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Inter-Simple Sequence Repeat Markers Variation among Natural Accessions of Moroccan Carob Tree (*Ceratonia siliqua*)

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ABSTRACT

We adapt an efficient maxi-preparation for the extraction of bulk DNA from leaves of the carob tree (*Ceratonia siliqua*). The bulk DNA quality and quantity were suitable for molecular analysis. Ten Moroccan accessions of carob were evaluated for molecular diversity by using ISSR markers. 176 reproducible amplification fragments were generated with a total of 16 primers. 77.27% of bands were polymorphic. A high genetic diversity values were obtained with all the primers, except for primer F3, with an average of 0.57. Fourteen primers produced some 40 monomorphic bands which can be considered to be specific to the carob accessions studied. The clustering of the different accessions showed a tight relationship with their origin distribution. ISSR technology also proved useful in discrimination closely related natural carob accessions.

Key Words: Carob tree; DNA extraction; Molecular diversity; ISSR

INTRODUCTION

Ceratonia siliqua L. (carob tree; Cesalpinaceae), is a Mediterranean and southwestern Asian evergreen sclerophyllous species. But, it was successfully introduced in different periods in some warm semi-arid zones of Australia, California and Arizona (USA), Mexico, South Africa, etc (Batlle & Tous, 1997). In Morocco, with the exception of arid zones, carob is naturally present up to 1150 m in altitude (Emberger & Maire, 1941).

Carob tree is considered to be an important component of vegetation for environmental, economic and social reasons (Batlle & Tous, 1997). The fruits are used for diverse of purposes as fodder, but are more important in food industry and are a source of many products such as gum, sugar and alcohol (Carlson, 1986; Tous, 1992). World carob pod production is estimated about 310000 tonnes per year and the principal producing countries are Spain, Italy, Portugal, Morocco, Greece and Cyprus (Batlle & Tous, 1997).

The morphological and physiological traits are traditionally used for the identification and the management of carob cultivars (Tous & Batlle, 1990; Gharnit *et al*., 2004). These characters are often influenced by environmental factors and not able to differentiate very well between the different populations (Morsy, 2007). For example, the sex of this slow growing tree can be determined after at least a four year period of growth and the use of flower and fruit characters, which can be important for the determination of cultivars are limited to adult trees. The development of isoenzym markers as a method for cultivars identification in many crop trees, have revealed only a low level of polymorphism between cultivars of the same as well as of the different origins (Barracosa *et al*., 1996; Batlle *et al*., 1996).

To overcome these problems, DNA markers present a very effective tool for rapid analyses of the genetic diversity among cultivars. They are independent of the part and the development period of the plant used are un-affected by the environment and they require a small sample of plant tissue. In addition, the use of molecular markers is mostly contributing to the speeding up of plant breeding and classification, confirming or even reforming the systemic taxonomy of several groups of organisms (Talhinhas *et al*., 2003; Gŭzeldağ & Çolak, 2007). To our knowledge, the premium molecular analysis of carob tree using RAPD marker was reported by Konaté *et al*. (2007).

ISSR (Inter-simple-sequence-repeat) markers are based on size-polymorphism of inter-microsatellite spacers, which are amplifiable by a single-primer (Zietkiewicz *et al*., 1994). This technique is similar to that for RAPD, except that ISSR primers consist of a di- or trinucleotide simple sequence repeat with a 5' or 3' anchoring sequence of 1 to 3 nucleotides. ISSR is considered to achieve higher reproducibility than RAPD markers and is reported to detect a high portion of genomic variation than RFLP (Zietkiewicz *et al*., 1994).

The aims of the present study are: (1) to perform an

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efficient maxi-preparation for bulk DNA extraction from carob leaves and (2) to use ISSR makers for analysing the genetic diversity among Moroccan accessions of carob tree.

MATERIALS AND METHODS

Plant materials. Ten accessions of carob tree (*Ceratonia siliqua* L.) were collected in different regions in Morocco. They were named according to their collecting site origin and used for studying molecular diversity by using ISSR technique. The origin and annual rainfall of each collection site were noted. Some fruit characteristic of each collected accession were also recorded (Table I). Seeds were taken randomly from each accession and were scarified by H_2SO_4 at 95% for 30, 45 and 60 min., depending on seed hardness and were sown in pot in a greenhouse. After six moths of culture, the leaves were harvested and stocked at –80°C.

DNA isolation. The maxi-preparation of total genomic DNA extraction, from a mixture of young leaves of fifty plants from each accession was carry out as well as the method described by Konaté *et al*. (2007) with some modifications. 3 g of leaves were ground in mortar in the presence of 15 mL of the lyses buffer: 50 mm Tris-HCl (pH_8), 5 mm EDTA (pH_8), 300 mm Mannitol, 0.05% BSA, 1% PEG_{6000} and 0.5% β-mercaptoethanol. The mixture was transferred to a sterile tube containing 1.8 mL of SDS (20%) and 1.2 mL of sodium acetate (3 m, pH 8) and incubated for 30 min, at 65°C. The tubes were kept for 5 min., to allow them to return to room temperature. An equal volume (v/v) of chloroform-isoamyl alcohol (24:1) solution was added. The tubes were then mixed and centrifuged at 10000 g at 4°C for 10 min. The aqueous phase was transferred to a clean tube and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The tubes were then mixed and centrifuged at 10000 g at 4°C for 10 min. Another step with chloroform-isoamyl alcohol was repeated. The DNA precipitation was done by adding a same quantity (v/v) of cold isopropanol followed by incubation at –20°C for 1 h. After centrifugation for 20 min., the pellet was washed with 70% cold ethanol, dried under vacuum, suspended in TE buffer (10 mm Tris, 1 mm EDTA, $pH_{7.4}$), treated with proteinase K (20 μg mL^{-1}) then incubated at 37°C for 30 min. Another treatment with RNase (10 mg/mL) was also performed and incubated at 37°C for 30 min. The proteinase K and Rnase were removed by one extraction step with phenol-chloroform-isoamyl alcohol. After centrifugation, 1/10 volume of 5 m NaCl and an equal quantity of absolute cold ethanol were added on the superior phase then left overnight at –20°C. The DNA was pelleted by centrifugation for 20 min., washed with 70% cold ethanol, dried and dissolved in 600 μL TE buffer. The concentration and the quality of DNA were assessed by optical density.

ISSR amplification. Sixteen ISSR primers (Operon Technologies, Alameda, USA) were used in the polymerase chain reaction (PCR) amplifications. The PCR were performed as described in the FAO/IAEA FAO (2002) protocol with some modification in the reaction mixture, with total volume of 25 μL containing 10 x PCR buffer, 25 mm $MgCl_2$, 125 μm of each dNTP, 4 μm of primer, 50 ng of genomic DNA and 2 U of *Taq* polymerase (Promega). Amplifications were carried out as follow: 7 min., at 94°C, 32 cycles of 30 s at 94°C, 45s at 45°C and 2 min., at 72°C, with a final extension for 7 min., at 94°C. PCR products were separated on a 3% agarose gel in 1×TBE buffer at 80 V for 4 h. After electrophoresis, the gel was stained with ethidium bromide (0.1%) and photographed under UV light with Perfect Image v. 6.

Evaluation of diversity and data analysis. Polymorphic products from ISSR analysis were scored quantitatively for presence (+) or absence (-). Polymorphism information content (PIC) or genetic diversity was calculated by: $PIC = n\ (1-\sum P^2 ij)/(n-1)$, with n is the patterns size and Pij the frequency of the i-th pattern revealed by the ISSR primer j summed across all patterns revealed by the primer j (Botstein *et al*., 1980).

The electrophoresis images were analysed using Gel Compare II (v. 2.5) to generate a dendrogram for evaluating the inter-accession relationships.

RESULTS

The maxi-preparation of DNA isolation adapted to the leaves of carob tree was very efficient and produced genomic DNA suitable for amplification analysis (Table II). The average quality value assessed by the ratio of DNA/protein was 1.89 and the highest DNA concentration obtained by this procedure was 1765 ng μL^{-1}.

ISSR amplification was successful with all the 16 primers used. They all generated polymorphic markers, except primer F3 (Table III). The primers generated a total of 176 bands, of which 136 (77.27%) were polymorphic. The average number of polymorphic band per primer was 8.5. 17 polymorphic bands were identified with primer F12. The amplified fragments per primer ranged from 1 with primer F3 to 19 with F12, with an average of 11 per primer. DNA polymorphism detected with primer F7 with 10 carob accessions is shown in Fig. 1.

Genetic diversity was calculated for each of the ISSR markers in all accessions (Table III). The difference in diversity value revealed the degree of polymorphism. The 16 ISSR primers had an average diversity of 0.57 with a range of 0.32 for primer F4 to 0.93 for primer F16. However, primer F3 showed no diversity. On other hand, all primers, except F1 and F16, generated 40 monomorphic markers from which some could be considered specific to *C.* carob tree species.

The dendrogram constructed by UPGMA cluster analysis is represented in Fig. 2. Percentage of similarity between all possible pairs of accessions ranged from 82.5% to 95.5%. Groups of accession can be delimited at the level of similarity of 86%, with an individual line formed by

Table I. Origin, annual rainfall, and fruit characteristics of carob accessions

Accession and site origin	N° affected to each	Rainfall (mm)	Some fruit characteristics			
	accession		Pod size*	Pod shape	Seed yield (%)	Scarified times
Taourirte	1	350-600	Light-short	Straight	15.86	30 min
Al Houceima	2	600-1000	Light-long	Straight	15.86	45 min
Taounate	3	600-1000	Medium	Curved	28.54	30 min
Aïn Safa	4	350-600	Light-long	Straight	15.67	60 min
Akchort	5	600-1000	Light-long	Straight	16.02	45 min
Demnate	6	350-600	Light-short	Straight	27.04	30 min
Ouazzane	7	600-1000	Light-long	Straight	15.61	60 min
Sidi Bou Othmane	8	350-600	Light-long	Straight	23.00	30 min
Essaouira	9	350-600	Short	Straight	9.99	30 min
Tetouan	10	600-1000	Light-short	Straight	26.64	60 min

* Medium pod length about 14 – 15 cm.

Table II. Concentration and quality of total genomic DNA extracted from leaves of carob

Accession	1	2	3	4	5	6	7	8	9	10
DNA concentration (ng/µl)	1550	1095	1515	1765	1635	955	1500	1710	1425	1440
DNA quality (OD_{260}/OD_{280})	1.92	1.98	1.90	1.83	1.90	1.70	1.78	1.88	1.99	2

Table III. List of ISSR primers used, with the percentage and the degree of polymorphism generated by each of the ten carob accessions

Primer names	Séquence	AT (°C)	Molecular weight (range, pb)	Polymorphism (%)	No. of markers		
					P	M	PIC
F1	CACACACACACAAT	46.76	1808 – 1860	100	2	0	0.59
F2	CACACACACACAGC	52.61	213 – 1696	73.33	11	4	0.72
F3	CACACACACACAAG	49.61	707	0	0	1	0.00
F4	AGCAGCAGCAGCCT	51.67	227 – 1736	43.75	7	9	0.32
F5	$[AC]_8CA$	57.62	344 – 1273	75	9	3	0.50
F6	$[AC]_8CG$	59.9	173 – 351	60	3	2	0.59
F7	$[AC]_8CT$	57.62	355 – 1712	92.85	13	1	0.65
F8	$[AG]_8CC$	59.9	277 – 2636	78.57	11	3	0.51
F9	$[AG]_8CG$	59.9	288 – 1718	63.63	7	4	0.46
F10	$[CA]_8AG$	57.62	219 – 1537	81.81	9	2	0.73
F11	$[CA]_8AC$	57.62	429 – 1278	75	6	2	0.51
F12	$[GA]_8CC$	59.9	251 – 1532	89.47	17	2	0.70
F13	$[GA]_8CG$	59.9	257 – 2048	78.57	11	3	0.61
F14	$[GA]_8CT$	57.62	295 – 2307	72.72	8	3	0.61
F15	$[GT]_8CC$	59.9	422 – 1486	90.90	10	1	0.69
F16	$[GT]_8CG$	59.9	208 – 1159	100	12	0	0.93
Total					136	40	0.57±0.205

AT: annealing temperature. P: polymorphic. M: monomorphic. PIC: Polymorphic information content

Ouazzane accessions. Group 1 included 5 accessions: Akchort, Aïn Safa, Taounate, Demnate and Sidi Bou Otmane. These accessions are situated in a continental region of Morocco. Group 2 consisted of 2 accessions, Taourirt and Al Houceima, which are situated in northeast of Morocco. Group 3 consisted of the accessions: Essaouira and Tetouan, which are situated at sea coastal of Morocco.

DISCUSSION

A suitable DNA extraction procedure is prerequisite for performing DNA-based markers studies on a new plant species. Since this was a continuous work on carob tree (Konaté *et al*., 2007), we tested several protocols: that of Dellaporta *et al*. (1983), Doyle and Doyle (1987) and Ouenzar *et al*. (1998). The best results were obtained with the method reported by Ouenzar *et al*. (1998), with the modification of the concentration of some lyses buffers and the procedure. The yield of bulk DNA was considerable with the highest amount about 1.765 ng μL^{-1} for Aïn Safa accession (Table II). The DNA extract quality for all accessions assessed by the ratio DNA/protein was 1.88. This allows us to provide a good and suitable material for molecular diversity analysis of carob tree.

DNA analysis using ISSR markers on bulk DNA was a useful tool to assess the genomic complexity of Moroccan accession of carob. The variations of carob accession revealed by ISSR markers confirm the high level obtained by RAPD markers (Konate *et al*., 2007) with few different. According to Zietkiewicz *et al*. (1994), ISSR markers can detect effectively very low levels of genetic variation. So, they have been successfully used to assess genetic diversity of many plants such as *Sorghum bicolour* L. (Yang *et al*., 1996), *Lupinus* spp. (Talhinhas *et al*., 2003), *Asparagus acutifolus* L. (Sica *et al*., 2005) and *Bombyx mori* (Pradeep *et al*., 2005). The total number of polymorphic markers

Fig. 1. ISSR patterns generated by primer F15 with 10 carob accessions

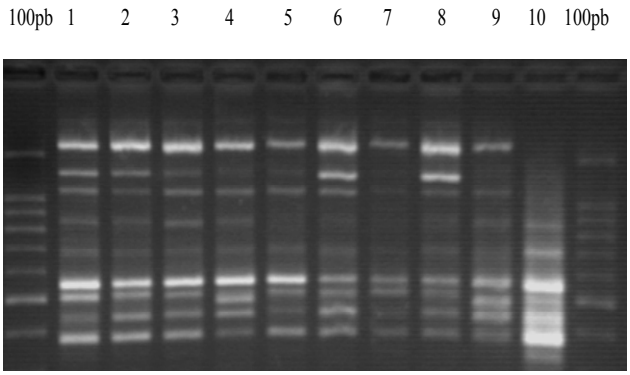
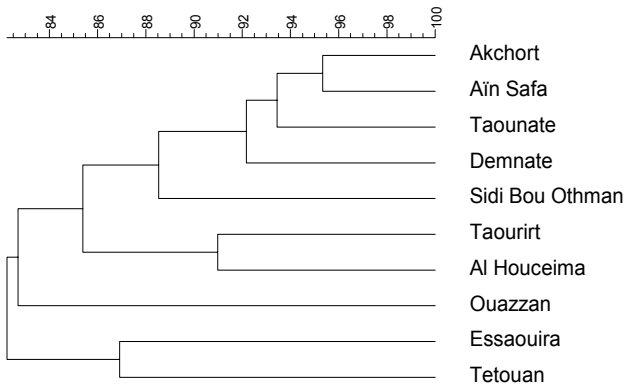


Fig. 2. UPGMA tree of the 10 *Ceratonia siliqua* L. accessions used in the ISSR analysis



obtained was 136 for the 16 primers used. This number is higher than 25/7 obtained for *Bombyx* by Pradeep *et al*. (2005), but much lower than 228/23 (*Asparagus*) and 557/41 (*Sorghum*) as reported by Sica *et al*. (2005) and by Yang *et al*. (1996), respectively.

The genetic diversity values calculated for all primers ranged from 0.32 (F4) to 0.93 (F16) with an average of 0.57, except primer F3 that showed no diversity (0.00). In this analysis, we identified with each accession 14 ISSR primers, which produce some monomorphic bands that seem to be specific to the carob tree accessions tested. These primers could be also useful for several genetic studies such as identification of ISSR marker linked to sex, in occurrence of carob tree which is a dioecious species. In *Pistacia vera*, dioecious tree, a RAPD marker linked to sex was identified using bulked segregant analysis (Hormaza *et al*., 1994).

The dendrogram constructed on UPGMA based analysis confirmed the high degree of diversity between the carob accessions and showed that these accessions can be classified into tree groups with an individual accession. This classification showed a high relatedness with their geographic distribution with the exception of Ouazzane accession that is rather distinct from the others. To our knowledge, this is probably the second report on the development of DNA markers for carob tree genetic studies. However, techniques such as flow cytometry and isoenzymes were already reported for genetic differentiation between wild and cultivated or inter and intra-population of carob (Bureš *et al*., 2004; Makrem *et al*., 2006).

CONCLUSION

Our results ended up with a suitable DNA isolation method for carob tree, which may offer a great opportunity for several molecular studies such as genetic identification and diversity. The use of ISSR markers was useful in distinguishing the Moroccan accessions of carob and confirmed the importance of genetic studies for designing collections and germplasm conservation strategies.

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