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Article

Genetic diversity of *Capsicum pubescens* by functional genomic markers of CYP450

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Abstract

The research work was developed at the Plant Genetic Resources Laboratory of the 'Presidente Juárez' Faculty of Agrobiology of the Michoacán University of San Nicolás de Hidalgo, in 2019 and 2022. The aim of this research was to evaluate the usefulness of functional markers derived from CYP450 for genetic diversity studies in *Capsicum pubescens*. The genetic material consisted of 31 cultivated varieties of *C. pubescens* from three different localities in the state of Michoacán, Mexico. Genomic DNA was obtained based on the protocol of Huang *et al*. (2013) and two combinations of CYP450 primers were included in the analysis. The amplification products were separated on 8% acrylamide gels and stained with silver nitrate. A total of 85 loci were detected: the CYP2B6F/CYP2C19R combination detected 34 polymorphic loci, while the CYP2C19F/CYP1A1R combination detected only 27. The diversity analysis of *C. pubescens* identified 1.54 alleles per locus, 1.33 effective number of alleles per locus, Shannon index of 0.3, a heterozygosity index of 0.2 and 60.39% of polymorphic loci. The results obtained show that markers derived from CYP450 are an efficient and low-cost alternative for studies of genetic diversity in plant species.

Keywords:

Capsicum pubescens, genetic variability.

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Introduction

The species *C. pubescens*, commonly known as perón, manzano, canario, cera or rocoto pepper (Escalera-Ordaz, 2019), is native to the highlands of South America, flourishing in elevated areas between 1 200 and 3 000 masl. In Mexico, it is found in the states of Mexico, Puebla, Veracruz, Michoacán, and Chiapas (Aguirre and Muñoz, 2015).

This species has a diversity of uses: medicinal, it is consumed fresh, dehydrated, or as canned food and is a source of natural dyes (Escalera-Ordaz, 2019).

For greater understanding, studies have been carried out on genetic diversity, population structure and phylogenetic relationships. In this sense, molecular markers are considered an important biotechnological tool for the evaluation of genetic diversity thanks to the characteristics they possess (Gil-Langarica, 2008). Among the techniques that have been used are AFLPs (Vos *et al*., 1995) and microsatellite sequences such as ISSRs (Zietkiewiez *et al*., 1994) and SRAPs (Li and Quiros, 2001).

However, these techniques currently have certain disadvantages that can become an obstacle: the requirement of large amounts of DNA (Rentería, 2007), reproducibility (Ríos *et al*., 2009) and prior knowledge of DNA sequences, among others (Carvalho *et al*., 2015). Research on this species has been mainly limited to varieties, hybrids, and lines of different *Capsicum* species (Castañón-Nájera *et al*., 2011; Contreras-Toledo *et al*., 2011; Mahmoud, 2013; Carvalho *et al*., 2015; Toledo-Aguilar *et al*., 2016; Xiao-min *et al*., 2016; López-Espinosa *et al*., 2018). In contrast, *C. pubescens* has a lower level of research (Pardey and García, 2011; Lijun and Xuexiao, 2012).

Cytochrome CYP450 was identified in 1958 as a cellular pigment (Jaimes-Santoyo *et al*., 2014), it is found in low concentrations in different plant organs and in some cellular organelles such as endoplasmic reticulum, plasma membrane, vacuole, mitochondria, and Golgi apparatus. In this regard, different cytochrome CYP450 molecules have been identified in a single organism (Valencia-Quintana *et al*., 2009) with different functions within plants: synthesis of various compounds of defense against insects and pathogens, in the biosynthesis of gibberellins, as well as in the process of fruit ripening.

In *Arabidopsis*, 270 genes belonging to 45 different families of CYP450 have been identified (Gonzáles-Mendoza, 2009), making CYP450 one of the largest families of enzymatic proteins in plants (Bak, 2011). Genes of the CYP88A family have also been isolated from *Cucurbita maxima* (Helliwell *et al*., 2000) and *Zea mays* (Winkler and Helentjaris, 1995), of the CYP73A9v1 family from *Pisum sativum* (Whitbred and Schuler, 2000) and CYP71A1 from *Persea americana*.

Functional genomic markers based on CYP450 (Shakeel *et al*., 2019) were originated from a study that evaluated the polymorphism detected by CYP450 in mammals and subsequently used as universal tools to assess the genetic diversity of plant species. Around 51 species have been evaluated with this marker (Yamanaka *et al*., 2003) and successfully in different plant species: *Musa* spp. (Wan *et al*., 2005), *Withania coagulans* (Gilani *et al*., 2009), *Curcuma amada* (Shakeel *et al*., 2019), *Eleusine coracana* (Panwar *et al*., 2010), *Moringa oleifera* (Saini *et al*., 2013), *Oryza sativa* (Yamanaka *et al*., 2011), *Sechium edule* (Machida-Hirano *et al*., 2015), among others.

The polymorphism levels determined range from 0.28% to 88.25%. Given the characteristics and results obtained with the CYP450 marker in studies of genetic diversity in different species, it was proposed to use them for preliminary studies of genetic variability in cultivated varieties of *C. pubescens*.

Materials and methods

Plant material

In the present research, 31 cultivated varieties of *C. pubescens* were used: 11 of them from the municipality of Tingambato (TIN) and 20 from two localities in the municipality of Uruapan: 11 from

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Toreo el Bajo (TOB) and 9 from Tiamba (TIA). The sites of origin of the analyzed materials range between 1 623 and 2 282 masl.

Obtaining genomic DNA

DNA was obtained based on the procedure described by Huang *et al*. (2013). Some modifications made consisted of the lyophilization of leaf tissue without the previous use of liquid nitrogen and the substitution of chloroform and isoamyl alcohol with dichloromethane (CH_2Cl_2) and ethanol, respectively. The concentration of the isolated DNA was determined with the help of a NanoDrop 2000c (Thermo Scientific®).

Amplification, separation, and identification of amplicons

To obtain amplicons, 10 µl of the following PCR reaction mixture were used: 2.5 µl ddH_2O, 0.3 mM MgCl, 0.6 µM of each primer, 3.5 µl 2X Taq network (0.7 X) and 2 µl of a DNA solution (25 ng μl^{-1}). The oligonucleotides used were two combinations of cytochrome CYP450-derived primers: CYP2B6F (5' gac tct tgc tac tcc tgg gtt 3')/CYP2C19R (3' cca tcg att ctt ggt gtt ct 5') and CYP2C19F (5' tcc ttg tgc tct gtc tct ca 3')/CYP1A1R (3' aag gac atg ctc tga cca tt 5') (Inui *et al*., 2000). For amplification, a 3 PrimeG techne® thermal cycler was used.

For the combination of CYP2B6F/CYP2C19R oligos, it was as follows: an initial stage of 5 min at 94 °C, followed by 35 amplification cycles as follows: denaturation 60 s at 94 °C, hybridization 60 s at 47 °C and extension 60 s at 72 °C. For the combination of CYP2C19F/CYP1A1R oligos, hybridization was performed for 60 s at 54 °C. For both combinations, a final extension of 10 min at 72 °C was included. The amplicons were separated by 8% acrylamide gel electrophoresis in 200 ml vertical systems (Enduro™ Power Supplies 300V).

The molecular reference markers were 20 bp and 100 bp. The detection of the amplified products was carried out by silver nitrate staining and they were visualized on a Carestream® Gel Logic 112 imaging system (Sanguinetti and Simpson, 1994).

Molecular data analysis

For this procedure, three groups of varieties were organized, considering the place of origin as a grouping criterion. Data were analyzed with the GenAlex statistical package (Peakall and Smouse, 2012) to calculate genetic diversity parameters: number of alleles (N), average number of alleles/ locus (Na), effective number of alleles (Ne), Shannon index (I), heterozygosity (He) and percentage of polymorphic loci (% P). From a matrix of averages, the matrix of genetic distances between varieties was calculated and the clustering analysis was performed with the Neighbor-Joining method. The dendrogram was generated using the MEGA5 program (Tamura *et al*., 2011).

Results and discussion

Determined level of polymorphism. The combinations of markers used generated bands between 40 and 500 bp. This contrasts with what other authors (Mahmoud, 2013) have obtained in studies carried out on *C. annuum*; in the latter case, they used codominant markers of the ISSR type. A total of 85 loci were detected, the CYP2B6F/CYP2C19R combination revealed 34 polymorphic bands, while the CYP2C19F/CYP1A1R combination revealed 27 polymorphic bands, for a polymorphism level of 60.39%, this value was 8.83% higher on average than that determined in other *Capsicum* species and 29.14% higher than that reported (Pardey and García, 2011) for *C. pubescens* using SSRs.

In general, CYP450 combinations detected a higher level of polymorphism, 41.9% and 35.59% higher than that detected with ISSRs (Lijun and Xuexiao, 2012) and RAPDs (Bobadilla-Larios *et al*., 2017), respectively. In contrast, studies on varieties of *C. frutescens* and *C. annuum* (Castañón-Nájera *et al*., 2011) and on landrace populations of *C. chinense* (López-Espinosa *et al*., 2018) have reported average polymorphism levels of 95.4% using ISSR and AFLP markers.

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It is worth highlighting the level of polymorphism (60.39%) determined in *C. pubescens* compared to the average (20.91%) detected with other markers (Pardey and García, 2011; Lijun and Xuexiao, 2012) in the same species. On the other hand, there are few studies on the use of markers derived from CYP450 in other plant species, with those carried out in *Musa* ssp. and in *Curcuma amada* (Shakeel *et al*., 2019) standing out, where polymorphisms of 65.2% and 94.6%, respectively, have been determined.

This type of markers is based on a multigene family that records diversity in functional regions of the genome, which explains the levels of polymorphism obtained and, therefore, it has been used to characterize the genetic diversity and variability of species of insects (Giraldo *et al*., 2011), hoofed mammals, and cetaceans (Irwin *et al*., 1991).

In addition, genes derived from CYP450, which are widely distributed within the plant genome, can be used universally; however, their use in plant species (Yamanaka *et al*., 2003; Wan *et al*., 2005; Gilani *et al*., 2009; Panwar *et al*., 2010; Yamanaka *et al*., 2011; Saini *et al*., 2013; Machida-Hirano *et al*., 2015; Shakeel *et al*., 2019) has been limited.

Genetic diversity analysis. The analysis identified an average of 1.54 alleles per locus, 1.33 effective number of alleles per locus, a Shannon index of 0.3, a heterozygosity index of 0.2 and a percentage of polymorphic loci of 60.39% (Table 1). The estimated heterozygosity value was low (0.2) compared to those reported by Xiao-min *et al*. (2016); Toledo-Aguilar *et al*. (2016), where values ranging from 0.36 to 0.59 were determined. The heterozygosity determined in *C. annuum* (Contreras-Toledo *et al*., 2011), *C. frutescens* and *C. chinense* (Carvalho *et al*., 2015; López-Espinosa *et al*., 2018) with SSR markers yielded average values of 0.42, higher than those reported in this research.

Table 1. Basic parameters of genetic diversity obtained by combining CPY450 markers in cultivated varieties of *C. pubescens* R. and P. from the state of Michoacán, Mexico.

Populations	N[a]	Na[b]	Ne[c]	I[d]	He[e]	P[f] (%)
Toreo el Bajo	11	1.66	1.38	0.34	0.23	67.06
Tingambato	11	1.55	1.31	0.29	0.19	60
Uruapan	9	1.42	1.3	0.27	0.18	54.12
Mean	10.33	1.54	1.33	0.3	0.2	60.39

[a] = number of alleles; [b]= average number of alleles/locus; [c]= effective number of alleles; [d]= Shannon's index; [e]= heterozygosity; and [f]= percentage of polymorphic loci.

Similar results have been obtained with the use of AFLPs (Guzmán *et al*., 2005) and ISSRs (Lijun and Xuexiao, 2012). It has also been observed that when the indicated species are related to *C. pubescens*, low values of genetic diversity are obtained (Pardey and García, 2011).

CYP450 functional markers have shown their feasibility for estimating high levels of genetic diversity, even in some plant species such as *Musa* spp. (Wan *et al*., 2005) and *Oryza sativa* (Yamanaka *et al*., 2011). In this sense, the He values determined in the populations studied in the three localities were similar, with an average of He= 0.2. This agrees with the levels of polymorphism estimated in each of the populations indicated.

The estimation of the Shannon diversity index showed that the richness of individuals in the population of Uruapan was lower (I= 0.27) compared to the populations of Tingambato and Toreo el Bajo (I= 0.29 and I= 0.34, respectively). The estimated average value for this parameter was I= 0.3 and may be a function of the homogeneity of the population and the frequency of alleles (Glasenapp *et al*., 2015). The analyses of genetic diversity parameters obtained with CYP450 are similar (Machida-Hirano *et al*., 2015) to those generated with other types of markers such as SSRs and RAPDs.

Kinship relationships. In Figure 1, it was observed that the markers derived from CYP450 identified two perfectly defined groups, both groups were not related to the morphological characteristics of

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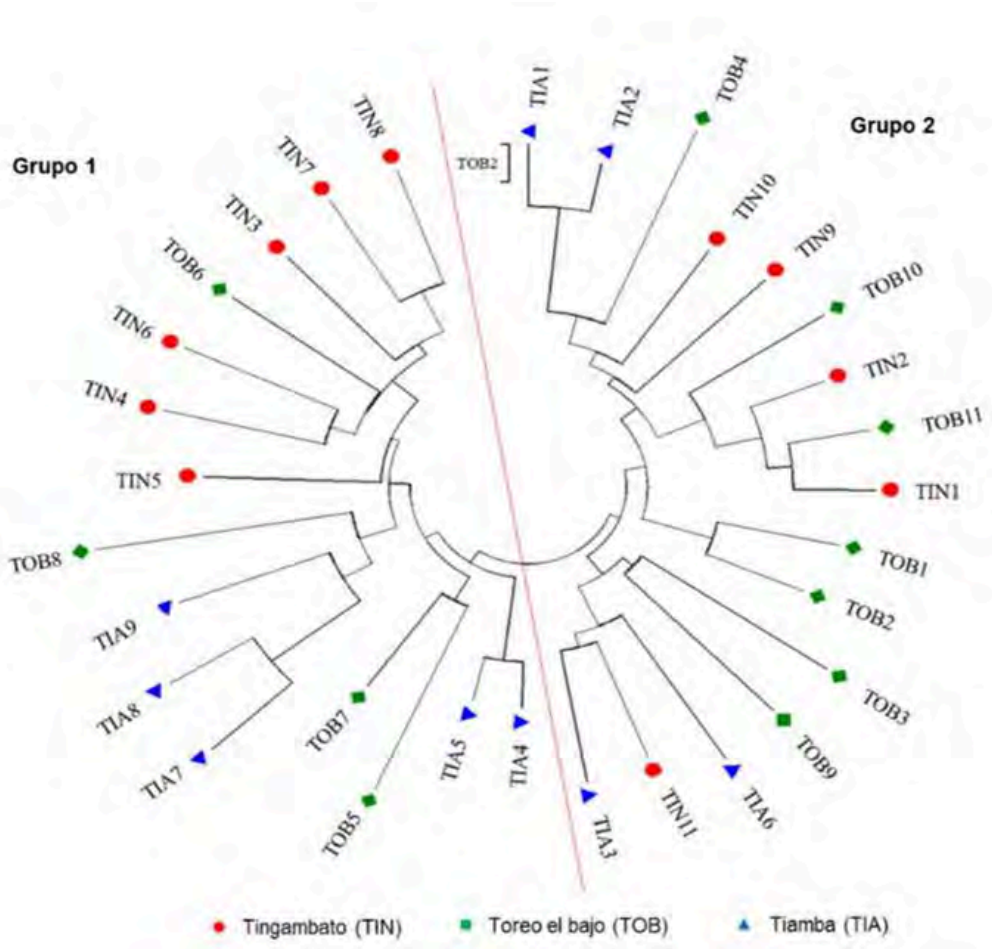
the fruits, but neither to their place of origin. A polytomy was observed in the dendrogram since there is not enough information to explain this relationship (Martínez, 2007). The formation of groups based on the polymorphism detected with CYP450-derived markers has not been explained (Wan *et al*., 2005; Gilani *et al*., 2009; Saini *et al*., 2013), it is likely that increasing the number of primers will lead to more information about these types of clusters (Wan *et al*., 2005).

Figure 1. Phylogenetic tree of 31 cultivated varieties of *Capsicum pubescens*, from three localities in the municipalities of Tingambato and Uruapan, Michoacán, Mexico, based on molecular data obtained from two combinations of primers derived from cytochrome CYP450.



In both groups, those that stand out for their greater genetic similarity and based on the sequences analyzed are the varieties from the locality of Tiamba: in group 1 the TIA4 with TIA5, and in group 2 the variety TIA1 with TIA2. However, in group 1 there is a predominance of cultivated varieties from Tingambato, while in group 2 those from the locality of Toreo predominate. The formation of groups based on the polymorphism detected with CYP450-derived markers has not been explained (Wan *et al*., 2005; Gilani *et al*., 2009; Saini *et al*., 2013), it is very likely that increasing the number of primers will lead to more information about this type of clusters.

The use of these markers in mammals (Giraldo *et al*., 2011) and in the identification of analogous sequences of CYP450 genes in different plant species (Gonzáles-Mendoza, 2009; Bak, 2011) allowed CYP450 to be considered as a universal tool for the assessment of genome genetic diversity in diverse plant species that do not have relevant genetic markers.

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These types of markers represent an ideal system (Yamanaka *et al*., 2011) to reveal the genetic diversity present in individuals, populations and species, since they allow the detection of a high level of polymorphism, are cost-effective, with limited budgets, without significant differences in terms of group formation, but useful in the estimation of genetic diversity and rapid assay (Panwar *et al*., 2010), this makes them a reliable alternative for studies of genetic diversity (Shakeel *et al*., 2019) in plant species.

Conclusions

The results generated in this research work, in cultivated varieties of *C. pubescens*, show the usefulness of functional markers derived from CPY450 in studies to generate information quickly and inexpensively on the variability and genetic diversity of individuals or populations of closely related plant species.

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