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Original Research Article

Varietal purity evaluation in sesame (Sesamum indicum L.) seeds by means of random amplified polymorphic DNA (RAPD) markers in Venezuela

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ABSTRACT

BACKGROUND: Sesame is an important crop due to the excellent quality oil derived from its grains. With as any other crop, the seed used as initial input in the agricultural process, is the first warranty of success that a farmer has. The quality seed must be evaluated during the multiplication process, to be sure that varietal purity, as component of genetic quality, is not affected. OBJECTIVES: The general objective was to determine ability of random amplified polymorphic DNA (RAPD) markers to evaluate varietal purity; two specific objectives were defined: to evaluate the ability of RAPD to detect seeds of non-target cultivars (seed contaminants), and to evaluate the ability of RAPD to detect troubles during seed multiplication. MATERIAL AND METHODS: For first objective seeds of cultivar UCLA-1 were mixed with seeds of another known accession, in different proportions: 99:1, 98:2, 97:3 and 95:5. RAPD markers were performed on these different proportions, and band pattern of each proportion was compared to UCLA-1 and to the other accessions band pattern. For second objective, RAPD was performed on samples from genetic seed, and seeds of the two following multiplication cycles. RESULTS: RAPD was able to identify seed contaminant in proportion as low as 1%. For second objective, RAPD identified the three samples from genetic seed with the same band pattern, but it was different to band pattern of the other two kinds of seed. Therefore, RAPD had the ability to identify that the multiplication of seeds was not totally suitable, because some non-target seeds appeared as source of contamination. CONCLUSION: RAPD proved to be an effective tool to monitor genetic quality, specifically varietal purity, in sesame seed.

Keywords: seed quality; genetic quality; molecular marker; DNA, random amplified polymorphic DNA; polymorphism

1. Introduction

Sesame is an ancient crop[1] widely cultivated in tropical and subtropical regions around the world[2]. Every year, 7 million hectares are used to produce approximately 3.5 millions of tons of sesame grains[3]. For this crop, commercial product is the grain, which is composed 50% of oil. Sesame

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is used as the source of excellent quality oil with an optimal balance between saturated and unsaturated fatty acids, and the presence of antioxidants, but sesame grains are also used in confectionary and for getting a sauce broadly known in the Arab cuisine called tahini.

The most important input in an agricultural process is the seed, because without seed there is no possibility of carrying out the agricultural process. In order to be efficient in the process, the seed to be used must have good quality, this quality is seen under four aspects: genetic quality, physiological quality, sanitary quality and physical quality[4]. Genetic quality has two attributes: genetic potential to have a suitable agronomic performance, which depends on the step of plant breeding to get the new cultivar, the new genetic seed; and varietal purity which is the warranty that all the seeds belong to the desired genotype, and depends mostly on the step of seed multiplication and seed conditioning. Seed quality is evaluated during the certification process, in which one of the steps is seed multiplication which implies generally three multiplication cycles, obtaining each cycle seed with a named category, so, in multiplication seed there is one first step sowing genetic seed (the given by plant breeder) to get foundation seed (this is the name of the seed category given in Venezuela to the seed produced when the genetic seed is sown), the second step sows foundation seed to get registered seed (this is the name of the seed category given in Venezuela to the seed obtained in the second cycle of multiplication), and the third step sows registered seed to get certified seed. After each cycle, seed is conditioned. The objective of each cycle (including conditioning) is to increase the seed quantity while keeping the quality, but there are threats to this objective, either in the field or in the conditioning process. In the field, it is necessary to avoid non desired pollination by means of isolation either in location or in time, also presence in the multiplication field of non-target seeds must be avoided to avoid spontaneous germination of these seeds, which will produce non-target seed and can be harvested together with the target seed. In the conditioning process is necessary to clean carefully all the equipment before conditioning starts, to avoid mechanical addition of non-desired seeds. Despite all standard measurements had been taken, it is necessary to evaluate seed quality in each step, and varietal purity is one of the main aspects to evaluate. Conventionally, varietal purity is evaluated by taking samples from the seed lot, and examining visually the presence of off-type seeds (by color, size, shape), but this method could be considered ineffective, due to examination is based on morphological attributes and they have strong environmental influence[5], furthermore is a method very time-consuming, this situation should be considered to look for other methods more effective and with less resources needed to perform it. Methods based on DNA could be more expensive, but could be more effective and practical to evaluate varietal purity. The aim of this study was to evaluate the ability of random amplified polymorphic DNA (RAPD) markers to estimate varietal purity in sesame seeds.

2. Material and Methods

2.1. Laboratory assay

Plant material

Seeds of cultivar UCLA-1[6] were used as target. DNA was extracted from three samples of genetic seeds of this cultivar to perform RAPD, obtaining a band pattern per sample. Band pattern of this cultivar was defined by means of the bands reproducible in the three samples. Other band patterns were obtained from accessions UCV, 43x32, Maporal, UCLA 90, UCLA 249 and [93-2113] Local Sesame[7] from the germplasm bank of Universidad Centroccidental Lisandro Alvarado. Band pattern of all these accessions were compared to band pattern of UCLA1 to choose the one that differed the most.

Experimental design

Once the band pattern most different as compared to band pattern of UCLA1 was identified,

mechanical mixtures at four levels were made based on one hundred seeds, mixing 95 seeds of UCLA1 with 5 seeds of the accession with the most different band pattern to get a mixture "contaminated at 5%"; 97 seeds of UCLA1 with 3 seeds of the accession with the most different band pattern to get a mixture "contaminated at 3%"; 98 seeds of UCLA1 with 2 seeds of accession with the most different band pattern to get a mixture "contaminated at 2%", and 99 seeds of UCLA1 with 1 seed of accession with the most different band pattern to get a mixture "contaminated at 1%". RAPD was performed for each of the four mixtures, using three replications per mixture level.

DNA extraction

DNA extraction was carried out from seeds according to the procedure of Clarke et al. (1989) [8]. Briefly, 110 mg. of seeds were ground in mortar in presence of 1000 µL of CTAB buffer (100mM Tris HCl pH 8.0, 2100 mM NaCl 150 mM EDTA pH 8.0, polyvinylpolypyrolidone 2%, CTAB 2%) containing 0.4 mg. proteinase K and 10 µL mercaptoethanol. The homogenates were transferred to tubes of 1500 µL, and incubated for 20 minutes at 65°C shaking each 5 minutes, and cooled to room temperature for 10 minutes. Samples were subjected to centrifugation for 10 minutes at 10000 rpm, and 500 µL of supernatant were transferred to another tube, adding 800 µL of phenol-chloroform-isoamylalcohol(25:24:1). Phases were separated by centrifugation for 10 minutes at 12000rpm. 300 µL of superior phase were transferred to other tube, adding 70 µL of NaCl 5M, DNA was precipitated with 2 volumes of cold ethanol 100% followed by incubation in ice for 60 minutes, samples were subjected to centrifugation for 10 minutes at 12000rpm. Supernatant was discarded, and DNA pellets at the bottom of the tube were washed twice with ethanol 70%, and dried at room temperature overnight. To dissolve the pellets, one hundred microliters of buffer TE pH 8.0 (10 mM Tris-HCl, 1 mM EDTA pH 8.0) were added to each tube, and they were incubated at room temperature for 18 hours. DNA concentration was determined by electrophoresis in a 0.8% agarose gel with lambda DNA standard, running the samples on a chamber Thermo EC Minicell Primo EC 320 for 60 minutes at 100V, staining the gel with ethidium bromide and visualizing in a transilluminator TM36E, with UV light and photographed by a camera Canon Power Shot SD11001S. Once DNA integrity was verified, 1 µL of RNAase was added to each sample, and they were incubated for 1 hour at 37°C. DNA was kept at 4°C until it was used.

RAPD markers

Twenty five nanograms of DNA were used for each reaction. Each reaction was performed in a final volume of 20 µL with 5 U of Taq polymerase (NeoTherm), 10 µM of one primer (primers used are listed in Table 1), 500 µM of dNTPs, 10mM gelatin, 1.5 mM MgCl2. Polymerase chain reaction was performed for 45 cycles in a thermocycler PTC-100 (MJ Research). Each cycle consisted in 1 minute at 94° C, 1 minute at 36° C and 1 minute at 72° C. When the 45 cycles were completed, samples were kept in the thermocycler at 72° C for 5 minutes. PCR products were resolved by electrophoresis in agarose (1.5%), loading 6 µL per sample and running for 70 minutes at 100V in a horizontal chamber Thermo EC Midicell Primo EC 330. A DNA ladder Promega 100bp was loaded in each electrophoresis. Gels were stained with ethidium bromide for 10 minutes, and they were washed with distilled water for 10 minutes. They were visualized with a transilluminator TM-36E with UV light and photographed with a camera Canon Power Shot SD11001S.

2.2.Field assay

Plant materia

Varietal purity was evaluated for cultivar UCLA-1, on genetic, foundation and registered seed, the last two categories multiplied and obtained by a private enterprise. Genetic seed was the comparison pattern and it was obtained from Germplasm Bank. For each seed category, 1 sample of 1 kilogram was taken, and disinfected with Tiametoxam. From each

of these samples, three subsamples were taken to perform DNA extraction and RAPD.

DNA extraction

DNA extraction was performed as explained in the section Laboratory assay.

RAPD markers

RAPD markers were performed as explained in the section Laboratory assay.

Statistical analysis

Bands on the gel were transformed to a binary matrix (1 representing the presence of the band, 0 representing the absence of the band). From this binary matrix, cluster analysis was performed using Jaccard similarity coefficient and UPGMA algorithm; principal coordinates was also performed. Both analyses were performed to visualize if grouping according RAPD was consistent as compared to seed categories, and in this way to infer two aspects: if RAPD was reproducible within seed categories, and if multiplication and/or conditioning process altered varietal purity. These analyses were performed with the software NTSYS pc. V. 3.01[9].

3.Results

The band pattern of UCLA 1 was defined, it is indicated in Table 2. Using 4 primers, 20 bands were obtained, 3 with primer OPC-11, 7 with primer OPL-05, 5 with primer OPO-016 and 5 with primer OPP17. From the six accessions in which the band pattern was obtained to be compared to the band pattern of cultivar UCLA1, accession [93-2113] Local Sesame was chosen because it presented a band pattern easily recognizable as different to the band pattern of UCLA1. This pattern presented 14 bands (Table 3). Therefore, seeds of UCLA1 were mixed in four proportions with seeds of accession [93-2113] Local Sesame as explained in section of Methods. For the three replications within each level of mixture of seed (5%, 3%, 2% and 1% of seed different of cultivar UCLA1), the band pattern was exactly the same. At the four levels, the 14 bands of seed of [93-2113] Local Sesame were identified. Due to there was no variation in percent of bands visualized among the treatments, no analysis of variance was performed.

From the field assay, the three samples of each seed category had identical results within seed category, but they differed when band patterns were compared to the other band patterns between categories. Figure 1 indicates that relationship among the nine samples could be explained with the formation of two main groups or nodes. The first node includes the three samples of genetic seed and the three samples of foundation seed, whereas the second node includes the three samples of registered seed. A cophenetic correlation value of 0.97 indicates a very good graphical representation of the similarity coefficients between pair of samples. Figure 2 indicated also the relationship among seed samples according to RAPD, where the two firsts axis explain 62% of the total variation; and it shows that foundation seed samples are more similar to genetic seed samples, than registered seed samples to genetic seed samples. In this biplot, registered seed samples appear as the most different samples.

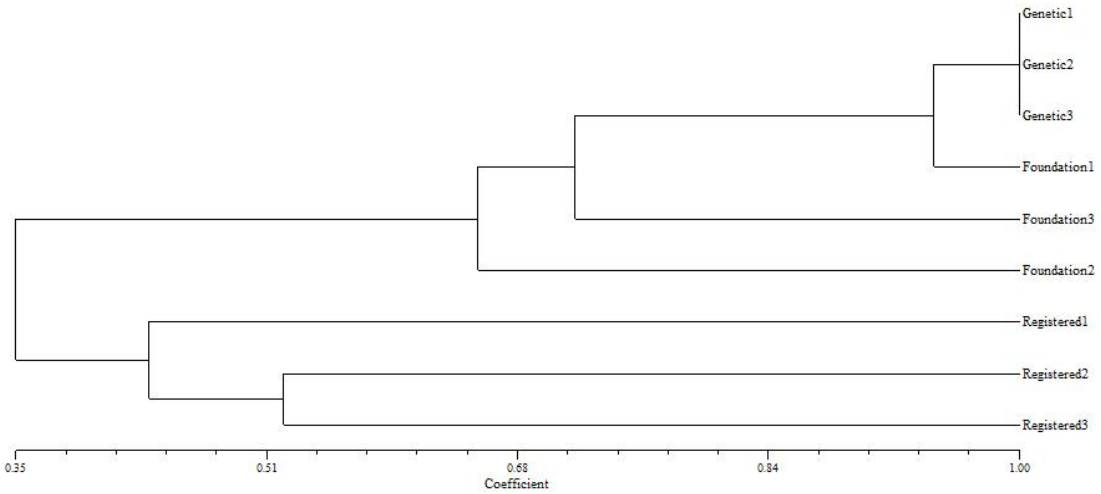

Figure 1. Phenogram (Jaccard similarity coefficient and UPGMA algorithm) for samples of genetic, foundation and the registered seed of UCLA1 subjected to RAPD. The correlation cophenetic value resulted in 0.97.

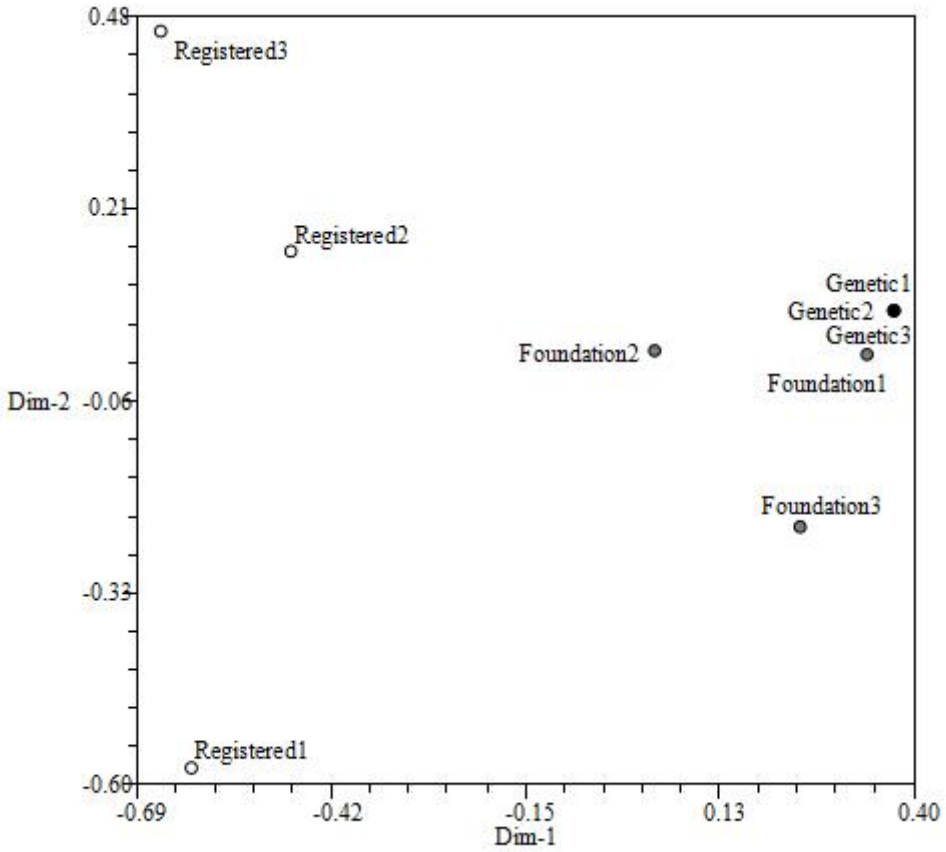

Figure 2. Biplot displaying the distribution of three samples of the genetic seed of UCLA1 (black circles), three samples of foundation seed of UCLA1 (grey circles) and three samples of registered seed of UCLA1 based on RAPD using four primers. Note that only one black circle appears because the three samples of genetic seed resulted exactly the same.

4.Discussion

RAPD was shown to be sensitive enough, to be able to identify non-target seeds from target seeds, even at levels as low as 1% of non-target seeds in a group of target seeds, similar results were reported in maize, but using microsatellites[10], it indicates the usefulness of this technique to be incorporated as routine work in seed programs to evaluate seed purity in each multiplication cycle. Whether some non-target plant grows in the field in which target seed is being multiplied, some non-target seeds will be harvested together with the target seeds, and proportions of non-target seeds – target seeds in the seed lot would be expected very low, but RAPD could detect these few seeds. Similar situation could occur if few non-target seeds are in the conditioning line of the target seed. When band pattern of the target seed is known, any different band to this pattern will be considered non-target seed, or more generally non-target DNA. If band pattern of other seeds, belonging to another cultivar is known, direct visualization of some band belonging to its pattern will be evidence of the presence of non-target DNA. RAPD is a technique that has the advantage on a conventional way to evaluate seed purity (with morphological traits) that is not so time demanding, and it is not influenced by the environment[11].

RAPD promotes the identification of presence of non-target seeds or non-target DNA, but it does not quantify it, RAPD just indicate if the DNA extracted belong only to target seed or not.

Figure 1 and 2 indicate that seed multiplication process or conditioning process did not guarantee to keep genetic purity, especially the process from foundation seed to registered seed. The three registered seed samples are too dissimilar as compared to foundation and genetic seed samples. What it should be expected, is that all the nine seed samples had the same band pattern, but grouping and ordination carried out by cluster analysis and principal coordinates respectively, indicate that there are differences in the band pattern among the samples, especially the band pattern of registered seed as compared to the other two seed categories, which can be explained by harvesting of non-target seeds in the field, or by adding non-target seeds during the conditioning process to the target seed lot. As expected if the conditions of isolation and/or cleaning in conditioning equipment were not ideal, varietal purity is perfect in genetic seeds, but inadequate in foundation seed (these seeds are similar to genetic seeds, but not equal), and very inadequate in registered seeds. Due to genetic seed samples were totally similar, it is discarded that genetic seed could be the origin of the problem. Due to foundation seeds come from multiplication of genetic seeds, if foundation are dissimilar to genetic seeds, and genetic seeds have no problem of purity, the only causes are multiplication and/or conditioning process. Whereas registered seeds come from foundation seeds, if foundation seeds were not totally similar to genetic seeds, it is expected that registered seeds obtained were more dissimilar to genetic seeds, it was expected to obtain foundation seeds more similar to genetic seeds (because it represents the first multiplication cycle) than similarity between registered and genetic seeds (because it represents the second multiplication cycle, where foundation seeds were the input).

No studies about varietal purity evaluation in sesame using molecular markers is known. However, in other crops such as maize[10], SSR markers were used successfully to test varietal purity. RAPD markers were reported to evaluate successfully hybrid seed purity in Capsicum annuum[12, 13], cotton[14], tomato[15], and barley[16].

Plant genetic resources conservation plans can also use RAPD for evaluating seed purity. Although the objective of evaluating seed purity in plant genetic resources conservation is totally different to multiplication seed programs objectives, both can take the advantages that RAPD offers to evaluate seed purity. Whereas in seed programs the objective is to verify that the seed to use is suitable for use in productive processes due to its quality (specifically genetic quality, especially varietal purity), in plant

genetic resources conservation the objective would be to verify that genetic identity of the accession is preserved after multiplication. RAPD has been used in plant genetic resources conservation, but to identify duplicate accessions[17], and to characterize genetic diversity in crops such as rice[18], maize[19], and wheat[20] among others.

5. Conclusion

RAPD technique has proven to be sufficiently sensitive and efficient to evaluate varietal purity in the different seed categories during the multiplication process. Deficiencies in multiplication and conditioning conditions can be inferred by results of varietal purity evaluated by RAPD.

RAPD is a very sensitive technique, because with just a few nanograms of DNA the amplification reaction can be carried out, therefore if RAPD is incorporated in routine work for evaluating varietal purity, this has to be carried out with samples already disinfected, to avoid DNA coming from microorganisms such as fungi, the idea is to warrant that extracted DNA comes only from seeds.

Authors Contributions

Edickson Frances executed the molecular biology work, he performed DNA extraction, RAPD and electrophoresis, he also took part on the writing of the manuscript. Hernán Laurentin planned the work, performed statistical analysis and took part on the writing of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Table 1. Primers used to determine varietal purity of cultivar UCLA-1, with its sequence and annealing temperature

Primer name	Primer sequence	Annealing temperature in ºC
OPC-11	AAA-GCT-GCG-G	36
OPL-05	ACG-CAG-GCA-C	36
OPO-016	TCG-GCG-GTT-C	36
OPP-17	TGA-CCC-GCC-T	36

Table 2. Bands obtained using RAPD for genetic seed of sesame cultivar UCLA1

Primer	Name of band	Approximate size of the band (pb)
OPC-11	OPC11-1	650
	OPC11-2	700
	OPC11-3	1500
OPL-05	OPL05-1	300
	OPL05-2	450
	OPL05-3	550
	OPL05-4	800
	OPL05-5	1000
	OPL05-6	1100

	OPL05-7	1300
OPO-016	OPO016-1	150
	OPO016-2	400
	OPO016-3	600
	OPO016-4	700
	OPO016-5	900
OPP-17	OPP17-1	100
	OPP17-2	200
	OPP17-3	450
	OPP17-4	1400
	OPP17-5	1500

Table 3. Bands obtained using RAPD for seed of sesame accession [93-2113] Local Sesame

Primer	Name of band	Approximate size of the band (pb)
OPC-11	OPC11-1	850
	OPC11-2	950
	OPC11-3	1100
OPL-05	OPL05-1	100
	OPL05-2	125
	OPL05-3	150
	OPL05-4	250
OPO-016	OPO016-1	450
	OPO016-2	1000
	OPO016-3	1400
OPP-17	OPP17-1	300
	OPP17-2	650
	OPP17-3	850
	OPP17-4	1000

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