VARIATION IN RAPD MARKERS OF *ARGANIA SPINOSA* TREES AND THEIR PROGENIES [1]

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ABSTRACT

Using the technique of Random Amplified Polymorphic DNA (RAPD), we were able to identify total 54 bands generated by eight primers in argan (*Argania spinosa* (L.) Skeels) tree leaves. The probability that two trees present identical multiband fingerprint was low, therefore the probability of discriminating between genotypes was high. This has enabled to identify genotype profiles with distinguished RAPD markers of parents and open-pollinated offsprings, thus establishing the discriminating power of RAPD to recognise argan tree progenies.

Key words: *Argania*, RAPD, Genetic diversity, polymorphism

INTRODUCTION

Argan, *Argania spinosa* (L.) Skeels, a non-timber forest tree of southwest of Morocco, has an undisputed ecological and economical value as a species resistant to arid conditions. Argan has a large potential for forestry as well as for domestication as an oil tree of medical, dietetic, cosmetic and industrial uses (BOUDY, 1952; EHRIG, 1974; MAURIN, 1992; FERRADOUS *et al.*, 1996; ALAOUI *et al.* 1997; CHAROUF & GILLAUME, 1999). Recent interest for its conservation and development increased following UNESCO declaration of argan area of distribution a MAB (Man and Biosphere) Reserve of Biosphere.

A morphological database is being established on argan and a breeding programme is emerging where the molecular markers can assist efficiently establishing markers such as QTLs. These help to speed up the process of recognition and selection of valuable genotypes; especially that argan tree is a slow growing perennial, which is a potential source of valuable genes of resistance or tolerance to aridity.

RAPD proved to be an efficient method for DNA polymorphism detection between genotypes (SINGSIT & OZIAS-AKINS, 1993), varieties (SCHILIRO *et al*, 2001), genomes (HOWELL *et al.* 1994) or species (BESNARD *et al*, 2001; M'RIBU & HILU, 1994). The aim of the present study is to identify genotype profiles with distinguished RAPD markers of three parents and their open-pollinated offsprings thus establishing the discriminating power of RAPD to recognise argan trees and their progenies.

MATERIALS AND METHODS

Plant material

Samples were collected from three argan fully grown trees and their five months old progenies grown under nursery conditions. One tree (B) is from Ait Baha located further south, 50 km from the Atlantic Ocean at 550 m altitude on the northern slopes of Anti Atlas. Two trees (R and R0) are from Argana 60 km next to the Atlantic Ocean at 620 m altitude on the southern slopes of High Atlas Mountains.

Template DNA preparation

Total cellular DNA from leaf material of each single genotype was extracted following the methods of BANI-AAMEUR & BENLAHBIL (2002).

Approximately 100 mg of refrigerated clean whole leaves, completely covered by 400 µl CTAB solution (CTAB extraction buffer hexadecyltrimethylammonium bromide 2 % w/v, 100 mM tris base, 1.4 M NaCl, 20 m M Na-EDTA, pH 8), were ground in a pre-cooled mortar (6 cm diameter) (–20 °C) with 10 % PVP w/v, 15 % SDS w/v and 0.2 % β mercaptoethanol v/v. The slurry was trans-

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ferred in a 1500 µl Eppendorf vial, washing the mortar with 400µl CTAB and transferring it again to the tube. The mixture was mixed thoroughly by gentle shaking. After incubation 800 µl chloroform was added and bath at 60 °C for 30 min. The tubes were centrifuged at 10 000 rpm for 10 min at 4 °C and the supernatant (approximately 640 µl) was transferred to a new centrifuge micro-tube. A half volume of 5 M NaCl (320 µl) was added and mixed gently to precipitate DNA before to add 500 µl of cold isopropyl alcohol 100 % and to refrigerate at –20 °C for 15 to 20 min. Thereafter the tube was centrifuged at 10,000 rpm for 5 min to precipitate DNA to the bottom of the tube. The alcohol layer was discarded and the tube walls were rinsed with 500 µl of 70 % ethanol to wash the pellet, quick-centrifuged (30 seconds) before to discard the supernatant. The DNA was air dried for 10 min and dissolved in 50 µl TE buffer. (Tris-EDTA pH 8).

PCR amplification

Eight 10 bases oligonucleotide primers made by Operon Technologies, primers OPA 1, 2, 4, 7, 10, 13, 15, 18) (Tab. 1) were used at random. The RAPD procedure set by BANI-AAMEUR & BENLAHBIL (2002) was used. Each reaction mixture (25 µl) contained 17.95 µl sterile bi-distilled water, 2.5 µl of 10 X reaction buffer, 2 µl magnesium chloride, 1 µl each of dATP, dCTP, dGTP and dTTP, 0. 375 µl of single PCR primer (Promega), approximately 0.1 ng of genomic DNA template and 0.175 µl Taq DNA polymerase (Promega). Amplification program was carried out in a Perkin Elmer Cetus thermal cycler as 35 cycles of 92 °C for 30 sec, 42 °C for 1 min and 72 °C for 30 sec for better amplification. On completion the samples were saved at 4 °C before electrophoresis.

Amplification products were analysed by electrophoresis on a 1.5 % agarose gel. Approximately 20 µl of sample plus 2 µl loading dye were loaded on the gel and run in Tris-acetate-EDTA (TAE) buffer at 95 V for 1h: 45 min. The one-kilobase DNA marker (EcoR I and Hind III digested l DNA) was used as a molecular standard. DNA was stained with ethidium bromide, visualised under UV light and photographed with a Polaroid apparatus.

Data analysis

Each sample was coded by 1s for presence of a band and 0s for its absence, the matrix representing the tree RAPD multiband fingerprint. We computed the probability for two seedlings of a mother tree to have an identical multiband RAPD fingerprint X^m, where X is the average proportion of common bands and m the mean number of amplified bands (YEH *et al.* 1995). Because we assumed a random association among RAPD fragments, X^m estimate is the minimum estimate of resemblance between seedlings.

Because we scored the bands as 1s for presence and 0s for absence, the percentage of trees amplifying a band being the frequency of the considered DNA fragment in the population was considered as an estimate of polymorphism. (YEH *et al.* 1995). Genetic similarity between seedlings and mother tree genotypes estimates were sought through the analysis of the generated rectangular matrix described above, where mother-trees were included. As the similarity measure the Dice coefficient was used (DICE 1945). It considers only shared presence of fragments and which is equivalent to equation 21 of NEI & LI (1979). The analysis was performed using NTCYS-pc package of computer programs (Version 1.5, ROHLF 1988) and genotypes were grouped using UPGMA (the Unweighted Pair Group Method Analysis).

RESULTS & DISCUSSION

RAPD variation in Argan

The RAPD primers produced reproducible banding patterns when replicated samples were tested, variations depending only on the primer and plant (tree or seedling) genotypes. The eight random primers out of twenty tested generated a total of 54 loci. The mean was 6.75 markers per primer varying each from four to nine (Table 1). In average, a genotype would amplify 2.25 bands of a given primer varying between 0.4 for OPA15 and 4.1 for OPA7. Not all primers produced scorable polymorphism, some giving more polymorphism than others. As it is OPA 18 had higher polymorphism than OPA4 or OPA10. Indeed, the first, although gener-

Table 1. Nucleotide sequence of the eight primers and number amplification bands.

Primer	Nucleotide sequence	Number of generated RAPD loci
OPA1	CAGGCCCTTC	9
OPA2	TGCCGAGCTG	6
OPA4	AATCGGGCTG	4
OPA7	GAAACGGGTG	9
OPA13	CAGCACCCAC	5
OPA15	TTCCGAACCC	7
OPA18	AGGTGACCGT	10
Mean		6.75

ating 10 bands had a comparable mean number of amplified bands to the second (Table 1 and 2).

Percentage of genotypes amplifying individual bands generated by a given primer, which we may use as an estimate of polymorphism of the population, depended on the concerned primer but were not related to the total number of bands generated by the primer. Thus, over all, OPA18 with 10 bands had a lower polymorphism than OPA2 with six bands (Table 2). Therefore primers had different probabilities X^m of producing identical multiband RAPD fingerprints. This probability decreased exponentially when m, the average number of amplified bands per tree, increased arithmetically. The primer OPA7 had the least probability of producing identical multibands X^m (0.04), and then its power of discriminating among trees ($P = 1 - X^m$ was 0.96) was the highest. Considering the 54 bands generated by the eight primers, the probability of that two seedlings have the same multiband finger print becomes so low (product of eight primers

Table 2. Characterisation of random primers amplification bands from ten progenies of each mother tree and over all progenies.

Primer	Tree family	Average number of amplified bands	Average proportion of chaired bands (X)	Mean number of amplified bands per genotype (m)	Probability for two seedlings to present an identical multiband RAPD finger print (X^m)
OPA1	AB100	2.67	0.27	2.40	0.04
OPA2		3.33	0.33	2.00	0.11
OPA4		2.00	0.20	0.80	0.28
OPA7		4.44	0.44	4.00	0.04
OPA10		5.50	0.55	2.20	0.27
OPA13		4.80	0.48	2.40	0.17
OPA15		0.57	0.06	0.40	0.32
OPA18		2.30	0.23	2.30	0.03
Mean		*3.20*			
OPA1	AR22	0.89	0.09	0.80	0.14
OPA2		4.67	0.47	2.80	0.12
OPA4		3.75	0.38	1.50	0.23
OPA7		4.11	0.41	3.70	0.04
OPA10		1.75	0.18	0.70	0.30
OPA13		3.00	0.30	1.50	0.16
OPA15		3.43	0.34	2.40	0.08
OPA18		4.00	0.40	4.00	0.03
Mean		*3.20*			
OPA1	AR98	2.78	0.28	2.50	0.04
OPA2		4.50	0.45	2.70	0.12
OPA4		5.00	0.50	2.00	0.25
OPA7		4.56	0.46	4.10	0.04
OPA10		4.25	0.43	1.70	0.23
OPA13		5.80	0.58	2.90	0.21
OPA15		5.00	0.50	3.50	0.09
OPA18		0.60	0.06	0.60	0.18
Mean		*4.06*			
OPA1	Over all	6.33	0.21	1.90	0.05
OPA2		12.50	0.42	2.50	0.11
OPA4		10.75	0.36	1.43	0.23
OPA7		13.11	0.44	3.93	0.04
OPA10		11.50	0.38	1.53	0.23
OPA13		13.60	0.45	2.27	0.17
OPA15		9.00	0.30	2.10	0.08
OPA18		6.90	0.23	2.30	0.03
Mean		*10.46*			

probabilities) that the probability of discriminating between genotypes using the eight primers is basically equal to one. Thus, RAPD fingerprinting even with eight primers and a limited population (3 × 10 seedlings), appears as an efficient tool for assessing genetic variability of argan.

Discrimination between trees and seedlings genotypes

The banding patterns of each genotype, tree or seedling, were different for each primer. As an example, out of the 54 marker loci generated by eight primers, only 24 were observed in the profiles of the three parents (Table 3). Among these, using presence / absence scores, we could distinguish for each tree a different fingerprint for five primers out of eight. More over, only three primers generated three unique bands for three seedlings only (Table 4). Indeed, at this stage of our investigation we were not yet able to determine bands that may characterise a particular genotype.

Table 3. Identification of markers (by their position from top to bottom of the gel) present in the profile of each mother tree for different primers.

	Mother tree		
Primer	AB100	AR22	AR98
OPA1	7	7	*
OPA2	*	4, 5, 6	5
OPA4	*	4	*
OPA7	2, 4, 9	*	3, 4, 5, 6, 7, 8, 9
OPA10	3	3, 4	4
OPA13	3, 4, 5	1, 2, 3, 4	1, 2, 3, 4, 5
OPA15	*	*	*
OPA18	*	4, 9	8

Table 4. Primers that give rise to markers unique to certain genotypes.

Primer	Number of unique band (genotype)
OPA1	1 (R07)
OPA2	–
OPA4	1 (R2)
OPA7	–
OPA10	–
OPA13	–
OPA15	–
OPA18	1 (R04)

As it is, some primers did not produce scorable markers from any of the parents (Ex: B and OPA2) but segregation in the open pollinated progeny was observed. Because of dominance of RAPD markers this could be explained only by the input of dominant alleles from foreign pollen as argan is a cross-pollinated species (Figure 1) (BENLAHBIL AND BANI-AAMEUR, 1999). In the other hand, some markers, which showed in the parent profile, were missing in few seedlings profiles. These are probably due to the test cross of a heterozygous parent (Aa) and its segregation into recessive (aa) and heterozygous (Aa) offsprings. These observations may contribute in further developments to increase the database concerning the extent of cross-pollination in the species.

OPA 13 and parent R0 plus its progeny is being used here for illustration of a pattern encountered with all primers although with different frequencies (Table 5). Considering all markers generated by a primer, it was possible to distinguish the parent from the offspring with a few exceptions. For R0 and OPA 13, only progeny R02 was matching R0, showing the extent of segregation. In the other hand, no differences were observed between progenies R03, R04, R06, R07, R08, although they differed from their parent.

Seedling genotypes of a family had distinguished profiles (Figure 1). There was no strictly common band to the 30 genotypes that we may consider as a species-specific marker as defined by HADRYS *et al.* (1992); even though band number 9 generated by OPA7 showed in every fingerprint except for four seedlings of different mother-trees.

Cluster classification generated seven end groups (Figure 1). The first end group with lower similarity contained only seedling B7. The second and the third groups were adjacent and included besides one seedling of parent R, parent R0 and six of its progenies. The fourth group contained two seedlings of parent B and eight seedlings of parent R. Parent R was included in the sixth group with its offsprings, two offsprings of parent R0 and was adjacent to two more of its descents in the fifth group. Finally the seventh group contained parent B and seven of its progenies. As it is, despite few exceptions, trees and their progenies tend to cluster together. More than that, trees from Argana geographical origin tend to group together, although the limited number of parents in this experiment may be a limitation to further interpretation.

Research on argan genetics and diversity was mostly concerned with diversity and variability at the population level (BANI-AAMEUR & FERRADOUS, 2001; ZAHIDI 1997; MSANDA *et al.* 1994, EL MOUSADIK & PETIT, 1996). This study established the discriminating power of Random Amplified Polymorphic DNA (RAPD) technique for recogni-

Table 5. Number of bands that will distinguish between pairs of genotypes of B its progenies using OPA 13.

Genotype	Genotype									
	1	2	3	4	5	6	7	8	9	10
B	2	0	2	2	1	2	2	2	3	2
1		2	3	2	1	2	2	2	1	2
2			2	2	0	3	3	3	3	3
3				0	3	0	0	0	3	2
4					3	0	0	0	3	2
5						3	3	3	3	3
6							0	0	3	2
7								0	3	2
8									3	2
9										3

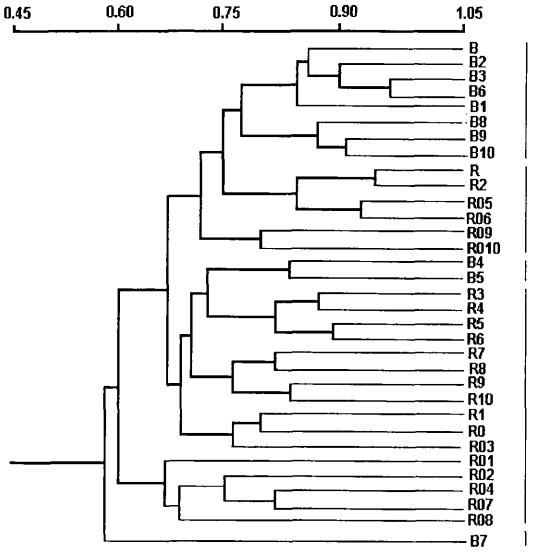

Figure 1. UPGMA dendrogram derived from similarity coeffi-cients showing the grouping (underlined) of three mother trees (B, R, and R0) and their open pollinated progenies designated by the parent symbol attached to seedling number (1 to 10).

tion of argan (*Argania spinosa* (L.) Skeels) genotypes at the tree or the seedling but also at the family level. Segregation of RAPD markers confirmed that argan has a cross-pollinated. The probability that two genotypes present identical multiband fingerprint was low, therefore the probability of discriminating between the genotypes was high. This has enabled to identify genotype profiles with distinguished RAPD markers of parents and open-pollinated offsprings.

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