



Molecular characterization of cultivars of *Capsicum annuum* L. and *Capsicum frutescens* L. (Solanaceae) from Valle del Cauca, Colombia by SSR-HRM technique

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Abstract The genus *Capsicum* includes plants known as chili, paprika or chili peppers, which are widely cultivated throughout the world. Its cultivation is of great importance because its fruits are widely used in gastronomy and industry. In Colombia, the species *C. annuum*, *C. chinense* and *C. frutescens* are cultivated. However, the Colombian production of chili peppers has not yet been recognized at the global level. Hence, in order to explore the genetic diversity and variation present in this genetic resource, it is important to molecularly characterize the material used in crops in Colombia. Thus, this study focused on the estimation of genetic diversity and variation of cultivated lines of *C. annuum* and *C. frutescens* species in Valle del Cauca using microsatellite markers and High-Resolution Melting (HRM) technology. No heterozygous individuals were found in the evaluated

cultivars of either species. This is consistent with the mostly autogamous mode of reproduction of the genus and the selection process to which this genetic resource has been subjected. The allelic diversity of the cultivars was low, with most loci having a maximum of 3 alleles. However, for both species, the original cultivar maintained greater diversity than the derived cultivar. The analysis of molecular variance (AMOVA) identified the comparison of individuals within lines as the level with the highest variation (55% and 75%). This was followed by the variation between the original line (before selection) and the derived lines (37.25 and 11.6%). Furthermore, three useful microsatellite systems were identified for the differentiation of individuals of three species (*C. annuum*, *C. frutescens* and *C. chinense*) by HRM. Finally, the TL-8 line of *C. frutescens* is postulated for agronomic evaluation in different environments due to its genetic homogeneity, while the original lines of both species still have genetic variation to continue a breeding program.

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Introduction

The genus *Capsicum* of the family Solanaceae includes plants commonly known as chili or paprika.

It is widely distributed in Central and South America with 43 species (Barboza et al. 2022), although some species of the genus have been spread around the world since the time of Spanish colonization and are cosmopolitan. Of all the species, five have been domesticated by humans (Eshbaugh 1980; Barboza et al. 2022) and are used to produce chili peppers. These are *C. annuum*, *C. chinense*, *C. frutescens*, *C. bacatum*, and *C. pubescens*. Plants of the genus *Capsicum* are agriculturally important because their fruits are rich in vitamins (A, C, and B6), antioxidants, β -carotenes, flavonoids, anticarcinogens, antimicrobials, pigments, carotenoid oils, oleoresins, and alkaloids with insecticidal potential (Liu et al. 2013). This makes chili growing an important part of the economies of many countries. China, Mexico and Turkey are among the world's major producers. Their production exceeds 41,125,952 tons per year (FAOSTAT 2023). Fresh fruit and chili paste are the main forms of import, with the United States, Germany, France and the United Kingdom being the main consumers (FAOSTAT 2023).

Colombia cultivates *C. annuum* (Cayenne), *C. frutescens* (Tabasco) and *C. chinense* (Habanero), with an annual national production of approximately 28,368 tons (Ministerio de Agricultura y Desarrollo Rural 2024). However, the production of chili peppers in Colombia has not yet been recognized by the world. This is particularly important considering that the country contains many chili pepper cultivars that can be a potential source of genetic resources, given that it contains two of the diversification centers of the genus, the Amazon and the Andes (Moscone et al. 2006; Carrizo-García et al. 2016). Therefore, it is important to molecularly characterize the material used in crops in Colombia. This will allow the study of the existing genetic diversity and variation in order to better conserve this resource, study the possibility of introducing new genetic variation, and plan new breeding programs (Sackville-Hamilton 1998; Boller and Green 2010).

Microsatellites are among the most widely used molecular markers for the characterization of genetic diversity. Some of their most important characteristics are their co-dominance, a high mutation rate and, in general, a high number of alleles per locus (Goldstein and Schlötterer 1999). In this work, the HRM technique was used for the genotyping of individuals with SSR markers. HRM is a real-time PCR

technique using a fluorophore that emits fluorescence when it is incorporated into double-stranded DNA. As the DNA helix is denatured under the influence of temperature, the fluorophore is released, and the instrument detects the gradual decrease in fluorescence as the reaction proceeds. This information is used to construct dissociation curves unique to each type of molecule, since dissociation mode is a property of each DNA sequence, depending on its base pair size and nucleotide composition (Simko 2016). Thus, the HRM technique allows to distinguish the alleles of a microsatellite locus not only by their size but also by their nucleotide composition (Ganopoulos et al. 2011).

This study focused on the estimation of genetic diversity and variation of cultivated lines of *C. annuum* and *C. frutescens* species in Valle del Cauca. This was done in order to provide molecular markers for the identification and classification of the material of the bank, to allow its conservation and, in addition, to help in the decision-making process of genetic improvement, complementing the morphological work done so far on this genetic resource.

Materials and methods

Collection of samples

Material from the seed bank of Hugo Restrepo y Cía. of Valle del Cauca, Colombia, represented by two species of *Capsicum* (*C. annuum* and *C. frutescens*), was used. The seeds were sown only from five lines of the species *C. frutescens* and from four lines of the species *C. annuum*. In each species, there is one line called O (original). It represents the initial material from which the other lines were created through breeding programs. The *C. frutescens* lines have been coded as TL-O, TL-23, TL-19, TL-08 and TL-05 and the *C. annuum* lines were coded as CL-40, CL-44, CL-50 and CL-O. Leaf tissue was collected for DNA extraction from 30 individuals for each line.

DNA Extraction

The procedures of DNA extraction, quantification and amplification were carried out in the Laboratory of Molecular Biology of the Department of Genetics of the Universidad del Valle, Meléndez, Cali, Colombia.

For DNA extraction, the CTAB method developed by Doyle & Doyle (1987) and modified by Stewart (1997) was used. Its quantification was performed in a Nanodrop device (Thermo Scientific, USA) to determine its concentration and purity.

Genotyping by HRM technique

For the molecular characterization of the samples, 10 microsatellites were used for *C. frutescens* and 8 for *C. annuum*, all markers standardized and tested by Guzman et al. (2020) (Table 1), according to the work of Lee et al. (2004) and Nagy et al. (2007). See Supplementary Data 1 for the nucleotide sequences of the primers. Based on these microsatellites, RT-PCR (Real-Time PCR) was performed on the CFX96 Touch™ Real-Time PCR machine for each sample with a final volume of 10 µL consisting of 5 µL Precision Melt Supermix 2X, 0.7 µM of each primer (forward and reverse), 0.1 µL deionized and distilled water and 35 ng DNA. The amplification program consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, hybridization (Table 1) for 30 s, and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 5 min. In addition, the HRM protocol was added, which consisted of increasing the temperature to 95 °C for 1 min, followed by cooling to 60 °C for 30 s and ending with a gradual temperature increase

of 0.2 °C per step, from 65 °C to 95 °C, holding the temperature for 10 s and taking the corresponding fluorescence measurement at each step.

The data obtained from the instrument were analyzed using Precision Melt Analysis™ v1.2 software, leaving all parameters at default. It was checked that the confidence of assigning the groups (genotypes) for each sample was not less than 95%. If it was, the sample was repeated. Samples were considered comparable only if their amplification take-off values (cq value) were within ± 4 cycles of each other and did not exceed 30, otherwise the sample was repeated with more or less DNA as appropriate. In addition, samples were filtered based on relative fluorescence units (RFU). Any sample with a final RFU lower or higher than the interval between the mode (RFU) and $\pm 30\%$ of it was repeated. The above parameters ensured that the samples were comparable in the subsequent HRM analyses and that real differences between the samples could be detected. Finally, to construct the multiallelic matrix for data analysis, the data were exported to spreadsheet format.

For each microsatellite system, there was confirmation that the amplification was a single fragment. To do this, the amplifications of some random samples were run on 1.8% agarose gels. The gels were stained with EZ-Vision to visualize the bands in the UV transilluminator. This was done to confirm the result of the dissociation curves. A single dissociation peak corresponds

Table 1 Microsatellite (SSR) markers used for genotyping two *Capsicum* species. A: Microsatellite used only in *C. frutescens*. B: Microsatellite used only in *C. annuum*. C: Microsatellite used in both species. Ng: Marker obtained from Nagy et al. (2007). Bd: Marker obtained from Lee et al. (2004). ^a-1 °C in each cycle

SSR	Locus	Species	Annealing temperature	Cycles	Annealing temperature	Cycles
Ng4	CA515275	A	60	35		
Ng6	CA515275	B	60	35		
Ng7	CA525390	B	60	35		
Ng8	CA516044	A	60	35		
Ng9	BM061910	B	60	35		
Ng10	CA516334	C	60	35		
Ng15	CA515055	A	63 ^a	5	61	30
Ng18	CA517699	A	65 ^a	5	60	30
Ng20	BM061461	B	60	35		
Ng33	CA516439	A	60	35		
Ng35	GPMS 187	B	60	35		
Bd3	Hpms1-173	C	65 ^a	5	60	30
Bd12	Hpms1-139	C	65 ^a	5	60	30
Bd17	Hpms1-165	A	54	35		
Bd22	Hpms1-214	A	58	35		

to a single amplification (band). A random 10% of the samples for each microsatellite system were repeated to test the repeatability of the technique. These replicates were compared to their respective samples for verification of the accuracy of the genotype assignment of the HRM technique.

Data analysis

Microsatellite information content (PIC) (Botstein et al. 1980), discrimination power (D) (Waits et al. 2001), average number of alleles per locus, proportion of polymorphic loci, number of effective alleles, observed and expected heterozygosity and their standard deviation, standard deviation of allele frequencies per line per marker (Falconer and McKay 2006) were calculated. For each species studied, these estimates were calculated using the GenAlex program version 6.503 (Peakall and Smouse 2012). In addition, an analysis of molecular variance AMOVA was performed for each species using the program Harlequin 3.1 (Excoffier and Lischer 2010). The levels were: between groups (original line vs. derived lines), within groups (variation between lines), between individuals within lines (variation within lines), and within individuals (allelic variation).

Wright's F-statistics were calculated, and based on the paired F_{ST} between lines, a dendrogram was constructed using the average linkage method (Tamura et al. 2021). The coefficient of co-phenetic correlation between the matrix of paired F_{ST} and the dendrogram was also calculated.

In addition, to evaluate the genetic structure of the cultivated lines, a principal component discriminant analysis with $K=4$ and $K=5$ was carried out for *C. annuum* and *C. frutescens*, respectively, and a probability plot of the assignment of each individual to the groups was made. Finally, to investigate the discriminatory ability of three SSR markers to identify the *annuum* complex species from this seedbank, 30 individuals of *C. chinense* were included in this study.

Results

HRM parameters

For all microsatellites analyzed, the curves generated by the Precision Melt Analysis™ v1.2 software

allowed the assignment of individuals to a specific genotype with a reasonable level of confidence ($\geq 95\%$). The random replication of samples was successful by assigning 100% of the replicates to the genotype previously assigned in their respective original sample. Supplementary Data 2 shows the microsatellites used for genotyping with their average cq value (Cq), T_m , RFU (relative fluorescence units) and size interval reported by Guzman et al. (2020).

Informativeness of the markers

Table 2 shows the main informativity indices of the microsatellites under evaluation for each species. Bd17 (*C. frutescens*) and Ng9 (*C. annuum*) were the most informative markers (with the highest number of alleles, PIC and discriminatory power). Markers Ng7, Ng20 and Bd3 also showed moderate PIC and discriminatory power in *C. annuum*. In *C. frutescens*, the remaining markers had low and similar informative values. Out of the 15 microsatellites, five were monomorphic, five of them in *C. annuum* and two of them in *C. frutescens*. Although

Table 2 Informativity indices for the microsatellites (SSR) evaluated in the lines of *Capsicum frutescens* and *C. annuum*. PIC (polymorphic information content), D (discrimination index). ¹SSR evaluated only in *C. frutescens*. ²SSR evaluated only in *C. annuum*. ³SSR evaluated in both species, data left corresponding to *C. frutescens* and right corresponding to *C. annuum*

Marker	Number of alleles	Number of effective alleles	PIC	D
Ng4 ¹	3	1.03	0.03	0.053
Ng6 ²	1	1	0	0
Ng7 ²	4	1.24	0.186	0.346
Ng8 ¹	3	1.04	0.04	0.077
Ng9 ²	7	2.59	0.674	0.838
Ng10 ³	3/1	1.03/1	0.03/0	0.052/0
Ng15 ¹	2	1.09	0.08	0.145
Ng18 ¹	2	1.03	0.03	0.051
Ng20 ²	4	1.83	0.501	0.766
Ng33 ¹	3	1.03	0.03	0.053
Ng35 ²	1	1	0	0
Bd3 ³	1/6	1.00/2.26	0.00/0.537	0/0.758
Bd12 ³	1/1	1.00/1.00	0/0	0/0
Bd17 ¹	5	1.65	0.46	0.676
Bd22 ¹	5	1.12	0.10	0.180

the remaining markers had more than two alleles, the number of effective alleles was found to be low (close to one). Even the most diverse markers of each species, Bd17 and Ng9, had less than half the number of effective alleles. This shows that loci can have multiple alleles, but one or two alleles are the most representative of the population. The allele frequencies with their standard deviation for each marker for each of the lines evaluated in the two species are shown in Supplementary Data 3.

Genetic diversity

The original *C. frutescens* line was found to have the highest diversity with about 14 genotypes, six polymorphic loci and an average of 2.33 alleles/locus when comparing the genetic diversity among the different lines (Table 3). It was followed by lines TL-19 and TL-23 with 9 and 10 genotypes, respectively. The number of polymorphic loci and the average number of alleles were similar. Finally, lines TL-08 and TL-05 were found to have the lowest genetic diversity. They had the fewest genotypes (5 and 6) and a low number of polymorphic loci. On the other hand, for *C. annuum*, none of the evaluated lines showed a higher genetic diversity than the others. This was true for the number of genotypes, the expected heterozygosity and the number of polymorphic loci. Absence of heterozygous individuals is reported in the lines of both species, which means that all alleles found are in homozygosis, hand in hand with FIS and FIT indices equal to 1 found for both species.

Genetic structure

The analysis of molecular variance (Table 4) showed that most of the genetic variation was observed when comparing individuals within lines, with 55.49% of variation for *C. frutescens* and 75.43% of variation for *C. annuum*. There was no variation within individuals in either species, while the variation when comparing the original line and the derivatives was greater in *C. frutescens* (37.19%) than in *C. annuum* (11.6%).

The dendrogram based on F_{ST} (Fig. 1) for *C. annuum* shows that the original line differs from the derivatives, but the differentiation values not only of this comparison but also of the others are below 0.15, indicating low differentiation. For *C. frutescens*, the dendrogram shows that line TL-5 is the most differentiated, followed by line TL-8, and the original line is grouped with the remaining lines. In this case, the differentiation values are higher than for *C. annuum*. In some comparisons, they are higher than 0.4, indicating strong genetic differentiation.

Finally, the information obtained from the dendrogram and AMOVA was supported by a discriminant analysis. For *C. annuum*, more than half of the individuals within each line are assigned to different groups. Line CL-50 is the one with more individuals distributed among the proposed groups. In the case of *C. frutescens*, the discriminant analysis shows the homogeneity of the derived lines. For example, in TL-8 all individuals are grouped in the same group. Similarly, TL-23 and TL-5 have less than 6 individuals in different clusters. While the assignment of individuals to clusters is more heterogeneous in the original line. For both species, the analysis was performed with clustering number $k=2$ (Supplementary Data

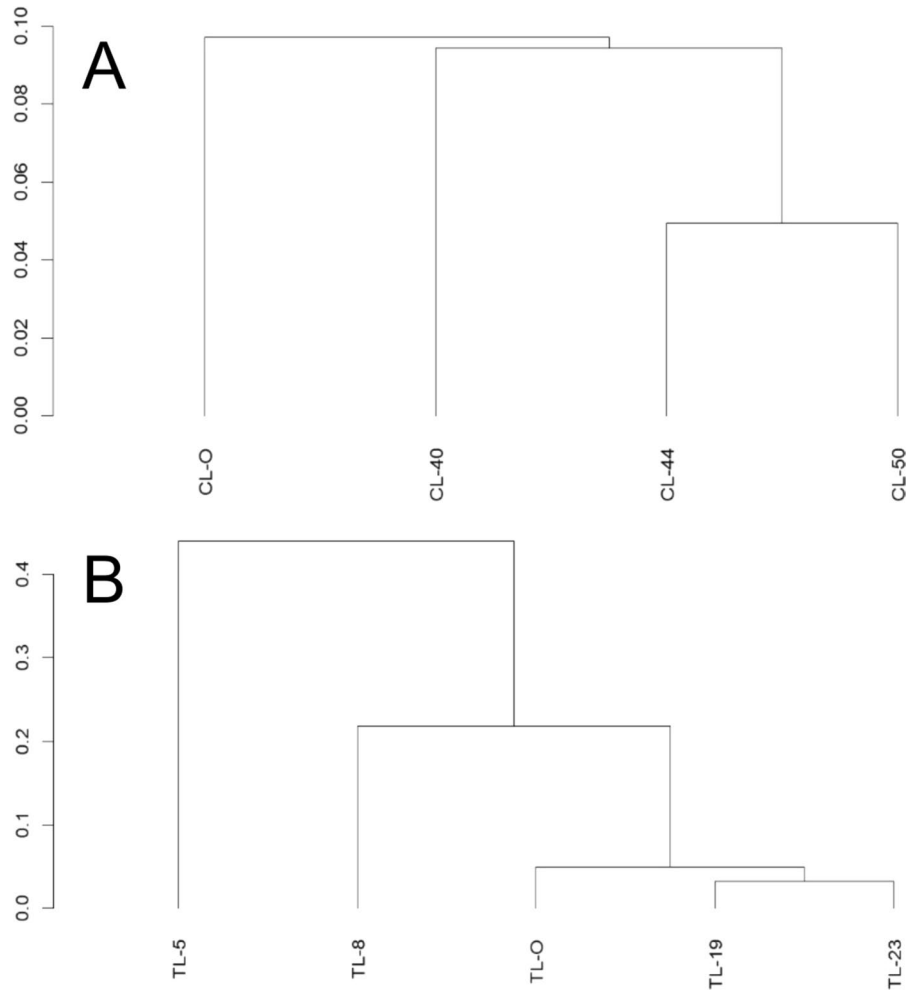
Table 3 Genetic diversity indices for the evaluated *Capsicum* lines. SD: Standard deviation

Line	Number of genotypes	Expected heterozygosity $\pm$ SD	Number of polymorphic loci	Average number of alleles per loci	Private alleles
TL-O	14	0.157 $\pm$ 0.064	6	2.33	7
TL-19	9	0.074 $\pm$ 0.059	3	1.44	1
TL-23	10	0.049 $\pm$ 0.032	2	1.44	0
TL-08	5	0.036 $\pm$ 0.019	3	1.22	1
TL-05	6	0.027 $\pm$ 0.018	0	1.55	3
CL-O	10	0.222 $\pm$ 0.005	4	1.75	1
CL-40	8	0.156 $\pm$ 0.004	4	1.75	1
CL-44	10	0.228 $\pm$ 0.005	3	2.00	2
CL-50	11	0.194 $\pm$ 0.005	3	1.75	0

Table 4 Molecular variance analysis for *Capsicum frutescens* and *C. annuum* cultivars. *p-value < 0.05

Variation source	Square summatory	Mean square	Variation percentage (%)
<i>C. frutescens</i>			
Between original and derived lines	16.533	0.14925	37.194*
Between derived lines	6. 618	0.02933	7.309*
Between individuals within lines	64.260	0.22270	55.497*
Within individuals	0	0	0
Total	87.411	0.40128	100
<i>C. annuum</i>			
Between original and derived lines	27.847	0.1330	11.600*
Between derived lines	21.422	0.1490	12.9700*
Between individuals within lines	207.021	0.8690	75.4300*
Within individuals	0	0	0
Total	256.290	1.1530	100

Fig. 1 Dendrogram of (A) *C. annuum*, (B) *C. frutescens* calculated with the average linkage method and the paired F_{ST} matrix



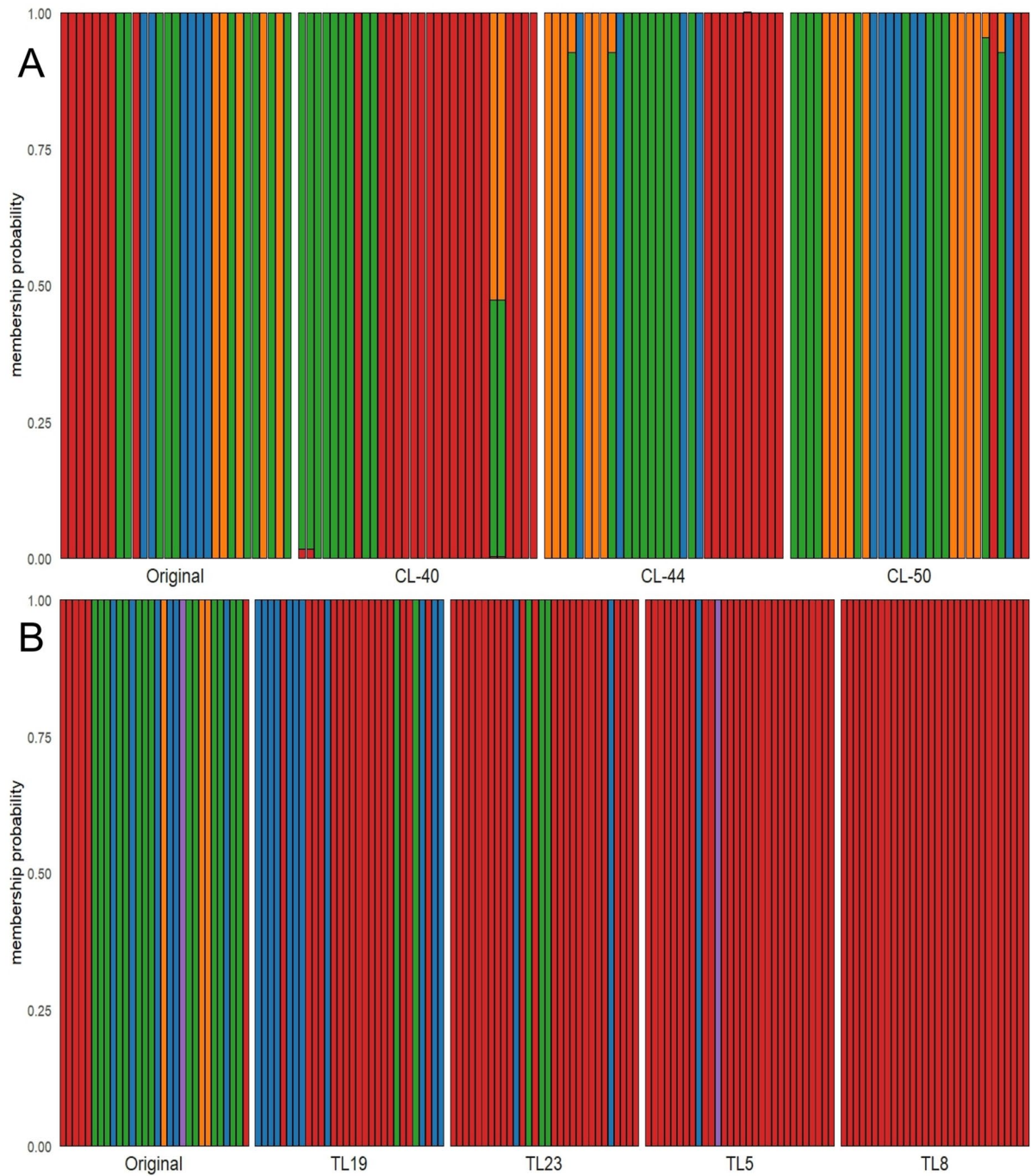


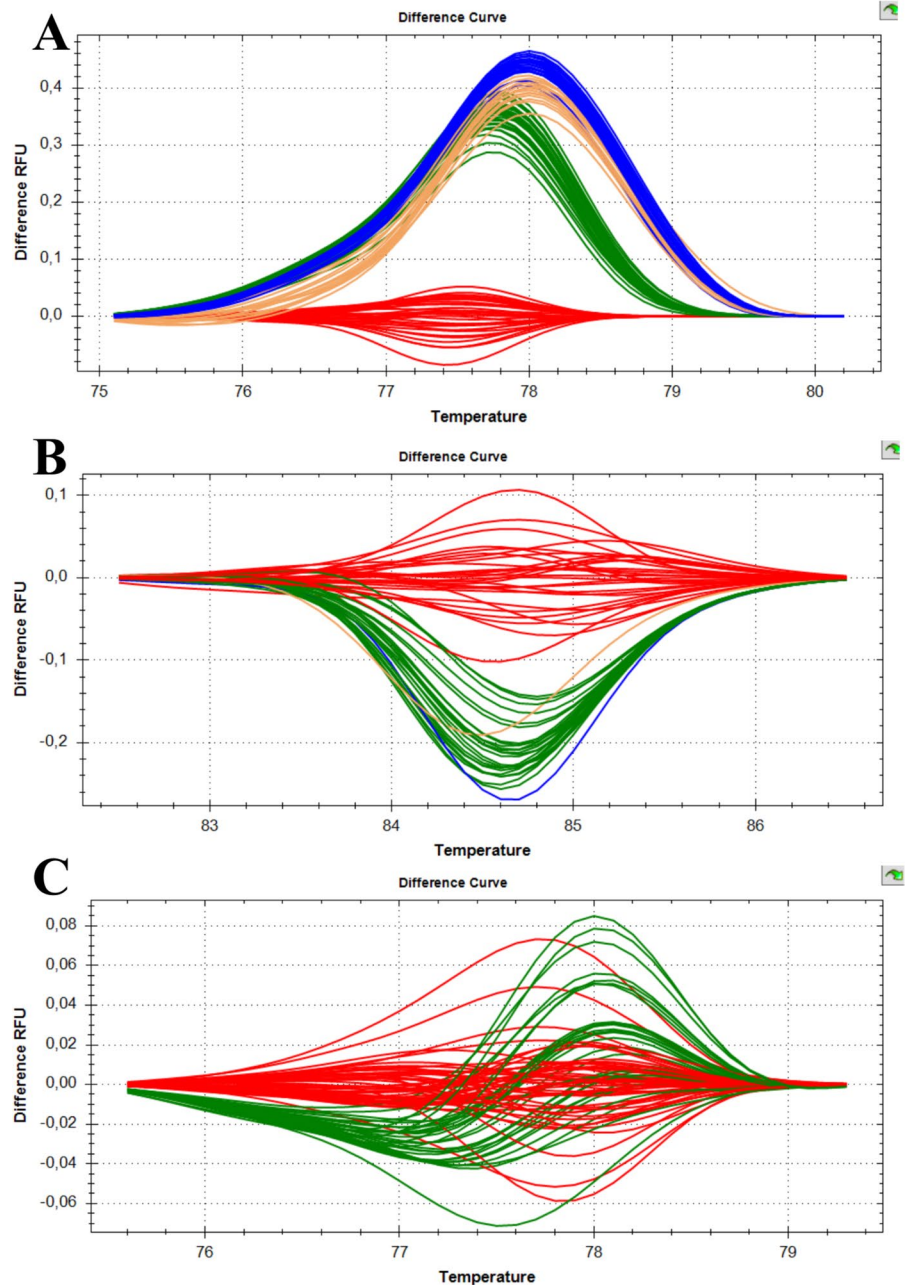
Fig. 2 Discriminant analysis for individuals of two *Capsicum* species lines and cluster assignment probabilities. (A) *C. annuum*, K=4. (B) *C. frutescens*, K=5. Each color represents a different cluster

4) to compare the original line with the derived ones. The results are similar to those presented previously (Fig. 2).

Microsatellite markers and HRM analysis

Based on the HRM analysis, three of the evaluated markers (Ng 10, Ng 18 and Ng 33) were able to discriminate the species of the *annuum* complex (*C. annuum*, *C. frutescens* and *C. chinense*). Ng 18 discriminated the three species of the complex by forming a group for each of them (Fig. 3A), Ng 10

Fig. 3 Temperature vs. relative fluorescence unit (RFU) difference plots for three microsatellite systems of *Capsicum* spp. A) Ng18 marker: red group represents *C. frutescens*, green *C. chinense* and blue and orange *C. annuum*. B) Ng10 marker: the green group represents *C. chinense* and the red group represents *C. annuum* and *C. frutescens*. C) Marker Ng33: the green group is *C. frutescens* and the red is *C. annuum* and *C. chinense*



discriminated two groups by its HRM curves, one composed of *C. chinense* and the other of *C. annuum* together with *C. frutescens* (Fig. 3B), while Ng 33 formed two groups, but one composed of *C. annuum*-*C. chinense* and the other with *C. frutescens* only (Fig. 3C).

Discussion

In both species, no heterozygous individuals were found for any of the loci evaluated. Several investigations in the genus *Capsicum* have reported very low heterozygosities for several molecular markers (Yumnam et al. 2012; González et al. 2014; Zhang et al. 2016; Lee et al. 2004; Rivera et al. 2016; Taranto et al. 2016). Very low heterozygosities have also been found for most of the loci used in research of wild Colombian populations of different species of the genus (Pardey 2009).

Since HRM is able to detect heterozygous samples (natural or artificial mixtures) regardless of the region or marker used, a technique effect can be excluded (Mader et al. 2008; Arthofer et al. 2011; Cheng et al. 2011; Ganopoulos et al. 2011). Thus, the low level of heterozygosity that was found can be explained by the high rate of autogamy. Odland and Porter (1941) and Justino et al. (2018) reported autogamy rates of 90 and 95% for the genus. This, along with self-compatibility for most species (Onus and Pickersgill 2004), explains the low proportion of heterozygous individuals found in most molecular research of the genus. The effect of autogamy on the proportion of heterozygous individuals in a population was shown by Falconer and McKay (2006). They showed that with 5% autogamy, the expected percentage of heterozygotes is 10% of the expected heterozygosity. In this study, the highest expected heterozygosity was recorded for the original *C. annuum* line ($H=0.222$), in which case the expected heterozygosity is 2.2%. This value is quite low to be recorded for the size of the sample that was used in this study. This explains the absence of heterozygous individuals in this study, considering that the expected heterozygosity for the other lines is lower.

The selection process and pollination control to which the evaluated lines were subjected, together with autogamy, acted as enhancers of the heterozygosity reduction process and created highly inbred lines.

The greatest differentiation is found when comparing individuals within each line, since individuals with different combinations of homozygous genotypes are compared. As a result, the results of the AMOVA for both species show the greatest variation at the level between individuals. This, in turn, influenced the discriminant analysis, since the low informativeness of the markers used, together with the high genetic homogeneity found in the *C. frutescens* lines (mainly TL-08), did not allow the discrimination of individuals in the proposed groups (lines).

In addition, the diversity indices showed that the original line of *C. frutescens* has greater genetic diversity than the other lines evaluated (Table 3). This could be explained by the fact that it is the only line that has not been subjected to any artificial selection process. Therefore, this genetic resource still has genetic potential for developing future breeding programs. On the other hand, although the differentiation between the original and derived lines is also present in *C. annuum*, it is less than in *C. frutescens* and the diversity estimators indicate that the other lines have a genetic diversity very similar to the original line. In this case, each line is a good candidate for a breeding program and its use is a matter of interest to the farmer.

Comparing the genetic diversity found in this study with research of natural populations and other accessions of *Capsicum* species, it can be noted that it is not high. For example, Villela et al. (2014) found 4.77 alleles/locus in *C. baccatum* populations, Tsaballa et al. (2015) found 6 alleles/locus in *C. annuum* populations, Marcos-Perez (2013) found 6.15 alleles/locus in *C. annuum*, and Ibarra-Torres et al. (2015) found 4.91 alleles/locus in *C. annuum* accessions. However, when compared to research in which samples had some type of selection, the average number of alleles is quite similar to this study (1.22–2.33 alleles/locus); Marcos-Pérez et al. (2013) found a range of 1.93–2.73 alleles/locus in commercial lines of *C. annuum* from Spain, while Rivera et al. (2016) found an average of 3 alleles/locus in inbred lines of *C. annuum*.

In this study, the line with the highest genetic homogeneity was TL-08. These results are consistent with the morphological study by Peñuela et al. (2021), which evaluated the same line but based on agronomic traits to estimate strict heritability using progenitor-progeny regression. In that study, heritability was estimated to be zero, indicating that the

phenotypic variation observed in the traits is mainly influenced by environmental components. Therefore, a selection program in TL-08 is not recommended because there is not enough variation to exploit in order to obtain a significant genetic gain in the selected traits (Falconer & Mackay 2006; Abbot & Pistorale 2010). Therefore, an evaluation of the agronomic performance under different environmental conditions is recommended. This will help to find the environment that gives the best performance of this line.

It was not possible to make a correct assignment of individuals to each line based on the discrimination indices estimated for each marker and the discriminant analysis. Although it is true that there were private alleles in 7 of the 9 lines under study, their frequency is not sufficient for a reliable assignment, especially in *C. frutescens*, where lines TL-8 and TL-5 have genetic homogeneity. Therefore, to identify the individuals of each line, it is necessary to increase the number of loci. However, it was possible to distinguish the species *C. frutescens* from *C. chinense* and *C. annuum* found in the seed bank using three microsatellite systems (Ng 10, Ng 18 and Ng 33). These markers are proving to be a useful tool for identifying plant material at the seedling or vegetative stage. This saves time compared to morphological identification, which can take one to two months depending on the species.

Conclusions

Molecular characterization has allowed us to detail a genetic differentiation between the original line and the derived lines in both species, resulting from the selection process the latter have undergone. Therefore, the original line can be postulated as a possible source of genetic resources for future breeding programs, due to the presence of a greater genetic diversity. Based on these data, an evaluation of the TL-08 line in different types of environments is recommended. This will help to find the combination of environmental variables in which it performs best. Finally, it was possible to discriminate the evaluated *Capsicum* species using the HRM technique to genotype individuals with microsatellite markers. In cases where morphological markers are absent or

ambiguous, this represents a useful tool for species differentiation.

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Authors' contribution RAVV conceived and designed the experiment, analyzed the data, prepared the figures and tables, drafted the manuscript, and revised and approved the final paper. HGC conceived and designed the experiment, analyzed the data, drafted the manuscript, and reviewed and approved the final paper. HC conceived and designed the experiment, reviewed and approved the final paper, and managed and supervised the project. HGC conceived and designed the experiment, analyzed the data, drafted the manuscript, and reviewed and approved the final paper. HC conceived and designed the experiment, reviewed and approved the final paper, and managed and supervised the project.

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Data Availability The data that support the findings of this study are available from the corresponding author, RAVV, upon reasonable request. No datasets were generated or analysed during the current study.

Declarations

Conflicts of interest The authors declare no competing interests.

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