 | 0.5 |
| p9-MP11 | N | 5 | 5 | 5 | 5 |
| | 215 | 0.8 | 0.7 | 0.5 | 0.5 |
| | 225 | 0.2 | 0.3 | 0.5 | 0.5 |
| p10-MP3 | N | 5 | 4 | 4 | 5 |
| | 190 | 0.1 | 0 | 0 | 0 |
| | 200 | 0.9 | 1 | 0.875 | 0.5 |
| | 240 | 0 | 0 | 0.125 | 0.5 |

### G. Allele Frequencies by Populations Over Locus

The analysis of codominant allele frequencies across 10 loci reveals that primer p6 exhibits the highest amplification efficiency, demonstrating consistent and robust allele expression in all populations (Fig 5). This suggests that p6 is the most reliable primer for genetic diversity and population structure studies. Other loci, such as p3, p5, and p10, are also effective but less consistently so, whereas p1, p8, and p9 show weaker performance and greater variability.



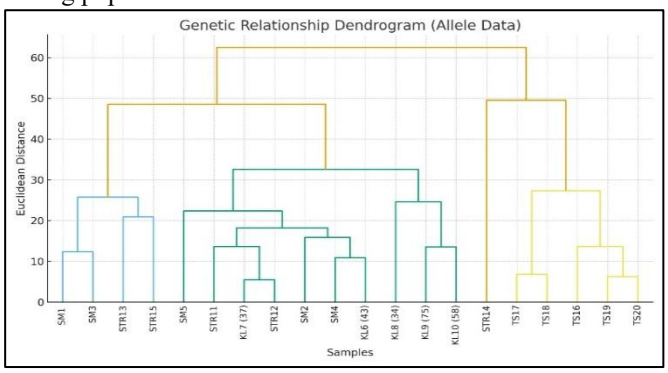

**[Figure 5: Allele Frequency Distribution Across Loci]**

### H. Chi-Square Test for Hardy–Weinberg Equilibrium

The Chi-Square test for Hardy–Weinberg Equilibrium shows that most loci across the SM and KL populations are in equilibrium, reflecting genetic stability and random mating. However, STR (locus p9) and ATR (loci p8, p9, and p10) deviate significantly from equilibrium ($P < 0.05$), suggesting that evolutionary forces such as selection, drift, or population structure may be influencing these loci. Monomorphic loci (e.g., p5, p6, p10) indicate fixation of alleles and thus no heterozygosity.

### I. Dendrogram

The dendrogram constructed from allele data using Euclidean distance illustrates that the distance matrix based on SSR marker frequencies reveals three main clusters of populations. Cluster 1 comprised SM 1, SM 3, STR 13, and STR 15, showing close genetic similarity and originating from the same or closely related populations. Cluster 2 includes SM 2, SM 4, SM 5, STR 11, and STR 12, and KL 6, 7, 8, 9, and 10, representing moderate genetic variation within the group. Cluster 3 comprises STR 14 and ATR 16, 17, 18, 19, 20, which form a distinct group with a relatively high Euclidean distance from the other cluster, implying substantial genetic divergence (Fig. 6). The clustering pattern demonstrates the existence of considerable genetic diversity among the studied samples, resulting from geographical separation limited gene flow or adaptive differentiation among populations.




**[Fig.6: Dendrogram for Selected Populations of *M. Parvifolia* Based on the Genetic Distance Method using SSR Markers]**

## IV. DISCUSSION

The genetic diversity assessment of *Mitragyna parvifolia* across four natural populations (SM, KL, STR, and ATR), using codominant SSR markers, revealed pronounced intra- and inter-population variation—consistent with patterns expected in outcrossing tree species. The genotypic distance analysis showed a wide range (6–30), reflecting both closely related and highly divergent individuals. The occurrence of low genetic distances (e.g., between SM and KL populations) suggests either recent gene flow or shared ancestry, whereas large divergence values (e.g., between KL7 and ATR18) point to genetically distinct lineages, perhaps due to geographic isolation or ecological differentiation.

The AMOVA results further supported this: 72 % of the total genetic variation lay within individuals, whereas only 12 % occurred among populations. Such variance partitioning mirrors observations in other cross-pollinated forest trees, where extensive gene flow and recombination maintain high within-population heterozygosity in Populus

tomentosa: ~79% within individuals and ~8% among populations [11]. Nm≈1.768 suggests that while gene exchange among populations is ongoing, it is not sufficiently strong to eliminate genetic structuring—again echoing general expectations under Wright's rule that Nm>1Nm > 1Nm>1 helps prevent divergence by drift [22]. The moderate inbreeding coefficient FISF_{IS}FIS (0.176) indicates some degree of local non-random mating among proximal individuals. At the same time, the overall FITF_{IT}FIT (0.278) reflects cumulative genetic differentiation across all hierarchical levels. Shannon's diversity index (sHtot=0.714) corroborates these findings: approximately 78 % of total genetic variation is within populations, leaving ~22 % distributed among them. The allele overlap coefficient (O′=0.476) shows that although populations share a substantial fraction of alleles, distinct allelic combinations persist and contribute to overall diversity. This pattern underscores that *M. parvifolia* populations retain a considerable adaptive potential due to rich within-population diversity.

Pairwise relatedness estimates (mean r=–0.030) confirm that most individuals are genetically independent, with few showing moderate relatedness (maximum r=0.462). The negative mean suggests low overall kinship, consistent with sampling from a broad genetic base rather than from clonal or closely related groups.

The allele frequency data across ten SSR loci revealed locus-specific variation in amplification and diversity. Locus p6 showed consistent strong amplification across all populations, making it a highly informative candidate for future studies. In contrast, p1, p8, and p9 showed lower frequencies and greater variability, possibly reflecting primer inefficiency or genuine allelic differences among populations. Deviations from the Hardy–Weinberg equilibrium at certain loci (especially in STR and ATR populations) may be driven by selection, non-random mating, or genetic drift in smaller or isolated populations.

Multivariate analyses, including PCoA and dendrogram clustering, further elucidated population structure. The first three principal coordinates explained ~45.97 % of total variation, effectively distinguishing three principal genetic clusters. These clusters corresponded to those inferred from the dendrogram: SM and STR individuals were grouped closely (Clusters 1 and 2), whereas ATR individuals formed a distinct Cluster 3. The distinct separation of ATR from the others suggests limited gene flow, potentially due to geographic distance, physical barriers, or local adaptation.

These findings position M. parvifolia within a well-established pattern: outcrossing forest trees typically exhibit high within-population diversity, low to moderate population differentiation, and some degree of structure when gene flow is constrained (e.g., by distance or barriers [11]). In comparison, studies in *Stephania tetrandra* using 26 SSR loci found F_ST values spanning 0.021–0.547 (average moderate differentiation), gene flow often exceeding 1, and clustering largely independent of geographic populations by [19], and *Lindera glauca* populations showed low genetic diversity and evidence of skewed sex ratios, despite SSR-based structure analyses reported by [20]

## V. CONCLUSION

The genetic analysis of *Mitragyna parvifolia* revealed high intra-population diversity and moderate differentiation among populations, consistent with its outcrossing reproductive system. Most genetic variation (72%) was found within populations, whereas moderate Fst (0.124) and Nm (1.768) values indicate ongoing but limited gene flow. The moderate inbreeding coefficient (Fis = 0.176) and deviations from the Hardy–Weinberg equilibrium in some loci suggest localised mating or drift effects. Despite this, the high Shannon's diversity index ($sH_{tot}$ = 0.714) and low relatedness (r = –0.030) confirm substantial genetic diversity and a broad genetic base across populations. The PCoA and dendrogram analyses revealed three genetic clusters, with the ATR population showing notable divergence, likely due to geographic or ecological isolation. These findings highlight the need to conserve multiple populations and maintain habitat connectivity to ensure continued gene flow and preserve the species adaptive potential.

## DECLARATION STATEMENT

After aggregating input from all authors, I must verify the accuracy of the following information as the article's author.

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