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*Research Paper*

# Morphological and molecular evaluation of the genetic diversity of Tunisian local date palm pollinators

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**ABSTRACT**

*Phoenix dactylifera* L. is widely distributed and plays an important socio-economic role in southern Tunisia. The objective of this work was the search for morphological and molecular markers useful for the identification and analysis of genetic diversity of date palm pollinators in the region of Djerid in Tunisia. Thirty one (31) morphological traits were used for the morphological study of 38 date palm pollinators. Therefore, the pollinators showed a significant difference between them, with a similarity index ranging between 0.161 and 0.548 that allows grouping them into six groups. The molecular study, using 6 ISSR primers, gave 41 reproducible bands in which 37 were polymorphic with the Percentage of 90%, which indicate the discriminating power of ISSR. The statistical analysis showed a significant genetic diversity with genetic distances ranging from 0.512 to 0.975 that allow grouping the date palm pollinators studied in two groups. The results obtained show that the ISSR is an effective tool for the detection of molecular polymorphism in date palm pollinators.

**Key words:** Date palm, pollinators, genetic diversity, morphological traits, molecular markers (ISSR), PCA.

## INTRODUCTION

*Phoenix dactylifera* L. (2n = 2x = 36) is dioecious, perennial, monocotyledon fruit tree that belongs to the family Arecaceae. Date palm has been domesticated since 3000 BC in Mesopotamia (Nixon, 1959) and it's mostly grown in the arid regions of Africa, the Middle East and South Asia (Khan et al., 2012). In Tunisia, oasis detects an important genetic heritage that consists of nearly 250 date palm cultivars (Rhouma, 2005). There are more than 4 million trees growing approximately on 32,000 ha of oases. All commercial varieties are female especially and there is no method yet for producing male palms of these varieties. However, the effects of pollen on date quality through metaxenia are well documented, and male genotypes with desirable qualities are maintained in the plantations and commonly used to hand pollinate female trees.

Tunisian plantations, despite their greater genetic diversity, are characterized by the abundance of an elite variety called "Deglet Nour". This trend has contributed significantly to genetic erosion in this plant genetic heritage and it tends to accelerate its vulnerability to biotic and abiotic stresses. The extension of the monovarietal culture can have harmful repercussions. Deglet Nour currently comprises approximately 60% of the Tunisian palm plantations (Rhouma, 2005). Others constraints which restrict date palm culture and contribute to the decrease of genetic diversity of the palm groves in the Tunisia are drought, salinity, desertification, the old age of the palm trees and a vascular fusariosis caused by *Fusarium oxysporum* f. sp. albedinis, which remains the most serious disease (Haddouch, 1996).

Therefore, it is imperative to develop a strategy for evaluating genetic diversity and the preservation of genetic material of Tunisian palms. Many studies have been conducted to identify the genotype of Tunisian date palm

using either morphological traits or biochemical markers (isoenzymes) (Rhouma, 1994; Reynes et al., 1994; Ould Mohamed Salem et al., 2008). In addition, the data based on molecular markers such as RFLP and RAPD markers were also used to characterize genotypes of date palms (Sedra et al., 1998; Trifi et al., 2000; Trifi., 2001; Ameer et al., 2014).

However, the evaluation of pollinators and more specifically their morphological characterization are very rare and those using molecular tools are lacking in the literature. Only limited studies on the analysis of metaxenic effects have been reported (Ben Salah and hallali, 1996). In this context, it is essential to assess the genetic diversity of Tunisian male palms as they represent an important link in the dates production chains.

In this study, thirty eight local date palm pollinators collected front the region of Djerid were described using some morphological traits. Analysis of principal components (PCA) was also dealt. Morphological distance clustering and phenologic ISSR clustering were compared to estimate correlation between morphological traits and ISSR markers.

## MATERIALS AND METHODS

### Plant material

A set of samples consisting of thirty eight date palm pollinators was subject of this study. These individuals are planted in the experimental plot of Tozeur and so they are submitted to the same bioclimatic conditions.

The different studied pollinators are appointed by PN followed by a number according to their position in the experimental plot and they are PN2, PN3, PN8, PN9, PN10, PN11, PN12, PN14, PN15, PN16, PN17, PN18, PN21, PN49, PN55, PN81, PN93, PN124, PN129, PN132, PN172, PN178, PN179, PN182, PN183, PN184, PN189, PN190, PN192, PN193, PN194, PN195, PN196, PN197, PN199, PN201, PN202 and PN203.

### Morphological analysis

The different accessions were first characterized and evaluated based on phenotypic characteristic. Thirty one vegetative characters (Table 1) were selected from the descriptor of the date palm (*P. dactylifera* L.) (IPEGRI, 2005) for the morphological study. The various measurements should be made on healthy plants of the same age (not less than 5 years of production, 13 to 15 years of age). In this study, we are interested only in the growth descriptors and the palms descriptors.

### Extraction, purification and quantification of the DNA

Total nuclear DNA was extracted from young leaves using Qiagen Plant Mini Kit. After purification, the resultant DNA was quantified using 0.8% agarose gel electrophoresis as described by Sambrook et al. (1989). DNA was then quantified at 260 nm using a spectrophotometer (standard CECIL CE2501 series 2000/3000): 5 µL DNA samples was diluted in 995 µL of Tris-EDTA (TE) buffer and compared with a control containing 1000 µL of TE. The DNA concentration (C) was calculated as follows: C (µg µL-') = DO260 ×10. The DO260 / DO280 ratio was also calculated to determine DNA purity.

### PCR amplification and amplified product electrophoresis

DNA polymorphism was detected by polymerase chain reaction (PCR) using ISSR primers. Twelve primers were tested on DNA samples, DNA amplification was carried out in a final volume of 25 µL containing 12.5 µL of ready mix Promega (buffer with MgC12, dNTP and Taq polymerase), 40 µM oligonucleotide primers, 25 ng of genomic DNA. The program of amplification; using a thermocycleur (Bio radicycler) consisted of a pre-denaturation cycle of 1 min at 94°C, 25 cycles of a denaturation for 30 s at 94°C, an hybridization for 1 min at Tm of each primer, an extension for 2 min at 72°C followed by a post-extension cycle for 7 min at 72°C. The amplified DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. The amplified pattern was visualized on a UV transilluminator and photographed using Gel documentation system. Only clear and unambiguous bands were considered for the further fingerprinting scoring, study similarity and discus polymorphism among accessions. ISSR clustering was compared to estimate correlation between morphological traits and ISSR markers.

### Data analysis

Twelve primers were tested but only six primers showed reproducible and polymorphic bands. Clear and distinct amplification products were scored as '1' for presence and '0' for absence of bands (Table 2). The matrix was computed with the unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) by means of NTSYS 2.02 program Software (Rholf, 1993) using the formula of Nei and Li (1979) to generate the genetic distance matrix. The similarity matrices were used in the cluster analyses which were employed to generate dendrogram. PIC was calculated for each primer by formula:

$$PIC = 1 - \Sigma (Pij)^2$$

### Statistical analysis

In order to identify groups of inter-correlated variables for

**Table 1.** Morphological traits in *Phoenix dactylifera* L.

| | Characters | Code | Abbreviations |
|---|---|---|---|
| **Growth descriptors** | Sex | C1 | S |
| | Vigor | C2 | Vg |
| | Port | C3 | Pt |
| | Appearance of the crown | C4 | Ac |
| | Shape of stipe | C5 | Ss |
| | Persistence of cornaf | C6 | Pc |
| | Presence of air releases | C7 | Pa |
| | Presence of fluff mane | C8 | Pf |
| | Ability to produce releases | C9 | Apr |
| **Palm descriptors** | Leaf bending level | C10 | lbl |
| | Rotation of the palm | C11 | Rp |
| | Total length (m) | C12 | Tl |
| | Leaf width (cm) | C13 | Lw |
| | Spined part length (cm) | C14 | Spl |
| | Thickness of the spine | C15 | Ts |
| | Color of the petiole | C16 | Cp |
| | Width of the leaf at the base of the petiole (cm) | C17 | Wlp |
| | Average number of thorns | C18 | At |
| | Rigidity of thorns | C19 | Rt |
| | Pinnaes's Number | C20 | Pn |
| | Number of spine by type of grouping | C21 | Nsg |
| | Maximum thickness of the middle spine | C22 | Mts |
| | Maximal spine width at the middle (cm) | C23 | Mws |
| | Pinnaes's color | C24 | Pc |
| | Pinnaes's flexibility | C25 | Pf |
| | Pinnaes's grouping | C26 | Pg |
| | Apical divergence angle of pennes | C27 | Ada |
| | Maximal pinnae width at the middle leaf (cm) | C28 | Mpw |
| | Maximum length of penne in the middle of the palm (cm) | C29 | Mpl |
| | Pinnae length at the top leaf (cm) | C30 | Plt |
| | Maximal pinnae width at the top leaf (cm) | C31 | Pwl |

**Table 2.** Details of amplification obtained with different ISSR primers.

| Prime | Primer sequence | Annealing temperature | Total number of replicated alleles | Number of polymorphic bands | Percentage of Polymorphism | PIC |
|---|---|---|---|---|---|---|
| **ISSR4** | (GA) 21 | 51°C | 9 | 8 | 88% | 0.80650815 |
| **ISSR5** | (CT) 10A | 60°C | 6 | 4 | 66% | 0.7777715 |
| **ISSR6** | (CT) 10T | 57°C | 4 | 3 | 75% | 0.74747684 |
| **ISSR7** | (GA) 17 | 48.5°C | 8 | 8 | 100% | 0.84022999 |
| **ISSR13** | (TC) 10G | 60°C | 9 | 9 | 100% | 0.82835938 |
| **ISSR14** | (TC) 10C | 57°C | 5 | 5 | 100% | 0.70124178 |
| **Total** | | | 41 | 37 | 90% | 0.78359794 |

date palm pollinators, a PCA was carried out using the XLSTAT 2014 program on all individual. The genetic distances between the different accessions were calculated on the centered and standardized varieties using measured data. The Mantel test (1967) has been applied, through MxComp program (Rohlf and Fisher, 1968) from NTSYSpc

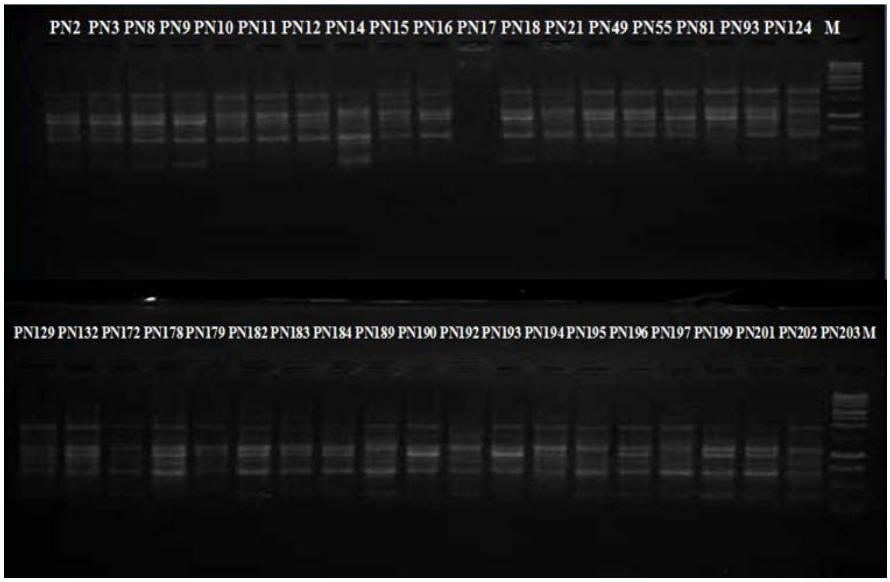


**Figure 1.** Example of an amplification profile obtained by the primer ISSR4. Legend: M: molecular weight markers (1 kb ladder Promega); Numbered wells correspond to the studied accessions.

2.02 software (Rholf, 1993), to establish the correlation between the genetic similarity matrix and the morphological matrix. The principle of this approach is to compare the observed Z - value or r-value with its permutational distribution according to null hypothesis (no difference between the distance matrix, Z = 0). In this comparison, 5000 random permutations were made.

## RESULTS

### Molecular study

Twelve (12) universal ISSR primers were tested in this study. Electrophoresis of the amplified DNA product showed that only 6 primers gave reproducible, polymorphic and intense bands (Figure 1). A total of 41 reproducible bands were obtained using the 6 primers selected, among which 37 are polymorphic with a polymorphism rate of 90%. For each primer, the bands number ranged from 4 to 9, with an average of 6.8. The size of bands ranged from 250 to 3000 bp. The PIC value enables us to measure the level of polymorphism of each marker. PIC values fluctuate from 0.840 for the most polymorphic locus ISSR 7 to 0.701 for ISSR 14, with an average of 0.783 indicating a high level of polymorphism for all loci.

The genetic distance between varieties ranged between 0.512 and 0.975 with an average of 0.743. This indicates that these pollinators are characterized by a high degree of polymorphism at DNA level. The lowest ratio was observed with the combination PN17 and PN193 which indicates the low molecular similarity between these two pollinators. The highest similarity 0.975 was observed between the combination PN3 and PN8. A dendrogram based on UPGMA analysis, using midpoint joining procedure of Nei and Li (1979) (Figure 2) similarity matrix, grouped the 38 pollinators into 2 main clusters, with a similarity rate of 70%. The dendrogram showed 2 main clusters with 20 and 18 genotypes respectively. Cluster A has 5 subclusters. Subcluster A1 is divided into 3 groups: the first set A1-1 has PN2, PN3 and PN8, the second set A1-2 has a single pollinator PN9 and the third set contains 4 pollinators PN11, PN12, PN14 and PN21. The subcluster A2 is divided into two sets; the first set A2-1has PN55 and PN81, the second set A2-2 contains only PN93. The subgroup A3 is divided into two sets: the set A3-1 has PN10 and PN40 and the set A3-2 has PN15 and PN16.
The subcluster A4 is also divided into two sets: a set A4-1 which has PN17 and the set A4-2 contains two pollinators PN19 and Pn18. The sub group A5 has only PN124.
Cluster B was further divided into 4 subclusters. The subcluster B1 is divided into three sets: the first set B1-1 includes a single pollinator PN129, the second set consists of 6 pollinators which are PN132, PN179, PN183, PN189, PN190 and PN184; the third set consists of 3 pollinators PN172, PN192 and PN182. The subcluster B2 has a single pollinator PN178. The subcluster B3 is divided into three sets: the set B3-1 is composed of PN193, PN195, the set B3-2 includes 4 pollinators PN199, PN196, PN197 and PN201 and the set B3-4 contains only PN203. The subcluster B4 is represented only by PN203.

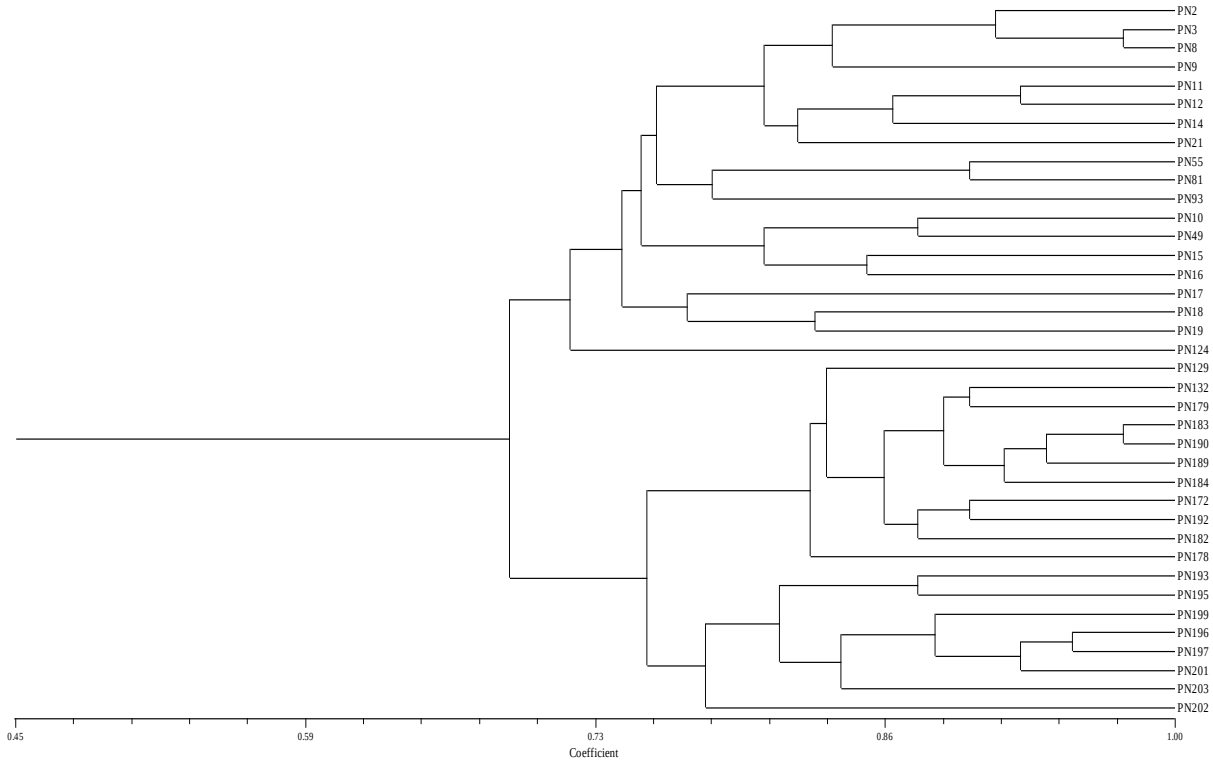


**Figure 2.** Phylogenetic similarity distance generated by ISSR markers using UPGMA procedure according to Nei and Li (1979) method.

## Principal components analysis

The morphological parameters are processed by a principal component analysis (PCA) using the XLSTAT software (2014). The two first axes were chosen: Axis 1 (F1) and axis 2 (F2), which absorbed a maximum of existing variability between the different date palm pollinators studied. They gave 24.88 and 11.97% respectively. The first principal component which explains 24.88% of variability received relatively high loadings from the variables the grouping of pennes (0.594), thickness of the spine (0.661), maximum thickness of the spine of the middle (0.672), maximum length of pennes in the middle of the palm (0.676), maximum width of pennes in the middle of the palm (0.683), number of pennes (0.839), width of the palm at the base of the petiole (0.847) and total length of the palm (0.849). The second component, which explains an additional 11.97% of the total variation, received a high positive loading from the average number of thorns (0.712) and number of spines by grouping type (0.768). The correlation analysis between morphological parameters using the coefficient of Person (n) shows that there are positive and negative correlations between these parameters: The total length (C12) is highly and positively correlated with the average number of penne (C20), with the width of the palm at the base of petiole (C17) and the spine thickness (C15).The number of pennes (C20) is positively correlated with the spine thickness (C15). A high correlation was observed between the average number of thorns (C18) and the number of thorns by group type (C21).

The principal components (Figure 3) assembly accessions into six groups. The first group consisted of 14 pollinators PN190, PN192, PN179, PN182, PN183, PN184, PN178, PN12, PN55, PN193, PN81, PN49, PN15 and PN11 that are negatively correlated with axis one. The second one is formed by PN21, PN19, PN124, PN17, PN10, PN2, PN14, PN197, PN16, PN132, PN201, PN189, PN9 and PN3 which are positively correlated with F2. The third group consisted of PN203, PN196, PN172 and PN93 which are positively correlated with the two axes. The fourth set composed of PN195, PN8 and PN202 are highly and positively correlated with the axis one. The fifth group consisted of two pollinators PN18 and PN129 which are negatively correlated with axis F1 and negatively with axis F2. The latest group gathered only PN199 which is negatively correlated with axis 2.

The application of NTSYS software version 2.0 Pc to all morphological data allowed us to get the genetic similarity matrix. The analysis of this matrix shows that similarity coefficients are ranging from 0.161 and 0.548. The lowest similarity (0.161) was observed between the combinations PN129-PN192. These coefficients reflect a weak similarity in morphological characters of these pollinators. Indeed,

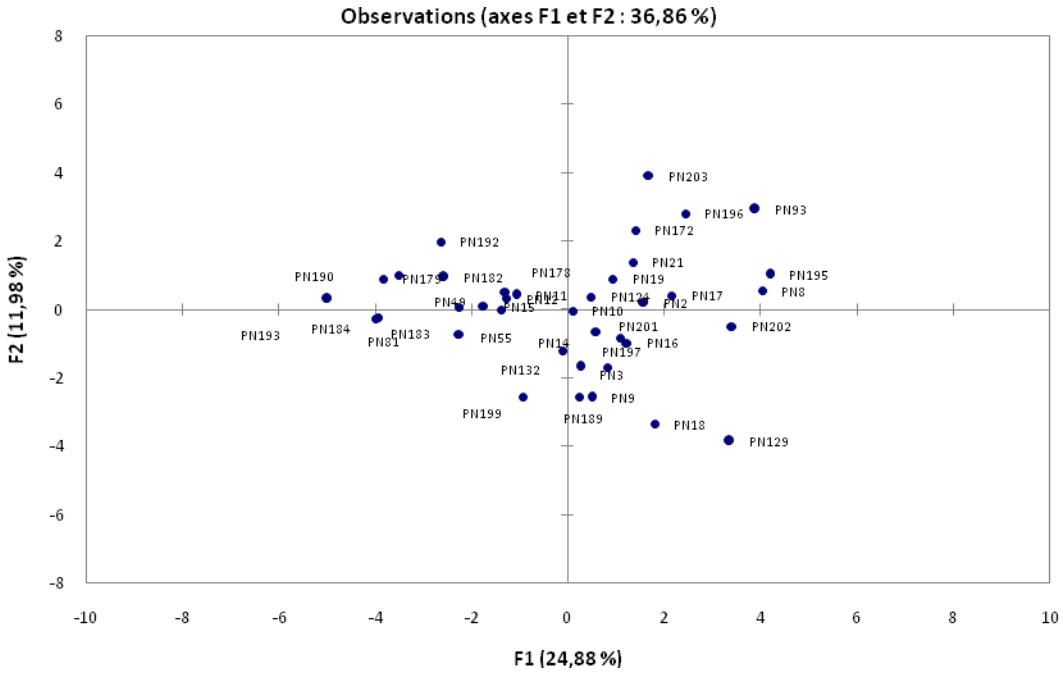


**Figure 3.** Principal components analysis of cultivars' morphological traits.

the vigor is low for PN192 while it is strong for PN129, the port is spherical for PN129 while it is falling for PN192. In addition to the color of the petiole which is yellowish for PN192 while it is mottled for PN129.

On the other side, the highest coefficient (0.548) was observed with the combinations PN15-PN19 and PN189-PN183 that resemble a high number of characters. Out of 31 characters studied, the pollinators PN183-PN189 differ only by the vigor, the port, the persistence of cornaf, the number of thorns per type of grouping.

For the thirty eight studied pollinators, 3 groups were distinguished with a threshold of 28% of similarity. Group 1 is represented by 21 pollinators and it is divided into 2 sub clusters. The sub cluster A1 is divided into two sets: the first set A1-1 contains only PN2 and the second set A1-2 contains two pollinators PN21 and PN49.The sub cluster A2 is divided into four sets: the set A2-1 is represented by 7 pollinators PN10, PN15, PN19, PN178, PN192, PN11, PN12, the second set A2-2 has only PN203, the third set A2 -3 comprises PN55 and PN81 and the last assembly is constituted by 8 pollinators which are PN124, PN182, PN189, PN179, PN193, PN183, PN184 and PN190.

Group B is composed of 17 pollinators and it is divided into 3 sub groups. The subgroup B1 is further divided in two set. The first set B1-1 is composed of 7 pollinators: PN3, PN201, PN18, PN14, PN132, PN197 and PN202. The second set is represented by PN16 and PN129, the third assembly includes PN8, PN17, PN196, PN195, PN9 and PN172. The second subgroup B2 is represented by a single pollinator PN93.

Group C includes only PN 199. This pollinator is characterized by the lowest maximum width of pennes in the middle of the palm (2 cm).

Despite that the PCA of accessions showed six groups and the dendrogram clustering showed only three groups, the most accessions gathered in the same group by PCA are also assembled in the same cluster. In fact, PN199 forms an individual group with the two methods.

## Relationship between morphological distance and ISSR markers distance

The relationship between morphological traits and the ISSR markers distances among studied accessions showed a very insignificant and weak matrix correlation coefficient (r= 0.02) with a probability p= 0.27. The treatment of molecular data through various methods has produced highly significant correlations. Indeed, the correlation coefficient between the MS and the Dice coefficient showed significant correlation with r = 0.76 and p = 0.0015.

## DISCUSSION

*P. dactylifera L,* a popular crop tree and functional food, is distributed over a wide range in Tunisia. Our field investigation revealed great diversity in its ecological environments. The aims of the present study were the search for useful morphological markers and provide

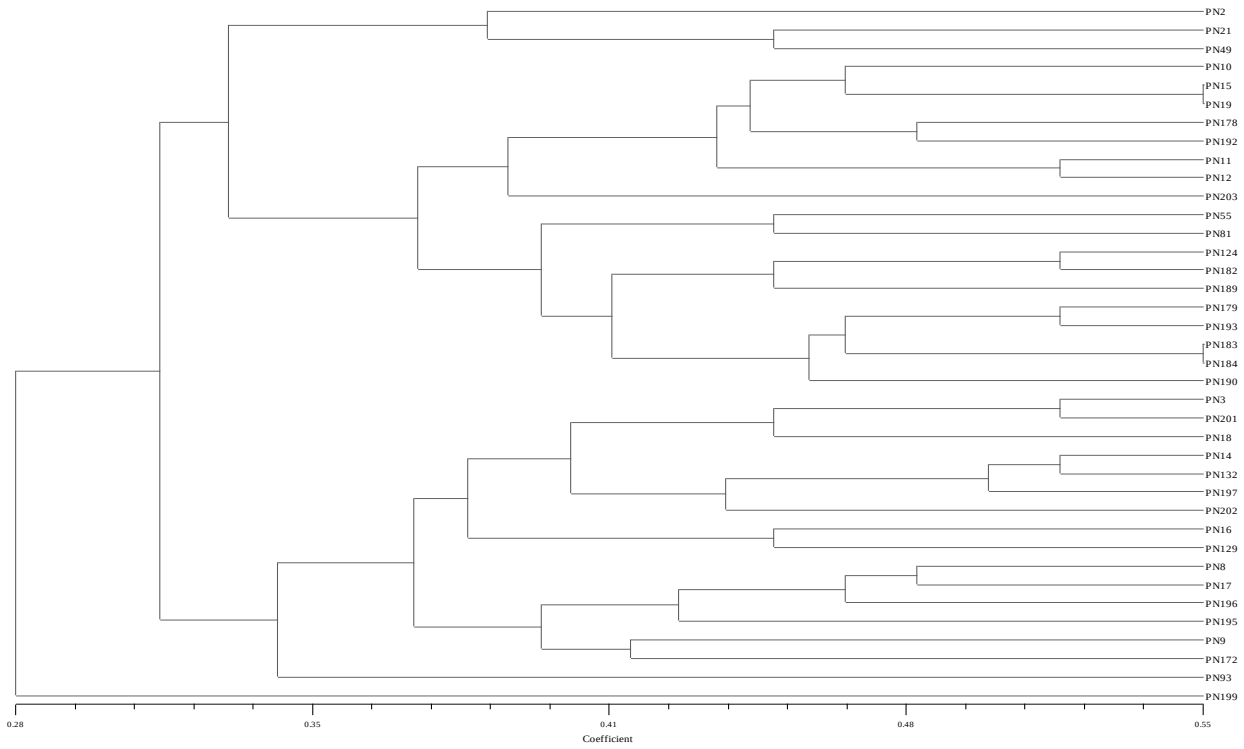


**Figure 4.** Morphological classification of the accessions based on weighted pair group method using arithmetic average using NTSYSpc 2.02.

polymorphic DNA markers suitable for the examination of the phylogenetic relationships among a set of Tunisian date-palm pollinators with the help of ISSR technology.

Morphological studies of date palms were still considered difficult to achieve since they require a large set of phenotypic data and because the date palms are quite varied because of the environmental effect (Munier, 1973). In this work, thirty eight male palms located in experimental plot in Djerid were studied. This training in the same environment minimized the influence of extrinsic factors (geographic, seasonal, and soil) on morphological characters. This present study portrayed the use of morphological parameters to examine the phenotypic variability in a set of Tunisian date-palm pollinator's. As a result, we assume that the studied cultivars are characterized by a high level of genetic diversity. This is strongly supported by projection of the cultivars in PCA plots as well as in UPGMA cluster analysis. In addition, taking into account the parameters studied a significant discrimination among cultivars has been observed. Most of the morphological characters are highly variable. According to PCA analysis, the most discriminating characters are Pinnaes's grouping, the thickness of the spine, the maximum thickness of the middle spine, the maximum length of penne in the middle of the palm, the average number of penne, the width of the palm at the base of petiole, the total length, the average number of thorns and the number of spines per type of grouping. Our data generally agree with those reported for the 11 Mauritanian females and one male studied by Ould Mohamed Salem et al. (2008) using 18 phenotypic markers. Similarly, the data are generally consistent with those reported for Moroccan date palm cultivars (Elhoumaizi et al., 2002) and those reported for date palm which originated from Adrar region, Mohamed Ahmed et al. (2011), studied 21 accessions by 30 phenotypic traits and found that the length of the palm and the length of the spined part are the most discriminating characters. Our results indicate that despite the fact that our samples are located in the same environment, we found a high genetic diversity. The study of polymorphism in this collection of pollinators through phenotypic markers was essential. However, morphological characters are often biased by environmental factors and the agro-morphological traits used to characterize date palm varieties present limited discriminatory power (Figure 4).

Molecular analysis of 38 date-date pollinators was preceded by the assessment of the quality and quantity of DNA. 6 ISSR primers used in PCR were found polymorphs. Thus, a total of 41 reproducible bands in which 37 are polymorphic were amplified with the percentage of 90% of polymorphism. This indicates the discriminating power of ISSR. Similar results were observed with Khierallah et al. (2014), in a study of 10 female and 7 male Iraqi date palm with 30 RAPD and 12 ISSR primers, they reported that the polymorphism rate was 94.4%. Similarly for Marsafari and Mehrabi (2013), who used both RAPD and ISSR markers for

genetic diversity study of 15 native palm date palm cultivars collected from the south and southwest of Iran, they concluded that polymorphism rate was 95% for ISSR and 97% for RAPD. On the contrary, Srivashtav et al. (2013), using 13 RAPD primers and 2 ISSR primers in the study of 8 cultivars palm date trees in the Kutch region of India, reported a low level of polymorphism for ISSR markers (23%).

The average bands per primer were 6.8 for all bands and 6.16 for the polymorphic bands. These results might be due to more genotypes and primers used in the study. The same results were obtained with Hamza et al. (2011) by studying 26 Tunisian cultivars using seven ISSR primers. A total of 43 bands were obtained of which 34 are polymorphic with the average number of fragments produced by the primer is 6.14. As for Hamza et al. (2013), they used both seven ISSR primers and five microsatellites to assess the genetic diversity of 26 Tunisian cultivars. The seven ISSR primers have given a total of 43 polymorphic fragments and the average was 6.1 / band. Instead, our data are less than those obtained with Hussein et al. (2004) in a study of 12 cultivars of Egyptian date palm, a total of 105 fragments were generated by 10 ISSR primers with an average of 10.5 fragments / primer.

The sizes of the bands obtained ranges between 250 and 3 kb. Our results are in agreement with Srivashtav et al. (2013), where the size of the amplification product varies between 300 and 4000 bp. The same results were found with Haider et al. (2012) by studying 23 accessions of date palm by 15 ISSR primers. Whereas, Elsheikh et al., (2014), following the study of 6 Libyan cultivars by 6 ISSR primers, reported that the amplified band sizes ranged from 100 to 1400 bp.

Statistical analysis showed high genetic diversity with genetic distances ranging from 0.512 to 0.975. This shows that the studied pollinators form genetically independent groups since the majority of genetic similarity index fluctuates between 0.6 and 0.7.

Our results showed that the ISSR is an effective and powerful molecular tool that can be used for the detection of polymorphism in date palm pollinators. Our results are in agreement with the results found by Zehdi et al. (2002); Hamza et al. (2011); Ahmed et al. (2013) and Kheirallah et al. (2014).On the contrary, Srivashtav et al. (2013) concluded that RAPDs are more powerful to detect the polymorphism than ISSR. Thus, Haider et al. (2012), in a study of 18 female date-palm and 5 pollinators by both RAPD and ISSR markers, found that the polymorphism level detected by RAPD analysis (58.5%) was higher than that observed for ISSR (50.6%). This shows that the RAPD is more discriminating but still remains the problem of non-reproducibility of RAPD marker.

The mantel test showed a low insignificant between morphological and molecular traits. This differentiation may be due to an eco-physiological adaptation of the studied cultivars associated with the conditions. Our data are generally consistent with those reported for Tunisian date palm cultivar (Hamza et al., 2011).

## CONCLUSION

As a conclusion, the phylogenetic tree constructed here revealed that male palm examined are not monophyletic and provide evidence of divergence among all tested genotypes since they were grouped in clusters. This present study indicates that morphological traits and ISSR markers individually have their own merits in the male palm cultivar fingerprinting. Furthermore, this study should contribute to cultivar conservation in southern Tunisia where male palm are now neglected and need to be conserved in the name of sustainable harvesting.

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