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REMEXCA

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Article

Genetic diversity and phylogeny of ITS regions in sweet orange genotypes from San Luis Potosí

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Abstract

The sweet orange is grown in several states of Mexico. Several research centers have citrus germplasm banks, including sweet oranges for genetic improvement. This work aimed to evaluate genetic diversity and perform a phylogenetic analysis based on the internal transcribed spacer (ITS) region of six sweet orange genotypes grown in San Luis Potosí, Mexico. In 2023, leaves of six varieties of sweet orange, known in the region as Sangre de Toro and Valencia, were collected. Genomic DNA was extracted, and ITS regions were amplified by PCR and sequenced. The percentage of identity and genetic distances were determined and a maximum-likelihood phylogenetic tree was generated. The results indicated that the intraspecific variability among *C. sinensis* genotypes in the ITS region is low to moderate (0-0.07). Particularly, among the population of *Citrus sinensis* (Sangre de Toro and Valencia varieties) from the regions of Axtla de Terrazas, Chimimexco-Tampacán and El Frijolillo-San Martín in San Luis Potosí, no differences were detected in their ITS regions. It is important to note that the number of samples analyzed was limited; therefore, it is necessary to evaluate a larger set of samples and varieties to obtain more robust conclusions regarding genetic variability in *Citrus sinensis* in Mexico. The phylogenetic analysis grouped the genotypes of Mexico into group G1, confirming the origin of the varieties with Asian countries (China, Korea and Vietnam), with which the percentage of identity was 100%.

Keywords:

Citrus sinensis, genetic variability, sequencing.

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Introduction

The sweet orange (*Citrus sinensis*) is one of the most consumed citrus worldwide. The fruits are highly appreciated for their organoleptic characteristics and high nutraceutical value, mainly for their high antioxidant content (Seminara *et al*., 2023). The genetic characterization of *C. sinensis* materials is important for breeding programs that seek to cross various materials to generate varieties with desirable genetic and commercial traits (Almeyda-León *et al*., 2023). In this sense, work has been carried out to determine the genetic variability and phylogenetic analyses of a wide range of *C. sinensis* varieties using various molecular markers, such as random amplified microsatellites (RAMs), random amplified polymorphic DNA (RAPDs), simple sequence repeats (SSRs) and start codon targeted (SCoT).

Abbaszadeh *et al*. (2023) determined low to moderate genetic diversity in 29 sweet orange genotypes from Iran using the SCoT molecular marker. The population showed a genetic structure influenced by geographical distance and climatic variables (temperature and precipitation). In another study, Shahnazari *et al*. (2022) conducted a genetic diversity analysis of 80 sweet orange trees of different varieties from Iran using SSR markers, finding high genetic variability that supports the use of these markers for identifying and classifying varieties. On the other hand, Tuwo *et al*.(2023) employed RAPD markers to analyze the genetic diversity of citrus species in five production centers in Indonesia, finding a high level of polymorphisms and close relationships among some genotypes.

In Mexico, Almeyda-León *et al*. (2022) carried out the genetic characterization of a population of 33 genotypes of sweet orange (*C. sinensis*) from the germplasm bank of the General Terán Experimental Field of the National Institute of Forestry, Agricultural and Livestock Research (INIFAP, by its Spanish acronym) in Nuevo León, Mexico, using RAM markers. All of these molecular markers produce binary matrices of the presence or absence of fragments to analyze genetic diversity, but their development can be laborious and error-prone. Alternatively, procedures such as DNA barcoding can be used, thus increasing the accuracy of varietal characterization.

In plants, nuclear regions (ITS, ITS1 and ITS2) and chloroplast regions (matK, rbcL, trnH-psbA, rpoB, rpoC1, trnL-trnF and psbK-psbI) have been used (Letsiou *et al*., 2024). These markers have been used for phylogenetic analyses with several citrus species, including *C. sinensis* (Li *et al*., 2010; Kyndt *et al*., 2010; Penjor *et al*., 2013; Hynniewta *et al*., 2014; Ito *et al*., 2014; Jin *et al*., 2018; Liu *et al*., 2021; Abbaszadeh *et al*., 2023). However, most studies are focused on Asian countries. In Latin America, there are no studies that have used the ITS region in *C. sinensis* to determine its diversity.

The objective of this work was to assess genetic diversity and perform a phylogenetic analysis based on the ITS region of six *C. sinensis* genotypes grown in San Luis Potosí, Mexico.

Materials and methods

Vegetative material

Young, healthy-looking leaves were collected from six sweet orange trees (Table 1). The leaves were wrapped in paper bags and placed in thermal coolers with refrigerant gels to be sent to the Biotechnology Laboratory of INIFAP’s Tecomán Experimental Field in Colima, where they were immediately processed.

Table 1. List of sweet orange samples used in this study.

Sample	Variety	Locality	Georeference
NarST M1	Sangre de Toro	Axtla de Terrazas	21 22 20-98 53 32
NarST M2	Sangre de Toro	Axtla de Terrazas	21 22 20-98 53 32
NarVal M3	Valencia	Chimimexco, Tampacan	21 24 24-98 48 14
NarVal M4	Valencia	Chimimexco, Tampacan	21 24 24-98 48 14
NarVal M5	Valencia	El Frijolillo, San Martín	21 14 49-98 36 02
NarVal M6	Valencia	El Frijolillo, San Martín	21 14 49-98 36 02

Extraction, quantification, purity, and integrity of plant DNA

The method used was that described by Bermúdez-Guzmán *et al*. (2016) with some modifications. Plant tissue (approximately 200 mg) was macerated in mortars and homogenized in 2 ml of CTAB buffer preheated at 65 °C for 60 min and treated with RNase A, followed by phase separation with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitation of DNA with cold isopropanol and sodium acetate. The DNA pellet was washed with 70% ethanol, dried at room temperature, and resuspended in water.

Quantification and purity were determined from 2 µl of each sample, measuring the 260/280 and 260/230 nm absorbance ratios using a NanoDrop 2000 spectrophotometer (Thermo Scientific). DNA integrity was verified by 1% agarose gel electrophoresis in 1X TAE buffer. Nucleic acid staining was performed with GelRed (Biotium) and visualized on a gel documentation system (UVP).

PCR amplification, purification, and sequencing of ITS regions

The oligonucleotides used were those described by White *et al*. (1990), modified by Viglietti *et al*. (2019): ITS4: 5’-TCCTCYRMTTAKYGATATGC-3’ and ITS1: 5’-TCCGTWRGTGAACCWGCGG-3’. PCR reactions were performed in a volume of 50 µl using REDTaq® ReadyMixTM PCR Reaction Mix (Sigma Aldrich), according to the manufacturer’s instructions.

The annealing temperature was standardized using a gradient: 50, 51, 52, 53, 54 and 55 °C in a VeritiTM thermal cycler (Applied BiosystemsTM). For all cases, the amplification conditions were 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 30 s at 50-55 °C, and 1 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products between 600 and 700 bp were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) and sent for sequencing to the National Laboratory of Agricultural, Medical, and Environmental Biotechnology (Lanbama, by its Spanish acronym).

Genetic diversity and phylogenetic analysis

The sequences were edited, a BLAST analysis was performed in the NCBI database, and 28 sequences of the ITS regions from *C. sinensis* were downloaded to generate a local database. As an external group, an ITS sequence from *Ruta graveolens* was included, and a multiple sequence alignment (MSA) was performed using the ClustalW algorithm.

To analyze genetic diversity, sequence-comparison matrices were generated based on the MSA. The distance matrix was calculated as the Jukes-Cantor corrected distance between two sequences. For the phylogenetic analysis, the MSA was analyzed with the ‘model test’ tool. A phylogenetic tree was constructed using the UPGMA construction method with the GTR + G + T nucleotide substitution model and a Bootstrap analysis with 1 000 replicates. All the aforementioned bioinformatic analyses were carried out using a valid license of the CLC Main Workbench software, version 24.0.2 (Qiagen).

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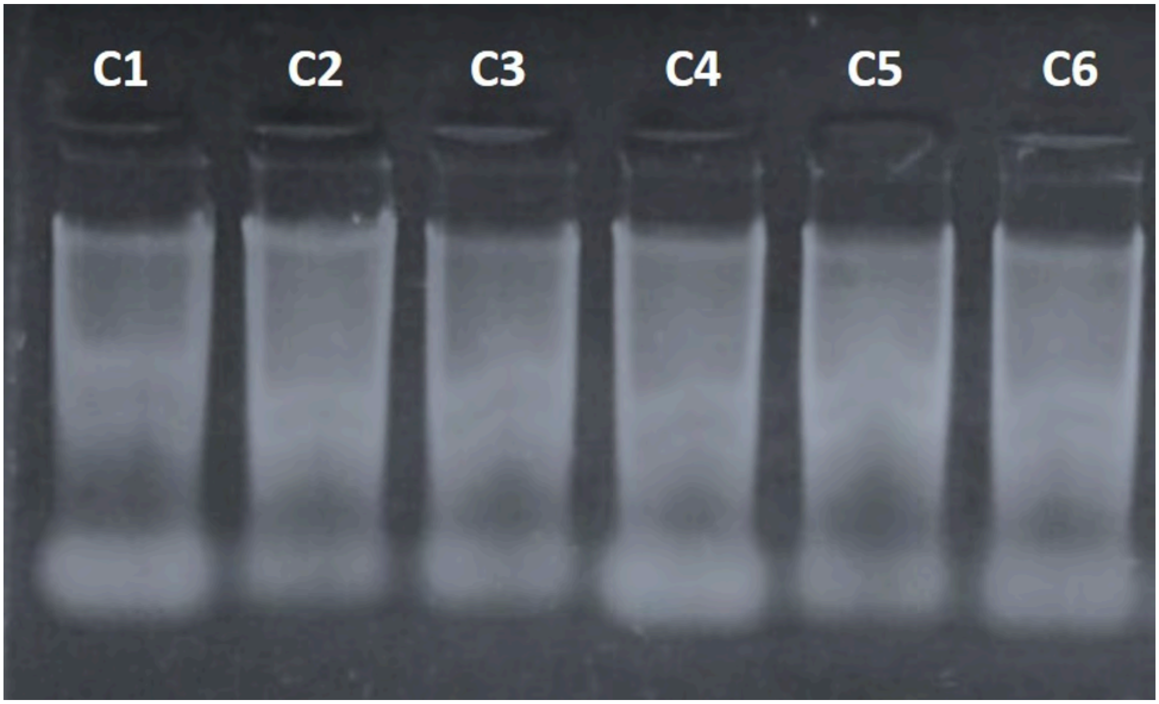
Results and discussion

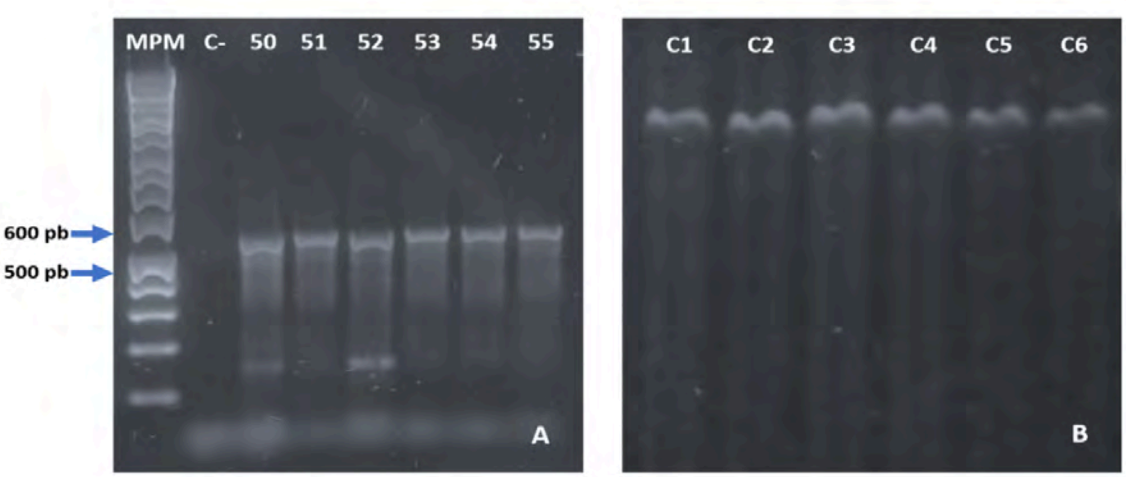
The average purity indices were 1.8 (A260/280) and 1.79 (A260/230). Figure 1 presents the agarose gel electrophoresis of the extracted genomic DNA, which shows partial degradation of the samples, likely due to the time elapsed since transport from San Luis Potosí to Colima. Nonetheless, in the upper part of the gel, high-integrity DNA bands were observed, sufficient for PCR amplification of the ITS region and for obtaining sequences.

Figure 1. Electrophoresis of genomic DNA extracted from *Citrus sinensis* leaves. Lanes C1-C2= samples of the Sangre de Toro variety; lanes C3-C6= samples of the Valencia variety. An average of 250 ng of DNA was loaded into each lane.



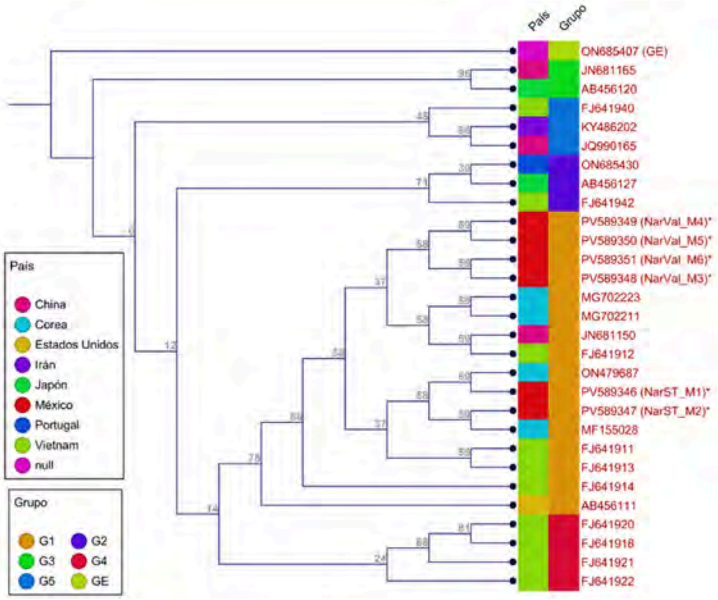
Gradient PCR amplification to assess the effect of temperatures of 50-55 °C is shown in Figure 2A. Temperatures of 53 to 55 °C showed unique bands, and the temperature of 54 °C was selected to amplify the six orange tree samples by PCR (Figure 2B).

Figure 2. Electrophoretic profile of the ITS region of orange trees. A) gradient PCR to standardize annealing temperature; B) PCR products (approximately 600 bp) from orange tree samples; MPM= molecular weight marker 100 bp (Sigma Aldrich); C= negative control water; 50-55= annealing temperatures; Lanes C1-C2= samples of the Sangre de Toro variety; lanes C3-C6= samples of the Valencia variety.



In the phylogenetic tree of several *C. sinensis* genotypes from different regions of the world, it is observed that the 28 genotypes were distributed in five groups (G1-G5) (Figure 3). The Mexican genotypes formed a group with others from Korea, China and Vietnam (G1), with some of which they share 100% identity (JN681150, MG702211, MG702223, MF15502 and ON479687), constituting the most closely related materials.

Figure 3. Maximum likelihood tree of the ITS region with *Citrus sinensis* genotypes. The names adjacent to the color code correspond to the accession numbers in NCBI; The numbers on the nodes correspond to the Bootstrap values; An asterisk indicates the genotypes of this study; GE= external group *Ruta graveolens*.



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Since increasing geographic distance often increases genetic distance (Abbaszadeh *et al*., 2023), in this study, the genetic variability in the ITS region among the six genotypes of *C. sinensis* was 0, with homologous sequences, probably due to the proximity of the sampling sites and the limited number of samples analyzed. This indicates high conservation between the Sangre de Toro and Valencia varieties in this region. The genotypes from Portugal, Japan and Vietnam, which form group G5 (Figure 3), showed greater genetic distance, with values of 94.1-95.71% of identity and a distance coefficient of 0.04-0.06.

The ITS sequence is widely used to identify plant species and conduct phylogenetic studies (Alvarez and Wendel, 2003; Graper *et al*., 2021). In citrus, it has been successfully used in species such as *C. sinensis*, *C. limon* and *C. reticulata* to evaluate genetic variability and phylogenetic relationships (Hynniewta *et al*., 2014). In this study, the genetic distance between all genotypes of *C. sinensis* (28 sequences) was low to moderate (0-0.07), indicating high conservation in the ITS region, whereas for *Ruta graveolens*, the values were 0.24-0.29. Similar results were obtained by Liu *et al*. (2021) in 22 samples of *C. reticulata* ‘Chachi’, where the ITS2 region showed a 100% homology, indicating high genetic stability in this region.

On the other hand, the genotypes of *C. sinensis* from Vietnam showed greater intraspecific variability (distances of 0.02-0.06) and were grouped into G1, G2, G4, and G5. Materials from China were in G1, G3, and G5, whereas those from Korea and Mexico clustered in G1. This diversity can be attributed to different varieties, climatic regions and cultivation and breeding practices (Abbaszadeh *et al*., 2023; Seminara *et al*., 2023). In contrast to these results, Almeyda-León *et al*. (2022) evaluated 33 varieties of sweet orange trees using RAMs, achieving a high percentage of polymorphisms and differentiating all the materials evaluated, which indicates a broad genetic base in their germplasm.

In other studies, using RAPDs, Tuwo *et al*. (2023) identified five groups with 175 citrus genotypes using cluster analysis with a similarity coefficient of 77%. On the other hand, SSR markers have also been used to identify hybrids for genetic improvement in citrus (Bermúdez-Guzmán *et al*., 2017; Gallego-Colonia *et al*., 2017; Sharafi *et al*., 2017; Carrillo-Medrano *et al*., 2018). This suggests that the use of molecular markers based on electrophoretic profiles, such as RAPDs, RAMs, SSRs and SCoT, among others, is still very useful for characterizing genetic variability in *C. sinensis* and other citrus species (Shahnazari *et al*., 2022; Abbaszadeh *et al*., 2023).

The use of these molecular markers for plant characterization dates back to the 90s (Williams *et al*., 1990; Morgante and Olivieri, 1993), but they are still widely used today because they are highly versatile, as they explore the entire genome. This study evaluated a small region of a genome, ITS, with a length of just over 550 bp, which represents a tiny part of the entire genome. For this reason, to make the diversity analysis more robust, it is advisable to use more than one region, such as nuclear regions (ITS, ITS1 and ITS2) or chloroplast regions (matK, rbcL, trnH-psbA, rpoB, rpoC1, trnL-trnF, psbK-psbI) (Letsiou *et al*., 2024).

On the other hand, it is important to consider that the costs of massive sequencing (NGS) have decreased significantly, so in the near future, it is desirable to migrate to this type of marker. With this technology, a large amount of information can be obtained on genetic variation, such as single-nucleotide polymorphisms (SNPs), insertion-deletions (InDels), structural variation (SV) and copy number variation (CNV), among others (Wenger *et al*., 2019; Ahsan *et al*., 2021).

Conclusions

The ITS region can be used as an exploratory and comparative tool to evaluate phylogenetic relationships in the genus *Citrus*; however, its intraspecific resolution was limited in closely related materials, as was the case with *C. sinensis* genotypes. In this study, with six samples corresponding to two varieties from San Luis Potosí (SLP), a low to moderate intraspecific variability (0-0.07) was observed between various genotypes of *C. sinensis* from various parts of the world. These findings are specific to the samples analyzed from the SLP region. The genotype sequences showed similarities with Asian accessions (China, Korea and Vietnam), but expanding genomic coverage is required to confirm these possible origins.

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