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Genetic, cytological and molecular characterization of chia (*Salvia hispanica* L.) provenances

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ABSTRACT

Chia (*Salvia hispanica* L.) is a native plant to southern Mexico and northern Guatemala although in these days also is cultivated in others South American countries such as Bolivia and Argentina. This study describes a genetic, cytological and molecular characterization of nine provenances of *Salvia hispanica*. The genome size was estimated to be C = 0.84 pg of DNA. Also a quantitative description of the karyotype is presented. The karyo-idiogram analysis shows that pairs 5 and 6 of the chromosomes are in the limit between submetacentric and subtelocentric types. When the nine provenances were studied using ISSR markers we were able to observe a very low genetic variability for the ISSR loci analysed (h = 0.08–0.15; I = 0.10–0.23; P = 9.3–23.5%). Selfing and reduced genetic background produced by genetic drift and human selection, might explain in part the scarce genetic differentiation found among provenances.

These genetic characterizations are part of the basic genetic information, which should be considered for improvement and germplasm conservation programs.

1. Introduction

Chia, *Salvia hispanica* L., is an annual herbaceous plant that belongs to the family Lamiaceae. Several studies have shown the distribution of the plant (Martínez-Gordillo et al., 2013) as well as cytological characterization (Masoud et al., 2010; Masoud and Alijanpoo, 2011; Ranjbar et al., 2015). It's native to southern Mexico and northern Guatemala although has achieved great development in neighboring countries such as Bolivia and Argentina (Beltran-Orozco and Romero, 2003; Ayerza and Coates, 2004; Capitani et al., 2012). The plants can reach 1–1.5 m in height. It has opposite leaves, 4–8 cm long and 3–5 cm wide. The flowers are hermaphrodite, presenting colors purple to white. They form terminal clusters which bloom from July to August in the northern hemisphere and late summer in the southern hemisphere (Baginsky et al., 2016). After the summer, the flowers give rise to a fruit in the form of indehiscent achene. The seed is rich in mucilage, starch and oil. It is about 2 mm long by 1.5 wide and is oval and shiny with black and grayish white color (Hernández-Gómez et al., 2008). The flowers are pedicellate and are clustered in groups of six or more, in whorls on the rachis of the inflorescence. The calyx is persistent and pubescent bilabiate. The corolla is purple or blue and bilabiate monopetalous; lip expands out and down and the top arches up in the form of helmet or galea (Ramamoorthy, 1985). The mechanism of pollination in chia is not known precisely. It has been suggested that it is allogamous (Hernández-Gómez et al., 2008) and insect pollinated because of the petal color, the “landing pad” shape of the lower lip of the corolla, the articulation of the stamens to the corolla and the presence of nectar at the base of ovary. However, *S. hispanica* is self-compatible and probably self-pollinated, since the flowers are very small and homostylic and even isolated plants produce seed being chia a predominant self-pollinated species (s = 92%; Cahill, 2004).

This species grows in light to medium soils, well-drained, not

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too humid; it tolerates acidity and drought (Silva et al., 2016), but cannot stand frost (Baginsky et al., 2016). It requires abundant sunshine, and does not develop fruits in the shade (Ramamoorthy, 1985; Cahill, 2004). Its economic potential lies in its seed, which produces oil for human and industrial use (Ayerza, 2009). The historical importance of the culture go back to the pre-Columbian populations of Central America where it was one of the basic foods, however was displaced by crops brought to America by the Spaniards. Nevertheless, at the end of the 20th century started again an interest in this crop, mainly due to the fact that the seeds are an important source of fiber, B-group vitamins and poly-unsaturated essential fatty acids, such as linolenic and linoleic acids that the human being cannot synthesize and must be ingested in the diet.

Although it is estimated that the greatest genetic diversity of this species is present in Mexico, there is little information available about basic genetic attributes such as the karyotype, nuclear genome size and molecular markers variability.

The number of chromosomes described for *Salvia hispanica* is $2n = 2x = 12$ (Estilai and Hashemi, 1990), but the genome size (C-value) is unknown. However in previous investigations carried out in other species of the genus, has been suggested that the mean of C value is 0.62 pg (Bennett and Leitch, 2011). On the other hand, genetic studies using molecular markers have been scarce. Cahill (2004) reported a high level of RAPD diversity among wild and cultivars of chia from Mesoamerica.

This study describes a preliminary genetic, cytological and molecular characterization of *Salvia hispanica*, using nine provenances of chia. The genetic variability was evaluated using inter simple sequence repeat (ISSR; Zietkiewicz et al., 1994); the cytological characterization were done through chromosome morphology studies using a karyio-idiogram that assign a value to the ratio "short arm/total chromosome length" and the genome content was estimated using the Feulgen reaction with tomato genomic DNA as standard through quantitative microdensitometry. These studies are part of the basic genetic characterization, which should be considered in breeding and germplasm conservation.

2. Materials and methods

2.1. Plant material

The following nine provenances of chia were used: Atlixco, Mexico (18°54′45N); Puebla, Mexico (20°39′58″N); Jalisco, Mexico (20°39′58″N); Arequipa, Peru (16°23′56″S); Arica, Chile (18°31′25″S); Antumapu, Santiago, Chile (33°27′S); Salta, Argentina (24°47′S) and Santa Cruz de la Sierra, Bolivia, white and black lines (17°47′S). These seeds represent the distribution of cultivars through Mexico and South America. Seeds were germinated and grown using the following greenhouse conditions: 20/15 °C day/night (±2 °C) with a light regime of 14/10 h day/night and 900 μmol m^{-2} s^{-1} light intensity.

2.2. Plant treatments

The root tips from germinated seeds were pre-treated for 12 h with 8-hydroxyquinoline 0.002 M at 8-10 °C, fixed in ethanol - glacial acetic acid (3:1) at 4 °C for 24 h, and stored in 70% ethanol at 4 °C until chromosome processing was done. The root tips were stained with the Feulgen reaction (hydrolyzed for 7 min in 1N HCl at 60°C, stained with Schiff reagent for 60 min and washed in sulphurous water) (Navarrete et al., 1983; Schulte, 1991). Then slides were made by squashing root meristems.

2.3. Cytological analysis

Chromosomes were observed with a Nikon Eclipse E-400 microscope equipped with a Moticam 2500 digital camera. In photomicrographs of at least five best metaphase plates (without overlapping) per provenance, the chromosomes were measured and arranged according to length and shape. The measurements of the short arms (SA) and long arms (LA) and the total relative length of each chromosome pair were expressed as a percentage of the total length of the haploid chromosome set. To obtain a detailed description of chromosome morphology, means and confidence intervals of relative arm lengths of each chromosome pair of all nine provenances of *S. hispanica* were plotted in a Karyo-idiogram according to Spotorno (1985) which assigns the chromosome shape on the basis of the centromeric index proposed by Levan et al. (1964) (ratio short arm/total chromosome length).

2.4. Genome content

Slides made by squashing root tips of chia germinated seeds not treated with antimitotic were prepared. These were stained with Feulgen reaction along with root tips of germinated seeds of commercial tomato (*Lycopersicon esculentum*). The genome size of tomato is known to be 2C = 2.06 pg DNA (Bennett and Smith, 1991) therefore we used it as a comparison standard to establish the genome size of *Salvia hispanica* by quantitative microdensitometry (Spencer et al., 1999; Vilhar et al., 2001; Jara-Seguel et al., 2008; Palma-Rojas et al., 2012). *L. esculentum* was used because its genome size is close to that reported for most species of the *Salvia* genus (Johnston et al., 1999; Bennett and Leitch, 2011). Slides were prepared as for the antimitotic treatments.

The Nikon Eclipse E-400 microscope, equipped with a Cohu 4912-200 black and white digital camera and image analyser software Image Pro-Plus (Media Cybernetic) was used to capture equivalent images of compact chia and tomato prophase. The software captures black and white images from the microscope and analyses the different structures visible on the images. Nuclear optic density (OD) is calculated by the software according to the formula $OD = \log_{10}(1/T) = -\log_{10}T$; where T = intensity of transmitted light/intensity of incident light. From this estimation, the computer integrates the values of OD obtained for each one of the pixels and it calculates the integrated optical density (IOD = ΣOD). For *Salvia hispanica* IOD values of 10 compact prophase from five individuals per provenances (two samples per individual) were determined. To evaluate the existence of significant differences between the IOD values of the nine provenances, a one-way ANOVA was performed. The IOD values were converted to absolute mass of DNA by comparison with 10 compact prophase from five individuals (50 in total) of *Lycopersicon esculentum* (2C = 2.06 pg). The 2C-value was determined using the equation CVu = CVs x (IODu/IODs). In the equation CVu = 2C-value of *S. hispanica*; CVs = 2C value of *L. esculentum*; IODu = average IOD of *S. hispanica*; IODs = average IOD of *L. esculentum*. (Vilhar et al., 2001; Hardie et al., 2002; Moscone et al., 2003).

2.5. Provenances characterization through the use of ISSR markers

Ten plants from each provenance were used for the ISSR genotyping. Genomic DNA was extracted from young leaves (0.5 g) of each plant using the method described in Tittarelli et al. (2009). The purified total DNA was quantified by spectrophotometry and gel electrophoresis (Klagges et al., 2013). DNA samples were stored at −80° C until use.

2.6. ISSR amplifications

A prescreening of 18 ISSR primers (ISSR set 100/8, Biotechnology Laboratory, University of British Columbia, Vancouver) was performed using the nine chia provenances which have early described (Table 1). Amplifications were performed in a total volume of 25 μL containing 40 ng genomic DNA; 1× PCR buffer (Gibco-BRL); 2 mM $MgCl_2$; 0.16 mM dNTPs; 0.24 μM oligodeoxynucleotide primer and 2 units of Taq DNA polymerase (Gibco-BRL). The following PCR conditions were used: initial denaturation step of 3 min at 94 °C; 40 cycles of 1 min at 94 °C; 1 min at the specific annealing temperature (Table 1), and 2 min at 72 °C. All PCR products were confirmed by electrophoresis on 2% agarose gels, run in TAE 1X buffer and visualized by ultraviolet fluorescence after staining with ethidium bromide (0.25 μg/ml staining solution).

2.7. Genotyping and data analysis

The ISSR data were analysed as dominant markers, where the band patterns were scored as 1 when the band was present and 0 when it was absent. Finally, a binary matrix containing the ISSR data was constructed. The genetic relationships among the cultivars were analysed as described in Carrasco et al. (2012, 2014).

The genetic variability parameter such as percentage of polymorphic ISSR loci (P); Nei's gene diversity (h) and Shannon's index (I) (Shannon, 1948; Nei, 1973, 1978; Lewontin, 1972) were estimated using the software Genalex version 6.5 (Peakall and Smouse, 2012). Nei's gene diversity corresponds to 1 - $\sum$ pi^2, where pi is the population frequency of each allele (1 and 0) at locus ith; it can reach values from 0 to 0.5. On the other hand, Shannon's index is -$\sum$ pi ln (pi), where pi is the proportion of the ith allele in the population; it can produces values ranging between 0 and 0.73 (Lowe et al., 2004).

3. Results

3.1. Chromosome characterization and genome size estimation

The nine provenances of *Salvia hispanica* showed 2n = 2x = 12 chromosomes with the same size and shape (Fig. 1). No satellites were observed, and there were no differences between the provenances in chromosome size or centromere position. The mean length of the haploid set of chromosomes was 19.32 ± 0.48 μm. The results of the chromosome measurements are detailed in Table 2.

The standardized means of chromosome arms, shown in Table 2, were used to construct the karyo-idiogram of Fig. 2, which shows the six chromosomes pairs of the nine provenances. The analysis of the karyotype shows that *S. hispanica* is composed of one metacentric pair, one submetacentric pair, three subtelocentric pairs and one telocentric pair (Fig. 3). The ANOVA results in Table 3, show that there are no significant differences between IOD value for the nine provenances (p = 0.120).

Table 4 shows the IOD values measured in prophase of *Lycopersicon esculentum* and *S. hispanica*. Given that prophase were measured, in both cases the values were estimate as 4C, which in the case of *L. esculentum* is equivalent to 4.12 pg of DNA. The genome size of the nine provenances of *S. hispanica* is estimated to be 0.84 pg DNA.

3.2. Genetic variability and genetic relationship

Among the 18 primers used (Table 1), four did not show any PCR product, seven were monomorphic, and others seven showed polymorphic and consistent amplification products. The seven ISSR primers analysed were UBC811, UBC812, UBC815, UBC835, UBC852, UBC853 and UBC854, which generated a total of 75 bands. The number of bands amplified by each primer ranged from 8 to 12, with an average of 10.7. Some examples of band pattern in chia provenances can be seen in Fig. 4.

Low genetic diversity was observed for the nine provenances studied (Table 5). Gene diversity (h), Shanon's index (I) and percentage of polymorphic loci (% P) ranged between h = 0.08 to 0.15; I = 0.10 to 0.23 and P = 9.33%–23.5%, respectively. The 90 individuals studied showed a high level of genetic similarity. Therefore, the genetic distances between provenances was scarce (0.006–0.070) being Bolivia white and Bolivia black almost identical (identity = 0.99). In contrast, the provenances Mexico and Bolivia black displayed higher genetic distance (Genetic distance = 0.070; Identity = 0.93).

Table 1
ISSR primers used in this study.

Primer name	T^o	Band size (bp)	Sequence 5 → 3′
UBC811	52	350-1100	$(GA)_8$ C
UBC812	52	300-1500	$(GA)_8$ A
UBC815	52	700-2500	$(CT)_8$ G
UBC820^m	52	700-2000	$(GT)_8$C
UBC825*	45	600-3000	$(AC)_8$CT
UBC834^m	54	450-2900	$(AG)_8$YT
UBC835	55	380-3500	$(AG)_8$YC
UBC840^m	54	380-3500	$(GA)_8$ YT
UBC844*	51	700-2900	$(CT)_8$ RC
UBC845^m	54	520-3000	$(CT)_8$ RG
UBC849*	52	300-1200	$(GT)_8$ YA
UBC852	52	280–5000	$(TC)_8$ RA
UBC853	52	650–4500	$(TC)_8$ RT
UBC854	52	500–3500	$(TC)_8$ RG
UBC855^m	52	600-2500	$(AC)_8$YT
UBC856^m	52	700–2500	$(AC)_8$YA
UBC857	55	500-3000	$(AC)_8$YG
UBC858*	52	350–2000	$(TG)_8$RT

T^o: annealing temperature; bp (base pairs); *not reproducible; m: monomorphic. In the sequences of degenerated primers: R can be either A or G; Y can be either C or T.

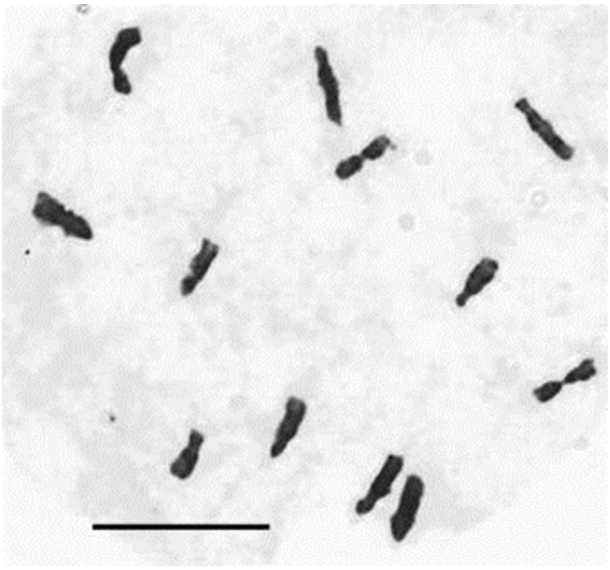

Fig. 1. Metaphase plate of *Salvia hispanica* representing the nine provenances of *Salvia hispanica* (1000 X) 2n = 2x = 12. The bar represents10 μm.

Table 2
Mean length of chromosome arms of *Salvia hispanica* measured in seven best metaphase plates per provenances (n = 63).

Pair	Long Arm (%)	Short Arm (%)	Relative Length (%)	Total Length (μm)
1	15.37 ± 0.97	4.17 ± 0.73	19.68 ± 0.82	3.90 ± 0.61
2	18.44 ± 1.20	0.00 ± 0.00	18.63 ± 1.07	3.61 ± 0.46
3	13.38 ± 1.01	4.05 ± 0.67	17.39 ± 0.64	3.36 ± 0.47
4	8.70 ± 0.71	7.43 ± 0.80	15.99 ± 0.90	3.10 ± 0.50
5	11.27 ± 1.34	3.63 ± 0.99	14.62 ± 1.00	2.81 ± 0.42
6	9.78 ± 1.44	3.70 ± 1.07	13.06 ± 1.53	2.54 ± 0.44

All values are ± SD representing 3 independent measures.

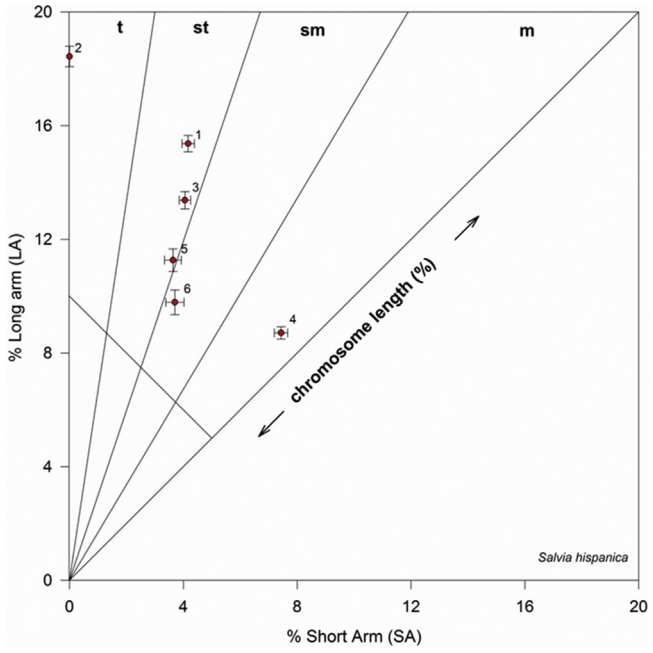

Fig. 2. Karyo-idiogram representing the nine provenances of *Salvia hispanica*. The bi-directional bars indicate 95% confidence intervals. m: metacentric; sm: submetacentric; st: subtelocentric; t: telocentric.

Table 4
IOD values in arbitrary units and C and 4C genome sizes of *Lycopersicon esculentum* and *Salvia hispanica*.

Species	C level	IOD (a.u.)	Genome size (pg DNA)
Lycopersicon esculentum	4C	202,726 ± 6091	4.12
Lycopersicon esculentum	C	50,680 ± 2534	1.03
Salvia hispanica	4C	165,627 ± 4968	3.36
Salvia hispanica	C	41,406 ± 1242	0.84

C: gametic nuclear content; IOD: integrated optical density (±SD); pg: picograms; a.u. arbitrary units.

4. Discussion

Even when chia is an important seed producing crop in terms of oil, protein and vitamin production, little is known at the molecular level of this crop. To start to understand this plant at the genetic and molecular level we decide to characterize the genome content as well as genetic relationship among some provenances to unveil how close or different they are. The chromosome number 2n = 2x = 12 found for *Salvia hispanica* provenances coincides with the number reported by Estilai and Hashemi (1990). However, these authors did not provide a quantitative description of the karyotype. The karyo-idiogram (Fig. 2) shows that chromosome pairs 5 and 6 are in the limit between submetacentric and subtelocentric types. This is not the case for the other four pairs, whose morphology is clearly defined according to the chromosome nomenclature proposed by Levan et al. (1964). Chromosome numbers in the genus *Salvia* vary extensively, from the 2n = 2x = 12 in *S. hispanica* (Harley and Heywood, 1992) to 2n = 8X = 88 in the octoploid *S. guaranitica* (Alberto et al., 2003). This variation occurs both due to polyploidy and to a wide diversity of basic numbers (x) of chromosome (Zhiyun et al., 2004). Basic numbers of chromosome such as 7, 8, 10, 11, 12 and 13 have been found for polyploid species of *Salvia* (Alberto et al., 2003). The genome size of C = 0.84 pg DNA estimated for *S. hispanica* is within the range of C values published in Plant DNA C-values Database of the Royal Botanic Gardens, Kew, London, UK (http://www.kew.org/). The minimum reported value is 0.43 pg for *S. broussonetii* and the maximum value is C = 1.07 pg for *S. glutinosa* (Temsch et al., 2010).

Several types of dominant molecular markers have been used to assess the genetic diversity in *Salvia* genus (Cahill, 2004; Echeverrigaray and Agostini, 2006; Wang et al., 2007; Farkas et al., 2008; Böszörményi et al., 2009; Song et al., 2010; Javan

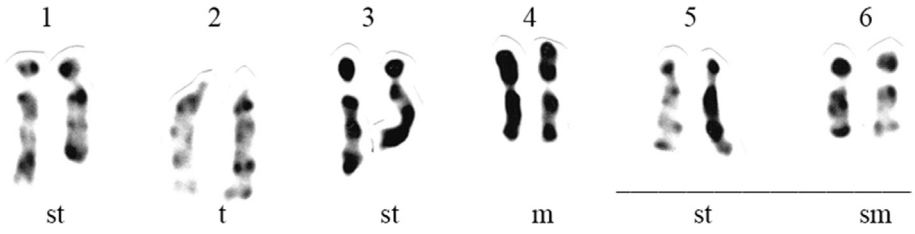

Fig. 3. Karyotype of *Salvia hispanica* (2n = 2X = 12) representing the nine provenances of *Salvia hispanica*. m: metacentric; sm: submetacentric; st: subtelocentric. The bar represents 10 μm.

Table 3
One way ANOVA between the IOD values of the nine provenances used in this study.

Source of Variation in the data	DF	SS	MS	F	P
Between Groups	8	323,031,335.289	40,378,916.911	1.666	0.120
Residual	81	1,963,670,469.200	24,242,845.299		
Total	89	2,286,701,804.489			

DF: degrees of freedom in the source; SS: sum of squares due to the source; MS: mean sum of squares due to the source; F: *F*-statistic; P: *P*-value.

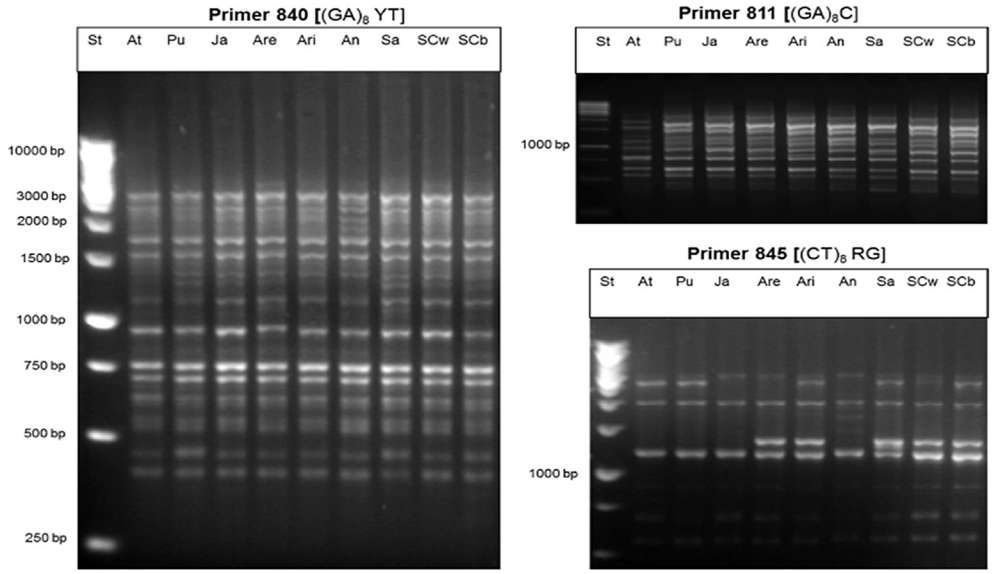

Fig. 4. Example of band patterns observed in nine provenances of chia. St = 1 kb generuler marker; At=Atlixco (México); Pu=Puebla (México); Ja = Jalisco (México); Are = Arequipa (Perú); Ari = Arica (Chile); Sa = Salta (Argentina); SCw = Santa Cruz de la Sierra, white line (Bolivia); SCb = Santa Cruz de la Sierra, black line (Bolivia).

Table 5
Genetic diversity among the nine chia provenances used in this study.

Provenances	h	I	P (%)
Atlixco (Mexico)	0.10 ± 0.02	0.15 ± 0.08	23.50
Puebla (Mexico)	0.11 ± 0.05	0.12 ± 0.08	19.70
Jalisco (Mexico)	0.08 ± 0.03	0.13 ± 0.03	20.50
Arequipa (Peru)	0.10 ± 0.04	0.10 ± 0.06	10.50
Arica (Chile)	0.13 ± 0.07	0.19 ± 0.05	15.70
Antumapu (Chile)	0.15 ± 0.08	0.23 ± 0.04	22.70
Salta (Argentina)	0.10 ± 0.03	0.15 ± 0.05	9.33
Santa Cruz de la Sierra, white line (Bolivia)	0.13 ± 0.04	0.19 ± 0.07	12.00
Santa Cruz de la Sierra, black line (Bolivia)	0.14 ± 0.06	0.21 ± 0.08	16.00

h: gene diversity (±SD); I: Shanon' s index (±SD); P (%): percentage of polymorphic loci and in bracket, standard error.

et al., 2012; Zhang et al., 2013; Penga et al., 2014). Among them, RAPDs have been a preferred molecular marker to evaluate the germplasm of *Salvia* sp. due to its ability to generate polymorphisms in a rapid and inexpensive way. However, RAPDs have shown to be less reproducible than other dominant markers such as ISSR (Javan et al., 2012). These markers use microsatellite sequences as primers which can usually be anchored at the 3′ or 5’ end with 1–4 degenerate bases, generating a multilocus pattern (Zietkiewicz et al., 1994). It is a simple, quick, cheap and highly reproducible approach compared with amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) (Pradeep Reddy et al., 2002).

In this study the *Salvia hispanica* provenances showed a low ISSR variability (h = 0.08–0.15; I = 0.10–0.23; P = 9.3–23.5%). These values are significantly inferior to those reported by Nybom (2004) for plants (h = 0.19–0.23) and for selfing species (h = 0.12), using dominant markers. Other species of *Salvia* genus have displayed at least twice RAPDs and ISSR variability than *S. hispanica* provenances used in this study. For example *S. officinalis* and *S. judaica have* shown between 10 and 59% of polymorphic bands for RAPDs markers (Echeverrigaray and Agostini, 2006; Farkas et al., 2008; Böszörmenyi et al., 2009). Similar tendencies have been seen for *S. multiorrhiza* and *Salvia* sp. using ISSR markers (93–100% of polymorphic bands; Javan et al., 2012; Penga et al., 2014).

Although not all existing provenances were evaluated in our study, at the intra-specific level the ISSR variability was higher than those reported by Cahill (2004) for modern cultivars of chia using RAPD (h = 0.02–0.08; P = 12.7%). However, the ISSR variability was similar to the one observed in wild germplasm (h = 0.15; P = 83.6%) and old cultivars (h = 0.1; P = 61.8%; Cahill, 2004).

The high selfing rate (s = 92%, Haque and Ghoshal, 1981; s = 98.5%, Hernández-Gómez et al., 2008) and genetic improvement, are considered some of the main factors that have affected the genetic structure of modern cultivars of *S. hispanica* (Cahill, 2004). The narrow genetic basis of new developed cultivars plus self-pollination and selection of pure lines, have allowed to stabilize different genetic combination at the homozygous state (Cahill and Provance, 2002). In that regards, these are frequently used breeding techniques to produce modern varieties of chia and they could explain in part the high level of similarity found among chia provenances. Cytology, genetics and molecular biology provide the basis for the assisted selection of chia genotypes with high content of Omega-3 depending on physiological parameters and how this content depends on the interaction between genotype and environmental factors.

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