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ABSTRACT

Fava bean is an important crop in Mexico, both for human and animal consumption. Small grain fava beans are an excellent source of protein for animal feeding and human consumption, while their seed size allows mechanical seed planting. However, despite their production value, little is known about genetic variability present within these groups. Then, in the present study, ten *Vicia faba* L., var. minor lines and five *Vicia faba* L., var. equina lines were characterized using 14 ISSR (Inter Simple Sequence Repeat) primers. A total of 117 amplified bands were obtained, 87 of them polymorphic (74%). Primers UBC-807, UBC-818, UBC-890 and ISSR9 had a high degree of confidence to identify genetic variability of fava bean and were able to separate tested lines according to their botanical type. Dice similarity coefficient (1 – S) between pairs of lines was used to construct a dendrogram. An analysis of molecular variance (AMOVA) showed that molecular variation between botanical types was higher (55%), however variation within types was also considerable, suggesting the convenience of keeping all tested materials for breeding purposes in this crop.

Keywords: molecular profiles, germplasm characterization.

Introduction

Fava bean (*Vicia faba* L.) is a worldwide important crop used as human food in developing countries and as animal feed, mainly for pigs, horses, poultry and pigeons in industrialized countries due to its high protein content; it is also used to improve soil fertility due to its ability for nitrogen fixation. Fava bean is an annual crop, diploid (2n = 2X = 12), and it is the seventh most produced legume, with a yield of 4.56 million tons of dry seed annually, planted in 2.4 million hectares, with an average yield of 1.85 tonha-1 (FAOSTAT, 2016).

It was one of the first domesticated legumes about 7,000 to 4,000 B.C., and it originated at the Mediterranean region and the Southwest of Asia; although it extended rapidly to Europe, North of Africa, Ethiopia throughout the Nile, India, China, and Mexico (Kosterin, 2014). Worldwide the main fava bean producer countries are China, Ethiopia, Australia, United Kingdom, and France. Mexico occupies the 34th place globally, with a planted area of 23,781 has, with an annual production of 36,970 ton, and an average yield of 1.55 tonha^{-1} (FAOSTAT, 2016).

Vicia faba specie has been divided into three botanical types based on seed size: small seed (*Vicia faba* L. var minor), medium (*Vicia faba* L. var equina), and large (*Vicia faba* L. var major). The major type is common in the South Mediterranean countries and China; minor is found in North Europe and equina types are grown throughout the Middle East, North Africa and Australia (Alghamdi et al., 2012). Two main fava bean types have been proposed for Europe: a Central and Northwest European gene pool, consisting of V. faba var. minor and V. faba var. major types, and a Mediterranean gene pool which includes the former types but also V. faba var. equina (Terzoupolus and Bebeli, 2008).

Botanical type *Vicia faba* L. var. minor (Harz) Beck has small seeds, with elliptical shape, and average weight of 0.3 to 0.7 g each. Pods are calendrical, from 8 to 15 cm long, and they contain 3 to 4 seeds; their length vary from 0.7 to 1.3 cm. Hundred seeds weight goes from 30 to 70 g. *Vicia faba* L. var. equina Pers has medium size seeds, with crushed shape and an average weight from 0.7 to 1.1 g. This botanical type has medium size pods, with moderate dehiscence and with 3 to 4 seeds, with 1.3 to 1.7 cm length. A hundred seeds weight goes from 70 to 110 g. Finally, *Vicia faba* L. var major (Harz) Beck is the most used type for human consumption at green stage; its seeds are the largest, and their reach an average weight from 1.2 to 1.8 g. Its pods are non-dehiscent, and they length varies from 12 to 35 cm, containing 4 to 5 seeds each. Their length goes from 2 to 3 cm, and the weight of a hundred seeds varies from 120 to 180 g.

Fava bean seeds could be oblong, oval, crashed or rounded, and their size varies widely among varieties. Seed testa color is one of the main traits associated to potential of germination, seed vigor and seed longevity. It could vary from white to green, beige, dark brown, purple, red and black. Seed color in fava bean is controlled by two independent loci with multiple alleles and sometimes epistatic effects. Spotted color is dominant over smooth colors, and among these, brown behaves dominant over black, green and beige; meanwhile, black and red behaves as recessive relative to other colors (Ryu et al., 2017). Moreover, zt-1 and zt-2 loci also control the amount and type of flavonoids produced; zt-1 is responsible for

flavonoids monomers (flavonols, etc,) while zt-2 locus controls flavonoids polymers (condensed tannins) (Webb et al., 2016).

Fava bean crop has nutritional and economic importance. Seeds from their pods can be consumed fresh (soft grains) or dried, with an average protein content of 25%. Uses of var major are mainly for human food in developing countries, while for industrialized countries it is used as fodder for pigs, horses, poultry and pigeons. It can be eaten as green vegetable or dried, fresh or canned. It is a frequent breakfast in Middle East, the Mediterranean, China and Ethiopia (Multari et al., 2005).

Vicia faba L. var minor with small seeds is used mainly for cattle fodder in countries where soybean is expensive (Gous, 2011; Tufarelli and Laudadio, 2015). *Vicia faba* L. var equina is also used as human food since immature seeds, green or dried can be eaten at the same manner as var. major. Boiled leaves can be eaten as spinaches. Fiber can be obtained from stems. Burned stems are rich in Calcium, so they can be used to produce soap. This type of fava bean is also used as green fertilizer planted at the fall or spring, since it is precocious, produces a lot of forage and also fixes nitrogen into the soil (Goyoaga et al., 2008).

The use of molecular markers has improved significantly the management and utilization of the genetic diversity of crops maintained at the germplasm banks (Laurentin, 2009). The large fava bean genome (13 Gb) difficult to obtain an effective characterization of diversity at the genomic level; however, there have been several studies dealing with molecular characterization of this crop.

One study conducted by Wang et al. (2012) on diversity and genetic relationships among 802 landraces and improved varieties from different geographic zones of China using ISSR (Inter Simple Sequence Repeat) molecular markers. With the use of 11 primers they obtained a total of 212 bands, 209 of them polymorphic. The obtained results allowed knowing that there is greater genetic diversity among cultivars from the North of China than those from the central regions; they obtained two different groups: autumn and spring cultivars, according to principal component analysis and conglomerate analysis through UPGMA method.

Abdel-Razzak et al. (2012) explored genetic variability among 10 fava bean varieties used at two distinct geographic regions in Egypt, also using ISSR markers. With 9 primers they detected 69.1% of polymorphism, which allowed clear separation of evaluated cultivars according to their botanical type: V. *faba* var. *major* or V. *faba* var. *minor*.

A study conducted by Terzopoulus and Bebeli (2008) to evaluate genetic variability among 57 local Greek varieties using ISSR, with only four primers they obtained a greater percentage of polymorphism, since from 192 total bands,190 were polymorphic (98.9%), probably due to a greater number of varieties considered.

Fava bean production and consumption have been disarticulated geographically, since in the latest years there have been important reductions on production precisely in countries with high demographic growth, which are the ones that depend strongly on fava bean crop as inexpensive source of protein (Webb et al., 2016). Landraces through time have fixed genes of interest; so, they

represent important sources of germplasm for present and future breeding programs that could lead to obtain new highly productive improved varieties (Yahia et al., 2012).

In Mexico there are few studies on genetic diversity of fodder fava bean cultivars; the majority of them are related to morphological characterizations; therefore, it is convenient to conduct studies on molecular characterization that allow knowing the genetic pool available for breeding purposes. Then, the aim of the present study was the molecular characterization of 10 *Vicia faba* L. var minor and 5 *Vicia faba* L. var equina lines through ISSRs molecular markers, in order to explore genetic variability present in these materials.

Materials and Methods

Experimental Material

The application of mass selection to different collections from 'Puebla' state, resulted in 10 *Vicia faba* L. var minor and 5 *Vicia faba* L. var equina lines, which were evaluated in this study (Table 1).

Table 1. *Name, Testa Color and Botanical Type of Vicia faba L. Lines under Study*

No.	Name	Color	Botanical type
1	CAN Morada 2016	Morado	Minor
2	CAN Morada intenso 2014-2015	Morado	Minor
3	CAN Café 2014-2015	Café	Minor
4	CAN Granos croma tipo Zac. 2015	Café	Minor
5	CAN Café CH. 2015	Café	Minor
6	CAN Morada 2015	Morado	Minor
7	Tipo CAN-MED 2015	Café	Minor
8	CAN Verde 2014-2015	Verde	Minor
9	CAN Café 2015	Café	Minor
10	CAN Crema 2015	Crema	Minor
11	CAN Parda 2015	Marrón	Equina
12	Meca Chica Morada	Morado	Equina
13	V4 EAM 2014/2015	Verde	Equina
14	Libanesa Morada	Morado	Equina
15	Meca	Rojo	Equina

DNA Extraction Method

Fava bean plantlets were obtained by sowing seeds into 200 cavities polystyrene trays containing vermicompost as substrate: They were kept under laboratory conditions until plantlets developed 4 true leaves. DNA was extracted from 3 plantlets per line using Dellaporta et al. (1983) proposal with modifications suggested by Salazar-Laureles et al. (2015). Healthy young leaves were cut, and

rinsed using alcohol at 70%. Afterwards, 0.3 g of plant tissue per sample was grounded inside a mortar using liquid nitrogen. The obtained powder was transferred to 1.5 mL microtubes containing 700 µL f extraction buffer (Tris-HCl 100 mM, EDTA 50 mM, NaCl 500 mM, 2-Mercaptoetanol, SDS 1.3%, pH 8.0). Tubes were agitated by inversion until homogenization; later, they were heated al 65^{o}C for 10 minutes. Then, 200 µL of potassium acetate 5M were added and they were cooled for 60 minutes. Next, microtubes were centrifuged for 20 minutes at 12000 xg; supernatant was transferred to new microtubes containing 600 µL of cooled isopropanol and DNA was precipitated for 60 minutes. Later, they were centrifuged at 6000 xg for 5 minutes; supernatant was decanted and 700 µL of STE dissolution buffer (Tris-HCl 50 mM, EDTA 10 mM, pH 8.0, NaCl 100 mM) were added. They were kept still for an hour and later DNA´s pellet was dissolved by agitation by inversion. Then, 4 µL of ARNase (10 mg/ml) were added and microtubes were incubated at 37^{o}C for one hour. DNA was precipitated again using 75 µL of sodium acetate 3 M and 500 µL of cooled isopropanol during two hours. Later, microtubes were centrifuged at 8000 xg for 5 minutes and then supernatant was eliminated and DNA pellet was washed using 70% ethanol. Microtubes were centrifuge again at 8000 xg for 5 minutes, supernatant was eliminated and DNA pellet were left to dry at room temperature. Finally, it was dissolved using 150 µL of TE pH 8.0 (Tris-HCl 10 mM, EDTA 1 mM) and microtubes were kept at 4^{o}C until further use.

DNA Quantification

Pureness and quantification of samples DNA were estimated using a Thermo Scientific Nanodrop model Lite. Afterwards, composed samples were obtained for each line under study by mixing the DNA of three plants. Purified water was added to obtain 10 ng/µL final concentration. A 100 µL aliquot was taken and putted into 1.5 mL microtubes.

DNA quality was measured by electrophoresis in agarose gels a 0.8 %, using TAE 0.25 X buffer (Tris-base 2 M, acetic glacial acid 50 mM, EDTA 50 mM), at 150 volts for 1.5 hours. Gel was stained for 15 minutes with ethidium bromide (0.5 µg/mL). Then, gels were documented using DigiDoc-It® Imaging System.

ISSR Patterns

In order to obtain ISSR patterns, initially 51 Sigma® primers were tested using a random sample and the 14 that produced well defined and clear bands were selected. Later, Taq DNA polymerase chain reactions were performed using the following protocol. First, original DNA solution was diluted using sterile water in order to get a uniform concentration of 10 ng of DNA·µL^{-1} for all samples.

Later, 22.5 µL of reaction mixture composed by PCR buffer (2.5 µL 1X), 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 purified water (5.2 µL), were added to 1.5 mL microtubes containing 2.5 µL of target DNA.

Microtubes were placed into a Techne TC-412 thermocycler and they were incubated under the following conditions: 1 pre-denaturation cycle at 93^{o}C for 8 minutes, 40 cycles of denaturation (1 minute at 93^{o}C), annealing (1 minute at temperature recommended for each primer), extension (1 minute at 72^{o}C), and 1 cycle of final extension (6 minutes at 72^{o}C). Amplified products were separated by electrophoresis in agarose gels, which were stained using ethidium bromide and afterwards they were documented as referred in the above section.

Statistical Analysis

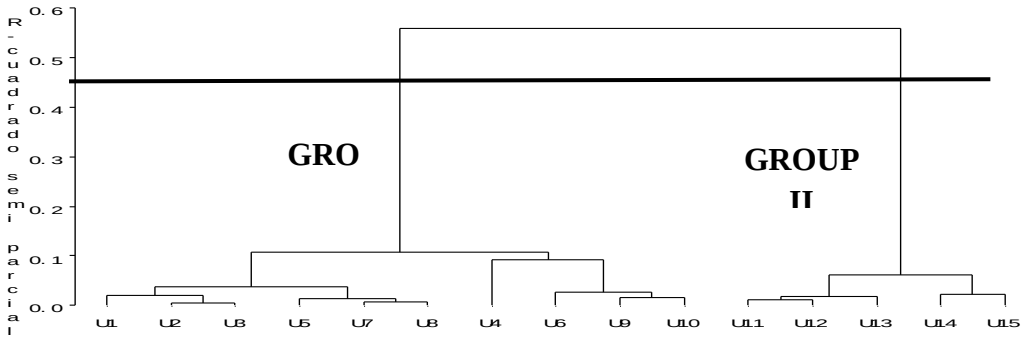
Lines comparisons were performed using similarities and differences among bands patterns; a value of 0 was assigned to the absence and a value of 1 to the presence of each particular band. For each primer, each amplified band was scored according to its migration distance at the gel. The ISSR patterns corresponding to each of the 14 selected primers were used to construct the basic data matrix (BDM).

With the aim to evaluate primer discriminant ability, a descriptive analysis on amplified products was performed; measured variables were: total amplified bands, mono and polymorphic loci, primer´s polymorphic information content, using InfoGen® (ver. 2011, Universidad de Córdoba, Argentina) software. Using BDM, Dice's genetic distance (1 – S) between pairs of accessions were estimated and a cluster analysis was computed using Ward's minimum variance method. A dendrogram was constructed; cut distance was defined based on T^2 Hotelling's pseudostatistic (Taylor, 2010) using PROC CLUSTER and PROC TREE of SAS® Ver 9.0.

Results and Discussion

Using BDM Dice genetic distance (1-S) between pairs of lines were calculated and afterwards a cluster analysis was performed using Ward´s minimum variance method with Infogen® software. The obtained matrix of distances was used to construct a dendrogram using PROC Tree from SAS®. According to the T^2 pseudostatistic cut distance was established at r2 semipartial value of 0.448, which clearly defined two groups (Figure 1).

Figure 1. *Dendrogram Constructed using Dice´s Genetic Distance (1-S) through Ward´s Minimum Variance Method*



Obtained results show that ISSR markers used were effective on separating the 15 tested lines according to their botanical type; since group I was formed by the 10 lines var. minor (U1 – U10), while group II was integrated by the 5 var equina lines (U11 – U15). These results agreed to those reported by Terzopoulus and Bebeli (2008) and Abdel-Razzak et al. (2012), who by the use of ISSR markers were able to separate *Vicia faba* varieties according to their botanical type.

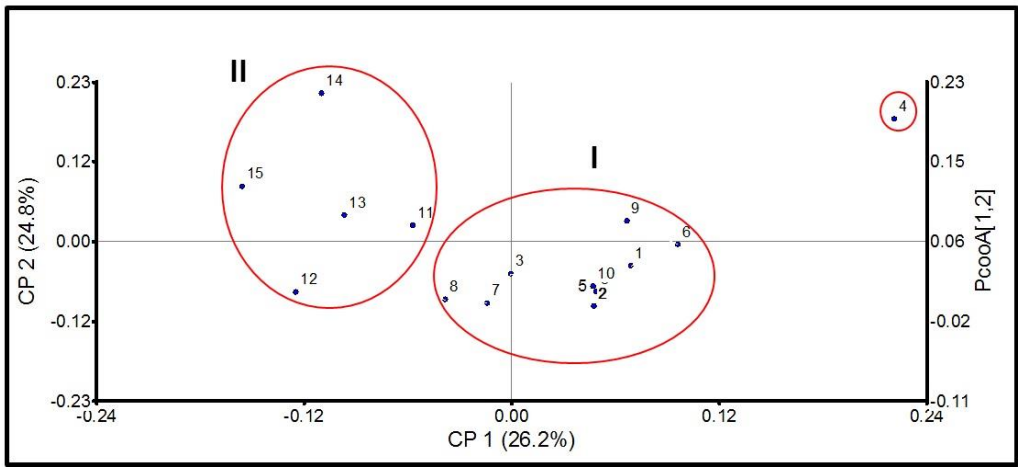
Related to testa color of evaluated lines, there were not subgroups formed based on this trait. According to Boukhanouf et al. (2016), main functional compounds in fava bean are phenols, specifically proanthocyanins or condensed tannins; in addition to flavonols and flavones, mainly mirecitine, quercitin and kaempferol. Their presence and concentration levels are influenced both by genetic and environmental factors (Baginsky et al., 2013). In previous studies in fava bean, a clear relationship between testa color and tannins content has been found. Black seeds contained less tannins than brown and purple seeds, while predominant pigments were mirecitine and anthocyanin (Kosinska et al., 2011). However, a recent study using liquid chromatography and mass spectrometry to analyze fava bean seeds with black, purple and different brown tones showed that there is not a clear relationship between testa color and tannins content; since there were not significant differences between testa color and prodelphinidin, epicatechin, and quercitine contents (Soon-Jae et al., 2018). Phenolic compounds, phenolic acids, catechin, flavonols, protoanthocyanins and anthocyanin show a big range of different structures; which in turn difficult their clear separation through analytic techniques (Siah et al., 2014). Then, the similarity on chemical composition of fava bean lines evaluated in the present study for these functional components, could have been responsible for the unclear separation of the evaluated materials according to their seed color.

Principal Coordinates Analysis

With the aim of exploring special distribution of evaluated fava bean lines, a principal coordinates analysis was performed with Dice´s genetic distances (1-S) using Infogen®. In Figure 2 it can be appreciated the formation of two clearly defined groups, group I formed by minor type lines and group II conformed by equina type lines, ratifying grouping obtained at the dendrogram. In addition, it

can be observed that line 4, from minor type, separated clearly from both groups; this suggests that this material could be genetically distinct from the remaining lines, and then, could be of interest for an hybrid breeding program in this crop, since expected heterosis of hybrids is a direct function of genetic dissimilarity between parental lines (Zeid et al., 2004).

Figure 2. *Two-dimensional Representation of 15 Fava Bean Lines through Principal Coordinates Analysis using Dice´s Genetic Distances (1-S) Obtained from ISSR Patterns*



Analysis of Molecular Variance

Hierarchical structure of genetic variability between fava bean of two distinct botanical types was explored by an analysis of molecular variance (AMOVA) (Table 2). It can be appreciated that even though genetic variability was greater between botanical types (55%), genetic diversity within types was also relevant (45%). These results agree to those reported by Terzopoulus and Bebeli (2008), Wang et al. (2012) and Oliveira et al. (2016); all these studies on molecular characterization of *Vicia faba* L. found greater genetic variation and differentiation within populations than among populations. These results were explained due to distinct responses of plants of the same population to biotic and abiotic stresses, agronomic practices or due to plant differences on adaptation ability to different environments. Likewise, if this crop highly allogamous habit is taken into account, a high genetic flow among lines within each botanical group is expected. The obtained results in this study suggest advisability on keeping all 15 studied lines in a breeding program since they represent distinct sources of genetic variation that could be of interest for the development of new improved varieties. However, there will be advisable to corroborate under field conditions their agronomic and production attributes.

Table 2. *Analysis of Molecular Variance of 15 Minor and Equina Fava Bean Lines according to their ISSR Molecular Profiles*

V.S.	SS	df	MS	P-value	Variance components	Variation %
Between types	453.03	9	50.34	<0.0001	13.17	54.86
Within types	216.67	20	10.83	<0.0001	10.83	45.14
Total	669.70	29	23.09		24.00	100.00

Molecular Markers Description

Table 3 shows the description of the 14 ISSR primers used to get molecular profiles of the 15 fava lines under study, obtained using Infogen® software. It can be noticed that none of the 15 patterns is duplicated, so all tested lines are genetically different and therefore it should be useful to keep them all in a germplasm bank for their eventual use in future studies on this crop.

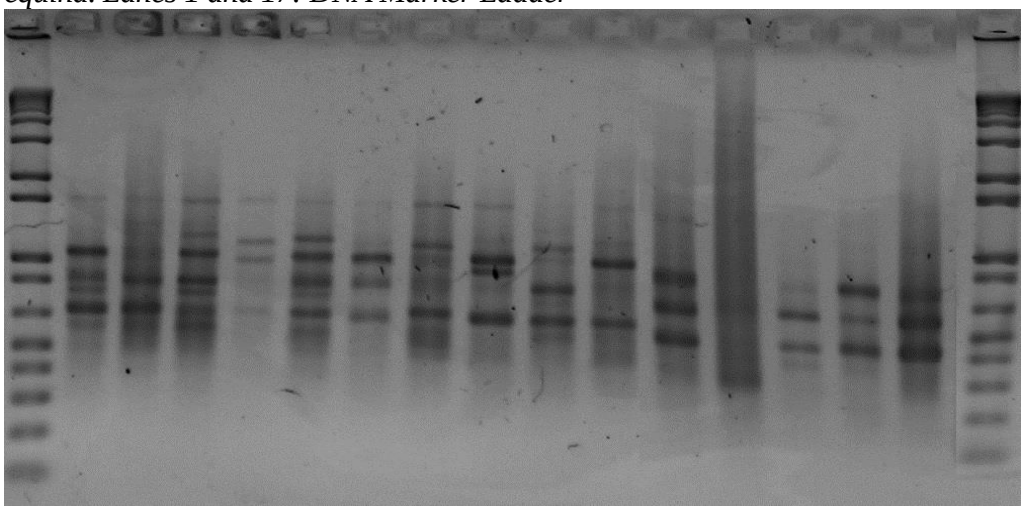
Table 3. *Number of Polymorphic, Monomorphic and Total Bands, Proportion of Polymorphic Loci, Polymorphic Information Content, Standard Error, Percentage of Amplification and Probability of Two Individuals Sharing the Same Allele by Chance Obtained Using 14 ISSR Primers in 15 Fava Bean Lines*

Primer	Sequence 5' - 3'	PB	MB	TB	POL(95)	PIC	E.E.	AMP	PTISMA
UBC807	$(AG)_8T$	6	3	9	0.67	0.33	0.02	36.30	1.20E-09
UBC812	$(GA)_8A$	7	2	9	0.78	0.23	0.04	53.33	1.80E-07
UBC818	$(CA)_8G$	6	3	9	0.67	0.32	0.02	49.63	1.30E-08
UBC842	$(GA)_8CTG$	10	1	11	0.91	0.33	0.02	53.94	8.00E-14
UBC866	$(CTC)_6$	4	4	8	0.50	0.21	0.03	66.67	5.30E-04
UBC890	$GACATCGAC(GT)_7$	4	2	6	0.67	0.26	0.02	80.00	1.50E-06
17898B	$(CA)_6GT$	6	3	9	0.67	0.25	0.02	32.59	1.90E-06
ISSR3	$(GA)_8CTC$	11	2	13	0.85	0.21	0.03	47.69	2.00E-06
ISSR4	$(AG)_8CTC$	6	1	7	0.86	0.28	0.01	56.19	3.30E-09
ISSR6	$(AC)_8CTG$	8	1	9	0.89	0.30	0.02	65.19	3.20E-10
ISSR9	$(AG)_8C$	8	1	9	0.89	0.31	0.01	56.30	1.40E-11
ISSR10	$(GA)_8T$	4	1	5	0.80	0.37	0.01	62.67	6.80E-13
PHV6	$CCA(CT)_8$	5	2	7	0.71	0.26	0.02	65.71	3.30E-07
PI03	$AGCT(GACA)_3$	2	4	6	0.33	0.23	0.02	60.00	2.70E-03
Total		87	30	117				54.64	6.20E-108

All 14 primers were polymorphic (100%). This high percentage of polymorphism agrees to results published by Terzopoulus and Bebeli (2008), which evaluated 20 fava bean Greek landraces using 4 ISSR primers and got a polymorphism of 98.9%. In a different study Wang et al. (2012), characterized 802 landraces and improved fava bean varieties coming from different regions of China using 11 ISSR primers. They also found a high percentage of polymorphism (98.6%). In the present study, primers that produced more bands were ISSR3 (13

bands) and UBC842 (11 bands), with an average percentage of amplification through all 15 lines of 47.7 and 53.9, respectively. High values of polymorphic information content (PIC) were obtained by ISSR10, UBC807, UBC842 and UBC818. The most informative primers had in common GA repetitions; these results agree to those reported by Salazar-Laureles et al. (2015), who evaluated 39 fava bean accessions and found that UBC-842 was one of the used primers that gave a high PIC value. Then, the four previously referred primers would be very useful for further studies on molecular characterization of fava bean resources. Related to low probability of two individuals sharing the same allele by chance (PTISMA), this was observed for UBC842, ISSR10, ISSR9, and ISSR6 primers. These results indicate a high degree of reliability for genetic differentiation on fava bean varieties. In this table it can also be noticed that primers UBC866 and ISSR3 showed the lowest discrimination ability and low PIC values, even though the last one produced the highest number of amplified bands.

Figure 3. *Molecular Profiles Obtained Using UBC848 Primer in 15 Fava Bean Lines. Lanes 2 to 11: Vicia faba L. var minor. Lanes 12 to 16: Vicia faba L. var equina. Lanes 1 and 17: DNA Marker Ladder*



Conclusions

The obtained results in the present study confirm the usefulness of ISSR molecular markers to clearly discriminate fava bean lines according to their botanical variety. In addition, these markers were also useful to estimate the degree of genetic variability present both, between and within botanical groups.

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