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Genetic relations among Moroccan *Opuntia* genotypes with different degrees of resistance to *Dactylopius opuntiae*

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Abstract. Genetic diversity and relationship among a set of 18 cactus pear genotypes, with different degrees of resistance to cochineal scale insect (*Dactylopius opuntiae*), was estimated using eight simple sequence repeat (SSR) markers. The genotypes used belong to four *Opuntia* species (*O. engelmanii, O. ficus indica, O. robusta,* and *o. dillenii*). The analysis revealed a total number of 56 alleles (Mean = 7) and an average genetic diversity index of 0.76 with genetic distances ranging from 0.00 to 1.00 at eight microsatellite loci in 18 Moroccan cactus pear genotypes. All microsatellites used were found to be highly informative, with mean polymorphic information content (PIC) estimated at 0.72. Genetic relationship estimated using the neighbor-joining (NJ) method and the principal coordinate analysis (PCoA), showed that the 18 genotypes were successfully assigned to four clusters, separated according to their taxonomy distribution and their levels of resistance to *D. opuntiae*. The results of this study demonstrated that the Moroccan cactus pear genotypes evaluated are highly divergent and that these genotypes will be useful for future crossing programs to improve the genetic diversity in *Opuntia* for resistance to *D. opuntiae.*

Keywords: *Cactus pear genotypes, tolerance, false carmine cochineal, genetic diversity, SSR markers*

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Introduction

The cactus pear (*Opuntia ficus-indica* L. (Mill.)) is a native plant of Mexico, which belongs to the *Cactaceae* family (El-mostafa *et al.,* 2014). This plant is very suitable for the ecological conditions of arid and semi-arid regions of the world (Tilahun and Welegerima, 2018). In Morocco, cactus pear culture plays a prominent role in the rural populations. It is a source of food for humans and feeds for livestock and an appropriate crop for land rehabilitation in the arid regions (Arba *et al.,* 2002). This drought-tolerant crop has various agro-inustrial uses such as cosmetics and pharmaceutical products (Barbera *et al.,* 1995). Unfortunately, during the last several years, the cactus pear has been subjected to the severe attack by the false carmine cochineal scale inset, *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae), which was first reported in Morocco in September 2016 in the Doukkala regions (Bouharroud *et al.,* 2016). This scale insect is characterized by high proliferation and rapid spread to other regions of the country. This insect is considered the most serious pest of cactus pear in several parts of the world (Menezes, 2005). Great economic losses due to *D. opuntiae* have been reported (Vasconcelos *et al.,* 2009) in many regions

of Brazil. Integrated pest management for this devastating pest explores host plant resistance as a promising strategy. The use of resistant cultivars reduces production costs, decreases insect populations, and simplifies the management of the pest by the farmer (Agrawal and Heil, 2012). In Brazil, some resistant varieties to *D. opuntiae* have been reported (Borges *et al.,* 2013; Santos *et al.,* 2006; Silva *et al.,* 2009; Vasconcelos *et al.,* 2009). Recently in Morocco, eight varieties resistant to *D. opuntiae* have been identified (Sbaghi *et al.,* 2019). In addition, some studies identified ten highly resistant genotypes of *D. opuntiae* under laboratory and greenhouse conditions (Akroud *et al.,* 2021).

Genetic diversity studies provide the analysis of a given trait by differences or similarities among the genotypes tested. Genetic diversity allows the grouping of the genotypes and simplifies the research of a common characteristic among these genotypes (Mergulhão *et al.,* 2012). Determining the level of genetic relationships among and within plant populations is a primary step for genetic resource conservation and breeding programs (El Kharrassi *et al.,* 2017). Various strategies have been used to assess the degree of genetic diversity based on morphological, biochemical, and molecular markers. Using molecular approaches can be quick and simple to start diversity an assessment (Velasco-Ramírez *et al.,* 2014). Particularly, molecular markers are a powerful tool in the characterization and evaluation of genetic diversity within and among species (Charcosset and Moreau, 2004). Microsatellites or simple sequence repeats (SSRs) have several advantages. They are simple to use, are inexpensive, require little DNA, and reveal a high degree of polymorphism (Powell *et al.*, 1996; Hokanson *et al.,* 1998). The characterization of the cactus genotypes by SSRs is useful to identify genotypes, estimate the genetic distance and relationships among accessions (Caruso *et al.,* 2010), and identify resistant genotypes (Tar'an *et al.,* 2007). In this context, the aim of our study was to evaluate using SSR markers the genetic diversity among 18 genotypes of cactus pear with differing degrees of resistance to *Dactylopius opuntiae*.

Material and Methods

Plant material

The analysis by SSR markers compressed a set of 18 genotypes of *Opuntia* from Morocco with three levels of resistance to *D. opuntiae*. The genotypes tested in this study were obtained from the Melk Zhar-Belfaa experimental station of the National Institute of Agronomic Research (INRA-Agadir), Morocco where a collection of more than 400 accessions has been maintained since1999.

The resistant plants did not show attachment of the insect colonies under greenhouse tests, and included ten genotypes (accession numbers 124, 189, 286, 319, 321, 251, 320, 311, 295 and 322) (Figure 2). For the tolerant category, the host-plant shows an ability to grow and reproduce itself or to repair injury to a marked degree in spite of supporting a population of pests equal to that damaging a susceptible accession. The tolerant category included four genotypes (accession numbers 003, 005, 006 and 007) and the susceptible accessions included four genotypes with accession numbers 022, 024, 034, and 049 (Table 1).

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Table 1. The cactus pear genotypes with different categories of resistance to the insect *Dactylopius opuntiae* and their collection sites.

Degree of resistance	Accession numbers	*Opuntia* Species	Origin or collection site
Resistant	124	*O. ficus-indica*	Dcheira Inezgane
	189	*O. ficus-indica*	Dcheira Inezgane
	286	*O. engelmanii*	Dcheira Inezgane
	319	*O. engelmanii*	Marrakech
	321	*O. engelmanii*	Marrakech
	251	*O. engelmanii*	Dcheira Inezgane
	320	*O. engelmanii*	Marrakech
	311	*O. robusta*	Bouznika irradiated
	295	*O. robusta*	Bouznika
	322	*O. dillenii*	Belfaa
Tolerant	003	*O. ficus-indica*	Cultivar "Israele monastra"
	005	*O. ficus-indica*	Cultivar "Millitello white"
	006	*O. ficus-indica*	Cultivar "White roccapalumba'
	007	*O .ficus-indica*	Bouskoura
Susceptible	022	*O. ficus-indica*	Tiznit
	024	*O. ficus-indica*	Ait Zkri –Anouguel
	034	*O. ficus-indica*	Cross
	049	*O. ficus-indica*	Ait Baamrane cultivar Aissa

Figure 1. *Opuntia ficus-indica* resistant genotypes to *Dactylopius opuntiae*

Sample prepration

One piece of each cladode (genotype tested) was cut, selecting the thick cuticle to avoid mucilaginous tissue. The vegetal material was separated out, lyophilized to complete dryness at -54 °C), and ground to a fine powder using a Geno-Grinder® 2010 grinder.

DNA extraction

Total genomic DNA was isolated from 50 mg of lyophilized-powdered tissue using the Saghai-Maroof *et al.* (1984) procedure of Cetyl Trimethyl Ammonium Bromide, with slight modification

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(Udupa et al. 1998). The quality and concentration of the extracted DNA were checked using 1% agarose gel with 1x TBE buffer and were stained with ethidium bromide. The intensity of the bands was visualized under UV and the concentration was estimated by comparing bands to known concentrations of lambda DNA. Using eight SSR markers (Caruso *et al.,* 2010; Table 2) to estimate the genetic diversity among the 18 genotypes selected.

The Polymerase Chain Reactions (PCRs) were performed in a total volume of 10 µL containing 1 µL template DNA (25-30 ng) and 9 µL PCR master mix composed of 4.95 µL sterile distilled water, 2 µL 5× GoTaq buffer (Promega), 1 µL 200 µM dNTPs (Promega), 1 µL 10 pmol /µL forward and reverse primers and 0.05 µL (0.25 U) Taq DNA polymerase (Promega).

The amplification reaction was generated in the Eppendorf Master Cycler with initial denaturation at 94 °C for 5 min followed by 35 cycles of each cycle with 60 seconds of denaturation at 95 °C, 60 s annealing at 58-60 °C depending upon T_m of individual prime pair (Table 2) and elongation step during 90 s at 72 °C. The final extension was carried out at 72 °C for 5 min followed by cooling at 4 °C for an indefinite period. Amplified products were separated on 8% (w/v) polyacrylamide gels. The amplified bands were detected by ethidium bromide staining and estimate the molecular sizes of the DNA fragments using a 100-bp DNA ladder.

Table 2. The list of eight SSR markers used for genetic diversity analysis in the cactus pear genotypes with different degrees of resistance to *Dactylopius opuntiae.*

Microsatellite loci	Repeat	Forward and reverse primer	T_m in °C	Expected size (bp)
Ops 9	$(TGA)_9$	F : AACTGCCTCACACGAGTTCC R : GCTACGAAATCTGCCGAGTC	60	163
Ops 24	$(CT)_{24}$	F : TCCTTCCATTTCCACCACAC R : CAAGACCCCTCATTCCAAAG	58	274
Opuntia3	$(AG)_{19}$	F : GTGAGTGCCCAGATGAAACT R :TCCTCAACTTTATTGTAGCAAGAG	57	317–344
Opuntia5	$(TAC)_5$	F : TATGCACAAAGCACCATGC R :CCAACCATACCAACTGTACTGAC	58	352–367
Opuntia9	$(AG)_{15}$	F: CTAGGCTTCATCCCACATTAGG R: TCCAAATTCACCTCCTCTGC	59	147–185
Opuntia11	$(CT)_{13}TT\ (CT)_2$	F : CCTACACCTGCTGCCAATC R : CGAGACAAACATCAGAGGAG	59	110–138
Opuntia12	$(TC)_4C\ (TC)_{12}$	F :TAATCTTATTCTCAGGTCAGTTAC R : GGTATCTTGTTATTCGTTCG	54	226–294
Opuntia13	$(AG)_{12}$	F : CCAAATACCCAGCCCATAC R : CGAGAACCTAACTTCCGATG	58	247–301

Data analysis

Alleles amplified by microsatellite primers for each genotype were scored. After the gel band readings, a binary matrix was constructed. The data matrix was analyzed using Power Marker software version 3.25 (Liu and Muse, 2005) to calculate the number of alleles, genetic diversity index (Nei 1987), and PIC (Botstein *et al.,* 1980) of each locus. A dendrogram was constructed based on a genetic distance (Nei, 1987) and NJ method (Saitou and Nei, 1987) and visualized

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using MEGA5 software (Tamura *et al.,* 2011). The PCoA was undertaken using GenAlEx 6.5 software (Peakall and Smouse, 2006).

Results

Efficiency of the primer

Based on the sizes of the amplified fragments of eight microsatellite loci in the 18 cactus genotypes, a total number of 56 alleles were identified, which varied in the number detected per locus from five (*Opuntia3*) to eight (*Opuntia11, Opuntia12,* and *Opuntia13*) with an average of seven alleles.

The microsatellite markers used in this study also showed different levels of genetic diversity, which varied from 0.65 (*Opuntia9*) to 0.82 (*Opuntia12*) with an average of 0.76 (Table 3). This confirms that the chosen primers were efficient enough to detect diversity in the 18 cactus pear genotypes tested. The *Opuntia12, Opuntia11* and *Opuntia13* showed the highest PIC value (0.80 and 0.76, respectively).

Table 3. Major allele frequency, the number of alleles, genetic diversity, and Polymorphism Information Content (PIC) at eight microsatellite loci in the 18 cactus pear genotypes studied.

Primer	Major Allele. Frequency	Allele No	Genetic diversity (H)	PIC
Ops9	0.33	7	0.75	0.72
Ops24	0.33	7	0.78	0.75
Opuntia3	0.39	5	0.71	0.66
Opuntia5	0.33	6	0.77	0.73
Opuntia9	0.56	7	0.65	0.63
Opuntia11	0.33	8	0.78	0.76
Opuntia12	0.28	8	0.82	0.80
Opuntia13	0.33	8	0.78	0.76
Total		56		
Mean	0.36	7	0.76	0.72
SD	0.08	1.07	0.052	0.05

Genetic relationships and PCoA analysis

The dendrograms showing genetic relationships among all cactus pear genotypes tested are presented in Figure 2. Using all the eight microsatellite markers, we were able to differentiate almost all the genotypes tested except (accession numbers 320, 251, 321, and 319) belonging to *O. engelmanii* and (accession numbers 7 and 22); (accession numbers 34, 24, and 49) belonging to *O. ficus-indica* that were similar for all eight loci and displayed the lowest genetic distance (0.00). The highest genetic distance (1.00) was observed between each pair of different species (Table 4).

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Table 4. Genetic distance based on shared allele frequency among 18 cactus pear genotypes of *Opuntia* with different levels of resistance against *Dactylopius opuntiae*.

Genotypes (Accession number)	***O. ficus-indica* (124)**	***O. engelmanii* (320)**	***O. ficus-indica* (003)**	***O. ficus-indica* (005)**	***O. ficus-indica* (006)**	***O. ficus-indica* (007)**	***O. ficus-indica* (022)**	***O. ficus-indica* (024)**	***O. ficus-indica* (034)**	***O. ficus-indica* (49)**	***O. dillenii* (322)**	***O. robusta* (311)**	***O. engelmanii* (286)**	***O. robusta* (295)**	***O. engelmanii* (251)**	***O. engelmanii* (321)**	***O. engelmanii* (319**	***O. ficus-indica* (189)**
***O. ficus-indica* (124)**	0.000																	
***O. engelmanii* (320)**	1.000	0.000																
***O. ficus-indica* (003)**	0.875	1.000	0.000															
***O. ficus-indica* (005)**	1.000	0.875	1.000	0.000														
***O .ficus-indica* (006)**	1.000	1.000	1.000	1.000	0.000													
***O. ficus-indica* (007)**	0.875	0.875	1.000	0.125	1.000	0.000												
***O. ficus-indica* (022)**	0.875	0.875	1.000	0.125	1.000	0.000	0.000											
***O. ficus-indica* (024)**	0.875	0.875	1.000	0.250	0.875	0.125	0.125	0.000										
***O. ficus-indica* (034)**	0.875	0.875	1.000	0.250	0.875	0.125	0.125	0.000	0.000									
***O. ficus-indica* (049)**	0.875	0.875	1.000	0.250	0.875	0.125	0.125	0.000	0.000	0.000								
***O. dillenii* (322)**	1.000	1.000	0.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000							
***O. robusta* (311)**	1.000	0.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000						
***O. engelmanii* (286)**	0.875	0.125	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.875	0.000					
***O. robusta* (295)**	1.000	0.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.125	0.875	0.000				
***O. engelmanii* (251)**	1.000	0.000	1.000	0.875	1.000	0.875	0.875	0.875	0.875	0.875	1.000	0.875	0.125	0.875	0.000			
***O. engelmanii* (321)**	1.000	0.000	1.000	0.875	1.000	0.875	0.875	0.875	0.875	0.875	1.000	0.875	0.125	0.875	0.000	0.000		
***O. engelmanii* (319)**	1.000	0.000	1.000	0.875	1.000	0.875	0.875	0.875	0.875	0.875	1.000	0.875	0.125	0.875	0.000	0.000	0.000	
***O.ficus-indica* (189)**	0.875	0.875	0.750	0.875	0.875	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.875	1.000	0.875	0.875	0.875	0.000

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The cluster analysis based on the NJ method had grouped the 18 cactus pear genotypes, with different degrees of resistance to *D. opuntiae*, into four groups at a genetic distance level of 0.5 (Figure 2). The first group grouped the two species, *O. engelmanii* and *O. robusta* genotypes, which are resistant to *D. opuntiae*. The second group consists of seven genotypes of *O. ficus-indica,* in which three and four genotypes were tolerant and susceptible to *D. opuntiae*, respectively. The third group also consists of *O. ficus-indica* genotypes, in which one and three genotypes were tolerant and resistant to *D. opuntiae* respectively. The fourth group consists of only one genotype of *O. dillenii,* which is resistant to *D. opuntiae*.

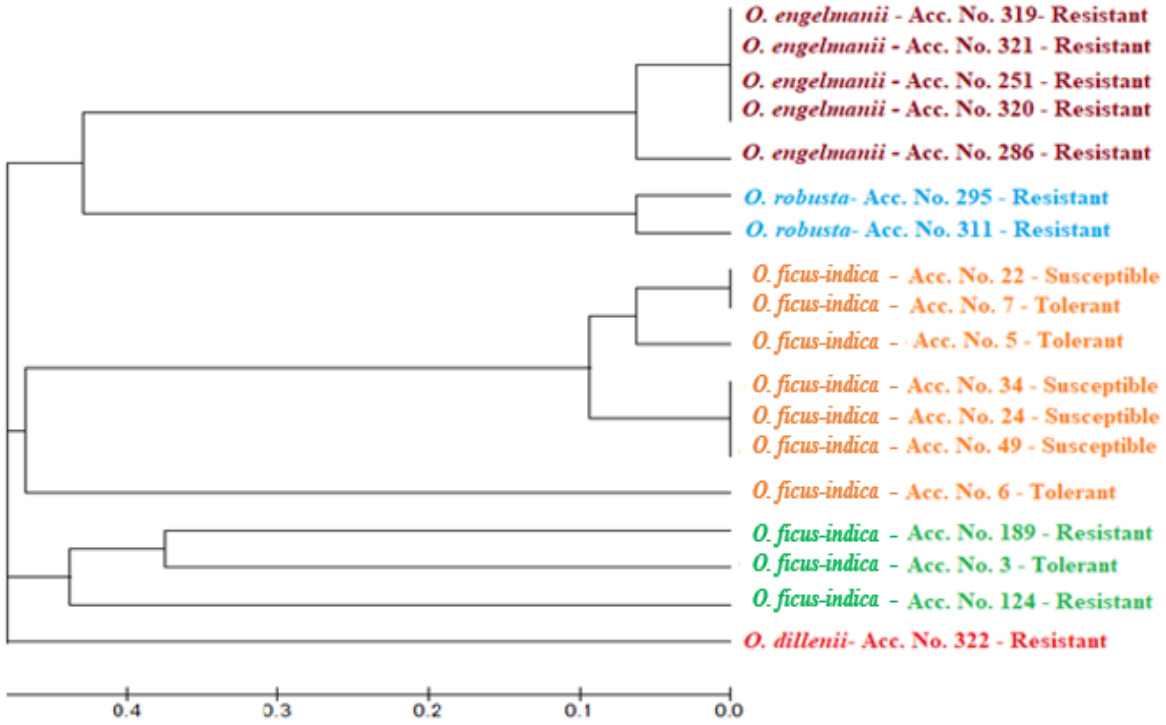

Figure 2. Dendrogram of 18 cactus genotypes of *Opuntia*, with different levels of resistance to *D*actylopius *opuntiae*, relationships revealed by Neighbor joining (NJ) method based on genetic distance (Nei, 1987).

Changes in genetic diversity of cactus pear genotypes over species

The highest genetic distance (1.00) was found between *O. engelmanii* and *O. dillenii, O. dillenii* and *O. robusta*, and *O. robusta* and *O. ficus-indica*, whereas the lowest (0.856) was observed between *O. robusta* and *O. engelmanii* (Table 5).

Table 5. Genetic distance of shared allele among four species of cactus pear

***Opuntia* Species**	***O. dillenii***	***O. engelmanii***	***O. ficus-indica***	***O. robusta***
O. dillenii	0.000			
O. engelmanii	1.000	0.000		
O. ficus-indica	0.960	0.875	0.000	
O. robusta	1.000	0.856	1.000	0.000

The dendrogram (Figure 3) resulting from the cluster analysis based on Nei (1987) genetic distance had separated the four species into two clusters. *O. ficus-indica* and *O. dillenii* were clustered together in one group and *O. robusta* and *O. engelmanii* were assembled in another group.

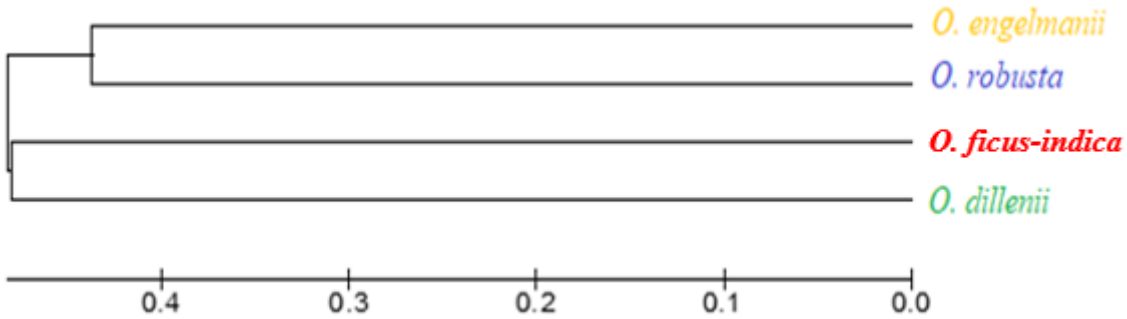

Figure 3. Dendrogram of four species of cactus pear, relationships revealed by neighbor-joining (NJ) method based on shared allele genetic distance.

The genetic structure was analyzed using the principal coordinates analysis (PCoA). The results of PCoA analysis corroborate those obtained from the cluster analysis of Neighbor Joining (NJ) method (Figure 4). The same grouping obtained by the NJ method was achieved by PCoA analysis, in which the resistant genotypes of *O. engelmanii* and *O. robusta* form one group. The susceptible genotypes of *O. ficus-indica* assemble into the second group and the resistant genotypes of *O. ficus*- indica and *O. dillenii* group into the third group.

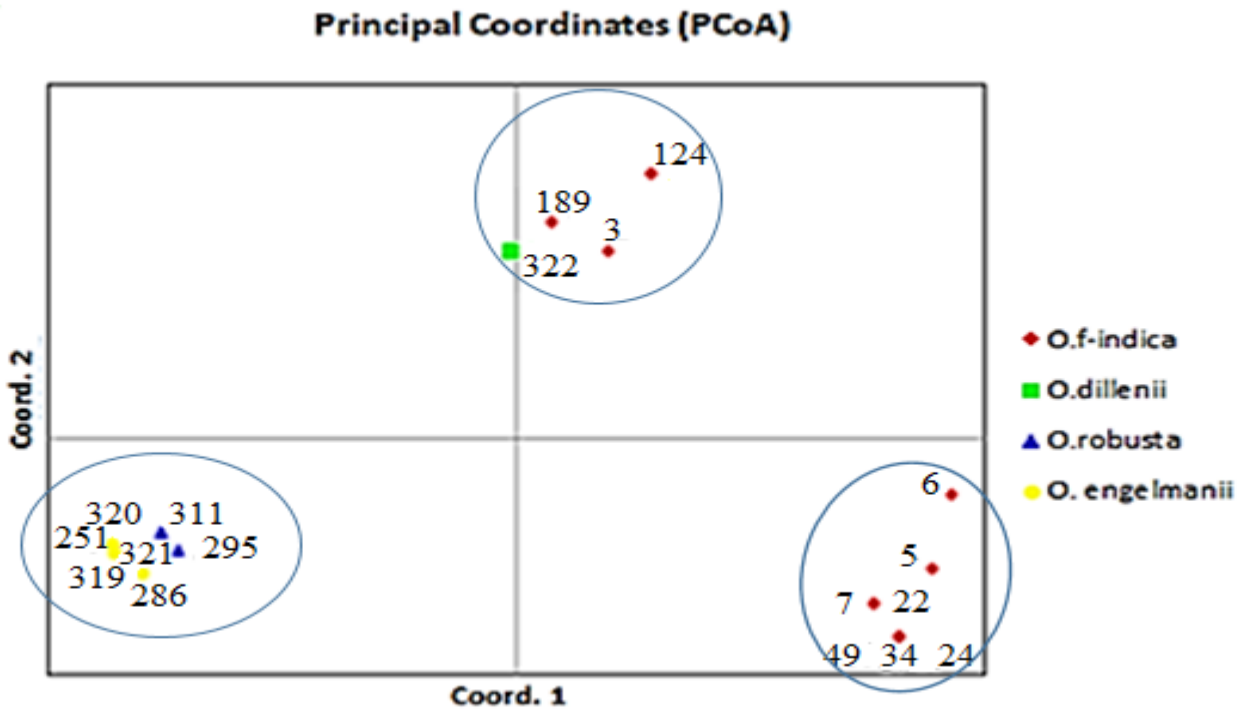

Figure 4 Principal Coordinate Analysis (PCoA) plot of the 18 cactus pear genotypes of *Opuntia*, with different levels of resistance to *Dactylopius opuntiae*, based on their genetic distance.

Discussions

The markers used in 18 genotypes revealed specific profiles which can be used for cultivar identification and showed polymorphism with a PIC value above 0.5. According to Botstein et al.

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(1980), PIC>0.5 is considered a highly informative marker, and therefore, the eight SSR markers used in this study were all highly polymorphic.

The total number (56) and mean (seven per loci) of alleles detected among 18 cactus pear genotypes at eight SSR loci, is slightly less than the total and mean number of alleles per loci reported by Nefzaoui *et al.* (2019). When the same markers were used by Nefzaoui *et al.* (2019) the obtained results revealed a total of 72 alleles with an average of nine alleles per locus. In fact, Nefzaoui's study tested a higher number of genotypes (*n* = 50) and more genetically diverse materials (Brazil and Morocco) compared to the current study that tested 18 genotypes and only Moroccan originated material. Indeed, El Finti *et al.* (2016) revealed a lower number with 45 alleles with a mean of 4.5 at ten SSR loci in 13 Moroccan origin cultivars. The current study, Caruso et al. (2010) and *Nefzaoui et al.* (2019) confirmed that (i) the primers tested were efficient enough to detect diversity in the cactus pear genotypes, belonging to different species, and (ii) they can be used as fingerprints for the cactus pear identification.

All the tested genotypes of cactus pear, *O. engelmanii* (five genotypes), *O. robusta* (two genotypes), and *O. dillenii* (one genotype) showed resistance, whereas only two genotypes of *O. ficus-indica* (accession No. 124 and 189). The rest of *O. ficus-indica* genotypes either showed tolerance reactions (four genotypes) or susceptible reactions (four genotypes). No polymorphism or very little polymorphism was evident among the susceptible genotypes (genetic distance ranged between 0-0.125) indicating that they are likely the same clones with very minor changes genetic changes arising from somatic mutations in vegetative cells.

Other studies have been carried out on the genetic diversity of the cactus pear using different molecular and methods have also been useful in categorizing the different species of *Opuntia.* For example, Zoghlami *et al.* (2007) have assessed the genetic diversity of *O. ficus-indica* in Tunisia using RAPD markers. In the same country, Bendhifi Zarroug *et al.* (2015) published on the genetic diversity of cactus species using RAPD markers. Valadez-Moctezuma *et al.* (2014) studied the genetic diversity within and among Mexican *Opuntia* species using RAPD and ISSR markers. In Italy, Caruso *et al.* (2010) investigated the level of intraspecific genetic diversity among *O. ficus-indica* cultivated varieties and some related species using SSRs markers. Also, Labra *et al.* (2003) assessed the genetic diversity in *Opuntia* species with cpSSR and AFLP markers. In Brazil, Mergulhão *et al.* (2012) studied the genetic diversity within five varieties of *O. ficus-indica* using ISSR and RAPD molecular markers.

Recently in Morocco, several genetic diversity surveys have been carried out on cactus pear, using different molecular markers (SSR, ISSR, and RAPD) providing more information on the biodiversity and genetic diversity of the local cacti *(EL FINTI et al., 2016; El Kharrassi et al., 2017; Nefzaoui et al.*, 2019). All these results show the relationships of varieties collected from different regions and compare the efficiency of the molecular markers used.

The species *O. dillenii*, in which we found only one resistant genotype was the most distant species. The last species *O. ficus-indica* that includes resistant, tolerant, and susceptible genotypes were subdivided into susceptible and tolerant, and resistant groups. Cluster analysis identified four groups of cactus genotypes according to their resistance to *D. opuntiae* and their taxonomy distribution. The species *O. engelmanii* and *O. robusta* that showed resistance to *D. opuntiae* were closest and were included in one group. This agrees with the results obtained by Bendhifi Zarroug et al. (2015), the first group was subdivided into 2 sub-clusters: subcluster A1 included *O. ficus-indica* cultivars and one species represented by *O. engelmannii*, and subcluster A2 grouped the

remaining cultivars such as *O. robusta*. Probably low genetic distance within species of *O. engelmanii and O. robusta* indicates that these genotypes were derived from the same clones and were planted in different locations.

This study is the first report on the genetic characterization of Moroccan susceptible and resistant cactus pear genotypes to *D. opuntiae using* SSR markers. The resistant genotypes to *D. opuntiae* were genetically divergent, among and within species. The use of these resistant genotypes, after selection by means of the relationship estimated by cluster analysis, will be useful for breeding programs to improve the genetic diversity and genetic basis for resistance to *D. opuntiae*. The low gene flow among these genotypes reinforces the idea of breeding to create more diverse genotypes resistant to *D. opuntiae*.

Conclusion

The results of this study showed the importance of SSR markers as an efficient tool to estimate genetic differences within species and among species of cactus pear genotypes, with different levels of resistance to the false carmine cochineal scale insect. The information generated in this study will be useful for the cactus breeding program towards increasing the genetic diversity and developing cochineal scale-resistant genotypes. Future studies should be conducted to (i) identify closely linked molecular markers for assisted breeding and (ii) investigate the genes involved in the defense responses against feeding by a cochineal scale.

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Conflict of interest disclosure

All the authors of this study declare they have no financial interests, which are associated with this manuscript.

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