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Molecular characterization, using RAPD markers, of *Ricinodendron heudelotii* (Euphorbiaceae) from two regions, West and South-East of Côte d'Ivoire, West Africa

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***Ricinodendron heudelotii*, a member of the Euphorbiaceae family, is a valuable plant in Africa, particularly for the people of Côte d'Ivoire, because of its numerous food, medicinal, and socio-cultural uses. A significant proportion of the income of local communities is derived from the sale of their kernels on national and international markets. Despite its importance, there is no national conservation policy for the sustainable management of this species in Côte d'Ivoire, and limited molecular research has been conducted on it. Using four Random amplified polymorphic DNA (RAPD) markers, this study aimed to assess the genetic diversity of *R. heudelotii* populations from two agro-ecological zones. Leaves from 118 trees collected in these zones, western Côte d'Ivoire (Man) and southeastern Côte d'Ivoire (Agboville) were used for genomic DNA extraction following the cetyltrimethylammonium bromide (CTAB) protocol. After DNA extraction, PCR amplification was performed, and the amplification products were visualized by 1% agarose gel electrophoresis at 100 V for one hour to confirm successful amplification. The effective number of alleles was 1.088, while the mean number of alleles ranged from 1.283 to 1.761. The mean genetic diversity was estimated at 0.060, and the mean Shannon diversity index ranged from 0.101 to 0.130. A total of 92 bands were detected, including 30 monomorphic and 62 polymorphic bands, corresponding to a polymorphism rate of 67.44%. Molecular analysis of variance (AMOVA) revealed that most of the genetic variation occurred within populations (98%). Cluster analysis of all sampled trees identified two distinct genetic groups composed of individuals from both populations. Genetic differentiation between the two populations was low (FST = 0.02). Group I comprised 24 trees from the Man site and six from the Agboville site, while Group II included 82 trees, consisting of 49 from Agboville and 33 from Man. *In situ* conservation is therefore essential to ensure the sustainable management of *R. heudelotii*. Furthermore, agroforestry policies that promote the use of trees from all genetic groups could reduce human pressure and help prevent the extinction of this species.**

Key words: *Ricinodendron heudelotii*, random amplified polymorphic DNA (RAPD), agroecological zones, Côte d'Ivoire.

INTRODUCTION

Ricinodendron heudelotii is a tree species native to Africa and is divided into two subspecies *heudelotii* and *africanum* (Orwa et al., 2009). This species is subject to persistent anthropogenic pressures, particularly deforestation resulting from agricultural expansion for cash crops such as coffee, cocoa, oil palm, and rubber. Various parts of *R. heudelotii*, including the seeds, roots, bark, branches, and wood, are widely used in traditional medicine and as ingredients in diverse food products (Akpovo et al., 2022). In addition, its seeds have considerable economic value, ranging from 8,000 to 10,000 CFA francs per kilogram during the peak harvest season and reaching up to 15,000 CFA francs toward the end of the season (Nikiema, 2021). Consequently, the commercialization of *R. heudelotii* seeds provides income and contributes to improving the livelihoods of both rural and urban communities. Like many forest tree species, *R. heudelotii* faces a high risk of extinction due to human activities and the impacts of climate change. This critical situation calls for urgent conservation measures, particularly given that the genetic structure of *R. heudelotii* has not yet been thoroughly investigated. Current research on the molecular genetic diversity of *R. heudelotii* seeks to address this knowledge gap. The objective of the present study is to generate data that can support the development of effective strategies for the regeneration, management, and sustainable use of this species.

A wide range of DNA-based molecular markers is available for genetic characterization and the management of plant genetic resources, including single-nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs or microsatellites), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD). RAPD analysis is based on polymerase chain reaction (PCR) amplification of random DNA fragments using primers with arbitrary nucleotide sequences (Williams et al., 1993). This technique offers several advantages: it requires only small amounts of DNA, does not require prior genomic information, and is simple, rapid, and relatively inexpensive (Bowditch et al., 1993). Owing to these advantages, RAPD markers have been widely used to assess genetic variability in several plant species, including *Haematostaphis barteri* (Sourou et al., 2020), *Pterocarpus erinaceus* (Goba et al., 2020), and *Garcinia kola* (Koffi et al., 2022).

However, to the best of our knowledge, no molecular genetic studies have yet been conducted on *R. heudelotii* populations in Côte d'Ivoire. Therefore, in this study, RAPD markers were employed to evaluate the genetic variation of *R. heudelotii* trees from two distinct agro-ecological zones of Côte d'Ivoire: Man in the western region and Agboville in the southeastern region. These markers enabled the generation of reliable information on the genetic diversity of this species.

MATERIALS AND METHODS

Sites and a collection of plant

Leaves used in this study were collected from two forested regions in southeastern and western Côte d'Ivoire, specifically in the departments of Agboville and Man. A total of 118 *R. heudelotii* trees were randomly sampled from 12 locations, comprising six villages in Agboville and six villages in Man. The number of trees sampled per location depended on the local density of the species, with a minimum distance of 100 m maintained between sampled trees to avoid selecting closely related or identical individuals (Bekessy et al., 2002). The geographic coordinates of each sampling site and individual tree were recorded using a Global Positioning System (GPS) device (Garmin eTrex 20x). For each sampled *R. heudelotii* tree, three healthy green leaves (a total of 354 leaves) were randomly collected and placed in labeled bags containing silica gel to ensure rapid dehydration. The samples were subsequently further dried in a laboratory desiccator prior to DNA extraction.

DNA extraction and purification

Genomic DNA was extracted from dehydrated leaves of *R. heudelotii* using a protocol originally described by Doyle and Doyle (1987) and later modified by Azmat et al. (2012). For each sample, 50 mg of leaf tissue was ground in a mortar with Fontainebleau sand and transferred to a 1.5 mL Eppendorf tube containing 700 µL of cetyltrimethylammonium bromide (CTAB) extraction buffer (20 mM EDTA, 0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, and 0.4% β-mercaptoethanol). The mixture was incubated in a water bath at 65ºC for 45 min, with vortexing every 15 min. Subsequently, 800 µL of a chloroform–isoamyl alcohol mixture was added, mixed gently by inversion, and centrifuged at 14,000 rpm for 15 min. After centrifugation, 500 µL of the supernatant was transferred to a new Eppendorf tube, to which 500 µL of chloroform was added. The mixture was inverted and centrifuged again at 14,000 rpm for 15 min. This step was repeated once more using 350 µL of the supernatant and 350 µL of chloroform, yielding a total volume of 700 µL. After centrifugation, 100 µL of the supernatant was transferred to a new tube, and 200 µL of cold isopropanol was added. The mixture was gently inverted and incubated at −20ºC for 1 h to precipitate the DNA, followed by centrifugation at 13,000 rpm for 10 min. The supernatant was discarded, and the resulting pellet was washed with 300 µL of 70% ethanol and centrifuged at 13,000 rpm for 5 min. This washing step was repeated twice. The pellet was then air-dried and resuspended in 100 µL of 0.1× TE buffer containing 3 µL of RNase, followed by incubation at 37ºC for 2 h. The purified DNA was finally stored at −20ºC until further analysis.

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DNA amplification using RAPD markers

The oligonucleotide primers used in this study were 10 nucleotides in length and contained no palindromic sequences. The selected primers had previously been evaluated by Nakkanong et al. (2008). For this study, eight RAPD primers supplied by PROMEGA (Madison, Wisconsin, USA) were initially tested on 12 DNA samples. Of these, only four primers produced clear, reproducible, and polymorphic banding patterns; these primers were therefore selected for the genetic diversity analysis. Genomic DNA samples were diluted to a final concentration of 20 ng/µL, resulting in a total volume of 100 µL per sample. PCR amplification was carried out in a final reaction volume of 30 µL per sample, consisting of 5 µL of template DNA and 25 µL of reaction mixture. The reaction mixture contained 0.25 µL of Taq DNA polymerase (5 U/µL; PROMEGA), 0.3 µL of primer (20 mM), 0.51 µL of dNTPs (10 mM), 1.8 µL of $MgCl_2$ (25 mM), 3 µL of colored buffer, 3 µL of colorless buffer, and 16.14 µL of sterile distilled water. A negative control was included for each PCR run and consisted of 5 µL of TE buffer and 25 µL of the reaction mixture. The PCR amplification protocol followed that described by Nakkanong et al. (2008). The thermal cycling conditions comprised an initial denaturation step at 94 °C for 2 minutes, followed by 41 cycles of denaturation at 94ºC for 30 s, annealing at 37ºC for 1 min (depending on the primer), and extension at 72ºC for 2 min. A final extension was performed at 72ºC for 5 min, after which the reactions were cooled to 4ºC. All PCR reactions were performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, USA). PCR amplification products were separated and visualized by electrophoresis on 1% (w/v) agarose gels at 100 V for 1 h to confirm successful amplification.

Statistical methods and molecular analysis

Molecular analyses were conducted using GenAlEx software version 6.503 (Meirmans, 2006; Peakall and Smouse, 2012), as well as STRUCTURE (Evanno et al., 2005) and its associated tool STRUCTURE HARVESTER (Earl and vonHoldt, 2012). Clear and reproducible DNA profiles were scored and compiled into a binary matrix, in which the presence of a band was coded as 1 and its absence as 0. This haploid binary matrix was used to calculate genetic distances, diversity indices, and genetic differentiation among populations through molecular analysis of variance (AMOVA) (Nei, 1978). The data matrix generated for each population was used to compute standard measures of genetic diversity and population structure (Wright, 1951; Nei, 1987). Indicators used to assess within-population diversity included the percentage of polymorphic loci (P), calculated at a 95% threshold, the mean number of alleles per locus (Na), which reflects allelic richness, the effective number of alleles (Ne), and Nei's genetic diversity index (h). The binary matrix was converted into a haploid data format to estimate additional parameters of genetic diversity, including genetic diversity (h), the Shannon diversity index (I), the number of observed alleles (Na), and the effective number of alleles (Ne) for each population.

Genetic differentiation among populations was evaluated using the fixation index FST, originally defined by Wright (1951) and later refined by Weir and Cockerham (1984). Statistical significance was assessed using permutation tests with 1,000 permutations. An FST value of 0 indicates no genetic differentiation between populations, whereas a value of 1 indicates complete genetic separation. Following the classification proposed by Mohammadi and Prasanna (2003), populations were considered weakly differentiated when $FST \leq 0.05$, moderately differentiated when $0.05 < FST \leq 0.15$, highly differentiated when $0.15 < FST \leq 0.25$, and very highly differentiated when $FST > 0.25$. To investigate genetic relationships among *R. heudelotii* populations, a Bayesian clustering analysis was performed using structure software version 2.3.4 (Pritchard et al., 2000), treating the two populations as independent. The distribution of genetic diversity was assessed based on allelic similarities to assign individual genotypes to genetic clusters using the admixture model, which allows individuals to have mixed ancestry, and assuming independent allele frequencies.

The analysis estimated the probability that each individual belonged to a given genetic cluster and determined the most likely number of clusters (K). Each simulation included a burn-in period of 10,000 iterations followed by 100,000 Markov chain Monte Carlo (MCMC) iterations. For each K value, ten independent runs were performed, with K ranging from 1 to 10. The optimal number of genetic clusters was identified using the ΔK method based on the second-order rate of change of the log-likelihood, as described by Evanno et al. (2005). Peaks in the ΔK distribution indicate the most probable number of genetic groups. In this study, emphasis was placed on the K value corresponding to the highest ΔK peak, as it reflects the strongest level of genetic structure. The output files generated by STRUCTURE were subsequently uploaded to STRUCTURE HARVESTER (Earl and vonHoldt, 2012), which enabled the calculation and graphical visualization of the ΔK statistic.

RESULTS

Marker polymorphism

Marker polymorphism is one of the most effective tools for detecting genetic structure and diversity. Of the eight RAPD primers tested, four (OPB-17, OPR-11, OPAD-01, and OPR-02) were polymorphic and produced multiple amplified fragments (bands) (Table 1). These primers were used to analyze the genetic diversity and population structure of 115 out of the 118 sampled *R. heudelotii* trees. Three individuals who failed to produce reproducible banding patterns were excluded from the analysis. The remaining four primers (OPAD-12, OPAD-10, OPZ-04, and OPN-16) were not retained for further analysis. Specifically, primers OPAD-12, OPZ-04, and OPN-16 did not yield any amplification, while primer OPAD-10 produced non-reproducible electrophoretic profiles. As RAPD primers are not species-specific, the number and molecular weight of the amplified fragments varied depending on the primer used. The molecular size of the amplified fragments ranged from 100 to 3,000 base pairs (Table 1). A total of 92 bands were detected, of which 62 were polymorphic and 30 were monomorphic, resulting in an average of 23 bands per primer. The overall polymorphism rate was 67.44%. The amplification profile generated using primer OPB-17 is presented in Figure 1.

Intra-population genetic diversity

Based on the binary matrix generated from the analysis of individual banding profiles, genetic diversity indices were calculated for each sampling area. These indicators are summarized in Table 2. Across all samples (N = 56 for Man and N = 59 for Agboville), the mean number of

Table 1. The number of bands and the percentage of polymorphism revealed by the RAPD markers used.

Primers' name	Nucleotide sequence of primers	Total No. of bands	No. of bands Polymorphs	No. of no Polymorphic	Polymorphism (%)	Band size (Kb)
OPB-17	AGGGAACGAG	23	17	6	73.91	100-3000
OPR-11	GTAGCCGTCT	19	12	5	63.16	100-3000
OPAD-01	CAAAGGGCGG	24	18	6	75	100-2000
OPR-02	CACAGCTGCC	26	15	11	57.69	100-2900
Total number of bands	-	92	62	30	-	-
Average	-	23	15.5	-	67.44	-

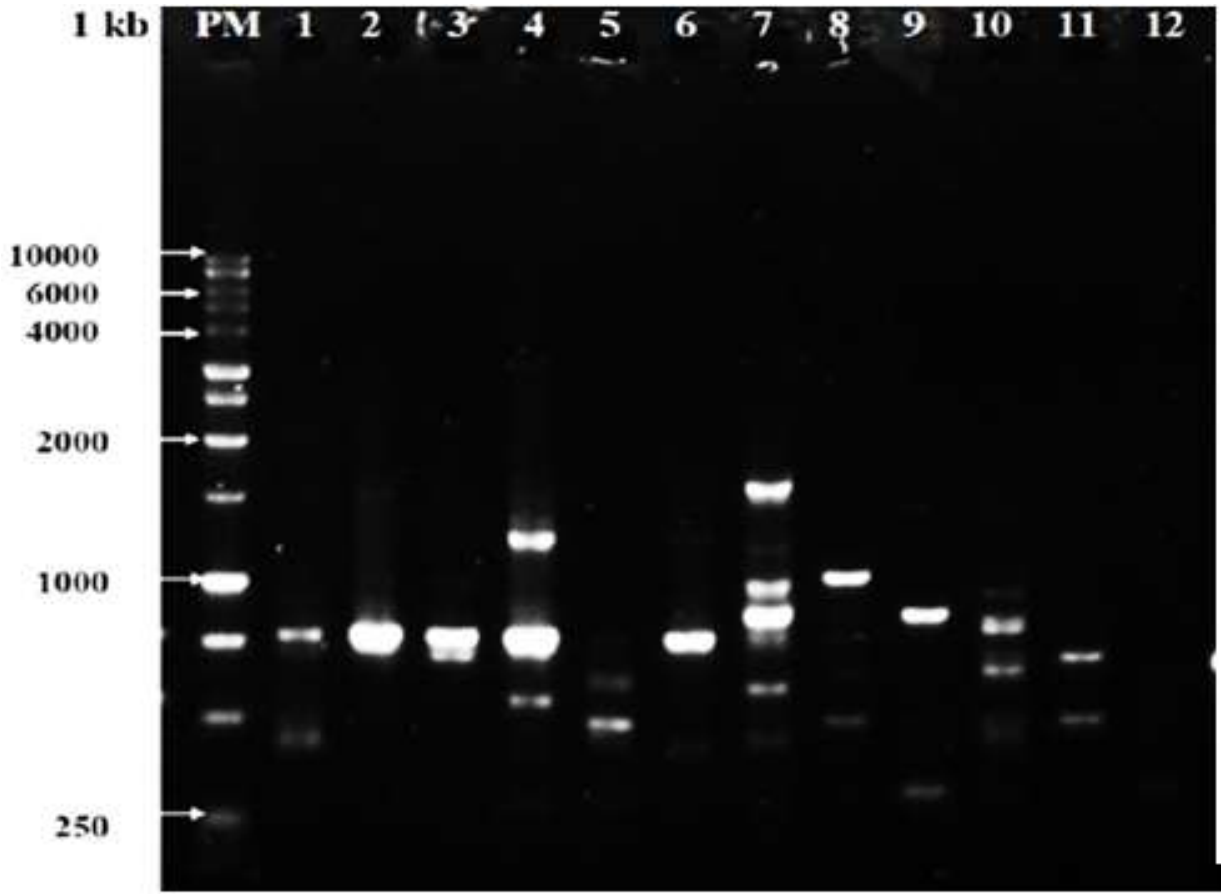


Figure 1. Example of polymorphism in *R. heudelotii*'s genomic DNA detected by the OPB-17 RAPD primer. MW: Molecular weight marker, 1 to 11: DNA samples used, 12: negative control.

alleles (Na) was 1.761 ± 0.068 for the Man population and 1.283 ± 0.101 for the Agboville population, yielding an overall mean of 1.522 ± 0.063 for the total population studied. The effective number of alleles (Ne) was 1.088 ± 0.017 in both Man and Agboville populations. The Nei genetic diversity index (h) was estimated at 0.067 ± 0.010 for the Man population and 0.055 ± 0.011 for the Agboville population. In contrast, the Shannon diversity index (I) was 0.130 ± 0.015 for Man and 0.101 ± 0.016 for Agboville.

Inter-population genetic diversity

Analysis of Molecular Variance (AMOVA) is a statistical method used to assess the distribution of genetic diversity within and among populations. It is based on the principles of classical analysis of variance (ANOVA). The

Table 2. Indices of intra-population genetic diversity (± standard deviation).

Zone	N	Na	Ne	I	h
Man	56	1.761± 0.068	1.088 ±0.017	0.13 ± 0.015	0.067 ± 0,01
Agboville	59	1.283 ±0.101	1.088 ±0.017	0.101 ±0.016	0.055 ±0.011
Average	57.5±0.111	1,522 ±0,063	1.082 ±0.012	0.115 ±0.011	0.060 ±0.007

N: Sample size; Na: number of alleles; Ne: number of effective alleles; I: Nei diversity index; h: Shannon diversity index.

Table 3. Analysis of molecular variance (AMOVA) of two populations of *Ricinodendron heudelotii.*

Source	df	SSD	MS	% variance	F_{ST}	P
Among populations	1	9.074	9.074	2	0.02	0.004
Within populations	113	505.186	4.471	98	0.903	0.004
Total	114	514.261		100		

df: Degree of freedom; SSD: sum of squares of the deviations; MS: mean squares; F_{st}: coefficient of differentiation; P: probability associated with the estimated Fst value after 999 resamples.

results of the AMOVA are presented in Table 3 and illustrate the overall partitioning of genetic variation as well as the level of differentiation between populations. The analysis revealed that only 2% of the total genetic variation was attributable to differences between the two studied populations, whereas the majority of the variation (98%) occurred within populations. The fixation index indicated low genetic differentiation between populations (FST = 0.02). Nevertheless, the genetic differences observed between sites in the two agro-ecological zones were significantly different from zero (p = 0.004). According to commonly used thresholds, this level of genetic differentiation corresponds to weak population differentiation.

The genetic diversity structure of *R. heudelotii*

A Bayesian clustering analysis was conducted to identify groups of genetically similar individuals within the two populations. Figure 2 presents the second-order rate of change of the log-likelihood values (ΔK) for simulations with K values ranging from 1 to 10. The likelihood analysis revealed a clear peak at K = 2, which was considered the optimal value for describing the genetic structure of *R. heudelotii* populations. This result indicates the presence of two distinct genetic groups. Using a membership probability threshold of 55%, 106 of the 115 analyzed trees were assigned to one of the two genetic groups. The remaining nine trees exhibited membership coefficients below 55% for both groups and were therefore classified as “unassigned” or “admixed,” reflecting genotypes resulting from genetic contributions from multiple groups. Most of these individuals displayed alleles common to both genetic clusters. Figure 3 illustrates the individual assignment profiles for all trees at K = 2, with each individual represented by a colored bar (red, green, or mixed). Analysis of the assignment proportions showed that individuals were distributed unevenly between the two genetic groups. The first group (red) comprised 24 individuals, including 18 from the Man site and 6 from the Agboville site. Membership coefficients within this group ranged from 0.558 to 0.963. The second group (green) consisted of 82 individuals, including 33 from Man and 49 from Agboville, with membership coefficients ranging from 0.557 to 0.959.

DISCUSSION

Polymorphism of RAPD markers used

Numerous plant species have been identified, and their genetic diversity has been widely assessed using dominant molecular markers (Indu et al., 2019; Sourou, 2017). Future breeding and conservation efforts aimed at safeguarding the threatened species *R. heudelotii* in Côte d’Ivoire depend on a comprehensive understanding of its genetic diversity. However, the limited genomic information currently available for *R. heudelotii* constrains the development of species-specific molecular markers. Consequently, RAPD markers remain an appropriate and effective tool for investigating genetic diversity and relationships within this species. In the present study, the four RAPD primers employed generated a total of 92 bands ranging in size from 100 to 3,000 bp, of which 67.44% were polymorphic. This level of polymorphism is comparable to values reported for other tree species, such as 65.43% in *Parkia biglobosa* (Mimosaceae) (Amusa et al., 2014) and 62% in almond (*Prunus dulcis*, Rosaceae) (Kadri et al., 2006). These results demonstrate the effectiveness and suitability of the RAPD markers

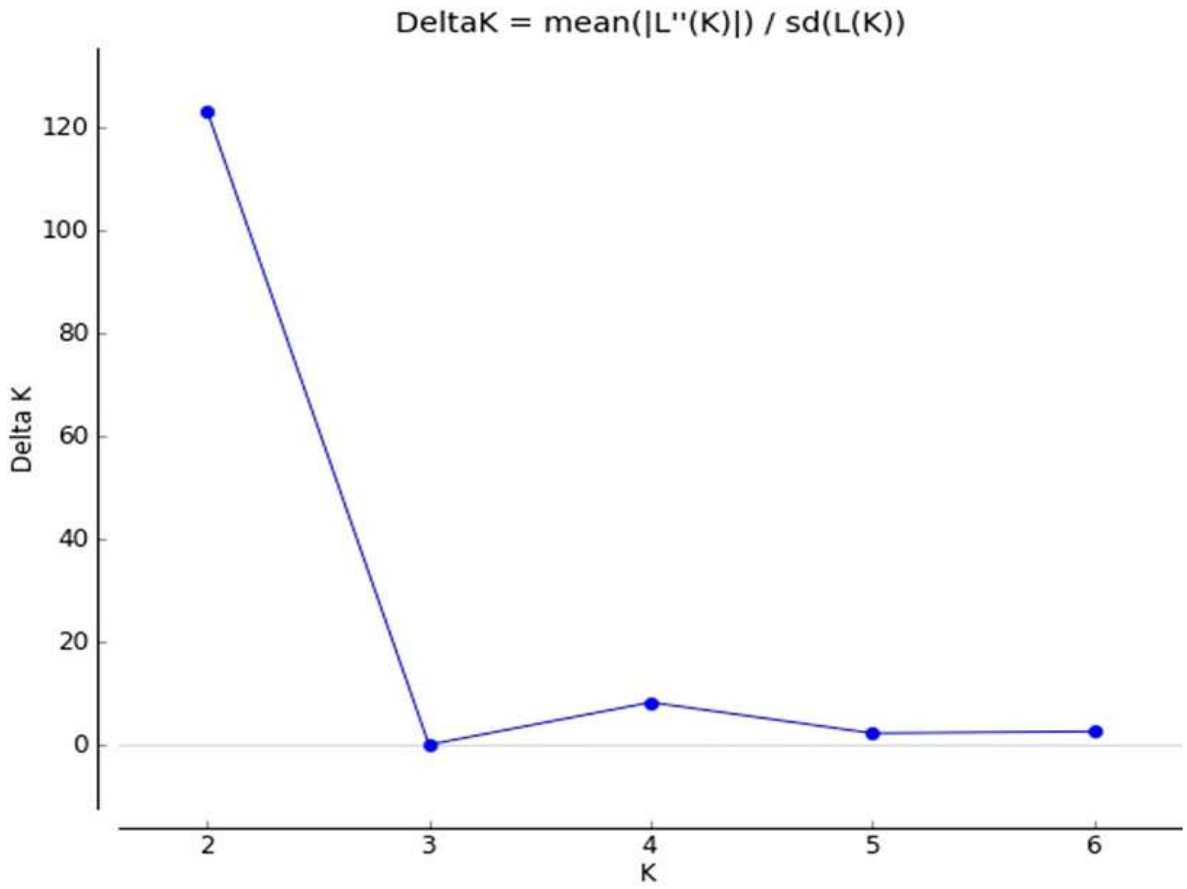


Figure 2. Determination of the most likely value of K according to the method of Evanno et al. (2005). K represents the number of groups (populations) formed.

used in this study. Polymorphism arises from the presence or absence of amplified fragments at specific loci, which is generally attributed to nucleotide substitutions or deletions in primer-binding regions.

Intra-population genetic diversity

AMOVA results revealed a mean genetic diversity value of 0.06 within *R. heudelotii* populations. According to Nei (1987), values ranging from 0 to 0.40 indicate low genetic diversity. The low genetic diversity observed in this study may be partly explained by the limited sample size (118 individuals), which itself reflects the intense exploitation pressure currently exerted on *R. heudelotii*. The mean genetic diversity detected in this study (h = 0.06) is markedly lower than values reported for other tree species, such as *Pterocarpus indicus* (h = 0.20) (Sulistyawati and Widyatmoko, 2017), *Pinus kwangtungensis* (h = 0.25) (Thuy et al., 2020), and *P. erinaceus* (h = 0.35) (Goba et al., 2020). Overall, the genetic diversity indices obtained indicate that the studied *R. heudelotii* populations possess low levels of genetic variation.

Inter-population genetic diversity

Analysis of Molecular Variance (AMOVA) revealed that the majority of genetic variation (98%) occurred among *R. heudelotii* trees within populations, whereas only 2% of the variation was attributable to differences in allele frequencies between sites. These findings are consistent with the results of Kremer (1994), who reported that forest tree species generally exhibit very high levels of intrapopulation diversity compared to other plant types. Similar patterns have been observed in other woody species, including *Argania spinosa* (Sapotaceae) (Pakhrou et al., 2016), *Populus tremuloides* (Salicaceae) (Yeh et al., 1995), *Araucaria araucana* (Araucariaceae) (Bekessy et al., 2002), *Eucalyptus microtheca* (Myrtaceae) (Li, 2000), and *P. erinaceus* (Fabaceae) (Goba et al., 2020; Koffi et al., 2022), supporting our results. The high level of intrapopulation diversity observed in *R. heudelotii* may be explained by its allogamous reproductive system. Indeed, *R. heudelotii* is a dioecious species, with male flowers borne on male plants and female flowers on female plants. Understanding the genetic diversity of a species is critical for

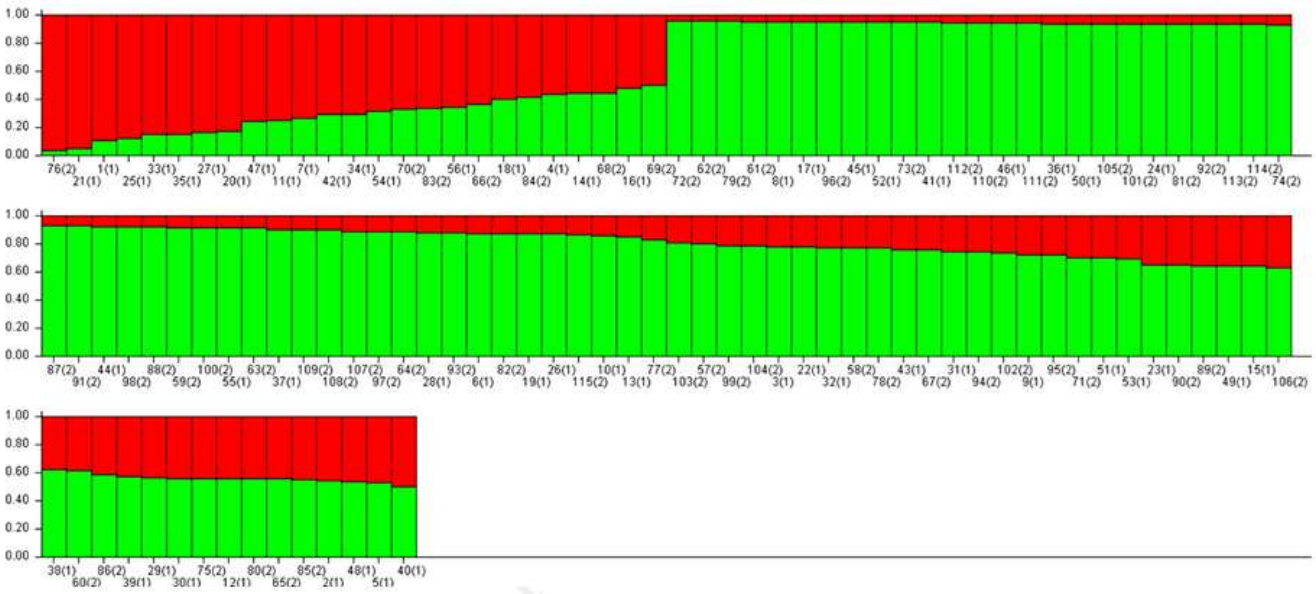


Figure 3. Histogram of genetic group assignment based on allele frequencies from RAPD genotyping of trees from two *Ricinodendron heudelotii* populations. The x-axis corresponds to the different trees from the two populations, while the y-axis indicates the probability of a tree belonging to a gene pool (K=2). Each color represents a gene pool. Populations are identified by a number in brackets (1 for Man and 2 for Agboville). Each tree is represented by a vertical line, colored according to gene pool membership, with lengths proportional to gene pool membership.

guiding conservation strategies and preservation efforts. *In situ* conservation, in particular, allows for the maintenance of existing genetic diversity and the continued expression of individual traits. Conversely, the low level of differentiation between populations suggests that individuals share a common genetic heritage and that the observed morphological variation is primarily influenced by environmental factors and tree age.

Genetic diversity structure of *R. heudelotii*

Bayesian clustering analysis divided the studied trees into two distinct genetic groups, with each group containing individuals from both populations. This shared genetic composition indicates the presence of a common gene pool between the two populations. The *R. heudelotii* populations analyzed, therefore, belong to the same genetic stock. These results provide important insights for future research on the domestication, breeding, and genetic improvement of *R. heudelotii* in Côte d'Ivoire.

Conclusion

This study enabled the genetic characterization of *R. heudelotii* in two provinces of Côte d'Ivoire, providing valuable information to guide decisions on the sustainable conservation of the species' genetic resources. The high genetic variability revealed by RAPD markers demonstrates the usefulness of this approach for assessing the species' genetic diversity. Analysis at two levels of organization showed that genetic variation is greater within populations than between them, emphasizing the importance of sampling as many individuals as possible within populations to ensure effective conservation. This work represents a preliminary study using RAPD markers to investigate the genetic diversity of *Ricinodendron*. The development and evaluation of species-specific markers, or the use of more informative molecular tools such as SSRs or SNPs, could provide a deeper understanding of the species' genetic structure. In addition, studies on seedling germination and growth would support the implementation of agroforestry strategies that incorporate trees from all genetic groups, thereby promoting the sustainable use and preservation of *R. heudelotii*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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