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Study on *Croton* sp. genetic diversity in the Department of Norte de Santander using the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA)

Estudio de la diversidad genética de *Croton* sp. en el departamento de Norte de Santander utilizando la región espaciadora transcrita interna (ITS) del ADN ribosómico (rDNA)

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***Croton draco* Schltdl., known as dragon's blood.**

Photo: G. Chaves-Bedoya

ABSTRACT

Genetic variability studies on species with a pharmacological potential are essential for conserving genetic resources. A genetic characterization of a species can guide efforts to collect and conserve germplasm for future breeding programs. The *Croton* genus belongs to the Euphorbiaceae family, which has approximately 1,300 species, is widely distributed in tropical and subtropical regions of the world, and has a wide range of ethnobotanical and medicinal uses. The aim of this research was to study genetic variability by analyzing sequences of the internal transcribed spacer (ITS) region (ITS1 5'-TCCGTAGGGAACCTGCGGC-3' and ITS4 5'-TCCTCCGCTTATGC-3') of ribosomal DNA (rDNA) of a *Croton* population from forested areas in the Department of Norte de Santander in the municipalities of Chinacota, Pamplona and El Zulia (Colombia). The results indicated considerable genetic variability in the *Croton* population, with a nucleotide similarity ranging from 54 to 99% and phylogenetical grouping according to the place of origin. The information gained from the ITS region can be a useful parameter for diversity evaluations and phylogenetic studies since there are no similar studies on *Croton* in this department in northeastern Colombia.

Additional keywords: Euphorbiaceae; medicinal plants; Drago's blood; Neotropics; molecular systematics.

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RESUMEN

Los estudios de variabilidad genética de especies con potencial farmacológico son esenciales para la conservación de los recursos genéticos. La caracterización genética de una especie puede guiar los esfuerzos para recolectar y conservar germoplasma para futuros programas de mejoramiento. El género *Croton* pertenece a la familia Euphorbiaceae, que comprende aproximadamente 1.300 especies, ampliamente distribuidas en regiones tropicales y subtropicales del mundo y se caracteriza por una amplia gama de usos a nivel etnobotánico y medicinal. El objetivo de esta investigación fue estudiar la variabilidad genética a través del análisis de secuencias de la región espaciadora transcrita interna (ITS, del inglés internal transcribed spacer) (ITS1 5'-TCCGTAGGGAACCTGCGGC-3' e ITS4 5'-TCCTCCGCTTATGC-3') del ADN ribosomal (rDNA) de una población de *Croton* presente en áreas boscosas del departamento de Norte de Santander en los municipios de Chinacota, Pamplona y El Zulia (Colombia). Los resultados indican que existe una considerable variabilidad genética entre los individuos de la población de *Croton* con una similitud de nucleótidos que oscila entre el 54 y el 99%, agrupándose filogenéticamente según el lugar de origen. La información generada en este trabajo desde la región ITS es un parámetro útil para la evaluación de la diversidad y los estudios filogenéticos ya que no existen estudios de esta naturaleza en *Croton* en este departamento del noreste de Colombia.

Palabras clave adicionales: Euphorbiaceae; plantas medicinales; Sangre de Drago; Neotrópico; sistemática molecular.

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INTRODUCTION

Croton is a genus in the Euphorbiaceae family with about 1,300 widely distributed species in tropical and subtropical regions, mainly in arid and semi-arid areas, with greater diversity in Brazil, Madagascar and the Caribbean (Luján *et al*., 2015). It is the second most numerous and diverse genus and encompasses herbs and trees with varied leaf forms, with glands at the base of the leaf or on the petiolo and female flowers with reduced or absent petals. Most *Croton* species have secretory structures such as floral and extrafloral nectaries (Salatino *et al*., 2007; Luján *et al*., 2015) and have abundant compounds with biological activity, mainly diterpenoids and active alkaloids (Milanowski *et al.,* 2002; Block *et al*., 2004). Taspine is a minor alkaloid found in the sap of mature trees (Perdue *et al.,* 1979).

A wide range of chemical compounds, such as terpenes, alkaloids and others with biological, medicinal and industrial properties, impart a high economic potential to the *Croton* genus. (Murillo, 1999). The *Croton* genus is characterized by species with a large number of uses at the ethnobotanical level, as validated by ancestral stories and the bibliography of recent decades. In Colombia, the first records date back to 1940, where 13 new species were reported along with a taxonomic and biogeographic discussion of related species. No new updated reviews of the genus *Croton* are known in Colombia (Coy *et al.,* 2016). *Croton* is frequently used by indigenous communities in South America. A red latex is obtained from trees that is popularly known as "Drago's blood", which is used to treat different diseases (Godoy *et al*., 2020). Several species are used in traditional medicine on different continents. Popular uses include treatments for cancer, constipation, diabetes, digestive problems, dysentery, fever, hypertension, malaria, and ulcers, among others (Salatino *et al*., 2007). More traditional and medicinal uses for *Croton* have recently been reported (Costa *et al.,* 2020).

Croton is widely distributed in the Department of Norte de Santander (Colombia). However, despite the importance of this genus at a cultural and medicinal levels and its pharmacological potential thanks to the high chemical diversity in the trees of this genus, no studies have been reported on genetic variability among *Croton* species in this department. Informative intraspecific genetic markers are important for evolutionary and conservation genetic studies. Nuclear ribosomal ITS has been the most used marker for evolutionary studies in different plant species. The ITS region is flanked by well-conserved rRNA genes and universal primers that can be used for widely different plant groups, avoiding the need for developing specific primer sets, such as the case of SSR markers (Mäder *et al.,* 2010). The objective of this research was to study genetic diversity and

phylogenetic relationships based on the ITS regions in *Croton* genotypes from Norte de Santander.

MATERIALS AND METHODS

Plant samples

Young leaves of *Croton* genotypes were collected in three different municipalities in Norte de Santander (Colombia): Chinacota, El Zulia and Pamplona (Fig. 1). From each locality, 10 individuals were sampled. The criteria for the collection of samples were those described by Farias *et al*. (2009): (1) healthy in appearance, (2) approximately the same height (12 m), and (3) approximately the same diameter at base height (30 cm). The leaves were packed in paper bags, labeled, stored in a polystyrene refrigerator with dry ice and taken to the FITOBIOMOL research laboratory at the Francisco de Paula Santander University, Cucuta, where they were stored in a Thermo Scientific™ Forma™ 88000 series freezer (Thermo, Waltham, MA) for preservation until processing. Altitude, latitude and longitude data were obtained with an eTrex 32X GPS (Garmin). The relative humidity and temperature were obtained with a digital TTH00 thermohygrometer (Halthen).

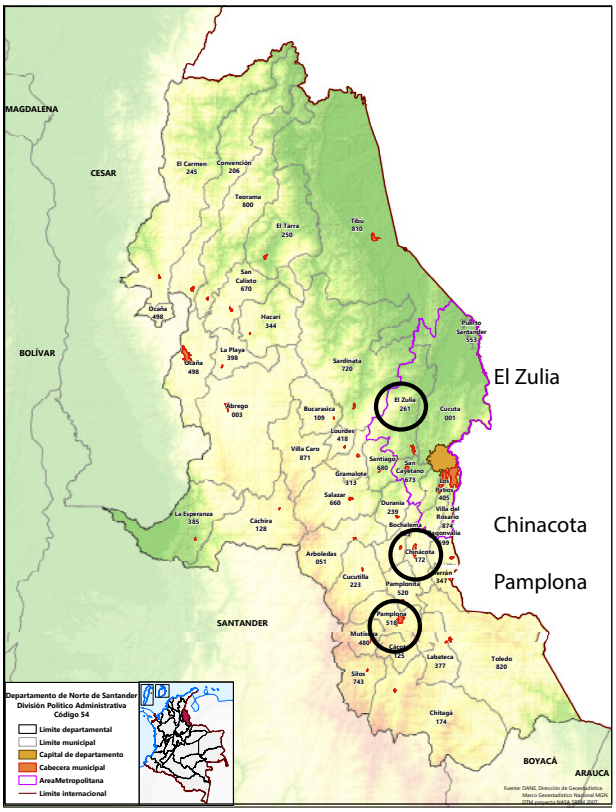

Figure 1. Location of *Croton* sampling sites in Norte de Santander (Colombia). The municipalities of El Zulia, Chinacota and Pamplona are indicated. Source: DANE.

DNA isolation

DNA from each *Croton* sample was extracted using a Monarch® genomic DNA purification kit (New England BioLabs) according to the manufacturer's instructions. The quality of the DNA was observed in a 1% agarose gel under ultraviolet light. Each sample was quantified in a UV Vis Spectrophotometer GenesysTM 10S (Thermo, Waltham, MA) at wavelengths of 260 and 280 nm and diluted to 10 ng uL^{-1} for PCR assays. The DNA was stored at -80°C for preservation and further analysis.

PCR amplification of ITS regions

Croton genomic DNA amplification was performed on a gradient thermal cycler LTCG-48-101 (Labocon, Leicester, UK) at a final reaction volume of 25 uL using the oligonucleotides ITS1 5'-TCCGTAGGGAACCTGCGGC-3' and ITS4 5'-TCCTCCGCTTATGC-3' (White *et al*., 1990). The reaction mixture was made using Taq polymerase from Biolobas® (Ipswichj, MA) following the manufacturer's instructions. The initial denaturation was at 95°C for 5 min, followed by denaturation at 95°C for 30 s, hybridization at a temperature of 52°C during 30 s, final extension of 72°C for 2 min, and 35 cycles. The verification of the amplified products was done in 1% agarose gels that were subsequently dyed with Gelred® (Biotium, San Francisco, CA).

Sequencing and phylogenetic analysis

The PCR products were purified from agarose gels using a Monarch® DNA Gel Extraction Kit from Biolobas® (Ipswichj, MA) according to the manufacturer's instructions. Each purified and verified PCR product was sent to Macrogen, Korea, for sequencing. The sequences were compared to the GenBank database (Clark *et al.,* 2016) using a BLAST analysis, aligned using MegAlign (LaserGene, DNASTAR, Madison, WI) and the ClustalW algorithm (Li *et al*., 2015). The phylogenetic analysis was performed using the Maximum-Likelihood method with 1,000

iterations to estimate the confidence of the grouping and the General Time Reversible model in MEGAX (Kumar *et al*., 2018). The nodes were collapsed using TreeGraph 2 (Stöver and Muller, 2010).

RESULTS AND DISCUSSION

Sampling regions

The three municipalities from Norte de Santander where *Croton* was sampled have different environmental conditions. The Chinacota samples were collected at an average altitude of 1,554 m a.s.l. and an average temperature of 21°C. The samples from Pamplona were collected at an average altitude of 2,276 m and an average temperature of 22.2°C. The samples from El Zulia were collected at an altitude of 157 m and an average temperature of 29.7°C. The collection information for some samples (Chinacota 5, Pamplona 9, El Zulia 7) and the codes assigned to each sample are shown in table 1.

PCR amplification

The PCR amplification with the oligonucleotides ITS1 and ITS4 (White *et al*., 1990) produced an expected band of approximately 800 bp, figure 2 shows the expected amplification product of *Croton* samples from three locations, suggesting the efficiency of the oligonucleotides selected to amplify ITS from plant samples, which may be used for future studies on other species.

Table 1. Geographical location of *Croton* samples collected in Norte de Santander.

Sample Id	Location	T°	RH	Latitude	Longitude	Altitude (m a.s.l.)
Chinacota						
M1CH	Chinácota Centro	26	48%	7.6216 N 7°37'17.47362"	-72.5964 W 72°35'48.01743"	1,232
M2CH	Cineral	24	73%	7.569867 (N7°34'11.52168")	-72.584099 (W72°35'2.75574").I4	1,568
M3CH	Iscala Sur	20	61%	7.510164 N 7°30'36.59121"	-72.566934 W 72°34'0.96209"	1,975
M4CH	Iscala Norte	17	47%	7.558293 N 7°33'29.85527"	-72° 34'28.15951"	1,678
M5CH	Manzanares	18	51%	7.609255 N 7°36´33.31822"	-72.592361 W 72°35'32.49893"	1,319
Pamplona						
M1PAM	Rio Negro	27.8	61%	7.485300 N7°29´7.07894	-72.634210 W 72°38´3.15442"	2,318
M2PAM	Curva	26	46%	7.468161 N 7°28´5.37805"	-72.634497 W 72°38´4.18743"	2,170
M3PAM	Entrada de Pamplona	27	44%	7.433168 N 7°25´59.40585"	-72.629223 W 72°37'45.20129	2,295
M4PAM	Parque Central	18	37%	7.376120 N 7°22´34.03044"	-72.648203 W 72°38'53.53178"	2,298
M5PAM	Barrio Cariongo	24	42%	7.358025 N 7°21'28.89022"	-72.659286 W 72°39'33.43027	2,383
M6PAM	Rio	22	40%	7.364537 N 7°21'52.33292	-72.664358 W 72°39'51.68978"	2,121
M7PAM	Tanques	19	44%	7.380099 N 7°22'48.35619"	-72.629460 W 72°37'46.05524"	2,278
M8PAM	Km 6	18	46%	7.398185 N 7°23'53.46643"	-72.619401 W 72°37'9.84331"	2,288
M9PAM	Escuela Chichira	18.5	45%	7.388406 N 7°23'18.26284"	-72.626124 W 72°37'34.04495"	2,338

Table 1. Geographical location of *Croton* samples collected in Norte de Santander.

Sample Id	Location	T°	RH	Latitude	Longitude	Altitude (m a.s.l.)
El Zulia						
M1ZUL	Alejandra	29	69%	7.961406 N7°57'41.06286"	-72.606063 W 72°36´21.82788"	204
M2ZUL	Cachamay	29	65%	7.984361 N 7°59'3.69850"	-72.605519 W 72°36´19.86983"	178
M3ZUL	Rancho Grande	29.5	67%	8.149730 N 8°8'59.02825"	-72.586857 W 72°35´12.68401"	126
M4ZUL	Quesera	30	66%	8.157913 N 8°9'28.48718"	-72.609057 W 72°36´32.60444"	158
M5ZUL	Salida de la Y	28.7	66%	8.147484 N 8°8'50.94411''	-72.580326 W 72°34´49.17358"	85
M6 ZUL	Angelita	30	70%	8.160695 N 8°9'38.50375"	-72.639477 W 72°38´22.11660"	147
M7ZUL	Finca la Esperanza	32	69%	8.138900 N 8°8'20.03999"	-72.641307 W 72°38´28.70409"	203

T°, temperature (°C); RH, relative humidity.

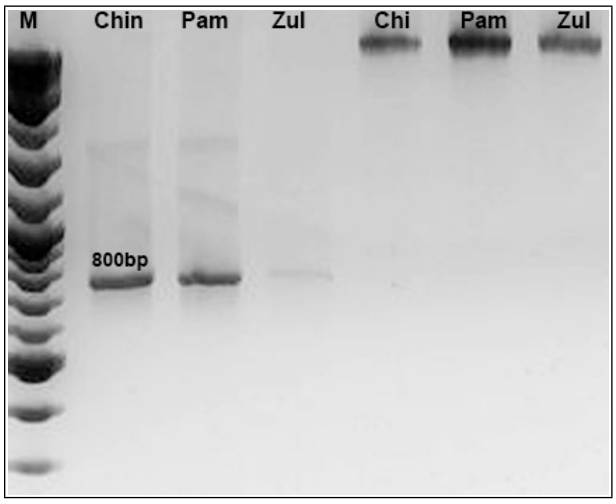

Figure 2. Verification of PCR amplification of nuclear ribosomal DNA using oligonucleotides ITS1 and ITS4. A band of approximately 800 base pairs was observed. The total DNA of each sample is indicated on the right side. Chin, Chinacota; Pam, Pamplona; Zul, El Zulia.

Nuclear ribosomal DNA (nrDNA) sequences

Internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA) contain ITS1, 5.8s rDNA, and ITS2. In recent years, nrDNA ITS sequences have been used to evaluate and analyze phylogenetic relationships between individuals of a species with PCR amplifications and sequencing. They are 18s-26s regions of the nrDNA encompassing spacer sequences with informational sites that have been widely used to investigate intra-specific differences and interspecies association in plants (Forough *et al.,* 2018; Yang *et al*., 2018). This approach was used to determine the sequences of *Croton* individuals in three locations in Norte de Santander and establish their phylogenetic relationships. The sequences were analyzed, cured and submitted to the GenBank database, where the corresponding accession numbers were assigned (Tab. 2).

Phylogenetic analysis

The new *Croton* ITS sequences from Norte de Santander (Tab. 2) were used to construct the phylogenetic tree and determine the evolutionary relationships between them (Fig. 3). The *Z. caribeaum* ITS sequence of was used as an outgroup.

As shown in figure 3, two groups are formed in the phylogenetic tree. The first contained the subgroups formed by the individuals from Chinacota and Pamplona, and the second group had the individuals from El Zulia, suggesting different origins in these populations and a greater relationship between Chinacota and Pamplona. The geographical temperature and altitude conditions of the collection areas in Chinacota and Pamplona were more similar to each other than with the conditions in El Zulia. At the anatomical level, differences in the cortex have been described, along with genetic variability in *Croton* individuals from different environments (Farias *et al*., 2009), as seen in this study.

Table 2. Accession numbers assigned to each of the new sequences generated with *Croton* from Norte de Santander.

Sample Id	GenBank accession number	Sample Id	GenBank accession number	Sample Id	GenBank accession number
M1CH	MZ820403	M3ZUL	MZ820411	M6ZUL	MZ820419
M1PAM	MZ820404	M4CH	MZ820412	M7CH	MZ820420
M1ZUL	MZ820405	M4PAM	MZ820413	M7PAM	MZ820421
M2CH	MZ820406	M4ZUL	MZ820414	M7ZUL	MZ820422
M2PAM	MZ820407	M5CH	MZ820415	M8CH	MZ820423
M2ZUL	MZ820408	M5PAM	MZ820416	M8PAM	MZ820424
M3CH	MZ820409	M5ZUL	MZ820417	M9PAM	MZ820425
M3PAM	MZ820410	M6PAM	MZ820418		

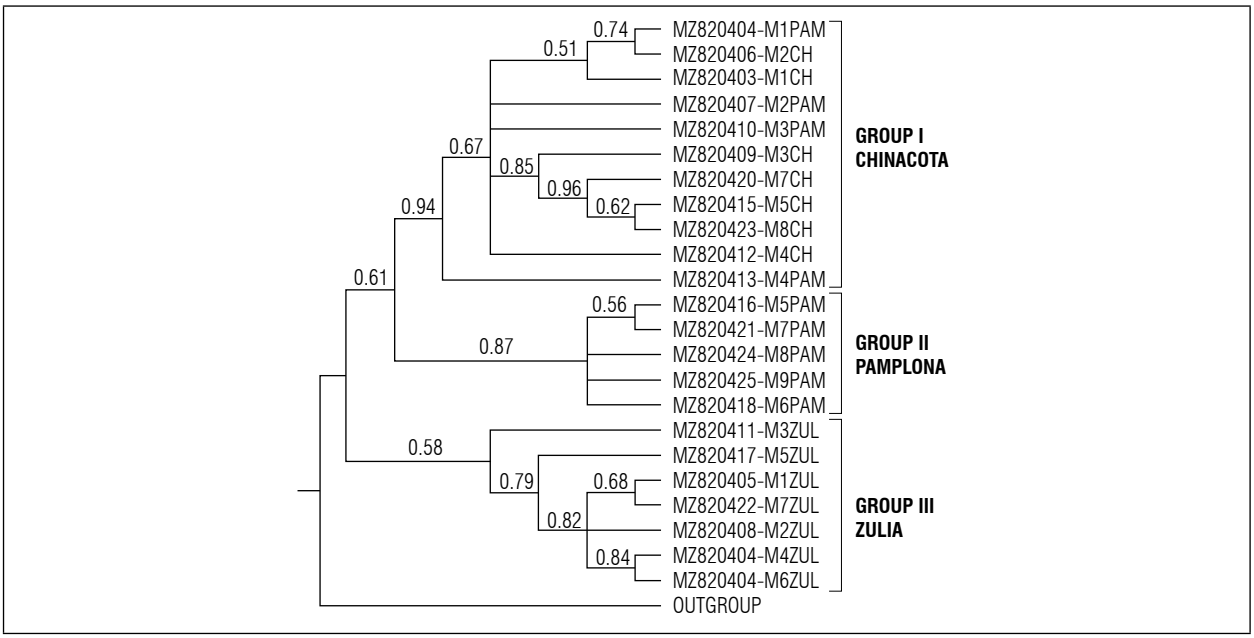

Figure 3. Phylogenetic tree of the *Croton* population in Norte de Santander.

Table 3. *Croton* ITS sequences from *Croton* species from Colombia reported in the GenBank.

Species	Location	Reference
Croton peltoideus Kunth	Colombia – Valle del Cauca	Van Ee and Berry (2011)
Croton malambo H.Karst.	Colombia-Bolivar	Berry *et al.* (2005)
Croton spruceanus Benth.	Colombia-N/A	Caruzo *et al.* (2011)
Croton ater Croizat	Colombia-N/A	Riina *et al.* (2009)
Croton purdiei Müll.Arg.	Colombia-N/A	Riina *et al.* (2009)
Croton magdalenensis Müll.Arg.	Colombia-N/A	Riina *et al.* (2009)
Croton pedicellatus Kunth	Colombia-Cundinamarca	Van Ee and Berry (2010)
Croton pachypodus G.L.Webster	Colombia-Nariño	Masa-Iranzo *et al.* (2021)
Croton rufolepidotus Caruzo & Riina	Colombia-Antioquia	Masa-Iranzo *et al.* (2021)
Croton amazonicus Müll.Arg.	Colombia-Caqueta	Masa-Iranzo *et al.* (2021)

In *Croton*, the ITS region has been used for taxonomic and phylogenetic studies, some of which include sequences originating in Colombia (Berry *et al.,* 2005; Riina *et al*., 2009; Van Ee and Berry, 2010, 2011; Caruzo *et al.,* 2011; Masa-Iranzo *et al.,* 2021). The search criteria *Croton*+transcribed spacer+Colombia in the Genbank at the time of writing this manuscript yielded 10 results that are shown in table 3.

The phylogenetic relationship of the new ITS sequences in *Croton* from Norte de Santander with ITS reported in Colombia for the departments Nariño, Antioquia, Cundinamarca, Bolivar, Valle del Cauca and Caqueta, and 87 sequences originating in other countries in the Americas were established. However, because of the high similarity between many of them and the scarce phylogenetic relationship with sequences from countries outside the Americas (data not shown), the analysis was limited to using 22 sequences from another country whose origin and corresponding species are indicated in figure 4.

Samples from Norte de Santander consistently grouped according to place of origin in three clusters.

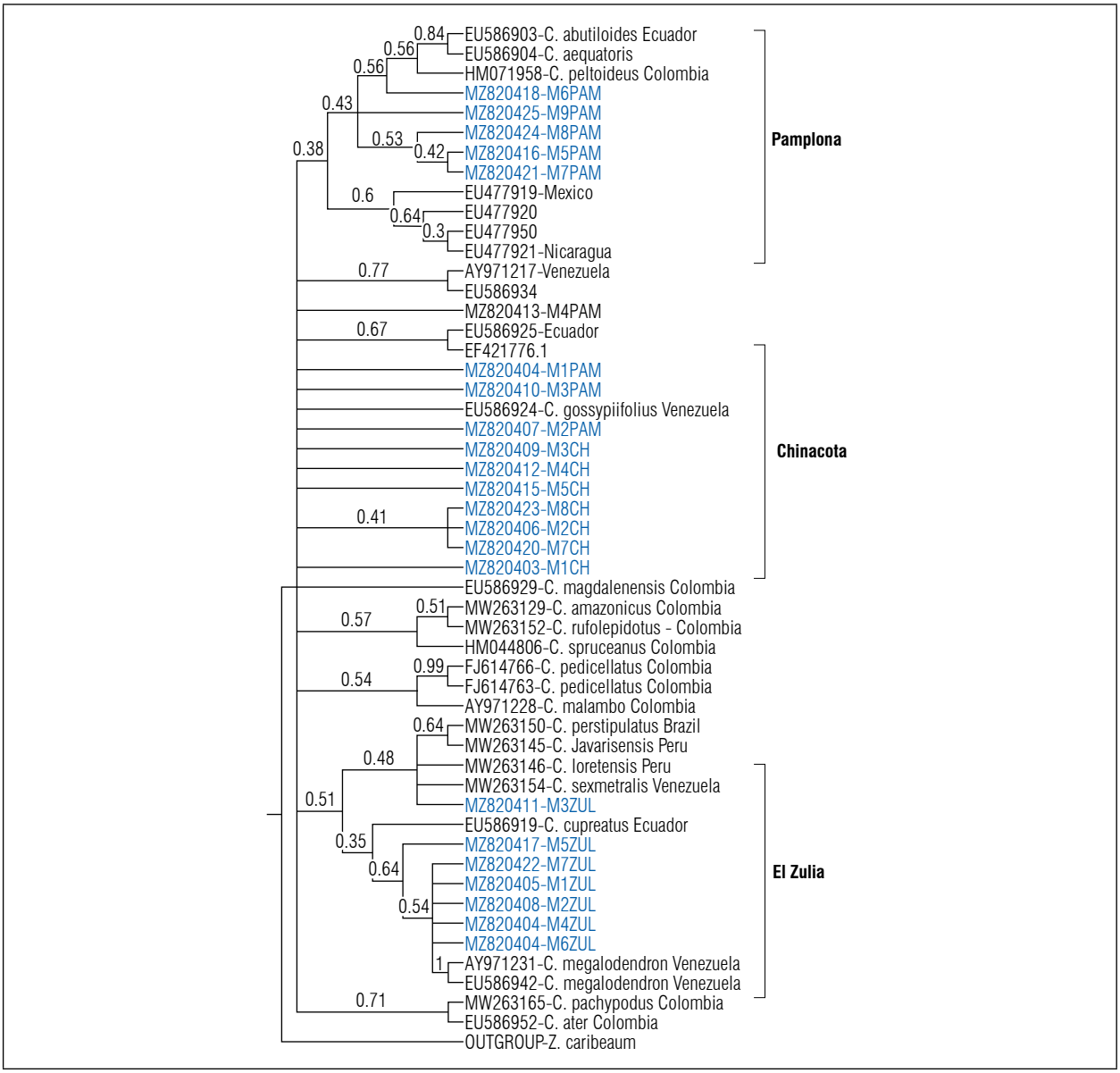

Figure 4. Phylogenetic relations of *Croton* from Norte de Santander with other sequences of Colombia and other countries in the Americas.

The groups from El Zulia and Pamplona were exclusively formed by individuals from those localities. The group from Chinacota had three samples that were collected in Pamplona, suggesting the same origin or a possible transfer of seeds or plant material between these two localities (Fig. 4). The grouping of the sequences of the three populations of Norte de Santander suggested high genetic variability. This hypothesis was reinforced by the distance matrix with a nucleotide similarity from 54 to 99% (data not shown).

The absence of a detailed botanical analysis and the limited availability of sequences meant it could not be conclusively stated to which *Croton* species the Norte de Santander samples belong. However, according to the results of the phylogenetic grouping indicated in figure 4, the samples collected in the municipality of Pamplona were more related to *Croton abutiloides* Kunth (EU586903) and *Croton aequatoris* Croizat (EU583904) from Ecuador and *C. peltoideus* (HC071958) from the Department of Valle del Cauca-Colombia. On the other hand, the data showed that the samples from Chinacota did not clearly group with other samples, including the other ones from Colombia, suggesting a different evolutionary origin. Finally, the samples from El Zulia were in two subgroups. The first one had more individuals with a higher phylogenetic relationship with *Croton megalodendron* Müll.Arg. from Venezuela and *Croton cupreatus* Croizat from Ecuador. The other subgroup only contained the individual M3ZUL (MZ820411) with a higher phylogenetic relationship with *Croton sexmetralis* Croizat from Venezuela and with *Croton loretensis* Riina & Caruzo and *Croton javarisensis* Secco from Peru.

CONCLUSION

The phylogenetic analysis and the distance matrix with the internal transcribed spacer region (ITS) of the ribosomal DNA (rDNA) for *Croton* individuals from three localities in Norte de Santander revealed high genetic variability. The Chinacota samples had the greatest nucleotide dissimilarity and the lowest phylogenetic relationship with the other samples. Because of the large number of species belonging to the *Croton* genus, botanical studies and a greater number of sequences are needed to conclude to which species the samples from Norte de Santander belong or whether they can constitute a new species in this genus. The results of this study represent an important contribution for future systematic studies, for establishing plant breeding programs, and for identifying *Croton* germplasms and the relationships between them.

Conflict of interests: The manuscript was prepared and reviewed with the participation of the author, who declare that there exists no conflict of interest that puts at risk the validity of the results.

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