Plant Gene 2 (2015) 29–33

Contents lists available at ScienceDirect

Plant Gene

journal homepage: www.elsevier.com/locate/plant-gene

Genetic diversity of Barbary fig (*Opuntia ficus-indica*) collection in Greece with ISSR molecular markers

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ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form 25 April 2015
Accepted 27 April 2015
Available online 30 April 2015

Keywords:
Opuntia ficus indica
ISSR markers
Genetic diversity
Greek germplasm

ABSTRACT

Barbary fig (*Opuntia ficus indica*) could be an economically important species as it could be an alternative crop extremely tolerant to dry condition and water deficiency. Moreover, it could be used in alternative sustainable cultivation systems and landscape conservation. In this work, we report the analysis of the genetic diversity of Greek Barbary fig genotypes using ISSR molecular markers. Six primers were screened to assess their ability to detect polymorphisms within twenty-two Barbary fig accessions and generated 57 markers (bands), with an average of 9.5 markers per primer. The percentage of polymorphic bands (50.21%) and the resolving power (R_P) (28.85) showed the efficiency of the used primers. Mean values for GD (gene diversity) and I (Shannon index) were found as 0.215 and 0.355, respectively. The revealed ISSR markers allow distinguishing all accessions analyzed except for one case. UPGMA dendrogram and PCoA (Principal Coordinate Analysis) were performed to access patterns of diversity among genotypes. The high genetic diversity existing in the Greek germplasm suggests that it would be beneficial to utilize this pool in Barbary fig breeding programs and germplasm management activities.

1. Introduction

Barbary fig (*Opuntia ficus-indica*) is a species of the genus Opuntia, which belongs to the Cactaceae family and has been documented to include a number of species, which vary from 160 (Gibson, 1990) to 250 (Britton and Rose, 1922). The genus taxa exhibit great variation in terms of ecology, reproduction (Scheinvar, 1995) and ploidy (from diploidy to octoploidy; (Felker et al., 2005)). Barbary fig is the most widely distributed species of the cactus family and at the same time the most economically important (Nobel et al., 2002). Although the ancestors of the species were native to Central America, Barbary fig was spread, through cultivation and trade, initially throughout the Caribbean and South America and subsequently into Mediterranean Europe, North America and other arid and semiarid regions of the world (Griffith, 2004). Natural hybridization, associated with polyploidy and geographic isolation, has lead to a great genotypic variability of Opuntia, displaying at the same time high levels of phenotypic plasticity (Wallace and Gibson, 2002). Thus, the taxonomical classification of the genus species is intriguing, despite the fact that many of the included species are commonly exploited, like Barbary fig which is cultivated for its large sweet fruit (Inglese et al., 2002), used as vegetable products (Stintzing and Carle, 2005) and as a host plant for *Dactylopius coccus* (cochineal insects) for the production of vivid red and purple dyes (Donkin, 1977).

DNA markers can provide a useful tool for the investigation of the genetic variation within the genus or species and assist with their identification or taxonomic classification. The most commonly used markers for such studies are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) or microsatellites and inter-simple sequence repeats (ISSRs) (Henry, 2012). Studies related to the genetic diversity of the genus Opuntia have already been conducted, using cpSSR and AFLPs (Labra et al., 2003), nrITS (Griffith, 2004), RAPDs (Zoghlami et al., 2007; Bendhifi et al., 2013), ISSRs (Valadez-Moctezuma et al., 2014) and microsatellites (Caruso et al., 2010). Each of these marker systems has many prons and cons. ISSR markers are extremely variable and have proven to be sensitive enough to differentiate cultivars and natural

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http://dx.doi.org/10.1016/j.plgene.2015.04.001

Table 1
Opuntia ficus indica accessions included in this study with their main area of origin in Greece and some morphological characteristics.

No	Accession	Region	End use	Plant size	Plant shape	Plant habit	Plant vigour	Cladodes Shape	Cladodes with thorns	Glochides	Cladodes Colour	Fruit Size
1	Dichova Manis	Mani	–	–	–	–	–	–	Moderate	Moderate	–	–
2	Israel	Israel	Fruit	Medium (height 1.6–2.0 m)	Elongate (width < height)	Upright	Medium	Elliptic	Few	Few	Yellow-green	Very small (<80 g)
3	Cyprus	Israel	–	–	–	–	–	–	Few	Few	–	–
4	KA5	Kalamata	Fruit	Medium (height 1.6–2.0 m)	Round (width = height)	Spreading	Strong	Elliptic	Many	Moderate	Deep green	Very small (<80 g)
5	KE1	Corfu	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Upright	Medium	Ovate	Few	Few	Green	Small (81–120 g)
6	KE2	Corfu	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Upright	Strong	Elliptic	Few	Few	Deep green	Very small (<80 g)
7	KE3	Corfu	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Spreading	Medium	Ovate	Many	Few	Green	Very small (<80 g)
8	RO1	Rhodes	Fruit	Medium (height 1.6–2.0 m)	Round (width = height)	Arborescent	Strong	Elliptic	Moderate	Few	Green	Small (81–120 g)
9	RO2	Rhodes	Fruit	Medium (height 1.6–2.0 m)	Round (width = height)	Arborescent	Strong	Elliptic	Few	Few	Green	Small (81–120 g)
10	RO3	Rethimno	Fruit	Medium (height 1.6–2.0 m)	Round (width = height)	Prostrate	Strong	Elliptic	Few	Moderate	Green	Small (81–120 g)
11	KA1	Kalamata	Fruit	Medium (height 1.6–2.0 m)	Round (width = height)	Spreading	Strong	Ovate	Many	Moderate	Deep green	Small (81–120 g)
12	KA2	Kalamata	Fruit	Large (height >2.1 m)	Elongate (width < height)	Spreading	Medium	Ovate	Many	Moderate	Green	Small (81–120 g)
13	KA3	Kalamata	Fruit	Medium (height 1.6–2.0 m)	Round (width = height)	Spreading	Strong	Elliptic	Many	Moderate	Deep green	Very small (<80 g)
14	KA4	Kalamata	Fruit	Small (height <1.5 m)	Elongate (width < height)	Medium	Strong	Elliptic	Few	Moderate	Deep green	Very small (<80 g)
15	RE1	Rethimno	Fruit	Large (height >2.1 m)	Flat (width > height)	Spreading	Strong	Ovate	Moderate	Moderate	Green	Small (81–120 g)
16	RE2	Rethimno	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Spreading	Strong	Ovate	Moderate	Moderate	Green	Small (81–120 g)
17	XA1	Chania	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Spreading	Very strong	Ovate	Moderate	Few	Green	Small (81–120 g)
18	XA2	Chania	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Spreading	Very strong	Ovate	Few	Moderate	Green	Small (81–120 g)
19	XA3	Chania	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Spreading	Very strong	Ovate	Few	Few	Green	Small (81–120 g)
20	XA4	Chania	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Spreading	Very strong	Elliptic	Few	Few	Green	Small (81–120 g)
21	XA5	Chania	Fruit	Medium (height 1.6–2.0 m)	Flat (width > height)	Spreading	Very strong	Ovate	Few	Moderate	Green	Very small (<80 g)
22	LA1	Lasithi	Fruit	Small (height <1.5 m)	Flat (width > height)	Shrubby	Strong	Ovate	Many	Moderate	Green	Very small (<80 g)

populations (Wolfe et al., 1998). The main drawback of this method is the dominant mode of inheritance of ISSR bands, which reduces the information provided by locus. Nevertheless, the intra-specific diversity of *O. ficus-indica* genotypes has to be further investigated. The aim of this study is to use ISSR molecular markers as a tool to estimate the genetic diversity in a collection of Barbary fig accessions from the Institute for Olive Tree and Subtropical Plants of Chania in Greece.

2. Materials and methods

Twenty-two Barbary fig (*O. ficus indica*) accessions, well representative of the *O. ficus indica* germplasm in Greece were used in this study. They were sampled from 9 localities, the most well known regions sheltering various morphological sorts of Barbary figs in the country (Table 1). All these accessions exist in the ex situ collection of the Institute for Olive Tree and Subtropical Plants in Chania (Table 1) and represent the richest germplasm bank in Greece resulting from a multiyear national survey. The descriptions of some of the Barbary fig accessions are available in Online European Minor Fruit Tree Species Database (EMFT Database, http://www.ueresgen29.unifi.it/netdbase/db1.htm). DNA was isolated from the outermost layer of mature cladodes using the NucleoSpin Plant II (Macherey-Nagel, Duren, Germany) kit according to the manufacturers guide. DNA yield was determined using a UV Spectrophotometer (JENWAY 6405UV/VIS, UK) at A260 nm, whereas DNA purity was estimated according to the A260/A280 ratio. Samples were then diluted to a 20 ng/μL working concentration.

ISSR amplification was performed in a total volume of 20 μL including 20 ng genomic DNA, 200 mM of each dNTPs, 40 pmol primers, 2 μL 10× KAPATaq DNA Polymerase buffer, and 1 U KAPATaq DNA Polymerase (KapaBiosystems, Cape Town, South Africa). PCR amplifications were performed in a MasterCycler (Eppendorf, Hamburg, Germany) as follows: an initial step of 5 min at 94 °C, followed by 35 cycles, each one including 30 s at 94 °C for denaturation, 90 s at 38 °C to 55 °C (depending on the primer) for annealing, and 90 s at 72 °C for elongation. A 5-min step at 72 °C was programmed as a final extension. PCR amplification products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. A 100-bp or 1-kb DNA ladder (Invitrogen, USA) was used as a size marker. The selected ISSR primers (Invitrogen; Table 1) were used for PCR amplification. Gels and images were analyzed using the UVIDoc software (UVItec, Cambridge, UK) to quantify signal intensity and band size. All the experiments were performed in duplicate.

The ISSR fragments were classified as present (1) or absent (0), and were typed into a computer file as a binary matrix. The matrix was then analyzed by FreeTree v. 0.9.1.50 software (Hampl et al., 2001). Similarity of qualitative data was calculated using the Nei and Li/Dice similarity index (Nei and Li, 1979), and similarity estimates were analyzed using UPGMA (unweighted pair group method using arithmetic averages). The matrices of mutual coefficients of similarity calculated by FreeTree were converted to MEGA 4 v.4.1 software (Tamura et al., 2007) and resulting clusters were expressed as dendrogram. The binary data for individuals was subjected to the PCoA (Peakall and Smouse, 2006), and the first two principal coordinates were plotted to indicate the multilateral genetic relationships among the Barbary fig accessions.

For statistical analysis, Nei's gene diversity (He) (Nei, 1973) and Shannon's information index (I) (Lewontin 1972) were estimated via the POPGEN 1.32 software (Yeh et al., 1997). Resolving power (Rp) of a primer is: $Rp = \Sigma\, IB$ where IB (band informativeness) takes the value of: $1 - [2 \times (0.5 - p)]$, p being the proportion of the 22 Barbary fig accessions analyzed containing the band (Prevost and Wilkinson, 1999).

3. Results and discussion

In this study we have demonstrated the reliability of ISSR markers to detect DNA polymorphisms and relationships within the richest collection of Barbary fig accessions in Greece. The set of six ISSR primers

Table 2
Details of ISSR primers used, number of markers obtained, polymorphism and genetic diversity indices of ISSR markers from twenty-two Barbary fig accessions.

Primer (UBC)	Primer sequence (5′–3′)	Annealing Temperature (°C)	Fragment size range (bp)	Fraction polymorphic fragments	Percentage polymorphism (%)	Gene diversity (GD)	Shannon index (I)	Resolving power (R_P)
807	AGAGAGAGAGAGAGAGT	53	800–3000	2/10	20	0.180	0.360	5.8
811	GAGAGAGAGAGAGAGAC	53	600–3000	6/12	50	0.087	0.267	2
827	ACACACACACACACACG	53	1200–4000	2/8	25	0.219	0.245	2.05
834	AGAGAGAGAGAGAGACYT	53	150–5000	8/13	61.5	0.294	0.505	8
841	ACACACACACACACYA	53	200–2900	6/13	46.1	0.346	0.447	6
891	HVHTGTGTGTGTGTGTG	53	850–4100	5/11	45.4	0.164	0.309	5
Mean	–	–	–	4.8/9.5	50.5	0.215	0.355	4.80

showed multiband patterns in each accession (Table 2). This primer set amplified a total of 57 reliable bands from the DNA of 22 Barbary fig genotypes tested. The total number of bands scored ranged from eight to thirteen and the number of polymorphic bands from 2 to 8 per primer. Primers UBC807 and UBC827 resulted in the smallest number of bands (two) and primer UBC834 generated the largest number of bands (eight). The average number of bands per primer was 9.5. The number of polymorphic bands generated by ISSR was slightly higher (4.80/primer) than those found in other studies, such as Wang et al. (1998), who reported an average of 4.31 bands/primer in Opuntia accessions collected in Texas, Mexico and Chile. Meanwhile, Zoghlami et al. (2007) found an average of 4.37 bands per primer among 32 Barbary fig accessions collected in Tunisia. Recently, Valadez-Moctezuma et al. (2014) characterized, using ISSR markers, fifty-two nopal varieties belonging to 12 species of the genus Opuntia and revealed an average of 14 polymorphic bands per primer which should be attributed to the increased number of Opuntia species used.

Band size ranged from 150 bp (UBC834) to 5.0 kb (UBC834). Among analyzed genotypes, 29 (50.5%) of the ISSR bands were polymorphic.

The diversity of the germplasm collection was represented with Nei's gene diversity (GD), as well as Shannon's information index (I). Data for GD and I for all the twenty-two accessions were analyzed using 6 ISSR markers and their corresponding mean values were found as 0.215 and 0.355, respectively (Table 2). The resolving power (R_P) provided a modest indication of the ability of ISSR primers to distinguish between accessions. The R_P of each primer was estimated in order to determinate the most informative ones for the discrimination between Barbary fig genotypes. The R_P of the 6 primers ranged from 2.00 for primer UBC811 to 8.00 for primer UBC834 (Table 2) with a mean value of 4.80. Three of the ISSR primers (UBC807, UBC834 and UBC841) possessed high R_P values (5.80, 8.00 and 6.00, respectively) and are the most efficient for surveying genetic diversity in the 22 cultivars of the collection. However, no single primer allowed the differentiation of all the accessions studied. Herein, the primers analyzed are characterized by high collective R_P rate of 28.85. This value is higher than the one reported by Zoghlami et al. (2007; $R_P = 15.56$) and by Bendhifi et al. (2013; $R_P = 24.17$), which analyzed Barbary fig accessions originating from Tunisia.

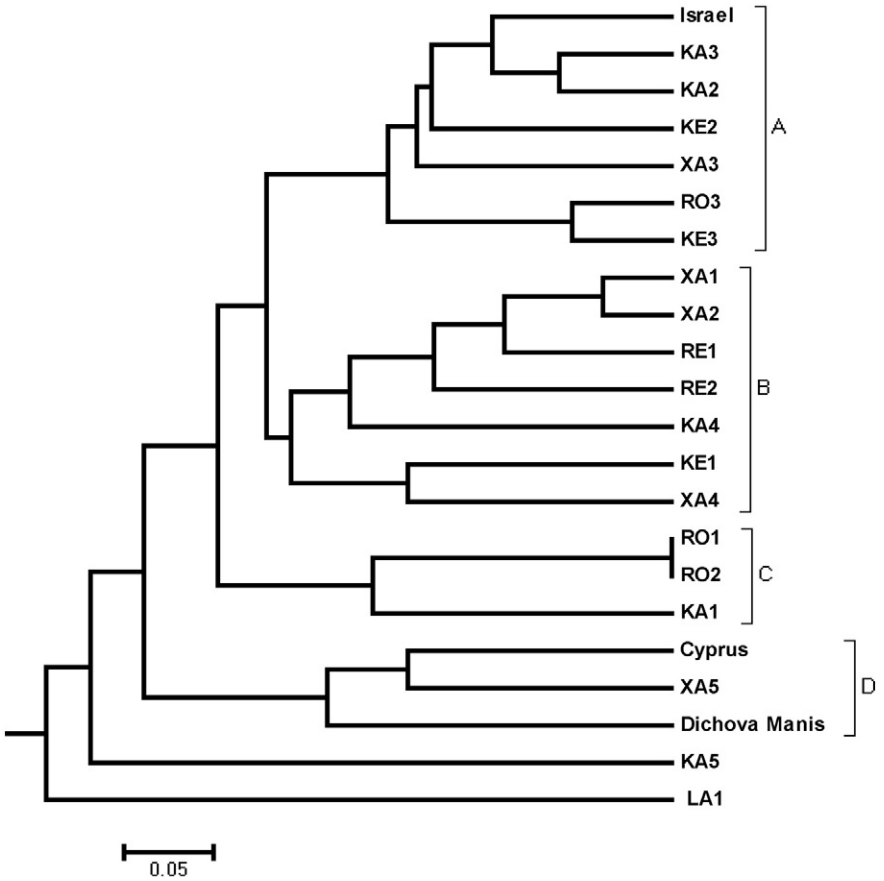

Fig. 1. UPGMA cluster analysis of twenty-two Barbary fig accessions with ISSR markers. The dendrogram was made using the Nei and Li/Dice coefficient; letters show clusters.

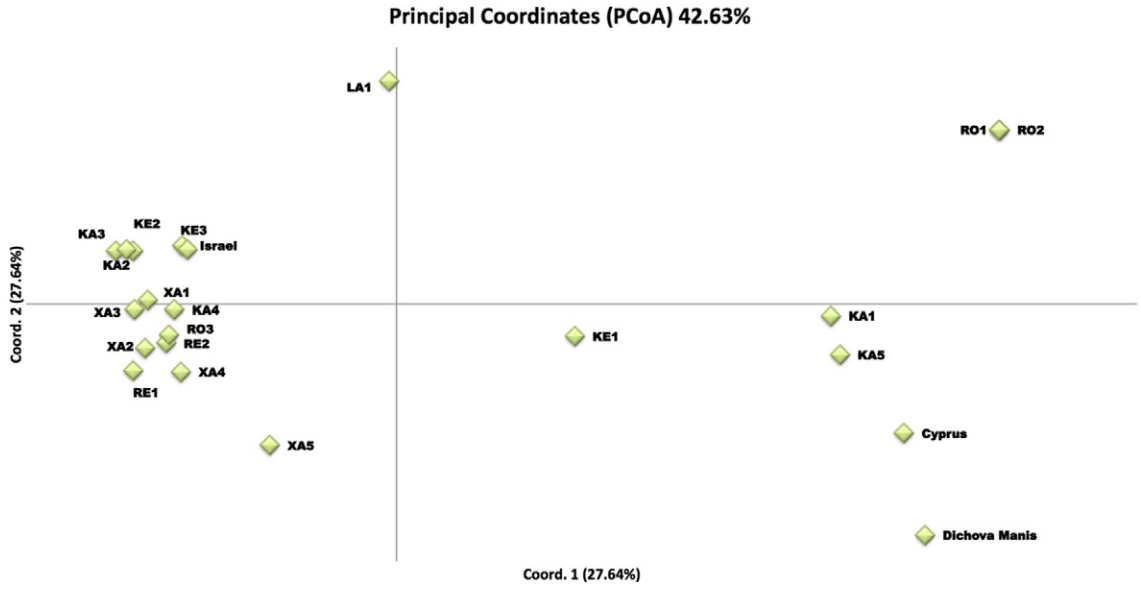

Fig. 2. Principal coordinate scatter plot of twenty-two accessions based on ISSR markers.

Estimates of genetic relatedness were obtained from ISSR marker data using the similarity coefficient of Nei–Li/Dice. The genetic distance matrix varied between 0.181 and 1.000. Thus, the genotypes analyzed showed high genetic diversity. The smallest similarities were recorded between "LA1" and "XA3" while the largest similarities were recorded between "RO1" and "RO2" (0.100). Additionally, high similarities between the accessions "XA1" and "XA2" (0.923) were also found.

Results of the ISSR analysis have shown that the twenty-two accessions are classified in 4 groups named from A to D in the dendrogram depicted in Fig. 1. Genotypes "KA1" (from Kalamata, Greece) and "LA1" (from Lasithi, Greece) are not placed in clades, probably as a result of isolation. Accessions from Rethimno region, "RO1" and "RO2", were identical at the analysed loci suggesting that these two genotypes should be considered synonyms.

The PCoA scatter plot (Fig. 2) further supported the dendrogram (shown in Fig. 1) results in a robust way, considering the high percentage of the total genetic diversity (PC-1 & PC-2 = 42.63%).

The selected ISSR primers showed informative and clear profiles; therefore they are useful to estimate intraspecific variability in the Opuntia species. These results agree with those obtained by Valadez-Moctezuma et al. (2014) in Opuntia and by Ganopoulos et al. (2011a), Ganopoulos et al. (2011b) in other species using ISSR markers.

In conclusion, the results presented above allow the efficient use of the ISSR markers for the genetic analysis of Barbary fig. The collection of the ISSR primers used herein produced an adequate number of bands which allows for the genetic diversity analysis. Based on the results, the genetic diversity among 22 accessions has been also discussed. This study shows once more the importance of genetic analysis using molecular markers in order to both confirm the differentiation of Barbary fig varieties and also provide the means for certification purposes of the Barbary fig varieties. Hence, we used the ISSR molecular markers in order to investigate for the first time the genetic background of the Greek Barbary fig germplasm. Moreover, we have successfully fingerprinted and differentiated at the DNA level all Barbary fig accession serving towards their preservation in a reference collection. Thus, the molecular characterization and the preservation of such crops could assist breeders through a definitive taxonomic sorting and the selection of interesting ecotypes that could be included in human and animal diets. For example, human and/or animal consumption of Barbary fig fruits could substantially increase by breeding for glochid (specialized short hair like spines) free cultivars with reduced seed content.

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