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REGULAR ARTICLE

Potential of random amplified microsatellites (RAMS) to typify and discriminate varieties of *Physalis ixocarpa* Brot. ex Hornem

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ABSTRACT

Physalis ixocarpa is an edible species of Solanaceae. This is one of the few cultivated and economically important species of the genus in Mesoamerica. In Mexico, several varieties and landraces have been developed, which have not been molecularly characterized. In the current study, five RAMS primers were used to characterize and assess the genetic variability of two varieties and three landraces of this species. The capacity of these markers to discriminate between them was also evaluated. With comparative aims, *Physalis peruviana,* the most economically important species of the genus in South America, was analyzed in the same manner. The results revealed that the varieties and landraces of *P. ixocarpa* conserve important levels of genetic variability (21.75% > Polymorphism < 42.75%), which were higher than that found for *P. peruviana* (10.75% Polymorphism). RAMS were useful specific markers, as *P. peruviana* and *P. ixocarpa* were clearly distinguished one from each other by both cluster analysis and principal components analysis. Close genetic relationships were found between the landraces San Isidro Chihuiro and Verde Puebla, and between the varieties Diamante and Rendidora. In spite of the genetic closeness, the RAMS amplification profiles had a clear varietal-specific tendency, in such a way that they may represent varietal fingerprints, which can be used as authentication tool for varieties and landraces of *P. ixocarpa*.

Keywords: Authentication tools; Genetic variability; Husk tomato; RAMS markers

INTRODUCTION

Green tomato, Husk tomato, and "tomatillo" are some of the common names of *Physalis ixocarpa* Brot. ex Hornem. This is a species of Solanaceae, whose edible fruits are appreciated culinary elements in Mexico and other Mesoamerican countries. The recent increase in the international demand of this species (Ramírez-Godina et al., 2013) has stimulated the development of new and high-yield varieties (Valerio et al., 2012). Besides, in Mexico, many landraces of tomatillo have been selected and conserved by farmers (Peña-Lomeli et al., 2014). Both, varieties and landraces are sources of valuable alleles, which are important to conserve. Molecular characterization and typifycation may contribute to conserve the varieties and landraces of *P. ixocarpa*.

Due to the high morphological variation and the abundance of wild populations, *P. ixocarpa* is considered as a species in a current domestication process (Zamora-Tavares et al., 2015). Furthermore, at present there are several taxonomic controversies concerning the specific delimitation of this species. Some authors consider that *P. ixocarpa* and *P. hiladelphica* Lam. are synonymous names (Santiaguillo and Yáñez, 2009), whereas others suggest that they are separate taxonomic entities (Zamora-Tavares et al., 2015; Lagos et al., 2005).

Most varieties of husk tomato have been typified by their morphological and agronomical attributes (Valerio et al., 2012; García-Osuna et al., 2015). Unlike *P. peruviana*, for which specific molecular markers have been developed (Simbaqueba et al., 2011), for *P. ixocarpa*, molecular characterization is still insufficient.

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Estimations of genetic variability of any, species, subspecies, varieties, or populations allow to characterize them and determine their biological potential (Sosa et al., 2002). Besides, genetic variability estimations have taxonomic and evolutionary implications that support the establishment of specific limits (Medrano et al., 2014). This, in turn, assists to develop tools of quality control concerning the origin and authenticity of plant cultivars and varieties. In this way, molecular characterization and genetic variability estimation are priority issues to develop authenticity and quality control tools for the emerging varieties and ancient landraces of Green tomato.

Different molecular markers have been used to assess the intra- and inter-specific variability of several plant species, like *Agave durangensis* (Almaraz-Abarca et al., 2013) and *Phaseolus vulgaris* (Bitocchi et al., 2013), as well as to determine the conspecific status of populations (Ellstran 2014; Medrano et al., 2014). Molecular markers also facilitate the selection of desirable features in crop improvement (Hocdé 2006; Quiroz-Chávez et al., 2012), as well as represent a valuable authentication tool of cultivated plants varieties (Masi et al., 2003).

Molecular markers, such as ISSR (Intersequence Simple Repeats), have been used to assess the genetic variability of some species of *Physalis*, like *P. virginiana* Miller, *P. cordata* Miller, *P. cinerascens* (Dunal) Hitch., *P. lignescens* Waterfall, *P. sulphurea* Waterfall, *P. angulata* L., *P. lagascae* Roem. and Schult. (Vargas-Ponce et al., 2011), and *P. philadelphica* (Vargas-Ponce et al., 2011, Zamora-Tavares et al., 2015). These studies were mainly carried out in wild populations. The genetic variability of the cultivated *P. peruviana* has been assessed with several markers, like SNP (Single Nucleotide Polymorphism) (Garzón-Martínez et al., 2015) and RAMS (Random Amplified Microsatellites) (Espinosa et al., 2004).

Like microsatellites, RAMS are also distributed throughout the genome. This distribution confers RAMS the ability to detect high polymorphism levels, as revealed for some fungus species (Sillo et al., 2016). Besides, RAMS have been reported as reproducible markers, which were useful to distinguish populations of *P. peruviana* (Espinosa et al., 2004). In the current study, five RAMS primers were used to assess the genetic variability of two varieties and three landraces of *P. ixocarpa* from Oaxaca, Mexico. The potential of these primers as varietal-specific markers was also evaluated. With comparative aims, *Physalis peruviana* was analyzed in the same manner.

MATERIALS AND METHODS

Plant material

Farmers of San Isidro Chihuiro in Nejapa de Madero, Oaxaca, Mexico (Fig. 1) provided seeds of the landraces San Isidro Chihuiro (SI), Verde Puebla (VP), and San Martín (SM). Seed were expressly provided for the current study in November 2015. Seeds of the varieties Diamante (D) and Rendidora (R) were donated in November 2015 by the Sistema Nacional de Recursos Fitogenéticos para la Alimentación y la Agricultura–Servicio Nacional de Inspección y Certificación de Semillas (SINAREFI–SNICS) of the Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA), through the Red de Tomate de Cáscara. Dr. Sandy Molina from the Instituto de Estudios Avanzados (IDEA) of Venezuela, provided the seeds of *Physalis peruviana* (P) in November 2015. The seeds of each variety were germinated in germination beds containing peat moss (Cosmocel®, Nuevo León, Mexico) and perlite (5:1), at room temperature and a photoperiod of 13 h light. Seeds were watered every 24 h. DNA was extracted from the foliar tissue of 7-day-old seedlings. DNA from 30 seedlings of each of the varieties and landraces D, SI, VP, and SM was individually obtained and analyzed. For the variety R and for *P. peruviana*, DNA from 26 and 23 seedlings, respectively, was individually obtained and analyzed.

DNA extraction

Total DNA was obtained according to Espinosa et al. (2004). The estimations of purity and concentration for each DNA sample were carried out by standard spectrometric and electrophoretic procedures (Sambrook et al., 1989).

PCR and electrophoresis conditions

RAMS fragments were amplified using five primers reported by Espinosa et al. (2004). The respective sequences are shown in Table 1.

PCR amplifications were performed using a mixture containing 5 µL of 5X Green go Taq buffer, 1.2 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 10 µL of 10 mM primer, 0.2 µL of 5 U/µL Taq DNA polymerase, 7.1 µL of ultra-pure H_2O, 1 µL of 25 ng/µL DNA, in a total volume of 25 µL. Cycling conditions were according to Muñoz et al. (2008): 5 min at 95°C; followed by 37 cycles of 30 s at 95°C, annealing 45 s at 61°C for CGA, 50°C for AG, 55°C for TG, 41°C for CA and CT, and 55°C for CCA, and extension at 72°C for 2 min. A final extension

Table 1: RAMS primers used for characterizing and estimating the genetic variability of *Physalis peruviana* and five varieties and landraces of *Physalis ixocarpa*

Primer	Sequence
AG	5'- CTA AGA GAG AGA GAG AGA -3'
CA	5'- AGT ACA CAC ACA CAC ACA -3'
CGA	5' - TAG CGA CGA CGA CGA CGA -3'
TG	5' - CAG TGT GTG TGT GTG TGT -3'
CCA	5' - TAT CCA CCA CCA CCA CCA -3'

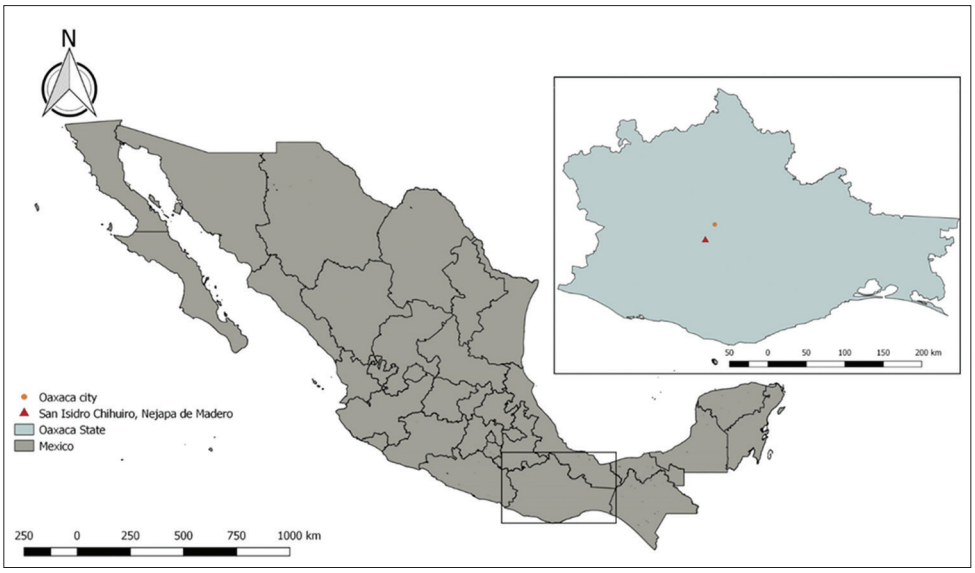

Fig 1. Location of Oaxaca, Mexico where *Physalis ixocarpa* seeds came from.

at 72°C for 7 min was the final step. DNA amplification was performed in a Bioer GeneQ (Hanzhou, China) thermal cycler. Amplified sequences were separated by 2% agarose gels electrophoresed at 95 V, constant power. Gels were stained with SYBR™ Green. Molecular size of each amplified loci was estimated using the 100 bp DNA ladder molecular size marker.

Data analysis

Only the consistent amplified products were recorded for each primer. Each fragment was characterized by size, in pair of bases (bp), and considered as a single molecular character. The amplified patterns for each sample-primer combination were assessed and used to construct a binary matrix coded by 1 (presence) or 0 (absence) with all individual samples vs. all amplified loci (169 individuals vs. 409 loci). For each primer, polymorphic information content (PIC) and proportion of polymorphic genes (P) at 95% were estimated using the software InfoGen V. 2013 (Balzarini and Di Rienzo, 2006). Genetic variability of *P. peruviana* and five varieties and landraces of *P. ixocarpa* was assessed with the following parameters: percentage of polymorphism (P), Shannon´s information index (I), Nei´s gene diversity (H), Nei´s genetic identity (GI), and genetic distance (GD), by using the software PopGene-1.32 (Yeh et al., 2000).

Genetic structure was evaluated using the software Structure V2.3.4 (Pritchard et al., 2000), applying a Bayesian analysis with 100 000 tree simulations, 100 000 replicates, and 10 iterations. Assuming (K=1 to K=9). At least 10 simulations were executed by each K value. The results were analyzed using the program of the web site Structure Harvester. The Evanno´s method was used to obtain the most probable value of K (Earl and vonHoldt, 2012; Evanno et al., 2005).

To estimate the genetic relationships between samples, the constructed matrix was submitted to a cluster analysis (Ward´s method) using Past 3.01 (Hammer et al., 2001) and to principal components analysis (PCA) using the software InfoGen V. 2016 (Balzarini and Di Rienzo, 2016).

RESULTS AND DISCUSSION

Purity and amount of obtained DNA

The relations A_{260nm}/A_{280nm} of the DNA samples were ≥ 1.7. According to Sambrook et al. (1989) values ≥ 1.7 indicate that the DNA purity is adequate. The DNA concentrations ranged between 99.18 and 462.06 ng/μL. According to Muñoz et al. (2008), DNA concentrations above 10 ng/μL are suitable for molecular analysis using RAMS.

RAMS informative capacity

The number of loci amplified by the five RAMS primers used across 146 individuals of five varieties and landraces of *P. ixocarpa* was 409. These same primers amplified 47 loci across 23 individuals of *P. peruviana*. The total number of loci (456) was higher than that obtained with 10 ISSR markers (229) for *P. peruviana* and six wild populations of *P. angulata*, *P. solanacea*, *P. patula*, and *P. subulata* by Medina-Medrano et al. (2016). The amplified sequences per sample per primer are shown in Table 2.

Size of fragments varied from 127 to 1488 bp with AG, from 239 to 1479 bp with CA, from 183 to 1444 bp with CGA, from 108 to 1495 bp with TG, and from 100 to

1433 bp with CCA. A representative example of the agarose gels obtained, corresponding to the combination Diamante-TG is displayed in Fig. 2.

All primers were highly polymorphic, P values ranged between 96.1% and 100% (Table 3). Comparatively, the five RAMS markers revealed similar polymorphism values to other types of molecular markers, as ISSR (Lüdtke et al., 2010; Vargas-Ponce et al., 2011). PIC is used to estimate the informativeness of a molecular marker (Guo and Elston, 1999). PIC values of the used markers ranged from 0.075 for TG to 0.1 for AG (Table 3), AG being the most informative.

Genetic variability

Table 4 shows the indicators of the genetic variability found for *P. peruviana* and five varieties and landraces of *P. ixocarpa*. The variety Rendidora was the most variable. The polymorphism (42.75%) and values of Shannon´s diversity (0.1021) and Nei´s genetic diversity (0.0545) calculated for this variety was higher than the values of the other analyzed samples. The results of Table 4 indicate that the registered varieties, Rendidora and Diamante, conserve higher genetic variabilities than the landraces of *P. ixocarpa*. The genetic variability values found for these varieties reflect efficient selective processes, which have not highly eroded the genetic variability. It has been reported that some traditional agricultural practices can promote gene flow to maintain variability in some special and desirable attributes of plants, as in *Stenocereus stellatus* in Central Mexico (Casas et al., 2006).

The polymorphism values of the five varieties and landraces of *P. ixocarpa* analyzed were higher or similar to the polymorphism reported for some populations of wild plants, like *Agave durangensis* (30.77%) and *Agave asperrima* (24.18%) (Almaraz-Abarca et al., 2013). *Physalis peruviana* was the less variable, what can be the result of the intense domestication process to which this species has been submitted for long time in South America (Pickersgill, 2007).

Bonilla et al. (2008) also reported a low genetic variability for *P. peruviana*, estimated from some of the RAMS markers used in the present study. The current Shannon´s diversity and Nei´s genetic diversity values calculated for the five analyzed samples of *P. ixocarpa* were lower than those reported for the wild species *Physalis solanacea* (Schltdl.), *P. patula* Mill., *P. angulata* and *P. subulata* Rydb. (4.35 to 4.48 and 0.32 to 0.42, respectively) by Medina-Medrano et al. (2016), calculated with ISSR markers. Low diversity values are expected for entities with some extent of domestication, like *P. ixocarpa*. The current Shannon's diversity values also were lower than those reported for a cultivated genetic pool of *P. philadelphica* (0.287 to 0.296) by Zamora-Tavares et al. (2015), calculated with ISSR markers.

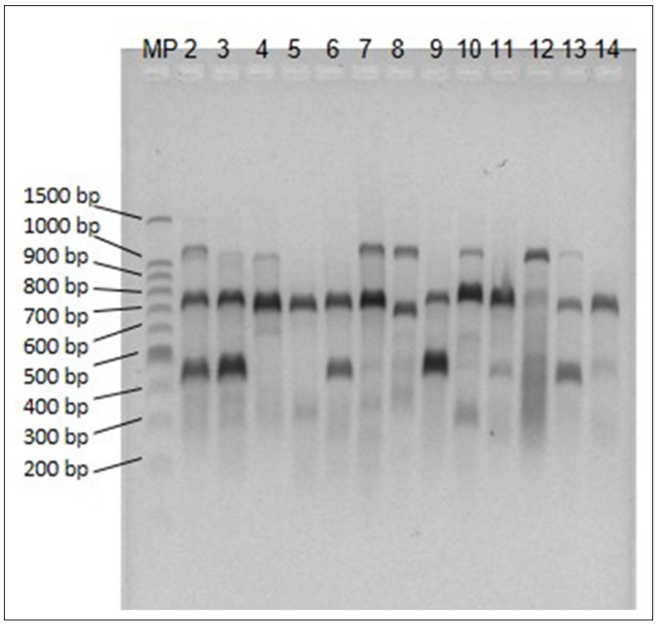

Fig 2. Representative example of the band patterns obtained from the RAMS primer TG on the DNA of 13 individuals of the variety Diamante of *Physalis ixocarpa* (lanes 2 to 14), on 2% agarose gel. MP: Molecular size marker.

Table 2: Amplified loci by five RAMS primers for *Physalis peruviana* and five varieties and landraces of *Physalis ixocarpa*

Variety or Landrace	Primer				
	AG	CA	CGA	TG	CCA
San Isidro Chihuiro (SI)	21	5	17	26	14
Diamante (D)	17	49	21	15	21
Verde Puebla (VP)	21	8	25	22	86
San Martín (SM)	23	17	21	30	27
Rendidora (R)	18	32	36	65	18
P. peruviana (P)	15	12	0	3	17

Table 3: Polymorphism (P) and polymorphic information content (PIC) for five RAMS markers in the genome of *Physalis peruviana* and five varieties and landraces of *Physalis ixocarpa*

Primer	P (%)	PIC
CGA	100	0.098
TG	100	0.075
CCA	100	0.078
AG	96.1	0.100
CA	100	0.093

The analysis of molecular variance (AMOVA) revealed significant differences between populations. The results indicated that 80.52% of variability was found within populations and 19.48% among populations (Table 5).

Genetic distance is the extent of genetic differences between populations or species and is inversely related to genetic identity (Nei, 1987). The values found for the samples analyzed are shown in Table 6. The lowest genetic distance (0.0032) and the highest genetic identity (0.9968)

Table 4: Genetic variability of *P. peruviana* and five varieties and landraces of *Physalis ixocarpa*

Sample	Polymorphic loci	Polymorphism (%)	H	I
San Isidro Chihuiro	87	21.75	0.0409	0.0691
Diamante	125	31.25	0.0490	0.0870
Verde Puebla	96	24.00	0.0359	0.0636
San Martín	123	30.75	0.0560	0.0965
Rendidora	171	42.75	0.0545	0.1021
Physalis peruviana	43	10.75	0.0300	0.0468

H: Nei´s diversity; I: Shannon´s diversity.

was found between Verde Puebla (VP) and San Isidro Chihuiro (SI), revealing a close genetic relationship between these two landraces.

Cluster analysis

The cluster analysis unveiled four major groups (Fig. 3). Group 1 included all individuals of *P. peruviana*, which were clearly separated from the individuals of *P. ixocarpa* (Group 2), as expected for independent species. Within the cluster formed by *P. ixocarpa*, three subgroups can be distinguished (2A, 2B, and 2C). Subgroup 2A included all individuals of San Martín (SM), this variety was the one that showed the highest genetic distance (53) respect to the other four analyzed samples of *P. ixocarpa*. The individuals of San Isidro Chihuiro (SI) and Verde Puebla (VP) formed group 2B. According to the results of Fig. 3, SI and VP were genetically close; however, a trend of grouping separately was observed, except for six individuals (SI8, SI26, SI27, SI28, SI29, and SI30). SI grouped independently of VP at a genetic distance of 0.28. Subgroup 2C was formed by all the individuals of Rendidora (R) and Diamante (D). However, inside the subgroup 2C, R and D clearly formed two independent clusters.

Genetic structure

The genetic structure analysis, according to Evanno´s method, indicated that the more probable number of groups for the analyzed samples was K=4 (Fig. 4). The results of Fig. 4 suggest that San Isidro Chihuiro (SI) and Verde Puebla (VP) are genetically similar varieties, both belonging to the same group (blue). Only few individuals of SI had a probability of 40% of belonging to the group formed by the varieties Diamante (D) and Rendidora (R) (red), whereas few individuals of VP had a probability of 20% to be genetically associated to the group formed by SM (green). These results, as those of genetic distance and genetic identity (Table 6) and those of the cluster analysis (Fig. 3) also suggested a high genetic relationship between D and R (Red). *Physalis peruviana* formed an independent and homogenous group. The results of the genetic structure analysis agreed with those of the cluster analysis (Fig. 3), which also revealed the formation of four groups, a genetic closeness between SI and VP, as well as a genetic closeness between D and R.

Table 5: Molecular variance analysis for a total of 169 samples of *Physalis peruviana* and five varieties and landraces of *Physalis ixocarpa*, based on RAMS markers

VS	df	p-value	VC	Variability (%)
Intra	5	<0.0001	0.02	19.48
Inter	159	<0.0001	0.07	80.52
Total	164		0.09	100

VS: Variability source, df: Degrees of freedom, VC: Variability composition

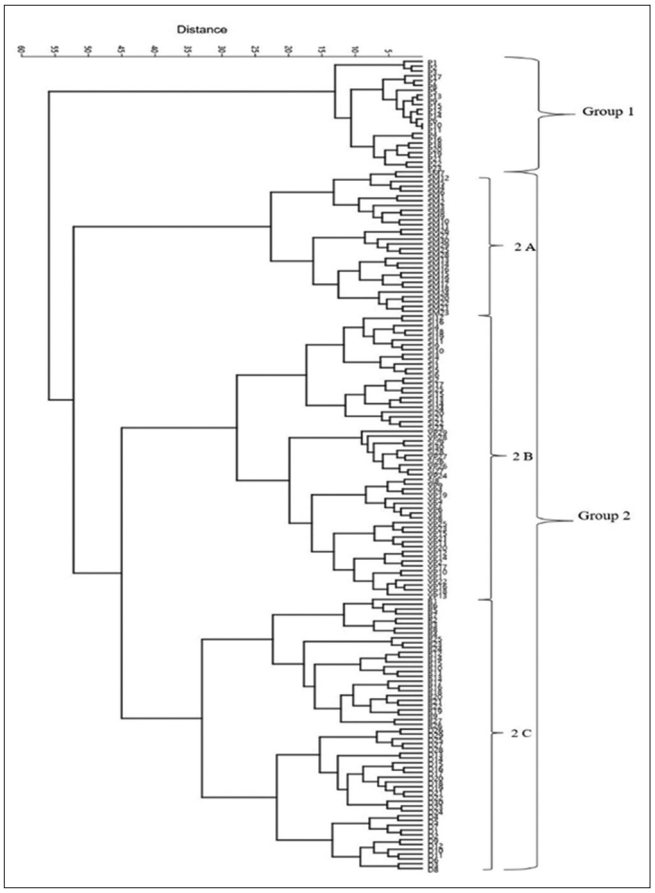

Fig 3. Dendrogram of genetic distance (Ward's method), based on RAMS markers, among *Physalis peruviana* (P) and five varieties and landraces of *Physalis ixocarpa* (San Isidro Chihuiro: SI; Diamante: D; Verde Puebla: VP; San Martín: SM; and Rendidora: R).

Principal components analysis (PCA)

The results of the PCA are displayed in Fig. 5. Five components had the highest discriminative potentials and eigenvalues, accounting for 27.25 % of variance (Table 7).

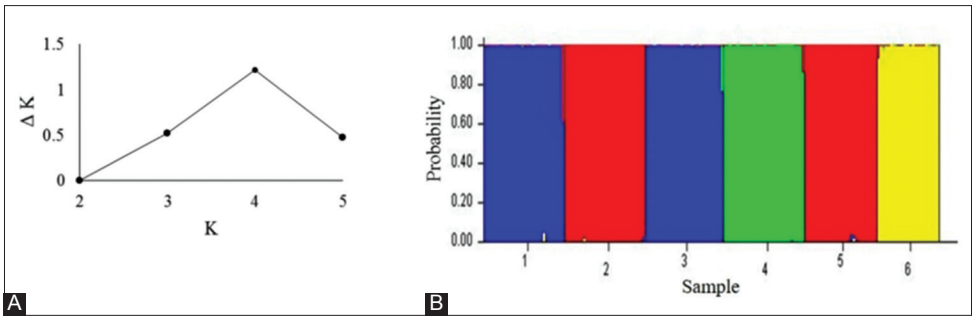

Fig 4. Results of a genetic structure based on a Bayesian analysis with 100 000 tree simulations. A) Selection of the most probable number of groups of 169 individuals of *Physalis peruviana* and and five varieties and landraces of *Physalis ixocarpa,* according to the model of Evanno et al. (2005). B) Inferred structure (1: San Isidro Chihuiro, 2: Diamante, 3: Verde Puebla, 4: San Martín, 5: Rendidora, 6: *Physalis peruviana).*

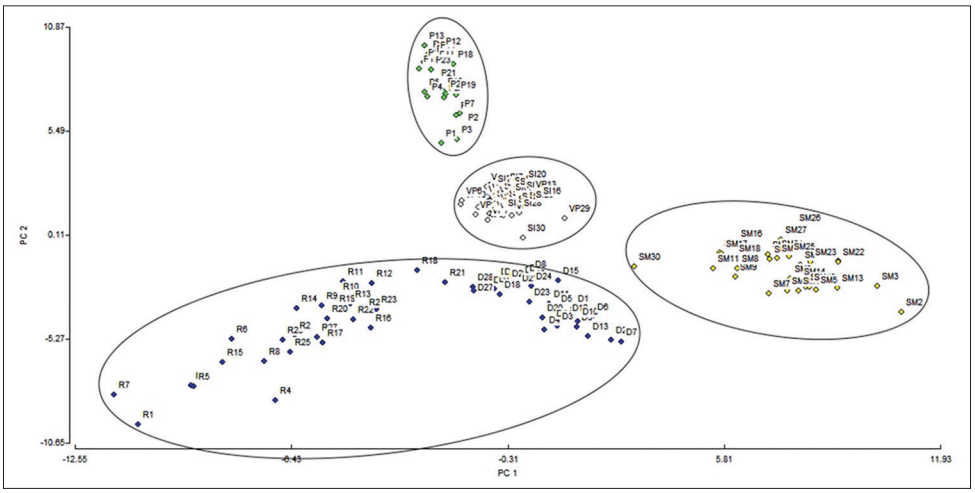

Fig 5. Results of principal components analysis (Jaccard's similarity measure) comparing the RAMS amplification patterns of *Physalis peruviana* and five varieties of *Physalis ixocarpa*. SI: San Isidro Chihuiro; D: Diamante; VP: Verde Puebla; SM: San Martín; R: Rendidora; P: *Physalis peruviana.*

The PCA results agree with those of genetic identity and genetic distance (Table 6), those of cluster analysis (Fig. 3) and those of genetic structure (Fig. 4). The principal components analysis also distinguished four groups. The green group included all individuals of *P. peruviana*. The white group was formed by the individuals of both SI and VP. The third group (blue) included the individuals of both R and D, whereas the group yellow was formed by the individuals of SM.

The results of the cluster analysis, genetic structure, and principal components analysis indicated that RAMS amplification profiles represent useful and reliable molecular fingerprints to distinguish species of *Physalis*, as well as quality indicators to typify and discriminate varieties and landraces of *P. ixocarpa*. The current results support those previously reported by Muñoz et al. (2008), who informed that RAMS are valuable specific markers, which also are useful to estimate genetic variability.

The cluster analysis (Fig. 3) and the genetic structure (Fig. 4) revealed that San Martín is the most defined among the five analyzed varieties and landraces of *P. ixocarpa*. According to Carstensen (2014), variety Diamante is a hybrid between Rendidora and Puebla (this last being the registered variety, not the analyzed in the current study, which was Verde Puebla), this fact could explain the close genetic relationship unveiled between Rendidora and Diamante. In spite of their close genetic relationship, the cluster analysis, based on RAMS clearly discriminated between them (Fig. 3).

Verde Puebla and SI are two landraces originated from Oaxaca, Mexico; the different genetic analysis revealed a high genetic relationship one to each other and none of them was clearly separated (Figs. 3-5). This suggests that both landraces are in a current process of definition of their varietal limits.

Several authors reported that *P. ixocarpa* is a species with a high genetic variability (Santiaguillo et al., 2004; García-Osuna et al., 2015). The current results revealed this species as more variable than other species of the genus, as *P. peruviana*, but less than other wild species also of the same genus, as *P. solanacea*, *P. angulata*, *P. patula*, and *P. subulata* (Medina-Medrano et al., 2016).

Table 6: Nei´s genetic identity (above diagonal) and genetic distance (below diagonal) between *Physalis peruviana* and five varieties and landraces of *Physalis ixocarpa*

Sample\Sample	San Isidro Chihuiro	Diamante	Verde Puebla	San Martín	Rendidora	*Physalis peruviana*
San Isidro Chihuiro	****	0.9928	0.9968	0.9910	0.9935	0.9822
Diamante	0.0073	****	0.9936	0.9918	0.9947	0.9812
Verde Puebla	0.0032	0.0064	****	0.9913	0.9947	0.9826
San Martín	0.0090	0.0083	0.0087	****	0.9914	0.9808
Rendidora	0.0065	0.0053	0.0053	0.0087	****	0.9826
Physalis peruviana	0.0180	0.0189	0.0175	0.0194	0.0176	****

Table 7: Eigenvalues and contribution to variance of the five most discriminative components

PC	Eigen value	% variance
1	1.477	7.93
2	1.267	6.81
3	1.035	5.56
4	0.749	4.02
5	0.544	2.92

CONCLUSIONS

The obtained variability values indicated that the varieties and landraces analyzed of *P. ixocarpa* conserve important levels of genetic variability. RAMS proved to be valuable molecular markers to discriminate between species of *Physalis*, as well as between varieties and landraces of *P. ixocarpa*. The amplification profiles had a varietal-specific tendency, so that RAMS markers may support the development of quality control tools, as fingerprintings, for the authentication of varieties of *P. ixocarpa*. Molecular fingerprinting is required to develop strategies to guarantee the authentication of varieties. The collected data contribute to the development of a kind of passport-ID of *P. ixocarpa* varieties for the commercial, safety, and integral use of this edible plant.

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AUTHOR'S CONTRIBUTION

E.A.D.A. designed the research and wrote the manuscript, N.A.A. participated in the design of research and manuscript preparation, C.E.T. carried out seed germinations and was involved is the literature collection, J.N.U.S. and J.A.A.R. were involved in the literature collection and analysis of results, R.T.R. made the statistical analysis, A.I.C.A. carried out most of the experiments.

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