ATINER CONFERENCE PAPER SERIES No: AGR2013-0765

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AGR2013-0765

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Email: info@atiner.gr URL: www.atiner.gr
URL Conference Papers Series: www.atiner.gr/papers.htm

Printed in Athens, Greece by the Athens Institute for Education and Research.

ISSN **2241-2891**
17/12/2013

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This paper should be cited as follows:

Peña-Ortega, M.G., Rodríguez-Pérez, J.E. and Martínez- Solís, J. (2013) **"Exploration of Genetic Variability of 'Mirasol' chili (*Capsicum annum* L.) Accessions through ISSR Markers"** Athens: ATINER'S Conference Paper Series, No: **AGR2013-0765**.

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Abstract

Chili is an important crop that has not been subjected to intensive breeding; therefore, there are not enough outstanding improved varieties available for producers. Then, exploration of genetic variability available for this crop results a valuable approach to identify useful materials that could enrich genetic diversity of the existing breeding programs. In this study, 30 chili landraces collected from two Mexican provinces were evaluated in order to estimate genetic diversity among them through their genomic fingerprints obtained from ISSR (Inter Simple Sequence Repeat) markers. Nine ISSR primers produced a total of 51 bands, and 43 of them were polymorphic (representing 84.3 percentage of polymorphism). UBC841, LOL12, and LOL10 primers showed high polymorphic information content, and therefore they could be very useful in further genetic studies in this crop. Gower's distances between pairs of accessions were used to define four groups according to their molecular diversity. The results obtained from this study proved the existence of important genetic variability among chili landraces collected at distinct geographical locations, and allow establishing the basis for conservation and utilization of evaluated materials for breeding purposes. ISSR molecular markers were able to differentiate genetically chili accessions even though their expected high genetic homogeneity due to its self pollination nature.

Keywords: molecular profiles, population genetic structure.

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Introduction

Chili (*Capsicum annuum* L.) is an important crop in Mexico due to its high demand for direct use to elaborate traditional spiced Mexican dishes, as source of oleoresins to be used by food industry as natural and safe colorants, to enhance cold meats flavor, and lately by the pharmacological industry due to their weight loss, anti-microbial, and anesthetic properties for human health (Breithaupt, 2004).

Among the distinct types of chili cultivated in Mexico, 30, 000 has are planted with 'Mirasol' annually, been the second most important after 'Jalapeño' type. The main production area is located at the North Central part of the country, with mean yields varying from 1.3 to 1.5 tha^{-1} of dried fruits, which are considered low compared to the productive potential observed for this crop. These low yields can be explained due to the fact that approximately 95 % of the production area is planted with local landraces with low yields, mixes of different types and subtypes of chili which result in turn result in great variability on type of plant, vegetative cycle, fruit shape, size, color, and number of fruits per plant. This variability causes low fruit quality and reduced yields (Aguilar *et al*., 2009). Therefore, it is necessary to generate improved chili varieties with high yield, fruits of uniform size and shape, and moderate pungency levels.

Genetic variability available for 'Mirasol' chili has not been extensively studied. Morphological characterizations in field conditions are laborious, slow, and expensive; in addition, expression of morphological traits is highly dependent on environmental conditions. Then, a molecular characterization of the different accessions that represent the initial genetic pool to start a breeding program signifies a more effective way to perform the first approximation to explore the genetic diversity available and rapidly detect repetitions that should be discarded for future studies. ISSR molecular markers have produced highly reliable, reproducible, and rapid results when exploring genetic variability in autogamous crops, such as chili, generating a significant number of polymorphic bands that help to differentiate between distinct genotypes, despite their genetic homogeneous nature, thus making them a very useful tool for breeding purposes in these species (Da Costa *et al.*, 2009; González *et al*., 2011; Patel *et al*., 2011).

In this study, in order to explore genetic variability available for this crop, molecular ISSR profiles were obtained for 30 'Mirasol' chili landraces, collected at different communities belonging to two of the mayor Mexican provinces dedicated to the production of this crop.

Materials and Methods

Geographical origins of the 30 accessions considered for this study are detailed in Table 1. Collections were performed during 2010 and 2011 at 'Zacatecas', and 'Durango' Mexican provinces. Plantlet production was

performed at lab conditions; seeds from each accession were sowed in 200 cavities polystyrene plastic germination trails filled with vermicompost as substrate. When plantlets reached 10 to 15 cm height (15 to 20 days after sowing), fresh and healthy leaves were cut for DNA extraction.

Table 1. *Geographical location, temperature and altitude conditions of collection sites for 30 'Mirasol' chili landraces*

No.	Locality	Latitude	Longitude	Altitude
1	La Joya, Dgo.	19° 29' 32.6"	98° 52' 20.8"	1,838
2	El Tobe, Dgo.	23° 48' 00.2"	104° 02' 35.1"	1,804
3	Vicente Guerrero, Dgo.	23° 48' 00.2"	104° 02' 35.5"	1,795
4	La Joya, Dgo.	23° 53' 0.45"	103° 54' 47.6"	1,820
5	La Joya, Dgo.	23° 53' 0.15"	103° 59' 24.6"	1,880
6	Vicente Guerrero, Dgo.	23° 47' 58.6"	103° 58' 31.2"	1,871
7	Nombre de Dios, Dgo.	23° 47' 19.5"	104° 00' 00.8"	1,840
8	Vicente Guerrero, Dgo.	23° 43' 31.3"	104° 00' 32.1"	1,800
9	Nombre de Dios, Dgo.	23° 43' 27.7"	104° 00' 24.7"	1,802
10	Nombre de Dios, Dgo.	23° 47' 35.2"	104° 06' 0.50"	1,807
11	Nombre de Dios, Dgo.	23° 47' 32.1"	104° 06' 11.2"	1,812
12	Vicente Guerrero, Dgo.	23° 39' 09.0"	104° 01' 12.3"	1,918
13	Vicente Guerrero, Dgo.	23° 39' 03.3"	104° 00' 59.4"	1,921
14	Vicente Guerrero, Dgo.	23° 39' 24.5"	104° 01' 15.8"	1,916
15	El Pardillo, Zac.	23° 47' 19.5"	104° 00' 00.8"	1,983
16	El Pardillo Segundo, Zac.	23° 08' 43.4"	102° 40' 58.9"	1,983
17	El Pardillo Segundo, Zac.	23° 08' 38.6"	102° 40' 21.6"	1,981
18	Bañón, Zac.	23° 12' 38.6"	102° 25' 13.2"	1,928
19	Bañón, Zac.	23° 12' 38.6"	102° 25' 13.0"	1,928
20	Bañón, Zac.	23° 12' 38.8"	102° 25' 13.4"	1,932
21	Santiaguillo, Zac.	23° 06' 20.4"	102° 28' 37.0"	2,028
22	Potrero de Ojuelos, Zac.	23° 06' 20.4"	102° 38' 37.4"	2,039
23	El Pardillo Segundo, Zac.	23° 08' 37.3"	102° 40' 42.0"	1,996
24	Las Auras, Zac.	23° 04' 29.6"	102° 38' 35.4"	2,005
25	Las Auras, Zac.	23° 05' 02.9"	102° 38' 28.8"	2,014
26	Las Auras, Zac.	23° 02' 01.4"	102° 38' 55.4"	2,029
27	Calera, Zac.	23° 02' 01.4"	102° 38' 55.4"	2,131
28	Calera, Zac.	23° 56' 27.6"	102° 44' 26.3"	2,145
29	Río Frío, Zac.	23° 56' 27.9"	102° 44' 26.4"	2,155
30	Río Frío, Zac.	23° 56' 27.9"	102° 44' 26.4"	2,155

DNA extraction. De la Cruz *et al*. (1997) protocol was used; 0.3 g of fresh leaf tissue was powder grounded with liquid nitrogen and then transferred to 1.5 mL plastic tubes preheated at 65^{o} C using a themoblock, containing 700 µL of extraction buffer (Tris-HCl 100 mM, EDTA-Na_2 50 mM, NaCl 500 mM, 2-mercaptoetanol 10 mM, SDS 1.3%, pH 8.0). Tubes were agitated for homogenization and reheated at 65^{o} C for 10 minutes. 200 µL of 5M potassium acetate were added to each tube and they were ice cooled for 30 to 60 minutes and then centrifuged for 20 minutes at 12,000xg. Supernatant was transferred to new plastic tubes containing 600 µL of precooled isopropanol which were kept at -20^{o} C for 30 to 60 minutes to precipitate DNA. Afterwards, tubes were

centrifuged for 5 minutes at 6000xg and decanted; DNA's pill was dissolved with 700 µL of solution and then 4 µL of RNase were added. Tubes were incubated at 37° C for 1 hour. DNA was reprecipitated using 75 µL of 3M sodium acetate and 500 µL of cooled isopropanol for 2 hours. Afterwards, tubes were centrifuged for 5 minutes at 8000xg, supernatant was disposed and the DNA's pill was washed with 70 % ethanol. Tubes were again centrifuged for 5 minutes at 8000xg, supernatant was discarded and the DNA's pill was dried at room temperature. Finally, it was dissolved in 50 µL of TE and then storage in a fridge at 4° C. Five repetitions per accession were performed, giving a total of 150 extractions.

DNA quantification and quality evaluation. DNA concentration for the different samples was determinate by a NanoDrop 1000 (Thermo Scientific Wilmington, USA) and dilutions were made in order to get a uniform concentration of 10 ngμL^{-1}. Later the five repetitions per accession were mixed to make one bulk per genotype. DNA quality was quantitated running a 8% agarose gel using 100 mL of TAE 1X buffer (Tris base 40 mM, pH 7.8; sodium acetate 20 mM and EDTA 2 mM, pH 8.0). Over a parafilm tape, 2 µL of load buffer (0.2% blue bromophenol, 0.2 xylene cianol, 25% glycerol, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0) were mixed with 10 µL from each sample, while in both extremes 3 µL of 1 Kb molecular ladder were loaded. Electrophoresis was performed at 90 volts for 1.5 hours. Afterwards the gel was removed and dyed using an ethidium bromide solution (0.6 µg·µL in TAE 1 X) for 20 minutes. Subsequently, the gel was drained and putted inside a UV transilluminator to be photographed using a Kodak EDAS 290 camera.

ISSR molecular profiles. 21 Sigma® primers were originally screened to evaluate their ability of producing clear and reproducible amplification products. From them only 9 primers were finally selected. The PCR protocol was as follows: 2.5 µL of DNA (10 ngμL^{-1}) were collocated into 1.5 mL plastic tubes containing 22.5 µL of reaction mixture integrated by PCR buffer (2.5 µL 10X), Taq DNA polymerase (0.3 µL, Invitrogen®), DNTP's (10 µL 500 µM), $MgCl_2$ (3 µL 50 mM), primer (1.5 µL 10 ng/µL) and water (5.2 µL) to complete a 25 µL total volume. Tubes were incubated in a Techne TC-412 thermocycler using the following program: one pre-denaturation cycle of 20 minutes at 93° C, 40 denaturation cycles (one minute at 93° C each), alignment (one minute at 36° C), extension (one minute at 72° C), and one cycle of final extension at 72° C. The amplification products were separated by electrophoresis in agarose gels, which were later stained with ethidium bromide, reveled inside the UV transilluminator and photographed using the Kodak EDAS 290 camera.

Statistical analysis. The similarities and genetic differences between pairs of accessions were computed from band patterns allocating the 0 value to the absence and a value of 1 to the presence of each band. Amplification products were recorded for each primer, and a consecutive number was assigned to them according their migration distance in the agarose gel. The registration of ISSR patterns obtained from each of the 9 selects primers for the 30 accessions created the basic data matrix (BDM). In order to identify the main factors that

influenced the distribution of genetic variability among accessions, an analysis of molecular variance (AMOVA) was performed using InfoGen® software (ver. 2011, Universidad de Córdoba, Argentina). To evaluate the discrimination power of tested primers a descriptive analysis of amplified products was obtained where quantitative variables such as number of amplified bands, proportion of mono and polymorphic loci, and polymorphic information content per primer were quantified. Due to the qualitative nature of used molecular markers, the graphical exploration of genetic variability was performed by a Principal Coordinates Analysis (PCA), from which a minimum distance tree (MDT) was constructed using Euclidean distances with InfoGen®. Because genetic distance can be used to compare similarity among different accessions, groups were constructed under the criteria of Ward's minimum variance using Gower's distance (1 – s) to construct a dendrogram. The cut distance was estimated by the cubic clustering criterion (r) and Hotelling's T^2 pseudostatistic (Taylor, 2010), using PROC CLUSTER and PROC TREE from SAS® Ver 9.0.

Results and Discussion

Genetic variability. The hierarchical analysis of molecular variance (AMOVA) (Table 2), where the 30 accessions were separated into two populations according to their geographical precedence, shows that the most important observe molecular diversity was within populations (95 %), suggesting that there are differences in allelic frequencies within the two populations which in turn are defining the population genetic structure (Oyama *et al*., 2006), while genetic differences due to geographical origin were less important for the detected genetic variability. These results could be explained if the collection sites within each of the two considered provinces were equally contrasting related to climate and elevation conditions, as can be appreciated in Table 1. The descriptive analysis of amplified products (Table 3) shows the 5'-3' sequence, and the annealing temperature for the 9 tested primers. Only 8 out of a total of 51 amplified bands were monomorphic, which represents 84.3 percentage of polymorphism. These results contrast to those achieved by González *et al*. (2003), who by the use of 16 primers in 24 commercial chili varieties obtained 36.8 percentage of polymorphism, approximately half of that obtained in this study.

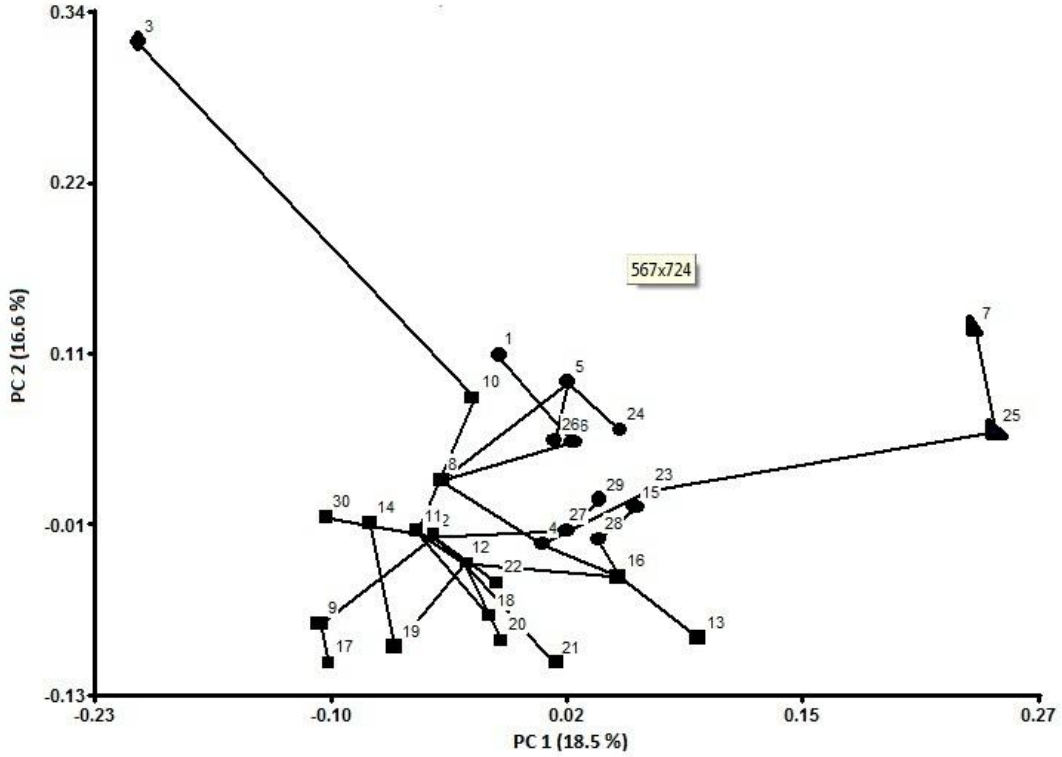
Even when UBC841 and LOL7 primers were the most efficient for production of amplified bands (6 bands each), UBC840 showed the highest percentage of amplification through all 30 accessions (94%). High polymorphic information contents were observed for UBC841, LOL12 and LOL10 primers, therefore being the most recommendable to be used in further studies of molecular characterization of chili. These results agree to those obtained by González *et al*. (2003), who also found that the primer UBC840 was one of the most efficient for amplification purposes in chili collections. The lowest probability for two individuals to share the same allele by chance

was found for LOL12 primer. This result suggests its usefulness to genetically differentiate between chili collections. By the way, even do UBC840 showed the highest percentage of amplification, it also showed the lowest discriminant power among tested primers.

Table 2. *Analysis of molecular variance of ISSR fingerprints from 30 'Mirasol' chili landraces*

Sources	Degrees of freedom	Mean squares	*P* value	Variance component	Variation %	Coefficients	Estimation
Between populations	1	8.38	0.0125	0.24	4.69	$Phi_{\_ST}$	0.05
Within populations	28	4.83	0.0150	4.83	95.31	$Phi_{\_Pob}$	0.05
Total	29	4.95		5.07	100.00		

Figure 1. *Minimum distance tree (MDT) using Euclidean distances among 30 'Mirasol' chili landraces*



Genetic differentiation among accessions. The ordination of accessions and further visualization in a graph in order to explore tendencies and possible relationships among them was obtained using Gower's distances (1-s) between pairs of accessions using the Principal Coordinates Analysis (PCA). Figure 1 illustrate the minimum distance tree (MDT) obtained for the 30 accessions. From the total genetic variability, 35% is explained by the two principal coordinates. At the left up side of the graphic PC1 clearly separates accession 3 from 'Vicente Guerrero' locality as the most genetically distinct material among

the 30 tested. This result could be explained considering information in Table 3. It can be noticed that this locality has the lowest altitude over the sea level (1,795 m), and therefore the warmest climate among the considered localities, with 17.4 ^{o} C annual temperature and between 500 to 600 mm of precipitation. PC1 also separates accessions 7 and 25 from the rest of the group and declares them genetically similar, even do they came from different localities at 172 km apart.

Table 3. *Primers, 5'-3' sequence, annealing temperature (AT), number of polymorphic bands (PB), monomorphic bands (MB), total bands (TB), proportion of polymorphic loci (PPL), polymorphic information content (PIC), standard error (SE), percentage of amplification (PA), and probability for two individuals to share the same allele by chance (PSSA) of ISSR fingerprints from 30 'Mirasol' chili landraces*

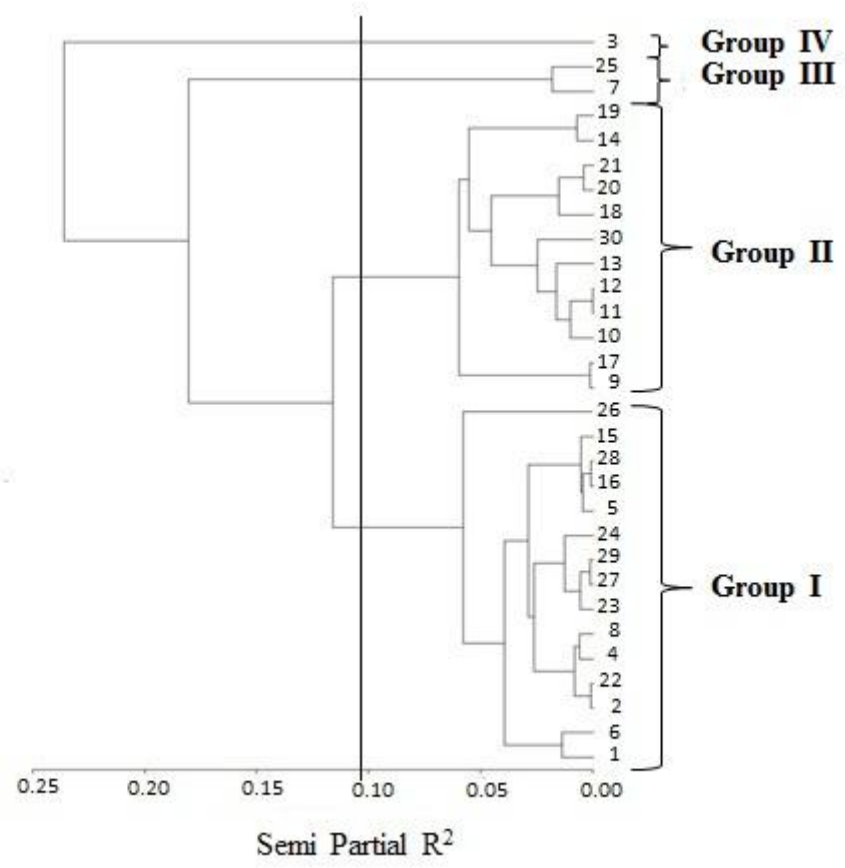
Name	(5' - 3') Sequence	AT	PB	MB	TB	PPL(95)	PIC	SE	PA	PSSA
UBC840	$(GA)_8YT$	51	5	0	5	0.40	0.10	0.02	94.0	7.9E-05
UBC841	$(GA)_8YC$	51	5	2	7	0.57	0.23	0.06	43.3	2.7E-07
UBC866	$(CTC)_6$	60	5	1	6	0.50	0.18	0.04	72.2	1.1E-05
LOL7	$(GAGA)_3CC$	53	3	4	7	0.29	0.18	0.08	35.2	1.8E-03
LOL8	$(GT)_6CC$	58	5	0	5	0.60	0.20	0.06	59.3	1.5E07
LOL10	$(GAG)_3GC$	56	5	0	5	1.00	0.21	0.04	78.0	1.5E-09
LOL12	$(GTG)_3GC$	61	4	0	4	1.00	0.22	0.01	68.3	2.9E-11
IS03	$(GA)_8CTC$	49	6	0	6	0.67	0.20	0.05	61.6	3.7E-08
IS07	$(AG)_8CTG$	53	5	1	6	0.67	0.15	0.05	58.3	4.3E-06
Total			43	8	51				61.4	4.6E-58

The remaining 27 accessions, located at the upper and lower central part of the graphic are separated into two groups, but both were formed by almost equal number of accessions coming from the two different provinces under consideration. These genetic similarities among collections of distinct geographical origin suggest that chili producers from colliding provinces could have been exchanging genetic resources between them, a common practice in Mexico.

Genetic relationships among accessions. After the graphical exploration of distribution of genetic variability among accessions trough PCA analysis, genetic distances between accessions pairs were estimated using Gower's similarity coefficient transformed to genetic distance as 1-s. Afterwards, clustering was performed using the Unweighted Pair Group *Method* with Arithmetic Mean (UPGMA). To ensure the obtained clustering was robust, 1,000 cycles of resampling by bootstrapping were performed and results were represented in a hierarchical dendrogram (Figure 2). The dendrogram cutting value (r = 0.11) was estimated using the cubic clustering criterion and Hotelling's T^2 pseudostatistic (first value inferior than 20% of the total number of accessions, which in this case was 6). The cutting point divided the

accessions into four groups, agreeing to the minimum distance tree (MDT) previously discussed. The accessions genetically more closely related were 11 and 12, both collected at different localities from 'Durango's province and 17 km apart, with a genetic distance value of 0.023; meanwhile, the most genetically distant accessions (0.525) were 3 and 7, also both coming from 'Durango's province, from localities separated by 4 km.

Figure 2. *Dendrogram generated from Gower's distances (1-s) for 30 'Mirasol' Chili landraces*



Group I was the most numerous integrated by 15 accessions, 6 of them from the 'Durango's province and 9 from 'Zacatecas', agreeing to space distribution of genetic variability observed at the MDT. These results suggest the existence of similarities among the molecular profiles of these accessions despite their different geographic origin. However, if genetic variation within the 'Durango's province is considered, most of the accessions came from 'Poanas' municipality, which was not represented in Group II. This result suggest a close genetic relationship among chili materials planted at this area; thus, if a crossing scheme was to be establish in order to get maximum heterosis in the hybrids top be obtained, it would be recommendable to cross these accessions to materials classified into the remaining groups, since genetic divergence is associated to greater heterosis (Birchler *et al*., 2006).

Group II was formed by 12 accessions where both provinces were equally represented. When genetic variability within 'Durango's province materials is considered, most of the accessions came from 'Calera's municipality, which in turn has the smallest extension compared to the other five sampled for this study. These results again suggest the existence of close relationships among materials collected at these locations. Within this group, accessions 11 and 12 were genetically closely related and came from localities at distinct municipalities, but located only 10 km apart. This result also confirms that for determination of genetic similarities among accessions the geographical origin was not the main determining factor.

Group III was integrated by only two accessions, one from 'Durango' and the other one from 'Zacatecas'. Then again no clear relationship between common geographical origin and genetic similarity was found. Finally Group IV was conformed only for accession 3, from 'Gabriel Hernandez' locality, which as discussed previously, was the collection site with lower altitude among the sampled sites and therefore showing the warmest climate, what in turn could explain the very particular and distinct molecular profile showed by this accession. Then again for a crossing scheme, it would be expected that crosses of accession 3 to accession from different groups would yield high heterotic chili hybrids due to allelic divergence among materials.

Results obtained in this study confirm the existence of genetic variability within the 30 'Mirasol' chili landraces evaluated. Non replicated genetic profile was found; therefore it is convenient to preserve all collected materials in the germoplasm collection for further use for breeding purposes since they represent different sources of genetic diversity. In addition, based on genetic distances estimated from ISSR patterns, a preliminary scheme for crosses is proposed looking for highly heterotic hybrids (Table 4). However, the following step must be a morphological characterization of proposed materials in order to corroborate that they have valuable agronomic traits such as high number of fruits per plant, and fruits of acceptable size, shape and pungency.

Table 4. *Potential progenitors of 'Mirasol' chili hybrids according to Gower's genetic distance estimated from ISSR molecular profiles*

Proposed Progenitors		**Genetic Distance**
3 (Vicente Guerrero, Dgo.)	x 7 (Nombre de Dios, Zac.)	0.525
	x 25 (Las Auras,Zac.)	0.523
	x 21 (Santiaguillo, Zac.)	0.488
7 (Nombre de Dios, Zac.)	x 30 (Río Frío, Zac.)	0.431
14(San Francisco Javier, Dgo.)	x 25 (Las Auras, Zac.)	0.422

Conclusions

The obtained results from this study provide the basis to establish general strategies for conservation and exploitation of evaluated materials. It had been confirmed the existence of useful genetic variability available for this crop

from distinct geographical origins. ISSR markers were effective to distinguish genetic differences for tested landraces despite the genetic homogeneity expected for an autogamous crop such as chili.

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