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ORIGINAL ARTICLE

Morphological and molecular characterization of cubios (*Tropaeolum tuberosum* Ruiz & Pavón) collected in two municipalities in Boyaca - Colombia

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Abstract

In this study, we aimed to determine the extent of genetic variation among cubios (*Tropaeolum tuberosum*) in a diversity micro-center in the department of Boyacá, Colombia, with morphological and ISSR (Inter Simple Sequence Repeats) molecular markers. Twenty-five cubio morphotypes were collected across different farms within the municipalities of Belén and Ventaquemada in Boyacá and were sown for subsequent morphological and molecular characterizations. Tuber features were evaluated according to descriptors of the International Potato Center, namely predominant tuber surface color, secondary tuber surface color, secondary tuber surface color distribution, predominant tuber pulp color, secondary tuber pulp color, distribution of secondary tuber pulp color, tuber shape, and depth of tuber eyes. Molecular variation was assessed via eight ISSR primers. Morphological and molecular characterizations allowed us to identify 25 cubio morphotypes with significant genetic variation in the study area. This finding is likely attributed to mechanisms maintaining genetic variability, such as naturally occurring crosses between morphotypes modulated by environmental conditions and local agricultural practices, including seed exchange and a preference for given morphotypes.

Keywords: Andean tubers; diversity; ISSR (Inter Simple Sequence Repeats); cubios; crop morphotypes.

1. Introduction

Cubios, the edible Andean tubers of the species *Tropaeolum tuberosum* Ruiz & Pavón, are declared by the Food and Agriculture Organization of the United Nations - FAO as marginalized or underused crops [1]. Cubios are native to the central Andes from northern Argentina to Colombia at altitudes of 2.400 to 4.300 meters above sea level [2]. They usually occur in association with other species, such as potatoes (*Solanum tuberosum*), ibias (*Oxalis tuberosa*), rubas (*Ullucus tuberosus*), broad beans (*Vicia faba*), and corn (*Zea mays*). In Colombia, cubios are grown in the departments of Cauca, Nariño, Cundinamarca, and Boyacá, yet their precise planted area and yield are not known [3]. Clavijo and Pérez (2014) reported a possible diversity microcenter for Andean tubers such as cubios, ibias, and rubas in the adjacent municipalities of Turmequé and Ventaquemada, within in the department of Boyacá [4]. Cubios and the other related tubers are tightly bound to this region's gastronomic culture. However, farmers cultivating tubers in this area are generally underpaid and endure poverty and marginalization conditions [4].

According to FAO's 1992 report, several circumstances lead to crop marginality, including traditional crop replacement with new species, loss of competitiveness to more productive crops, progressive demand decrease, and a combination of economic, cultural, political, and religious factors [1]. One important marginalization-triggering factor is the loss of knowledge about plant use and cultivation techniques, resulting from the disappearance of the ethnic groups possessing that knowledge. In contrast, social and biotic factors play equally important roles in crop conservation. Farmers are decisive for crop conservation since they select and disperse the various genotypes of crop plants [5].

Many underutilized species conserved today are the basis of local food systems around the globe. These crops are well-adapted to their local agroecological conditions and maintain strong links with their communities [6]. Underutilized crops can improve local food security, where they remain in use despite competition by crops grown at larger scales [7].

Cubios are one of the highest-yielding tubers and one of the easiest to grow; they thrive on marginal soils, develop quickly, and successfully outcompete weeds [8]. The properties attributed to cubios include nematicidal and insecticidal actions, which is why some farmers intersperse them among plants of other species [2]. Cubios also exert beneficial health effects supporting proper liver and kidney function and likely help with urinary and prostate disorders and eczema [9]. These properties are due to compounds such as isothiocyanate and anthocyanins, which occur in varying concentrations in tubers with diverse colors ranging from yellow to dark purple [10, 11, 12].

Cubio genetic variation has chiefly been studied in Peru and Bolivia, deemed as the tuber's centers of origin [13, 14, 15, 16]. These studies have highlighted the importance of furthering the knowledge of cubio genetic diversity since its agronomic, nutritional, and cultural features constitute a valuable genetic resource for the food security of high-altitude Andean peasant and indigenous communities. Assessment and monitoring of agricultural biodiversity are essential for planning and prioritizing effective conservation strategies [17]. A recent Colombian molecular characterization study of some cubio genotypes revealed the presence of variability among them [18].

In this context, our study's objective was to determine the genetic diversity of cubios from a diversity microcenter in Boyacá, Colombia, using morphological and molecular markers (ISSR). Our methods and results can become tools for future conservation strategies in the area.

2. Materials and methods

2.1. Plant material

Twenty-five morphotypes of cubios were collected from peasant fields in the municipalities of Belén and Ventaquemada, in the Colombian department of Boyacá, situated on the country's eastern Andean range (Cordillera Oriental). All the fields were located at altitudes above 2.700 m.a.s.l. Upon collection, the information recorded included farm names, morphotypes collected, georeferencing information, and accession numbers (**Table 1**).

Once collected, each morphotype was planted at the San Javier farm of the Pontificia Universidad Javeriana, in the municipality of Cogua, in the department of Cundinamarca, and an accession number was assigned to each planted morphotype (Table 1). Tubers from each sample planted were characterized morphologically and at the molecular level. This project is framed within a

Table 1. Seed tubers collected in the municipalities of Ventaquemada and Belén, Boyacá - Colombia.

Farm name	Morphotype (*i.e.*, tuber features)	Location	Latitude N	Longitude W	Altitude (m.a.s.l)	Accession no.
La Victoria	Light yellow	Ventaquemada	05°22´57.5"	073°30´21.6"	2786	AC001
El Pastalito	Yellow with irregular dark red bands	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC002
El Pastalito	Yellow with purple eyes	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC003
La Victoria	Purple with irregular bands and yellow dots	Ventaquemada	05°22´57.5"	073°30´21.6"	2786	AC004
El Pastalito	Yellow with dark red bands and eyes	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC005
El Morro	Yellow with purple eyes	Belén	06º 02´24”	72º 53´ 42”	3410	AC006
El Pastalito	Yellow with dark red bands, dots and eyes	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC007
El Pastalito	White with irregular bands and purple dots	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC008
El Pastalito	Yellow with irregular red bands	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC009
El Pastalito	Light yellow with purple eyes	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC010
El Pastalito	White with purple eyes and splotches	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC011
Casa Amarilla	Purple	Belén	06º 2´21.4”	072º 54´37.9”	3133	AC012
La Victoria	Yellow with red eyes and irregular bands	Ventaquemada	05°22´57.5"	073°30´21.6"	2786	AC013
El Morro	White with purple eyes	Belén	06º 02´24”	72º 53´ 42”	3410	AC014
El Pastalito	Yellow and orange	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC015
El Pastalito	Irregular red bands with secondary yellow coloring	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC016
Casa Amarilla	Light purple	Belén	06º 2´21.4”	072º 54´37.9”	3133	AC017
El Pastalito	Yellow with burgundy stripes and black splotches and dots	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC018
El Pastalito	Dark purple with white	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC019
Casa Amarilla	Yellow	Belén	06º 2´21.4”	072º 54´37.9”	3133	AC020
El Pastalito	Purple with pink and with irregular burgundy stripes	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC021
El Pastalito	Light yellow with red eyes	Ventaquemada	05°22´38.9"	073°31´47.3"	2794	AC022
Casa Amarilla	Dark purple	Belén	06º 2´21.4”	072º 54´37.9”	3133	AC023
La Victoria	Yellow with irregular red bands and purple dots	Ventaquemada	05°22´57.5"	073°30´21.6"	2786	AC024
El Pastalito	White with irregular pink bands and purple eyes	Ventaquemada	05° 2´38.9"	073°31´47.3"	2794	AC025

inter-institutional agreement to study Colombias genetic resources (*i.e.*, Contract for Access to Genetic Resources and their Derivative Products No. 256 of 2019 signed between the Colombian Ministry of Environment and Sustainable Development and Pontificia Universidad Javeriana).

2.2. Morphological characterization

Morphological characterizations were done following the morphological descriptor scale of the International Potato Center (CIP) [19], which accounts for the most important traits needed for identifying cubio morphotypes. The tuber characteristics used included predominant surface color, secondary surface color, secondary surface color distribution, predominant pulp color, secondary pulp color, distribution of secondary pulp color, tuber shape, and tuber eye depth. The sixth edition of the Royal Horticultural Society Color Chart [20] was used for all evaluations.

2.3. Molecular characterization

Morphotype molecular characterizations were conducted with leaf sample DNA from each individual accession planted in the San Javier farm. Samples were kept in self-sealing bags with silica gel and then processed at to the Plant Molecular Biology laboratories of Javeriana University in Bogotá. The Quick-DNA Plant/Seed MiniprepTM kit from Zymo Research was used for DNA extraction from each sample following the manufacturer's instructions. Amplification of a set of Inter Simple Sequence Repeats (ISSR) markers was done with 12.5 μl of PromegaTM Mastermix, 4 mM of primers and 25 ng of DNA at a final reaction volume of 25μl. The primers used are shown in **Table 2**.

Table 2. Inter Simple Sequence Repeats - ISSR primer information used in this study. The provided information includes ISSR primer name, sequence, optimum banding temperature (OBT), polymorphic bands (PB), total bands (TB), polymorphic information content (PIC), observed heterozygosity (Ho) and expected heterozygosity (He) in cubios (*T. tuberosum*)

Primer Sequence (5' a 3')	Complete Sequence	OBT (ºC)	PB	TB	PIC	Ho	He
(AG)8-T	AGAGAGAGAGAGAGAGC	48	4	5	0.15	0.36	0.15
VHV(GT)7-G	AGAGAGAGAGAGAGAGT	50	3	5	0.13	0.20	0.09
DDC (CAC)5	VHVGTGTGTGTGTGTGTG	51	2	4	0.11	0.12	0.06
BDB (CAC)5	BDBCACCACCACCACCAC	55	4	6	0.18	0.48	0.18
(AC)8-G	DDCCACCACCACCACCAC	55	4	5	0.30	0.72	0.36
(GA)8-A	GAGAGAGAGAGAGAGAA	50	4	5	0.17	0.24	0.16
(AG)8-C	ACACACACACACACACG	47	3	4	0.24	0.32	0.23
DHB(CGA)5	DHBCGACGACGACGACGA	53	2	4	0.28	0.48	0.23
Total		26	38				

PCR (polymerase chain reaction) assays were done in a Veriti™ thermocycler (Applied BioSystems™) under the following conditions: initial denaturation temperature of 94 ºC for ten minutes, followed by 35 cycles of denaturation at 95 ºC for one minute, annealing for 45 seconds, at varying temperatures according to each primer [Table 2], and a two-minute extension at 72 ºC. The program was completed with a final elongation step of five minutes at 72 ºC. Amplification products were separated on 1.8% agarose gels, which were stained with HydraGreen™ and visualized on a UV transilluminator. The HyperLadder™ 1 kb molecular weight marker was used for each electrophoresis gel.

2.4. Data analysis

A database was created to record the results of all variables evaluated for the morphological descriptors. These data were used for a principal component analysis, to reduce the size of the set of variables. Similarly, a cluster analysis using the statistical software R, version 3.6.0 (2019-04-26) was used to group morphotypes via euclidean distance. The results where then use to create a dendrogram.

Molecular diversity was assessed by identifying amplicon bands. Only those that were reproducible in several independent runs were taken into account. With these bands, a binary matrix based on the presence of one (1) and absence of zero (0) was created. Both polymorphic and monomorphic bands were included. The percentage of polymorphism for each primer used was determined using the number of polymorphic bands with respect to the total number of bands. The discrimination power of each primer was evaluated in order to characterize its ability to detect polymorphic loci among the accessions analyzed. For this analysis we calculated the percentage of polymorphism (% P) and the polymorphic information content (PIC) [21]. The genetic diversity, calculated heterozygosity (H), and unbiased heterozygosity (HI) were estimated using the molecular data from each one of the accessions. The genetic distances between the accessions were obtained with Dice's similarity coefficient. Then, a dendrogram was constructed with the UPGMA (unweighted pair group method with arithmetic mean) using Info-Gen software, version 2016.

3. Results and Discussion

3.1. Morphological analysis

Morphological trait data of the 25 cubio accessions planted on the San Javier farm were recorded (Table 1). Qualitative variables were coded, standardized, and used for statistical analysis. PCA showed that 74% of the variation among accessions is explained by the first three principal components (38%, 18%, and 17%, respectively).

Fig. 1. revealed groups defined by the first two components of their principal component analyses. These accession groups emerged from two shared principal traits, for instance predominant tuber surface color and secondary tuber surface color distribution. Predominant tuber surface color exhibited five states ranging from white to dark purple, whereby yellow and dark purple were most frequent among the accessions. Next, secondary tuber surface color presented six states. The combinations of the states of these two traits leads to ample variability of cubio accessions in these areas of Boyacá. The greatest morphotype variability was found among accessions from farms in Ventaquemada (Table 1).

The dendrogram in **Fig. 2** was constructed from the morphological data and shows the formation of four groups of accessions. Group A includes accessions whose predominant surface color is yellow with secondary surface colors ranging from dark red to purple, and yellow tuber pulp. Group B encompasses accessions of purple as the predominant surface color, with dark red as the dominant pulp color. Group C includes accessions with yellow as their secondary surface color, predominant pulp color, and secondary pulp color. Finally, group D includes accessions with tuber surface colors between white and yellow and secondary colors ranging from red to purple. This group contains one exception: accession AC023 has a dark purple surface. The accessions of this group had conical shaped tubers. In the other groups, the predominant tuber shape was elongated.

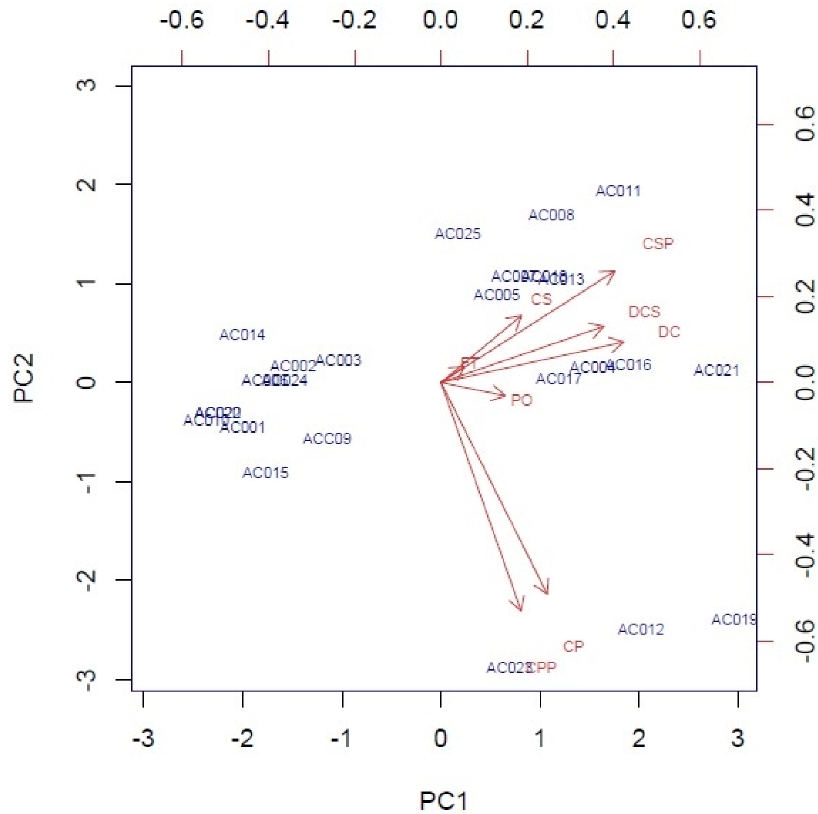

Figure 1. Principal component analysis of the 25 cubio accessions. Component 1 (PC1) explains 38% of the variability and component 2 (PC2) explains 18% of the variability.

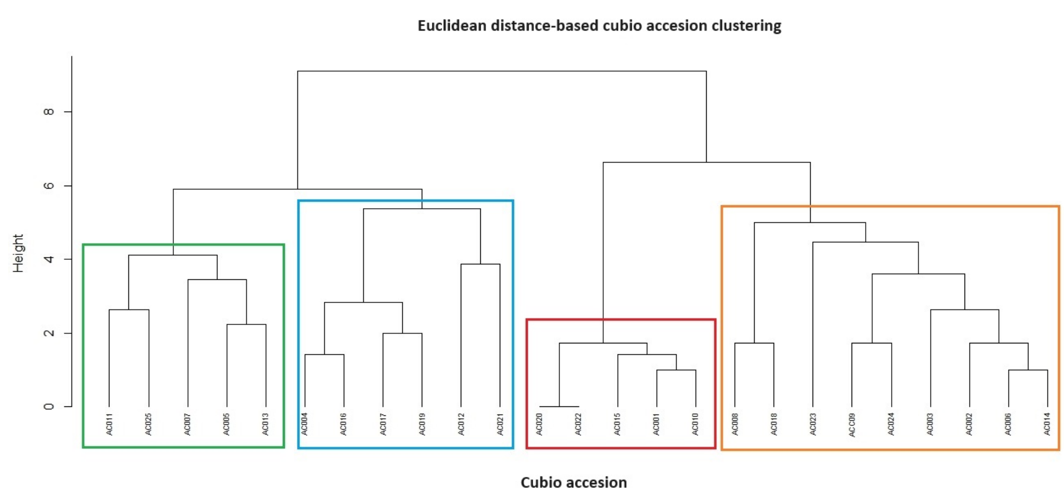

Figure 2. Dendrogram built with the euclidean distance for 25 cubio accessions based on a set of morphological traits. Accession group A (green box), Accession group B (blue box), Accession group C (red box), and Accession group D (and orange box).

The clusters formed in the PCA and the dendrogram are very similar. The most consistent group is group C which includes accessions whose tuber surface colors are all yellow. Previous studies that analyzed cubio diversity in Boyacá [3, 18] also reported highly diverse phenotypes. This can be attributed to the cubio conservation, propagation, and reproduction strategies by indigenous people and farmers in this area since pre-Columbian times [21]. Similarly, in this area agricultural biodiversity awareness-raising and conservation activities, together with rural development plans and strategies, have allowed these tubers to have greater importance in peasant communities in recent decades [4].

3.2. Molecular Analysis

The ISSR markers efficiently detected this species' polymorphisms. The eight primers evaluated (Table 2) generated a total of 38 reproducible bands. Twenty-six (68%) were polymorphic. Amplicon sizes ranged from 200 bp to 1500 bp. The number of polymorphic bands per primer ranged from two to four and the percentage polymorphism per primer ranged from 50% to 80%. The most informative primer for evaluating these accessions was (AC)8-G which had a PIC of 0.3 and 80% polymorphism. In this species, A, G, or C dinucleotide repeat motifs were abundant in the cubio genome, which makes them highly efficient markers, in revealing the highest levels of polymorphism [13].

The number of markers per primer and the percentage of polymorphism of each primer in the accessions evaluated (Table 2) indicated ample among-accession (*i.e.*, infraspecific) variability even though farmers in this area only propagate cubios asexually. The observed intermediate intraspecific variability (He = 0.36) in this species is likely explained by opposing situations. On the one hand, the propagation of the plants and the minimal importance of this product in markets (namely, no commodity chain for cubios exists in Colombia) lead to a loss of genetic diversity. On the other hand, sexual reproduction (cross- and self-pollination) occurs in this species. In plots where various morphotypes coexist, these can promote genetic exchange between varieties, thereby giving rise to new genotypes [13]. These genotypes are conserved and exchanged among farmers who progressively have attributed different properties and uses to them [21].

Dice's coefficient-driven analysis at a distance of 0.27 differentiated our cubio accessions into four groups (**Fig. 3**). Group A contained accessions 16, 18, 11, 10, 09, 24, and 21. These tubers' predominant surface color varies between yellow, and their secondary surface color varies between red and purple. All accessions in this group came from Ventaquemada. Group B contains accessions 14, 17, 12, 23, and 06, and their predominant surface color is purple. All accessions in this group came from Belén. Group C entails accessions 05, 08, 13, 20, 07, 04, 25, 19, 22, and 03. Their prevailing surface color transitions between white and purple, except for accessions 04 and 19. Their predominant outstanding surface color is purple. All the accessions of this group, except for 20, came from Ventaquemada. Accession 20 is from Belén. Finally, group D contains accessions 15, 02, and 01. Their predominant surface color is yellow, and they came from Ventaquemada.

These clusters suggest an origin-dependent differentiation among genotypes. The fact that there is no constant exchange of seeds between the two municipalities has probably allowed differentiation between these provenances. Most seed tubers were collected in Ventaquemada, within El Pastalito farm, where numerous morphotypes co-occur thanks to their owner's conservation effort over several years. Likely, genetic exchange through crosses between different morphotypes in this farm results in new cubio varieties.

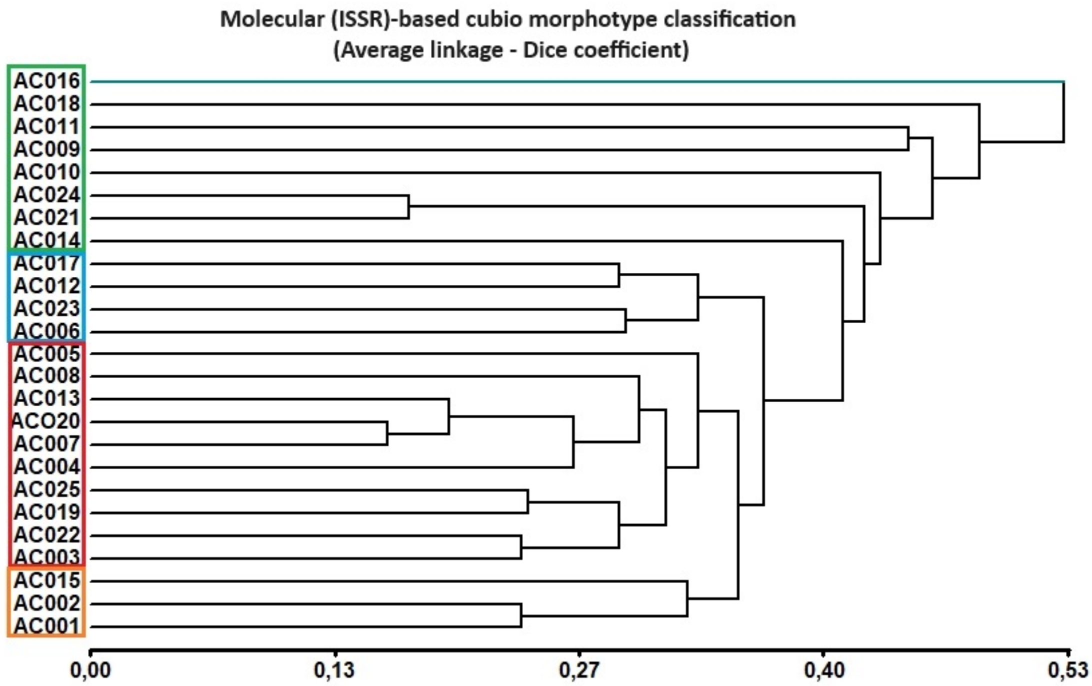

Figure 3. Dendrogram of 25 *Tropaeolum tuberosum* morphotypes based on ISSR markers with the Dice distance and the UPGMA clustering methods. Group A (green), Group B (blue), Group C (red) and Group D (orange)

The groups revealed by the dendrograms from morphological characters (Fig. 2) and genetic differentiation (Fig. 3) displayed no correspondence. Other studies comparing ISSR markers with morphological markers have highlighted the notably greater discriminatory capacity of ISSR markers and the difficulties arising from the use of morphological markers. Morphological markers are an easily applied indirect measure of diversity but are subject to biases and are modulated by environmental conditions [13].

4. Conclusions

Eight tuber morphological descriptors resulted in the identification of 25 morphotypes among the assessed cubios. These descriptors behaved differently in the principal component analysis. The first three principal components explained 74% of all morphological variation among tubers.

The morphological characteristics evaluated for this study suggest that cubios exhibit ample morphological variation within the study area and that multiple processes may be responsible for maintaining such variation.

The use of ISSR markers allowed us to differentiate all the accessions evaluated in this study and revealed significant genetic variability present in the study area.

The observed morphological and genetic variability levels among the studied cubio morphotypes suggest that this diversity is likely maintained by sexual reproduction events among morphotypes even though farmers propagate tubers asexually. Similarly, over the years, the seed exchange among farmers and the local preferences for given morphotypes have allowed the conservation of the genetic diversity of this tuber in these areas of Boyacá.

Although *T. tubersosum* is considered an underutilized species, its nutritional properties, rusticity, and resistance to pests and pathogens make it a species of strategic importance. The genetic diversity within the studied microcenter of diversity is of great value for this species' conservation and sustainable use.

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6. Conflict of Interests

The author(s) declare no potential conflicts of interest with respect to research, authorship, and/or publication of this article. The authors declare having no conflicts of interest.

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Caracterización morfológica y molecular de cubios (*Tropaeolum tuberosum* Ruiz & Pavón) recolectados en dos municipios de Boyacá - Colombia

Resumen: En este estudio, nos propusimos determinar el grado de variación genética entre los cubios (*Tropaeolum tuberosum*) encontrados en un microcentro de diversidad del departamento de Boyacá, Colombia, usando marcadores morfológicos y moleculares, específicamente ISSR (Repeticiones Simples Intersecuenciadas). Se colectaron veinticinco morfotipos de cubios en diferentes fincas dentro de los municipios de Belén y Ventaquemada en Boyacá los cuales se sembraron para su posterior caracterización morfológica y molecular. Las características de los tubérculos se evaluaron siguiendo los descriptores del *International Potato Center*, a saber: el color predominante de la superficie del tubérculo, el color secundario de la superficie del tubérculo, la distribución del color secundario en la superficie del tubérculo, el color predominante de la pulpa del tubérculo, el color secundario de la pulpa del tubérculo, la distribución del color secundario en la pulpa del tubérculo, la forma del tubérculo y la profundidad de los ojos del tubérculo. La variación molecular se evaluó mediante ocho cebadores ISSR. Las caracterizaciones morfológicas y moleculares nos permitieron identificar 25 morfotipos de cubios con una variación genética significativa en el área de estudio. Este hallazgo probablemente se debe a mecanismos que mantienen la variabilidad genética, como los cruces naturales que ocurren entre morfotipos, modulados por las condiciones ambientales y las prácticas agrícolas locales, incluyendo el intercambio de semillas y la preferencia por ciertos morfotipos.

Palabras Clave: Tubérculos andinos; Diversidad; ISSR (Repeticiones Simples Intersecuenciadas); Cubios; Morfotipos de cultivo.

Caracterização morfológica e molecular de cubios (*Tropaeolum tuberosum* Ruiz & Pavón) coletados em dois municípios de Boyacá - Colômbia

Resumo: Neste estudo, nos propusemos a determinar o grau de variação genética entre cubios (*Tropaeolum tuberosum*) encontrados em um microcentro de diversidade no departamento de Boyacá, Colômbia, usando marcadores morfológicos e moleculares, especificamente ISSR (Repetições Simples Intersequenciais). Foram coletados 25 morfotipos de cubios em diferentes fazendas nos municípios de Belén e Ventaquemada em Boyacá, estes foram plantados para posterior caracterização morfológica e molecular. As características dos tubérculos foram avaliadas de acordo com os descritores do *International Potato Center*, incluindo: a cor predominante da superfície do tubérculo, a cor secundária da superfície do tubérculo, a distribuição da cor secundária na superfície do tubérculo, a cor predominante da polpa do tubérculo, a cor secundária da polpa do tubérculo, a distribuição da cor secundária na polpa do tubérculo, a forma do tubérculo e a profundidade dos olhos do tubérculo. A variação molecular foi avaliada por meio de oito primers ISSR. As caracterizações morfológicas e moleculares nos permitiram identificar 25 morfotipos de cubios com variação genética significativa na área de estudo. Esse achado provavelmente se deve a mecanismos que mantêm a variabilidade genética, como os cruzamentos naturais que ocorrem entre morfotipos, modulados pelas condições ambientais e pelas práticas agrícolas locais, que incluem a troca de sementes e a preferência por certos morfotipos.

Palavras-chave: Tubérculos andinos; Diversidade; ISSR (Repetições Simples Intersequenciais); Cubios; Morfotipos de culturas.

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