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RESEARCH ARTICLE

SSR-based genetic diversity analysis of Tunisian varieties of melon (cucumis melo L.) and Fakous (cucumis melo var.flexuosus)

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Abstract

The genetic diversity is fundamental in order to provide information for genetic resources conservation and breeding programs. Genetic diversity of Tunisian melon varieties (*Cucumis melo* L.*)* and 'fakous' (*Cucumis melo var. flexuosus*) selected by the National Institute of Agronomic Research of Tunisia was evaluated using twelve microsatellite markers. Among 12 primer pairs, 11 SSR were polymorphic and reproducible. The number of alleles per locus varied between 2 (CMGAN25. CMCTN35. CMCTN86. CMAGN68. FR14G19) and 3 alleles (CMCTN5. TJ10. TJ27. CMGAN80. MU118. SSH6123), with an average of 2.54 alleles/primer. The Polymorphism Information Content (PIC) ranged from 0.43 (CMCTN35) to 0.92 (TJ10) with an average value of 0.75. The dissimilarity coefficient ranged from 0.090 to 0.818 with a mean value of 0.454. Our data provide evidence of high molecular polymorphism, showing that the varieties of melon and fakous constitute an important pool of diversity. The relationship between cultivars was evaluated through cluster analysis and separated the cultivars in two major groups with a clear divergence of 'fakous' (*Cucumis melo var. flexuosus*) from the other varieties.

INTRODUCTION

Melon (*Cucumis melo* L. ., 2n=24) is a major vegetable crops grown, which is consumed worldwide and therefore of economic importance (Kirkbride, 1993). The species is commonly called melon, sweet melon, muskmelon, casaba and cantaloupe (Nayar and Singh, 1998). A large diversity has been observed among melon genotypes. Munger and Robinson (1991) defined seven groups of melon: Cantaloup, Inodorus, Flexuosus, Conomon, Chito, Dudaim and Momordica. A recent taxonomy of melon identified 16 groups (Pitrat et al., 2000 a), five in subspecies agrestis (acidulous, chinensis, conomon, makuwa and momordica) and 11 in subspecies melo (adana, ameri, cantaloupensis, chandalak, chate, chito, dudaim, inodorus, flexuosus, reticulates and tibish). The origin of melon is in dispute, most authorities consider that it originated in Africa (Kerge and Grum, 2000). Recently phylogenetic data demonstrated that *Cucumis* originated from Asia (Sebastian et al., 2010; Telford et al., 2011). It is cultivated in tropical and subtropical regions, which are also grown extensively in temperate climates (Staub et al., 2004; Pech et al., 2007). *Cucumis melo* L. shows extreme variation especially in vegetative and fruit morphology (Kirkbride, 1993; Monforte et al., 2004).

In Tunisia, melon is one of the most consumed summer fruits. In 2011, 10447 ha were dedicated to this crop and its production amounted to 104482 tones (FAO 2013, http://www.fao.org). It is ranked fourth vegetable production after potato, pepper and watermelon which occupy 32%. 32% and 23% respectively (FAO 2013, http://www.fao.org). However, the Tunisian varieties of melon are frequently damaged by severe genetic erosion primarily due to the introduction of improved varieties improper management and inadequate regeneration

procedures of germoplasm collections. Therefore, a survey of the genetic diversity is necessary to encourage rational management and selection programs involving the local *Cucumis melo* germoplasm.

Different criteria for genetic diversity estimation can be used: pedigree records, morphological traits, biochemical markers and molecular markers (Eshghi and Akhundova, 2010). Diversity in melon based on morphological traits was implemented by many authors in the world (Staub et al., 2004; Laghetti et al., 2008; Escribano and Lazaro, 2009; Nasrabadi et al., 2012; Henane et al., 2013; Trimech et al., 2013). Molecular markers have been used as a valuable tool in the characterization and evaluation of genetic diversity within and between species and population such as amplified fragment length polymorphism (AFLP, Yashiro et al., 2005). random amplified polymorphic DNA (RAPD, Lopez-Sesé et al., 2003; Staub et al., 2004; Sensoy et al., 2007; Tanaka et al., 2007; Yi et al., 2009; Nhi et al., 2010; Soltani et al., 2010) and simple sequence repeat (SSR, Monforte et al., 2003; Raghami et al., 2014; Trimech et al., 2015).

The main objective of this work was to investigate the genetic diversity of Tunisian melon and fakous germplasm using SSR markers to determine the genetic diversity structure and identify relationships within and among five varieties of melon (*Cucumis melo* L.) and fakous (*Cucumis melo var. flexuosus*) selected by the National Institute of Agronomic Research of Tunisia.

Material and Methods

Plant materials

Five Tunisian varieties of melon (‘Maazoun’. ‘Galaoui’. ‘Stambouli’. ‘Trabelsi’. ‘Asli’) (*Cucumis melo* L.) and ‘fakous’ (*Cucumis melo* var. *flexuosus*) selected by the National Agronomic Research Institute of Tunisia (INRAT) were used in this study (Fig 1). The essay was carried out from March to August 2012 at the Manouba Support Station located in the North East of Tunisia (36°45’0”N. longitude 10°0’00”E). Seeds were germinated in polystyrene trays with a peat substrate. Twenty days after emergence, the most vigorous seedlings of each variety were transplanted to the field in three rows (replication) with an in-row spacing of 100 cm and a between-row spacing of 150 cm. The experimental area was fertilized before planting by 85 Kg Ammonitrate ha^{-1}, 70 Kg phosphoric acid ha^{-1}, 130 Kg potassium nitrate ha^{-1}, 80 Kg magnesium sulfate ha^{-1}. Other agronomic practices including irrigation, weeding and chemical insecticide treatments were conducted uniformly and as required in all plots.

Genomic DNA extraction

Genomic DNA was extracted from young leaf tissues (18 plants) by CTAB method (Saghai Maroof et al., 1984). The quality of DNA was examined and estimated using agarose-gel electrophoresis. DNA was quantified by spectophotometer (Beckman DU 650. Beckman Coulter. Inc., CA. USA). The concentration and quality of extracted DNA was determined by reading at 230, 260 and 280 nm. The purified DNA samples was diluted to 50 ng/µl using dH2O and stored at -20°C.

PCR amplification and electrophoresis running

Twelve SSR primer pairs described previously in the literature and distributed along the melon genome (Danin-Poleg et al., (2000, 2001, 2002); Katzir et al., 1996) were used (Table 1). PCR reaction for SSR analysis was performed in a total volume of 25 µl in a Perkin Elmer thermocycler (GenAmp 9700 (Perkin-ElmerCrop., Norwalk, CT)). The reaction mixture containing 50 ng DNA, one unit of GoTaq DNA polymerase (Promega), 0.2 mM dNTPs, 0.5 µM of each primer, 25 mM $MgCl_2$ and 10X PCR Buffer. The cycling parameters were: 1 cycle of 94° C for 2 min; 35 cycles of 1 min denaturing step at 94 °C, 1 min annealing temperatures between 53°C and 55°C depending on the different primer combinations and 1 min extension at 72° C, followed by 2 min at 72 °C (post-extension). PCR products were visualized by running 7 µl of each sample on 8% polyacrylamide gels, stained by ethidium bromide (0.5 mg/ml) and visualized under UV light.

Analysis of genetic Data

Molecular data were analyzed to reveal the genetic relationships among Tunisian varieties of melon (*Cucumis melo* L.) and fakous (*Cucumis melo var. flexuosus*). DNA fragments at all SSR loci were scored as binary data matrix with “present” as 1 or “absent” as 0 and used for analysis. Genetic dissimilarity (GD) between accessions was calculated according to the formula of Nei and Li (1979). Based on the matrix of (GD) values, the UPGMA (unweighted pair-group method with arithmetic averages, Sokal and Michener, 1958) clustering method was used to obtain the dendrogram, depicting genetic relatedness of the cultivars (DARwin software (version 5.0.158)). Branch robustness was tested using 1000 bootstraps. The Polymorphism Information Content (PIC) value ranged from 0 (monomorphic) to 1 (highly discriminative) and is an indication of discriminative power of a given marker, not only for the number of alleles at a locus, but also for the relative frequencies of those alleles in the genotypes under study (Botstein et al., 1980).

The PIC was calculated as:

$$\mathbf{PIC} = 1 - \sum_{i=1}^{n} fi^2$$

where *fi* is the frequency (*f*) of the *i*th allele. The PIC value provides an estimate of a marker's discriminatory power by accounting for the number and frequency of alleles at a given locus (Lubberstedt et al., 2000).

Results and discussion

Characterization of SSR polymorphism

To characterize and evaluate the genetic diversity of Tunisian varieties of melon (*Cucumis melo* L.) and fakous (*Cucumis melo var. flexuosus*), twelve primer pairs were used. Migration profiles generated ten polymorphic profiles, one monomorphic profile for primer (FR14G19) and one primer CMGAN21 that generated no band (Table 1). These microsatellites helped to amplify a total of 27 alleles, with number of alleles per locus ranging from 1 (FR14G19) to 3 (CMCTN5, TJ10, TJ27, CMGAN80, MU118, SSH6123). The average number of alleles per locus is 2.45, which was similar to that reported by López-Sesé et al. (2002) who analyzed the genetic relationships among 15 Spanish melons and using 12 SSR markers (2.4). However, higher values were reported by Raghami et al. (2014) who found an average of 4.38 alleles per locus using 18 SSR markers on 24 Iranian cultivated melons.

The number of amplified fragments is affected by primer sequences, cultivars, and protocol conditions (Wan et al., 2005). Casas et al. (1999) have shown the relationship between the degree of polymorphism and the primer sequence since the amplification will only occur when the primer is complementary at the target DNA.

Moreover, the Polymorphism Information Content (PIC) ranged from 0.43 for CMCTN35 to 0.97 for FR14G19, with an average value of 0.79. Nine SSR markers were very informative (PIC >0.5), including (CMCTN5 (0.75), TJ10 (0.92), CMCTN86 (0.88), TJ27 (0.91), CMAGN68 (0.78), CMGAN80 (0.74), MU118 (0.88), SSH6123 (0.77), FR14G19 (0.97)). Indeed, the very informative markers are extremely useful for genetic studies and determination of the level of polymorphism on a specific marker locus (Sundaram et al., 2007).). The 11 SSRs used herein were sufficient to distinguish all the tested cultivars, indicating the usefulness of the chosen marker set to study the genetic variability among the analyzed cultivars

Genetic relationship among melon varieties and fakous

The obtained profiles were transformed on dissimilarity matrix with Darwin 5 (version 5.0.158). The dissimilarity matrix was used to determine the level of relatedness among the studied cultivars (Table 2). The genetic dissimilarity coefficient (GD) ranged from 0.090 ('Stambouli'-'Trabelsi') and 0.818 ('Stambouli' - 'Fakous' and 'Trabelsi'-'Fakous') with a mean value of 0.454. The result revealed a great diversity between the studied varieties of melon and fakous.

Genetic distance analyses showed that the average genetic distance between group I ('Maazoun', 'Galaoui', 'Stambouli', 'Trabelsi and 'Asli') and group II ('fakous') were very large. From these results, SSR markers can be used effectively to estimate genetic distances among cultivars. The mean genetic distance was 0.674 between Iranian cultivated melon (Raghami et al., 2014) and was 0.285 between Spanish melon (Lopez-Sesé et al., 2002), while in the present study, it varied from 0.090 to 0818 with an average of 0.454. This genetic distance shows the importance of Tunisian melon varieties and fakous for conservation and use in breeding programs.

The dendrogram of genetic distances (Fig 2) was constructed based on UPGMA Method using midpoint joining procedure of Nei and Li (1979) dissimilarity matrix.

According to genetic distances dendrogram which is built on SSR markers data and referring to a dissimilarity coefficient among the varieties of melon and fakous, we distinguished two major groups (Figure 2). The first group (GI) included 'Maazoun', 'Galaoui', 'Stambouli', 'Trabelsi' and 'Asli'. The second one (GII) is only one cultivar 'fakous'. The first group (GI) could be subdivided into three subgroups:

- Subgroup A gathered three varieties ('Galaoui', 'Stambouli' and 'Trabelsi'). Their presence in the same group is the fact that they share the same seed color, the presence of fruit splitting, the low flesh acidity and the intermediate storage capacity of fruit. In this group, the highest genetic dissimilarity coefficient is shown between 'Stambouli' and 'Galaoui' (GD =0.454). Nevertheless, the lowest one is observed between 'Stambouli' and 'Trabelsi (GD= 0.090). This sharp similarity between 'Stambouli' and 'Trabelsi' makes one think of 'synonymy phenomenon': it is the same variety, but having undergone two different names depending on the collection area.
- The Subgroup B consisted only of the variety 'Asli'. This variety comes from a selection in the progeny of a F1 hybrid type 'Canary Yellow'. It is characterized by long terminal lobes and avoids fruit with light-yellow predominant skins.
- Finally the subgroup C contained just 'Maazoun'. It is a variety that represents the melon 'winter type' which differs from the other varieties by fruits with oblate shape, pale green predominant skin, dark green secondary skin and cream flesh.

The second group (GII) contains only one cultivar 'fakous' which has the highest dissimilarity with each of the other varieties of melon (GD : 'Stambouli'-'Fakous' : 0.818, GD :'Trabelsi'-'Fakous' : 0.818, GD : 'Asli'-'Fakous' : 0.727, GD :'Galaoui-'Fakous' : 0.727, GD :'Maazoun'-'Fakous' :0.727, GD :'Stambouli- 'Maazoun' : 0.636, GD :'Trabelsi'-'Maazoun' : 0.636). It is clearly stood apart from the rest of melon varieties studied.

Indeed, 'fakous' differs from the other varieties of melon by its taxonomy since it represents another group of melon (*Cucumis melo var. flexuosus*). It is characterized by an important leaf area with dark green color, elongate fruits with dark green skins, green flesh with an intermediate acidity and whitish seeds.

This result is in accordance with the work of Pitrat et al., 2000 (b) and Henane et al., 2013 that 'fakous' *Cucumis melo var. flexuosus*' or 'Snake melon' has a low similarity with each other varieties of melon and represented other group of melon.

The results obtained indicate that SSR markers are good tools for to identify genotypes and to evaluate genetic diversity in varieties of melon and Fakous.

Conclusions

Taken as a whole, the present study clearly show that Tunisian melon germplasm with this broad genetic diversity could play an important role in the preservation and the enhancement of melon genetic diversity. The genetic structure of melon constitutes a rich genetic heritage and a track of investigation can focus on genetic markers to detect useful alleles such as, for resistance to some diseases (such as odium) or for a better nutritional quality.

Table 1. Variability of simple sequence repeat marker (SSR) used for Tunisian varieties of melon and fakous genetic analysis.

Locus	Primer sequence	Tm	Na	Af	PIC
CMGAN25:F	TAGCCAATGTGAAGGATGAACA	55	2	0.85	0.46
CMGAN25:R	TGCAATTAGCCTCTTCTCTA				
CMCTN5:F	TCACCTTAAAGTTTAGCCCC	53	3	0.39	0.75
CMCTN5:R	AAAAATGCAATGAACTGAGCGC				
TJ10:F	TACGAGGAAAACGCAAAATCA	53	3	0.35	0.92
TJ10:R	TGAACGTGGACGACATTTTT				
CMGAN21:F	TGCTGTAAAACGAAACGGAGA				
CMGAN21:R	CGATCTTCTTTATTCTTCGCC				
CMCTN35:F	TCCAATAATGTAATCGTCTTGG	55	2	0.82	0.43
CMCTN35:R	GTTCCAAACTTTCTACCAATCA				
CMCTN86:F	TGTGACAGTTATCAAGGATGC	53	2	0.54	0.88
CMCTN86:R	AAGGGAATGCATGTGGAC				
TJ27:F	TAAGCGGAACAAGCTCATCTC	55	3	0.39	0.91
TJ27:R	CAAAAGCATCAATTGCTTGAA				
CMAGN68:F	TGGAAGGAAATTAGCATGCAC	55	2	0.54	0.78
CMAGN68:R	GCCACTCTGTCTTTCTTCC				
CMGAN80:F	TATATTGATTGCTGGGAAAGG	53	3	0.5	0.74
CMGAN80:R	CTTTTTTGGCTTTATTGGGTC				
MU118:F	TGTGTGCTGTACTCCTGAAA	53	3	0.54	0.88
MU118:R	CGGTTCTTTCTTCTCTTCCT				

SSH6I23: F SSH6I23:R	CCGCTTCTTCTTCTTCTTCT CTAGGACCGGAATCGTAATG	53	3	0.66	0.77
FR14G19:F FR14G19:R	TCTTTGTCTACCACCAAACC GTTTGAGAGGAGGAAGAGGT	53	2	0.5	0.75
Mean			2.54	0.46	0.75
Total			28	5.15	8.27

Tm: Annealing temperature (°C). Na: Number of alleles. Af : alleles frequencies. PIC : Polymorphic Information Content.

Table 2. Genetic dissimilarity values of melon varieties and fakous as determined by SSR markers using Jaccard similarity coefficient.

	Maazoun	Galaoui	Stambouli	Trabelsi	Asli	Fakous
Maazoun	0.000					
Galaoui	0.363	0.000				
Stambouli	0.636	0.454	0.000			
Trabelsi	0.636	0.363	**0.090**	0.000		
Asli	0.545	0.545	0.454	0.454	0.000	
Fakous	0.727	0.727	**0.818**	**0.818**	0.727	0.000

Fig 1. Tunisian varieties of melon (*Cucumis melo* L.) and fakous (*Cucumis melo var. flexuosus*)

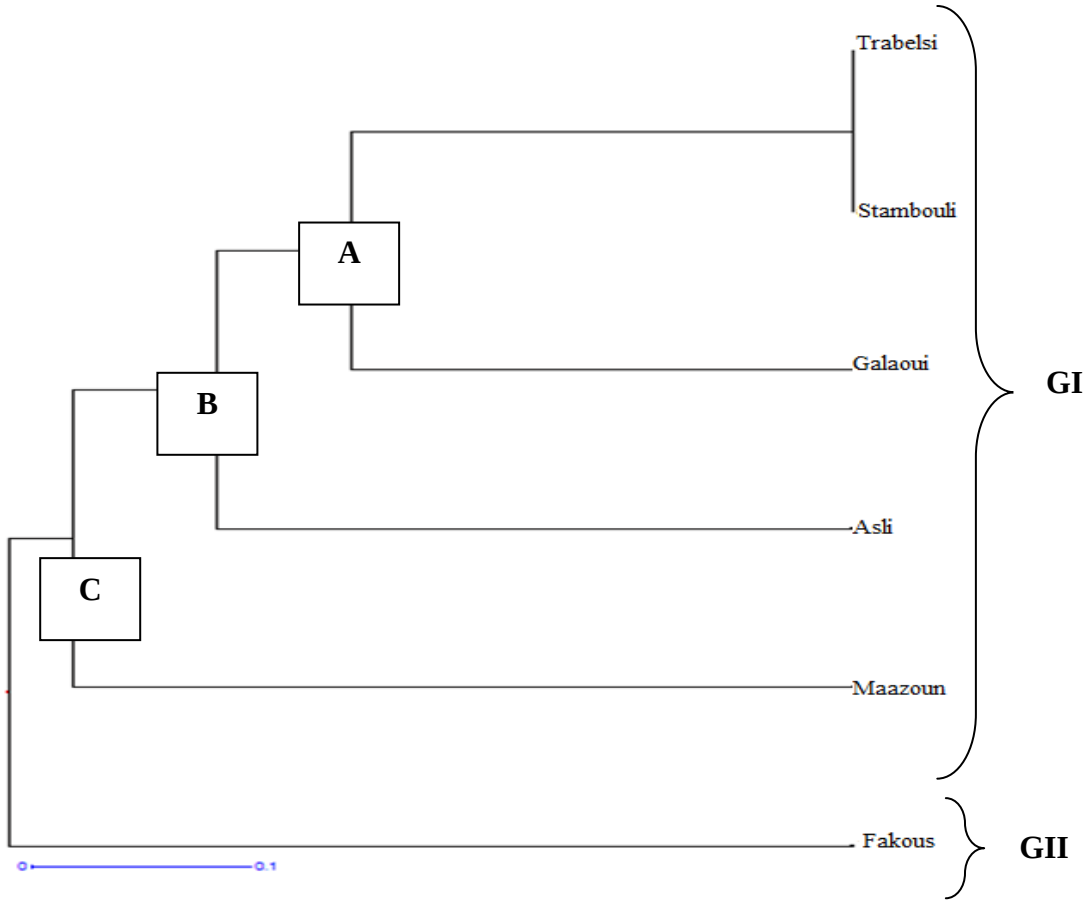

Fig 2. Dendrogram resulting from an UPGMA cluster analysis of varieties of melon and fakous and based on data of 11 microsatellite primer pairs.

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