ARTÍCULO DE INVESTIGACIÓN

# ISSR markers as a tool to differentiate genotypes of Cinchona hybrids propagated *in vitro* and *ex vitro*

# Marcadores ISSR como herramienta para distinguir genotipos de híbridos de Cinchona propagados *in vitro* y *ex vitro*

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**RESUMEN**

La diferenciación de genotipos híbridos de *Cinchona spp*. es un desafío debido a sus orígenes compartidos y similitudes morfológicas. En este estudio se utilizaron marcadores ISSR para distinguir entre los genotipos de *Cinchona spp*.: LF40, LC29, LF74, LF74GB y LF211, mantenidos en condiciones *in vitro* y *ex vitro* en Colombia. Se extrajo ADN genómico de los materiales vegetales y se utilizaron ocho cebadores ISSR para la amplificación por PCR. En total, se amplificaron 61 loci, de los cuales 37 (60,92%) fueron polimórficos. El número total de loci por cebafdor varió de 5 a 12, con un promedio de 7,62, y los loci polimórficos variaron de 3 a 6, con un promedio de 4,62 por cebador. El análisis de conglomerados basado en el método de grupos de pares no ponderados con media aritmética (UPGMA) agrupó los genotipos en distintos clusters, mostrando diferencias genéticas. El análisis de coordenadas principales (PCA) confirmó los patrones de agrupamiento, distinguiendo aún más los genotipos a pesar de sus orígenes compartidos. El cebador ISSR4 fue el más efectivo, con la mayor tasa de polimorfismo (75%) y valor PIC (0.473), seguido del ISSR6 que tuvo una tasa de polimorfismo del 71% y un valor PIC de 0.426, ambos cebadores permitieron la identificación de un grupo de plantas en condiciones de campo a partir de cultivos in vitro, con origen genotípico desconocido. Como resultado, fue posible confirmar un grupo de plantas pertenecientes al genotipo LF40 utilizando sólo dos cebadores. Los resultados demuestran la distinción genética de los genotipos de Cinchona seleccionados y subrayan la utilidad de los marcadores ISSR como una herramienta confiable para identificar diferencias genéticas de selecciones de plantas de Cinchona *in vitro* y *ex vitro*.

**Palabras clave:** Cinchona, ISSR, Identificación Molecular, Marcadores Moleculares.

**ABSTRACT**

The differentiation of *Cinchona* spp. hybrids genotypes are challenging due to their shared origins and morphological similarities. This study utilized ISSR (Inter Simple Sequence Repeat) markers to distinguish between genotypes of *Cinchona spp*.: LF40, LC29, LF74, LF74GB, and LF211 maintained *in vitro* and *ex vitro* in

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*Colombia*. Genomic DNA was extracted from the plant materials, and eight ISSR primers were used for PCR amplification. In total 61 loci were amplified, of which 37 (60.92%) were polymorphic. The total number of loci per primer ranged from 5 to 12, with an average of 7.62, and polymorphic loci varied from 3 to 6, averaging 4.62 per primer. Cluster analysis based on the unweighted pair group method with arithmetic mean (UPGMA) grouped the genotypes into distinct clusters, showing genetic differences. Principal coordinate analysis (PCA) confirmed the clustering patterns, further distinguishing the genotypes despite their shared origins. The primer ISSR4 was the most effective, with the highest polymorphism rate (75%) and PIC value (0.473), followed by ISSR6 which had a polymorphism rate of 71% and a PIC value of 0.426, both primers allowed the identification of a group of plants under field conditions from *in vitro* cultures, with unknown genotype origin. As a result, it was possible to confirm a cluster of plants belonging to the LF40 genotype using only two primers. The results demonstrate the genetic distinctiveness of the selected *Cinchona* genotypes and underscore the utility of ISSR markers as a reliable tool for identifying genetic differences of *in vitro* and *ex vitro* Cinchona plant selections.

**Keywords:** Cinchona, ISSR, Genotype differentiation, Molecular identification, Molecular markers.

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## INTRODUCTION

Plants of the genus *Cinchona* (family *Rubiaceae)* native from South America, have been recognized for centuries thanks to their therapeutic properties, particularly in the treatment of malaria, which is related to different alkaloids, including quinine, quinidine, cinchonine and cinchonidine, present in the bark of the tree (Andersson, 1998; Aslam *et al*., 2023; Jaramillo-Arango, 1949). The use of cinchona bark began with indigenous Andean people and was recognized and valued by Europeans in the 16th century, driving global demand for cinchona bark in the following centuries and the subsequent establishment of plantations in European colonies located in South Asia and Africa (Jaramillo-Arango, 1949; Schaepmeester, 2021). In addition, cinchona alkaloids are used today in the food and beverage industry, representing about 40% of global alkaloid production, while the remaining 60% are used for medicinal purposes (Schaepmeester, 2021). The most commonly cultivated species are *Cinchona ledgeriana, Cinchona pubescens* and *Cinchona calisaya,* each known for their high quinine content, growth characteristics and environmental adaptability. These species exhibit variable ploidy levels, with diploid forms common in wild populations, and polyploid forms found among cultivated hybrids and selections (Aslam *et al*., 2023; Canales *et al*., 2020; Holland, 1932; Taylor, 1943). Hybrids between these species were selected to combine the desirable traits of different parent plants, resulting in plant selections with greater disease resistance, faster growth rates, and higher alkaloid production (de Swiet, 2023; Ku, 2016; Steere, 1945; Theiler, 2014; Veale, 2010).

However, in the last 50 years Cinchona plantations in the Kivu area of the Democratic Republic of the Congo in Africa, were affected by the soil-borne plant pathogen *Phytophthora cinnamomi*, which causes severe losses in the productivity of plantations. Therefore, efforts were undertaken to select *P. cinnamomi* tolerant genotypes, in order to reduce the impact of the disease and were propagated through *in vitro* culture and established *ex vitro* for propagation purposes (Theiler, 2014). Subsequently, the identification of the genetic stability of the selected genotypes is necessary to ensure that they retain the desired characteristics over generations.

Tools for the identification of variations in the genotype of plants include molecular markers, which are DNA sequences used to detect and select specific traits in the plant genome (Amom & Nongdam, 2017; Hussain & Nisar, 2020). For *Cinchona spp*. different molecular markers have been used to assess their genetic diversity, including RAPD markers, nuclear microsatellite loci, the chloroplast rps16 intron, along with studies using or rbcL, ITS and matK genes (Perez Ocampo, 2021; Sasongko *et al*., 2021). Additionally, ISSR (Inter Simple Sequence Repeat) markers have been used to evaluate somaclonal variation in *Cinchona officinalis* regenerated through different in vitro protocols, together, these approaches provided valuable insights into the genetic variability and stability of cinchona species, supporting conservation, breeding, and propagation efforts by enabling precise monitoring of genetic fidelity and diversity (Cueva-Agila *et al*., 2019; Armijos-González *et al*., 2021) Furthermore, the use of codominant SSR markers—transferred from related species such as *Coffea arabica, Psychotria tenuinervis, Galium catalinense,* and *Antirhea borbonica* has provided valu-

able information on genetic variability within the genus (Aranha Trelles & León Piedra, 2013).

Additionally, genomic resources for *Cinchona* genus are limited, as only partially complete genomes of *C. calisaya* and *C. pubescens* have been published (Arbizu *et al*., 2021; Canales *et al*., 2022; Du *et al*., 2025). Given this, alternative molecular markers are ISSR, which are 100–3000 bp DNA sequences amplified by polymerase chain reaction (PCR) using a single primer made from genomic microsatellite sequences, generating multilocus markers that are highly polymorphic and informative for various genetic studies (Zietkiewicz *et al*., 1994). ISSR markers have been broadly used due to their cost-effectiveness characteristics, reproducibility, and capacity to identify polymorphisms throughout the genome in plant populations (Arya *et al*., 2022; Godwin *et al*., 1997).

While ISSR have been extensively applied to various plant species (Amruthakumar *et al*., 2024; Arya *et al*., 2022; Raji & Siril, 2021), their use in Cinchona hybrids remains underexplored, offering an opportunity to apply ISSR markers to Cinchona plant selections, which can provide a powerful tool to evaluate genetic differences and confirm the distinctiveness of selected genotypes. The main objective of this study was to assess the potential of ISSR markers for identifying four genotypes of *Cinchona spp*. maintained *in vitro* and grown *ex vitro* under field conditions, due to the difficulty of distinguishing phenotypic differences among them, for the accurate maintenance of these genotypes in the establishment of a Cinchona plantation in Colombia.

## MATERIALS AND METHODS

### *Plant Material*

Four different genotypes of *Cinchona spp*. seedlings were selected based on their tolerance against *P. cinnamomi*, derived from hybrids between *Cinchona calisaya* (formerly known as *C. ledgeriana*) and *Cinchona pubescens* (formerly known as *C. succirubra*) (Theiler, 2014). These genotypes were labeled as LC29, LF211, LF40, LF74, and LF74GB (*in vitro* selection of LF74 characterized by larger leaf blades). The LC29 genotype shares phenotype traits with *C. pubescens*, while LF211, LF40, LF74 and LF74GB exhibit morphological characteristics resembling *C. calisaya*.

Through multiple *in vitro* subcultures spanning decades, the selected cinchona genotypes were initially established *in vitro* at Agroscope Changins-Wädenswil ACW (Switzerland), and subsequently transferred to the Plant Tissue Culture laboratory at the Instituto de Biotecnología de la Universidad Nacional de Colombia (Theiler, 2014; Saavedra, *et al*., 2025), where they have been maintained and propagated *in vitro,* then transferred to soil and planted in the field *(ex vitro)*, providing a reliable source of plant material for genetic analysis and stock for plantation development. For the ISSR analysis, a total of fourteen samples were taken from genotypes grown *in vitro* and *ex vitro*, including the genotype S29, obtained from seed progeny derived from a LC29 *ex vitro* plant under field conditions (Table 1). A plant of *Coffea* sp. (a related genus to *Cinchona* within the Rubiaceae family), was added as a control as it differs taxonomically with *Cinchona* species. Additionally, a group of plants (10 samples) under field conditions derived from *in vitro* plants of unknown in vitro genotype origin was included, as we aimed to confirm their genotype. The experimental design consisted of three main groups: (i) *in vitro* maintained genotypes, (ii) *ex vitro* genotypes propagated from known *in vitro* lines, and (iii) *ex vitro* plants of unknown genotype. For the ISSR analysis, leaf samples were collected from each genotype, with each sample representing an experimental unit and analyzed in triplicate.

Table 1. Plant samples of Cinchona hybrids under *in vitro* and *ex vitro* conditions, and the plant sample used as a control.

| Genotype Name | *In vitro* / *Ex vitro* |
|---|---|
| LC29_IV | *In vitro* |
| LF74_IV | *In vitro* |
| LF74GB_IV | *In vitro* |
| LF211_IV | *In vitro* |
| LF40_IV | *In vitro* |
| S29_EV | *Ex vitro* |
| LC29_EV | *Ex vitro* |
| LF74_EV | *Ex vitro* |
| LF74GB_EV | *Ex vitro* |
| LF211_EV | *Ex vitro* |
| LF40_EV | *Ex vitro* |
| Coffea_Control | *Plant of Coffea sp from field conditions used as a control.* |
| Group of plants with unknown genotype origin | *Ex vitro* |

### *DNA Extraction*

For genomic DNA extraction of the cinchona genotypes, 100 mg of fresh leaves from *in vitro* and *ex vitro* plants of each genotype was placedinto a 2 mL extraction tube

Table 2. List of used inter simple sequence repeat (ISSR) primers, their sequence and annealing temperature.

| Primer Name | Primer Sequence 5´-3´ | Annealing Temperature (°C) | Reference |
|---|---|---|---|
| ISSR1 | AGAGAGAGAGAGAGAGT | 52 °C | *Armijos-González, R., Espinosa-Delgado, L., & Cueva-Agila, A. (2021)* |
| ISSR2 | AGAGAGAGAGAGAGAGC | 54 °C | |
| ISSR3 | AGAGAGAGAGAGAGAGG | 56 °C | |
| ISSR4 | AGAGAGAGAGAGAGAGYT | 56 °C | |
| ISSR5 | AGAGAGAGAGAGAGAGYA | 54 °C | |
| ISSR6 | GAGAGAGAGAGAGAGAYT | 51 °C | |
| ISSR7 | GAGAGAGAGAGAGAGAC | 52 °C | *Yan L., Ogutu, C., Huang, L. et al., 2019* |
| ISSR8 | ACACACACACACACACG | 51 °C | |

containing eight zirconia beads (0.5 mm), the tissue was ground and homogenize at 5.000 rpm for 2 minutes in a lab-built bead beater. Then total DNA was isolated using the modified CTAB (Cetyl trimethylammonium bromide) protocol defined by Inglis *et al*. (2018), with the inclusion of proteinase K (0.2 mg) during the initial incubation step. DNA integrity was assessed by electrophoresis on a 1% agarose gel, run at 100V/cm for 20 minutes. In addition, quality was checked with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). All DNA samples were diluted to a final concentration of 20 ng/µL and stored at -20°C until further ISSR PCR amplification.

### *ISSR molecular markers and analysis*

A total of 10 ISSR primers were chosen based on previous reports of ISSR profiling in Rubiaceae family plants, however only 8 of them showed polymorphism (Table 2). The PCR amplification reactions were performed in a volume of 10 µl, which contained 1X PCR buffer, 0.5 mM dNTPs, 1.5 mM MgCl2, 0.4 µM of each primer, 1 U of Taq DNA polymerase and 40 ng of genomic DNA. The conditions for PCR amplification was 94 °C for 4 min (Initial denaturation), followed by 35 cycles of 94 °C for 1 min (Denaturation), 51-56 °C for 1 min (Annealing), 72 °C for 2 min (Extension) and 72 °C for 7 min (Final extension), for each primer PCR conditions were standardized, and the corresponding annealing temperatures are shown in Table 2. Amplified DNA samples with all the 8 ISSR primers were run in 1.5% agarose gel at 80 V/cm for 60 min, and ISSR fragments generated in this experiment were documented using gel documentation (Bio-Rad, USA), molecular weights of the DNA fragments were determined using a DNA ladder between 500 and 5000 bp (Bioline - HyperLadder™ 500bp) (Supplementary Material).

For data analysis, reproducible clear amplified bands in the gel profiles were recorded as present (1) and absent (0), resulting in a binary matrix. Each primer was evaluated with regard to the total number amplified loci (NAL), total number of polymorphic loci (NPL), rate of polymorphism (RP) (RP) = (NPL / NAL) × 100 and the value of the content of polymorphic information (PIC) according to the equation proposed by (Roldán-Ruiz *et al*., 2000):
$PIC_i = 2fi(1-fi)$

where $PIC_i$ is PIC for primer i, fi is the frequency of amplified fragments and 1 – fi is the frequency of non amplified fragments. A dendrogram indicating the association between the genotypes was created by using the unweighted pair group method with arithmetic average (UPGMA) based on Jaccard's similarity coefficients (Sneath & Sokal, 1973). Besides, principal component analysis (PCA) was conducted to examine the existing genetic variation between cinchona hybrid genotypes, all of the analysis were performed in the software R Language V4.4.2 (R Core Team, 2021).

## RESULTS AND DISCUSSION

A total of ten ISSR primers were used to reveal genetic polymorphism in different genotypes of cinchona grown *in vitro* and *ex vitro*. Among these, eight primers successfully amplified reproducible DNA band profiles. A total of 61 loci were amplified, of which 37 loci (60.92%) were polymorphic (Table 3). The total number of amplified loci (NAL) per primer ranged from 5 (ISSR5) to 12 (ISSR3), with an average of 7.62 loci per primer. The total number of polymorphic loci (NPL) varied from 3 (ISSR2 and ISSR5) to 6 (ISSR3, ISSR4, and ISSR6), with an average of 4.625 polymorphic loci per primer. The rate of polymorphism (RP%) ranged from 50.0% (ISSR2)

Table 3. Total number of loci, number of polymorphic loci, rate of polymorphism and value of the polymorphic information content of eight ISSR primers in the Cinchona hybrids individuals.

| ISSR PRIMER | Total number amplified loci (NAL) | Total number of polymorphic loci (NPL) | Rate of Polymorphism (RP %) | Content of polymorphic information (PIC) |
|---|---|---|---|---|
| ISSR1 | 7 | 4 | 57,1 | 0,229 |
| ISSR2 | 6 | 3 | 50,0 | 0,369 |
| ISSR3 | 12 | 6 | 50,0 | 0,399 |
| ISSR4 | 8 | 6 | 75,0 | 0,473 |
| ISSR5 | 5 | 3 | 60,0 | 0,142 |
| ISSR6 | 7 | 5 | 71,4 | 0,426 |
| ISSR7 | 9 | 6 | 66,7 | 0,260 |
| ISSR8 | 7 | 4 | 57,1 | 0,408 |
| Average | 7,62 | 4,62 | 60,92 | 0,338 |
| Total | 61 | 37 | NA | |

*NA: Not Applicable*

to 75.0% (ISSR4), with an overall average of 60.92%. The polymorphic information content (PIC), a measure of primer informativeness that takes into account the relative frequency of the allele, ranged from 0.142 (ISSR5) to 0.473 (ISSR4), with an average PIC value of 0.338.

The moderate level of polymorphism detected is consistent with the expectations for hybrids genotypes, which typically exhibit intermediate variability due to their mixed parental origin and the monophyletic nature of the genus confirmed using molecular markers (Andersson & Antonelli, 2005). Notably, the primers ISSR4 followed by ISSR6 emerged as most informative, with the highest RP% and PIC values, making them useful for detecting genetic differences in cinchona genotypes. High polymorphism rates and PIC values mean that these primers can detect subtle genetic differences between closely related genotypes. This is consistent with studies in Coffea species (Kumar *et al*., 2023; Mishra *et al*., 2022), which demonstrated that certain primers can differentiate hybrids even when morphological differences are minimal.

In this study, it was included a plant of coffee as a control, which allowed for a comparative analysis with the *Cinchona* genotypes, offering a baseline to evaluate the genetic profiles of *Cinchona* samples in relation to a related genus within the *Rubiaceae* family. The results showed a clear difference in the DNA profile between the genus *Coffea* and *Cinchona*. Besides, the Cluster analysis using the UPGMA method and Jaccard coefficient, placed the 11 cinchona hybrid genotypes in two groups (Figure 1). The first group includes a cluster consisting of genotypes LC29_IV, S29_EV and LC29_EV. Group two consists of three sub-clusters: the first containing LF211_EV, LF211_IV, the second with LF40_IV, LF40_EV, and the third consisting of LF74EV, LF74GB_EV, LF74_IV and LF74GB_IV (Figure 1).

The PCA results revealed that the first two principal components (PC1 and PC2) explained a substantial proportion of the total variance, with PC1 accounting for 56.87% and PC2 contributing 23.26% (Fig. 2). Together, these two components explained 80.13% of the total variance in the dataset, suggesting that they capture the majority of the genetic variation among the genotypes. Specifically, the cinchona hybrids (LC29_IV, S29_EV, and LC29_EV) were grouped in the PCA plot, indicating their genetic similarity and reflecting their shared ancestry. It is important to note that the S29_EV genotype, which was derived from seeds produced by an LC29_EV under field conditions, was grouped in the same group.

In contrast, the separation of the remaining genotypes into three distinct sub-clusters indicates a greater degree of genetic differentiation compared to the LC29 hybrid group. Within this group, the first sub-cluster (LF211_EV, LF211_IV) represents a set of genotypes with closer genetic similarity. The second sub-cluster (LF40_EV, LF40_IV) and the third sub-cluster (LF74_EV, LF74GB_EV, LF74_IV, LF74GB_IV) are positioned closer

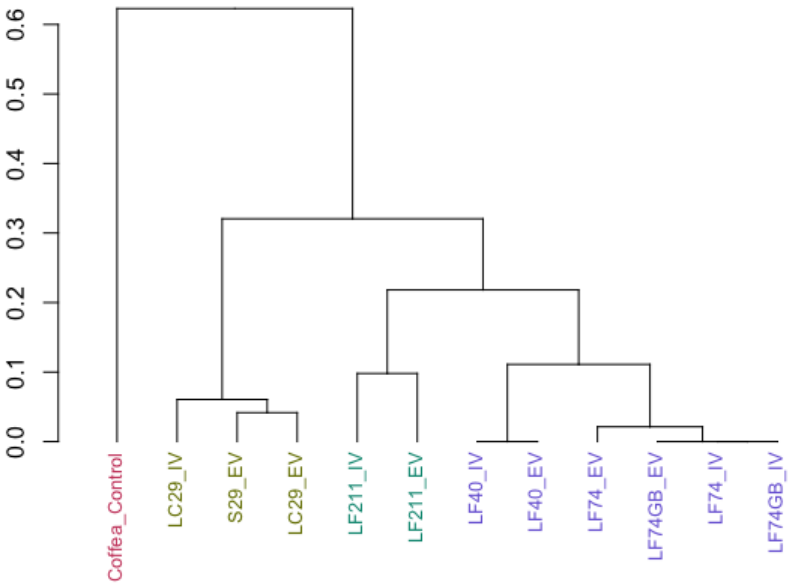


Figure 1. UPGMA Dendrogram of Cinchona genotypes *in vitro* and *ex vitro* based on jaccard similarity index.

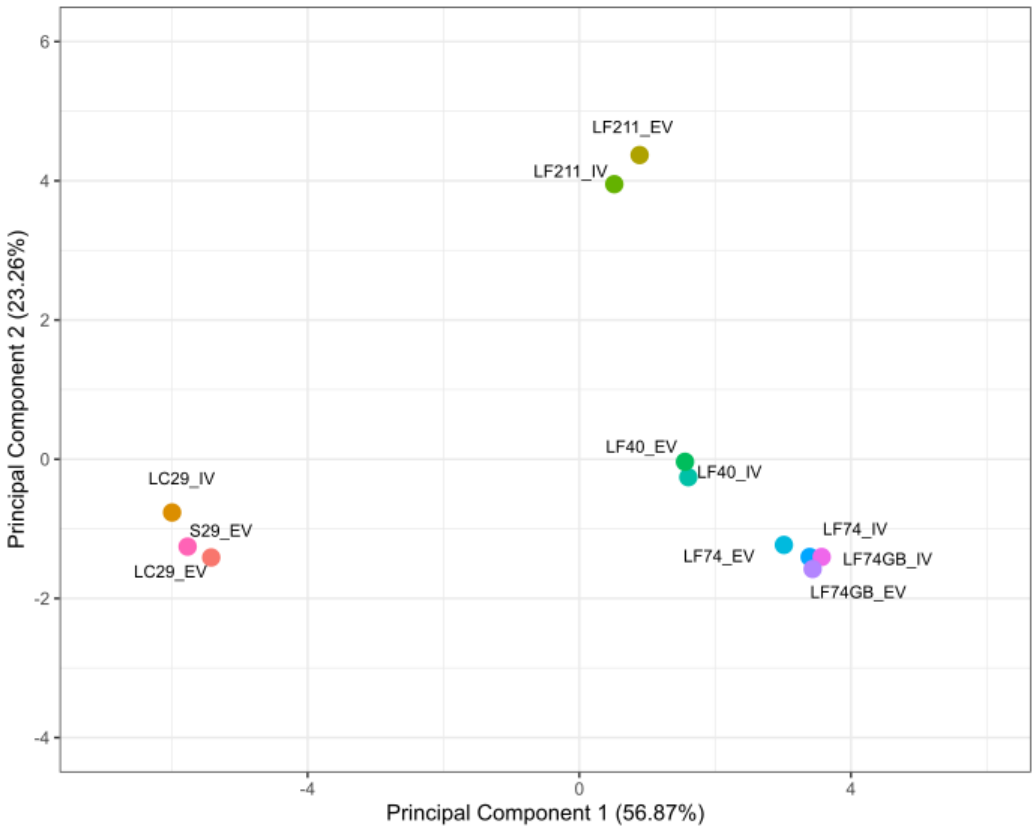


Figure 2. Principal component analysis (PCA) of Cinchona hybrids ISSR data, PC1 accounted for 53.43% and PC2 for 31.29% of variance.

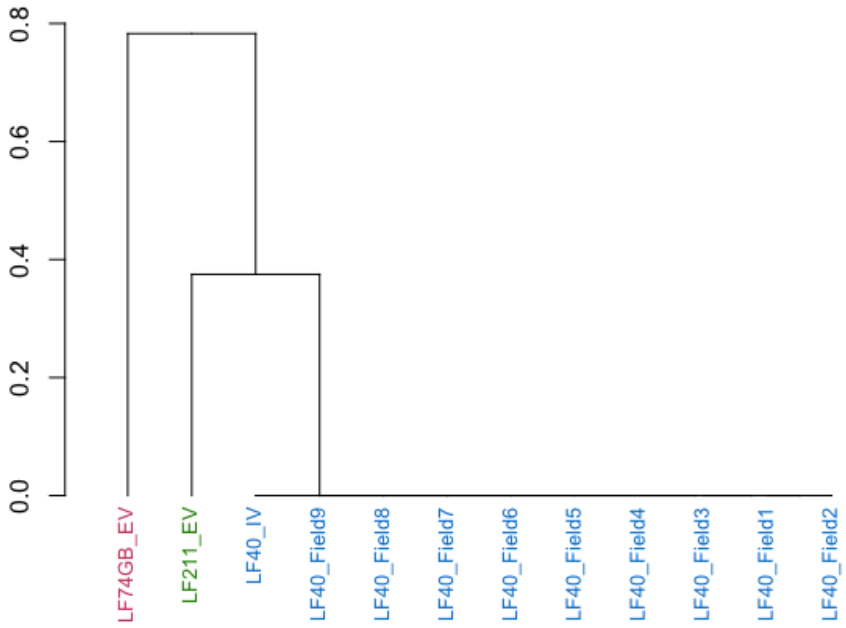


Figure 3. UPGMA Dendrogram of Cinchona genotypes from field based on Jaccard similarity index, using only the primers ISSR4 and ISSR6.

to each other, suggesting that these genotypes share a distinct genetic profile with only minor differences. Furthermore, the grouping of LF74 and LF74GB underscores the particularly close genetic relationship between these genotypes, reflecting their shared genetic origin. The overall clustering patterns confirm that the genotypes within each node or sub-cluster share a common ancestry and maintain a high level of genetic consistency under *in vitro* and *ex vitro* conditions.

The clustering pattern among the clones may reflect the morphological characteristics of the genotypes, and their genetic relation with *C. calisaya* and *C. pubescens*. Moreover, a previous study using RAPD markers found that *C. calisaya* is the most genetically distinct species among a collection of Cinchona accessions including *C. pubescens, and Cinchona sp.* from the Junghuhn Nature Reserve in Indonesia, detecting polymorphism levels as high as 75% (Sasongko *et al.*, 2021) Similarly, in this study ISSR markers revealed a separation between the genotype LC29 genotype which shares phenotypic traits with *C. pubescens*, and the LF211, LF40, LF74, and LF74GB genotypes related with *C. calisaya*, which could explain the genetic divergence found between the evaluated genotypes.

Furthermore, we selected the ISSR4 and ISSR6 primers based on their high level of polymorphism, to identify 9 field-grown plants (6–12 years old) derived from *in vitro* cultures of unknown genotype origin, since it was unclear whether they belonged to the genotypes LF40, LF211, or LF74. Using these primers, it was possible to classify all field plants belonging to the LF40 genotype and therefore labeled as LF40_Field (Fig. 3). Moreover, based on their genetic profiles, a clear genetic distinction from the LF211 and LF74 genotypes was observed. The ISSR markers evaluated in this study provide a useful tool for assessing the genetic diversity of native tree species *Cinchona calisaya* and *C. pubescens*, both distributed across different regions of Colombia (Aymard, 2019). Additionally, these markers could facilitate the development of genetic improvement programs involving these two species, given their ability to discriminate hybrids, this also opens the possibility of using them to certify plant material intended for propagation in commercial plantations.

Finally, the overall similarities found between the different cinchona genotypes under *in vitro* and *ex vitro* conditions, demonstrates a genetic consistency through the plant acclimatization process. Which is important for detecting the desirable traits of the genotypes. However, the detec-

tion of minor genetic differences between clones of the same genotypes, might potentially be attributable to somaclonal variation (Armijos-González *et al*., 2021), caused by spontaneous and uncontrolled genetic changes in plants during long-term *in vitro* propagation and different factors such as the exposure to plant growth regulators, and the stress associated with *in vitro,* manifesting changes as small polymorphisms, chromosomal rearrangements, alterations in gene expression or epigenetic changes (Armijos-González *et al*., 2021; Duta-Cornescu *et al*., 2023). However, to conclusively confirm the presence of somaclonal variation in the genotypes evaluated in this study, further research is required.

## CONCLUSIONS

This study demonstrates the utility of ISSR markers to identify and differentiate *Cinchona spp.* genotypes under *in vitro* and *ex vitro* conditions. The ISSR analysis revealed moderate genetic polymorphism and allowed the identification of the different genotypes: LC29, LF211, LF40, and LF74. The primers ISSR4 and ISSR6 with the highest rate of polymorphisms are capable of detecting subtle genetic differences between closely related genotypes, identifying a group of field-grown plants of unknown genotype origin, within the LF40 genotype group. In addition, minor genetic variations within clones of the same genotype are likely attributable to somaclonal variation due to the long-term *in vitro* subculturing practices, however further investigation is needed to assess the genetic stability of *Cinchona spp.* genotypes. The findings presented here enable more accurate genetic verification of *Cinchona* genotypes, facilitating improved quality assurance, certification, also contributing to the management and conservation of *Cinchona spp.* genetic resources through accurate genotype identification

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