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Original article

Agro-morphological traits assessment of Tunisian male date palms (*Phœnix dactylifera* L.) for preservation and sustainable utilization of local germplasm

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ABSTRACT

Date palm (*Phœnix dactylifera* L.) like other crop species in the arid Mediterranean region is being threatened by genetic erosion and climate change. Therefore, the understanding and assessment of the diversity extent of this species is a primary requisite for preserving these crop resources. This study was designed to quantify the potential of Tunisian male date palms using a set of agro-morphological characteristics i.e. flowering traits, inflorescence morphology and pollen quality. The flowering time traits exhibited a trend of precocious phenotype at emergence spathe trait and the dominance of the full-season phenotype at the opening date. At inflorescence morphology, all observed traits expressed wide ranges which reflected the broad variability of the evaluated male genotypes. Significant difference was recorded for the majority of the examined traits with a high significant variation in the tree quantitative traits: Spathe Total Length, Spathe Maximum Width and Length to the brunched part. Pollen viability ranged from 51.10% to 98.75% while the germination rate was between 0.90% and 70.50%. Our phenotypic investigation has allowed the identification of males with desirable agronomic traits which have been genotyped using 18 nuclear SSR markers and a chloroplast minisatellite for preservation and effective utilization purposes.

1. Introduction

Biodiversity resources are facing multiple biotic and abiotic stresses such as pathogen, drought and salinity (Fujita et al., 2006). Furthermore, the new challenge of changing climate has emerged these last few decades and represents a high risk for a decrease of our plant biodiversity and extinction of current species (IPCC, 2014).

Consequently, knowledge, estimation and monitoring the amount of biodiversity for contemporary germplasm collection are needed (Rao and Hodgkin, 2002). For these reasons, crop plants have been one of the main topics of research studies in attempt to survey and evaluate the level of their genetic variability. The set of this information is a passport data of fundamental research, conservation, and efficient utilization of these genetic resources at the different agricultural ecosystems (Rao and Hodgkin, 2002; Maulión et al., 2016). Generally, several approaches based on morphological and agronomic traits have been used for information acquisition (Ibrahim Bio Yerima et al., 2020; Mai et al., 2021; Pérez-Sánchez and Morales-Corts, 2021). Phenotyping is an effective tool in data collection and documentation of the genetic variability at species and ecosystems levels (Mijnsbrugge et al., 2016; Thaowetsuwan et al., 2017; Tripodi et al., 2018; Virga et al., 2020; Pérez-Sánchez and Morales-Corts, 2021).This approach is generally undertaken for one (or several) of three major reason; discrimination among accessions, identification potentially valuable traits for use in plant breeding, and genome-wide association studies

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(GWAS) for development of genetic makers for maker-assisted breeding (MAB) and marker-assisted selection (MAS) (Nybom and Lācis, 2021). Morphological, phenological and agronomic traits are some of the most answered methods. Studies of these markers provides information that is not only important to identify potential valuable traits for farmers but contribute to develop guidelines useful in addressing the challenging situation of agro-systems specifically in semi-arid and arid regions (Martínez et al., 2019; Martínez-Nieto et al., 2020; Sancho-Galán et al., 2020; Ouaja et al., 2021).

Oasian agro-system which is characterized by vegetation in layers represents the cultivated areas in the semi-arid and arid countries of North Africa and the Middle East. Date palm (*Phœnix dactylifera* L.) is one of the most valuable crops colonizing this area, with a huge richness; we count at least 250 varieties in Tunisia (Rhouma, 2005) and more than 200, 400, and 800 cultivars in Morocco, Libya, and Algeria respectively (Toutain et al., 1971; Benkhalifa, 1996; Racchi et al., 2014). On a global scale, the fruit production of this species exceeds 8 million tons thus guaranteeing millions of US dollars to local and national economies (FAOSTAT, 2021). Given its economic and ecological importance, several studies have been established to quantify and assess the genetic richness of this species. However, the knowledge of date palm agrobiodiversity remains incomplete since they have been principally directed towards female varieties. Consequently, the male genotypes are still poorly characterized despite their central role in pollination and date production yield given that the quality of pollen provided a major impact on quality and quantity of fruits (Nixon, 1927). Therefore, it is important to enrich our knowledge of this germplasm particularly those related to their adaptation capacity to these agroclimatic areas.

Flowering time and pollen quality of these male date palm genotypes are an important agronomic traits to ensure the production. As climate change progresses, the flowering phase is under pressure and can express in either an advance or a delay for both male and female date palms. Therefore, this situation leads to flowering asynchronism and non pollen availability for pollination which may intensify the disturbances of the oasis agro-system. In this context, studying the flowering time of the male genotypes provides an excellent tool to select interesting genotypes whose flowers are in synchrony with female accessions that can address these ecological shifts in the oasis agro-system and ensure the future date production in increasing climate change.

In date palm,the pollen grains influence not only the size and shape of the seeds, which is called the "xenic" effect, but also the size, shape, weight and rate of fruit ripening, which is defined by the "metaxenic" effect (Nixon, 1928). Thus, date palm pollen plays a non-negligible role in the expression of certain characteristics of the fruit. Therefore, it is necessary to undertake studies on pollen performance in order to study the variability that may exist in the phœnicicole heritage and to select from the existing collection a male genotypes donor of good reproductive quality pollen.

To increase our knowledge about this local germplasm, agro-morphological approach was conducted on Tunisian collection in order to select interesting male genotypes for their flowering time stability and good pollen quality. The set of the selected genotypes was perfectly identified with a patented SSR markers. This list can overcome climatic perturbations and provide desirable date production in global warming scenario in the oasis agro-system. The data will provide valuable information that would be exploited in an improvement program and conservation purposes for this specie.

2. Materials and methods

2.1. Plant material

The floral phenotypic study was based on 180 male genotypes which have been selected from Tozeur oasis based on framers's surveys and observations and have been conserved in the experimental station of the Centre Régional de Recherches en Agriculture Oasienne (CRRAO) de Degache, Tozeur, Tunisia. They were tagged and numbered from M1 to M180 where the GPS coordinates were determined using Garmin eTrex Venture HC GPS receiver (2007) (Table S1). The male genotypes collection belongs to the same age range according to the IPGRI descriptor of the date palm (IPGRI, 2005) and receives the same cultural practices (Trimming-pruning palms, irrigation and fertilization). From this collection, 49 male genotypes chosen for their flowering time phenotype were used for the inflorescence morphological approach and pollen quality evaluation.

2.2. Study site bioclimatic data

The bioclimatic data was collected from the Tunisian Meteorology National Institute (INM). The recorded data for the studied years included the minimum and the maximum temperature (°C), the minimum and the maximum of relative humidity (%) (Fig. S1). The minimum temperature varied from 5.71 °C to 16.98 °C in 2014, from 3.74 °C to 18.31 °C in 2015 and from 6.18 °C to 18.85 °C in 2016. For the maximum temperature, the values ranged from 21.31 °C to 37.73 °C in 2014, from 19.62 °C to 40.26 °C in 2015 and from 16.91 °C to 31.49 °C in 2016. For the studied period, the study site was characterized by a minimum relative humidity varying from 11.14 % in 2016 to 48 % in 2015 and a maximum relative humidity with a mean of 66.74 %, 67.81 % and 65.54 % in 2014, 2015 and 2016 respectively.

2.3. Monitoring of flowering traits and morphological measurements

The monitored flowering traits were the emergence and the opening of the first spathe. The observations were made weekly during the entire flowering period from January to April over three successive seasons 2014, 2015 and 2016. At every observation date, each male genotype was marked by noting the presence (1) or absence (0) of each flowering event (Castellana et al., 2010; Pintaud, 2012).

For morphological measurements, five matured spathes were sampled from different locations in the crown (Fig. 1A). The morphological studies were performed following IPGRI (2005). This included eleven inflorescence descriptors consisting of two qualitative traits: Spathe Shape (SS) and Spikelet Density (SD) and nine quantitative ones: Spathe Total length (STL) (cm), Spathe Maximum Width (SMW) (cm), Spikelet Number (SN), Longest Spikelet Length (LSL) (cm), Shorter Spikelet Length (SSL) (cm), Flower Number/Longest Spikelet (FNLS), Flower Number/Shorter Spikelet (FNSS), Length of the Branched Part (LBP) (cm) and Width at the middle of the Branched Part (WBP) (cm) (Fig. 1B). Male genotypes pollen was evaluated by the two qualitative characteristics: the color and the odor (IPGRI, 2005).

2.4. Pollen grains collection, viability staining and in vitro germination

For the two successive seasons 2014–2015, five spathes of the same male genotype were freshly harvested as soon as it cracked. The spikelets are detached and dried on paper sheets at room temperature. After 48 h, these spikelets are slightly shaken in order to

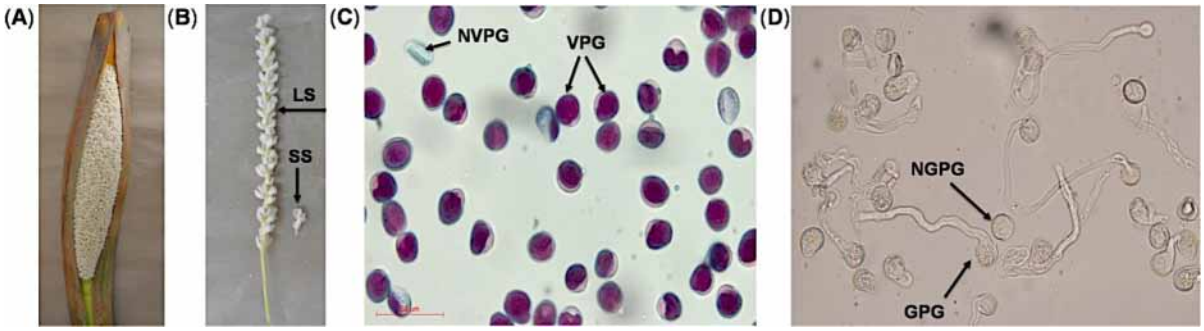

Fig. 1. Morphological measurement and pollen quality evaluation; **(A)** Collected spathe at cracking used for morphological measurement and pollen collection; **(B)** The longest spikelet **(LS)** and the shorter one (**SS**) used for morphological study; **(C)** Viability test with the Alexander staining, viable pollen grains **(VPG)**, Non Viable pollen grains **(NVPG)**; **(D)** Pollen germinated in liquid medium, Germinated pollen grains **(GPG)**, Non germinated pollen grains **(NGPG)**.

extract the pollen from the flowers. The collected pollen is recovered, sieved and stored at 4 °C.

Pollen grains were mounted on slides with one drop of 1% Alexander solution (Fig. 1C), purple stained indicate that they are viable. The percentage of viability was calculated for the average of five replications at the rate of 400 pollen grains per repetition. Pollen was germinated *in vitro* on liquid medium of Brewbacker and Kwack (1963) modified and adapted to date palm pollen by Furr and Enriquez (1966) (Fig. 1D). This germination medium was composed by 15% Sucrose, 50mg Boric acid, 300mg Calcium nitrate, 200mg Magnesium sulfate, 100mg Potassium nitrate and 100ml of distilled water. Five Petri dishes for each sample were incubated for 24 h at 27 °C (Boughediri and Bounaga, 1987, El Mardi and Bakheit, 1996). The blockage of germination is done with spraying of 45% Acetic acid (Mortazavi et al., 2010). To determine the pollen germination ratio in percentage, 400 pollen grains were counted per slide for the total of the five repetitions.

Microscopic examination of viability and germination was made with an Olympus BX51 microscope equipped with a Nikon D5100 camera. Images captured were analyzed with the program of Image.J (v.1.43) for counting.

2.5. Statistical analyses

Graphing of the flowering and climatic data were carried out using GraphPad Prism 7.04 and the *ggplot2* package of R (R Core Team v3.5.1.). A Principal Component Analysis (PCA) based on flowering pattern was performed with the packages *FactoMineR* and f*actoextra*. Furthermore, the flowering and climatic data were submitted to correlation test *via* the library *corrplot*.

The violin plots for each morphological trait were applied using the *ggplot2* library. Significant difference at the morphological parameters was run by the chi-square test ($\chi 2$) and the one-way analysis of variance (ANOVA). The multiple comparisons using Duncan's test with 95% degree of confidence were conducted by the discriminated traits and using the *Laercio* library. With *dudi.pca* function of the package *ade4*, a principal component analysis (PCA) was performed to represent the level of variability within this collection based on the quantitative morphological markers. The hierarchical clustering of the examined male genotypes based on the inflorescence morphological data was carried out using the function *hclust* implemented in R. The hierarchical clustering on the factor map was built by the *HCPC* function. Correlation analysis was carried out using Pearson coefficient via *cor.test* function to identify an association between pollen quality and the studied morphological variables.

2.6. SSR genotyping and identification key

Based on the phenotypic screening, 15 male genotypes were selected from the entire collection for their interesting traits (Table 1). Fresh and young leaves of each sample were collected and used for DNA extraction using the Chemagic DNA Plant Kit (Perkin Elmer) according to the supplier's instructions adapted to the workstation of the KingFisher Flex™ (Thermofisher) automated DNA purification. DNA concentrations and purity were determined using a TECAN-GENios plus spectrofluorimeter. A set of 18 nuclear SSR markers and a plastid decanucleotide minisatellite particularly useful for the certification of cultivars were used to genotyping the selecting male genotypes (Patent Application Publication: US2010337395A1, 2015; Alberlenc-Bertossi et al., 2014; Billotte et al., 2004; Henderson et al., 2006; Ludeña et al., 2011; Zehdi-Azouzi et al., 2015) (Table S2). Amplification reactions were performed as described by Zehdi et al. (2015). The resultant PCR amplicons were analyzed using the ABI prism 3130xl Genetic Analyzer and the GeneMapper V3.7 software (Applied Biosystems, USA).

Based on the SSR data, and as described by (Patent Application Publication: US2010337395A1, 2015) a molecular identification key was established to fingerprinting the selecting males'genotypes.

3. Results

3.1. Phenotypic diversity of flowering traits

3.1.1. Germplasm characterization

The flowering traits revealed a considerable degree of variability. At emergence level, the distribution of the male genotypes

Table 1
Details of the 15 male genotypes selected in the study.

Male genotypes	GPS
M4	N33°55,656′E008°08,121
M14	N33°55,689′E008°08,089
M19	N33°55,668′E008°08,115′
M24	N33°55,685′E008°08,098′
M31	N33°55,662E008°08,131′
M34	N33°55,673′E008°08,119
M44	N33°55,658′E008°08,140′
M45	N33°55,662′E008°08,137′
M49	N33°55,679′E008°08,120′
M69	N33°55,672′E008°08,141′
M79	N33°55,672′E008°08,150′
M109	N33°55,703′E008°08,131′
M123	N33°55,708′E008°08,133′
M148	N33°55,716′E008°08,139′
M178	N33°55,713′E008°08,188′

characterizing each emergence period underscored a great difference from one year to another (Table 2). In 2014, most plants flowered in mid-season emergence period. However, during the next two years, the majority of them recorded an early emergence event. The early flowering phenotype was the dominant one, with 74% for 2015 and 93% for 2016. Moreover, the initiation and the peak time of emergence spathe date was recorded two-week advancement during the three surveyed seasons (Fig. 2A).

At individual scale, the flowering traits of the studied collection is presented in the graphic series established for the examined male genotypes where the observation dates are coded from 1 to 16 (Fig. S2). As shown in these graphics, the timing of traits of this collection revealed a wide spectrum of variation among the individual male genotypes and the study years. As regards the opening of the first spathe, almost all the male genotypes have recorded a full season opening period with a distribution of 80.55%, 79.44% and 94.44% for 2014, 2105 and 2016 respectively (Table 2). Furthermore, this floral stage has conserved its optimal activity time at the last week of March during the three studied years (Fig. 2B). The set of this result reflects the potential stability of this flowering trait.

The emergence-opening duration exhibited variability during the three years of study (Table 2). Duration of one to six weeks has been recorded at this flowering trait. In 2014, about 64% of the collection displayed a duration of two and three weeks. In 2015 and 2016, the majority of them showed four and five weeks at their emergence-opening period. Remarkably, the examined trait revealed a prolonging trend throughout the study period.

3.1.2. Principal component analysis of the flowering pattern

The data set on flowering features was submitted to the multivariate analysis PCA. The first two axes explained 38.3% and 21% of the total variation, respectively (Fig. 3). This analysis discloses four distinct groups. The first one (A) consisted of 134 samples which flowered earlier. The second one (B) included 25 male plants with a mid-season flowering. The third group (C) grouped 10 male genotypes which are characterized by a late flowering period. The fourth group (D) gathered the 10 remaining plants with fluctuating flowering time. The observed distribution shows the high phenotypic variability of flowering traits during the three monitored seasons among the male genotypes of the examined collection.

3.1.3. Correlation among climatic variables and flowering traits

The Pearson correlation coefficient among climatic variables and flowering traits were calculated and presented in Fig. 4. The emergence of the first spathe (EFS) shows a significant positive correlation with all of the relative humidity parameters, the minimum relative humidity (HRmin) (r = 0.74, $p < 0.01$), the maximum relative humidity (HRmax) (r = 0.61, $p < 0.01$) and the mean relative humidity (HRmean) (r = 0.35, $p < 0.01$). Moreover, the correlogram exhibited a negative correlation between the emergence of the first spathe (EFS) and temperature variables. The mean temperature (Tmean) (r = -0.73, $p < 0.01$) and the minimum temperature (Tmin) (r = -0.73, $p < 0.01$) had the greatest correlation. Whereas, the opening of the first spathe (OFS) was not significantly correlated with the examined climatic variables.

Table 2
Proportion distribution (%) of the male genotypes characterizing each flowering traits for the entire collection during the three years of study.

Years	Monitoring period		
	2014	2015	2016
Flowering traits			
Emergence of the first spathe			
Early	25	74.44	93.33
Mid-season	61.66	17.77	6.11
Late	8.88	7.22	0
Opening of the first spathe			
Early	0	0.55	3.33
Mid-season	80.55	79.44	94.44
Late	28	19.44	1.66
Emergence-Opening duration			
One week	9.49	7.26	0.56
Two weeks	33.51	9.49	3.35
Three weeks	30.16	7.82	15.65
Four weeks	13.40	34.08	34.63
Five weeks	8.93	35.19	37.98
Six weeks	0.56	6.14	7.82

3.2. Patterns of inflorescence morphological variation

Inflorescence morphological study was conducted for 49 male genotypes chosen for their flowering phenological characterization across the studied seasons. This set of male genotypes was divided into five groups named from G1 to G5 with more details regarding its flowering phenotype (Table 3). The first group G1 included samples with early flowering stability. The group G2 is characterized by early flowering for two successive seasons. The third group G3 is formed by individuals with full- seasons flowering for two successive seasons. The fourth group G4 contains plants which are characterized by late flowering for two successive seasons. The last one G5 is represented by the male genotypes with flowering fluctuation.

3.2.1. Qualitative characters analysis

Qualitative characteristics have been used to evaluate reproductive organ morphology of the samples. The two inflorescence traits Spathe Shape (SS) and Spikelet Density (SD) have revealed a considerable variation among the five groups (Fig. 5A and B). The distribution indicated the dominance of the fusiform shape for the three groups G2, G3 and G4 with a frequency that scores 52%, 75% and 77%, respectively. At the group G1, the spathe shape for 50% is inflated, 33% lanceolate and 16% fusiform. However, the fusiform and lanceolate shapes represent each 40% for G5 male plants. As concerns the spikelet density, the result showed that G1 and G4 were overrepresented by the compact and the medium density, respectively. 63% of G2 individuals had medium density, 31% had compact density and 5% had loose density. For the G3 group, the spikelet density is 50% compact and 50% medium. At the group G5, the loose and medium density had the same frequency distribution with 40% and the compact density had the lowest frequency with 20%.

The two pollen qualitative traits color and odor exhibited a variation among the studied groups (Fig. 5C and D). The modality whitish showed the absolute dominance at the group G3 and predominance at the groups G1, G2 and G5. For the group G4, about 55% had yellowish pollen and 44% had whitish pollen. Concerning the odor, the low type had the highest frequencies for the three groups G2, G4 and G5, whereas the majority of the group G1 (66%) had the strong type. In return, the two odor types had recorded the same distribution frequency with 50% at the group G3.

To better appreciate the variability, the chi-square test ($\chi 2$) was performed for the examined qualitative traits. This test exhibited a high significant difference between the five studied groups for the inflorescence qualitative traits used in this study and only for the character of color at pollen level (Table S3).

3.2.2. Quantitative characters analysis

Information on morphological variability among the examined samples is estimated by the violin and box plots based on nine quantitative traits (Fig. 6). The box plot elements show that the greatest values of the most quantitative traits are surrounded

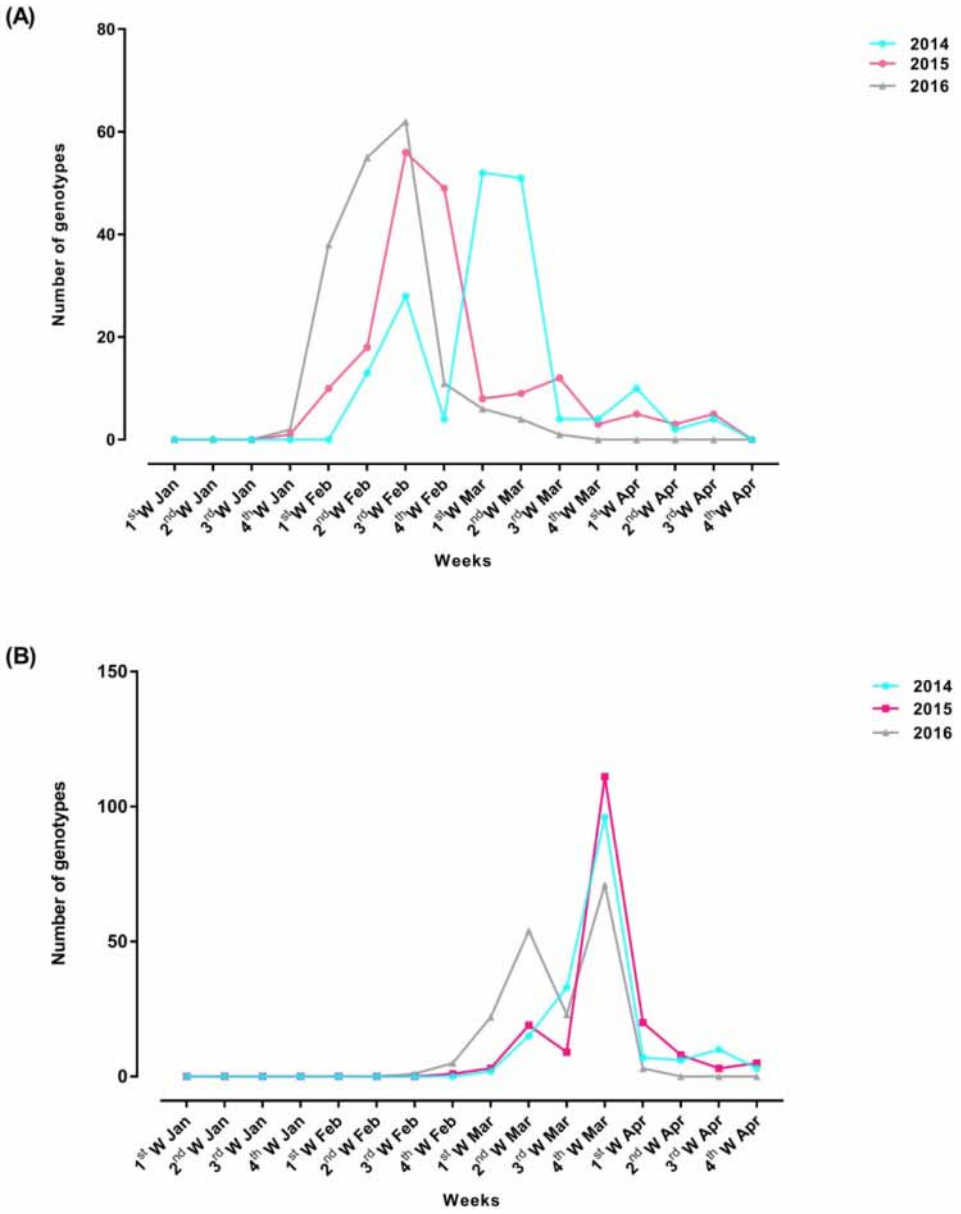

Fig. 2. Flowering patterns of the male genotypes collection in the three seasons considered (2014–2015-2016) in this study; **(A)** Number of male genotypes showing the emergence of the first spathe for each observation survey during the three studies years; **(B)** Number of male genotypes recording the opening of the first spathe was observed during the three studied years; **1st W:** First week**, 2nd W:** Second week**, 3rd W:** Third week**, 4th W:** fourth week**, Jan:** January, **Feb:** February, **Mar:** March, **Apr:** April**.**

between the G1, G2 and G3 groups whereas the G4 and G5 groups are characterized by the smallest values on the majority of examined traits. At the first group G1, the highest range of morphological variation was observed at Spikelet Number (SN). The wide range of variation at the three following traits: Spathe Total Length (STL), Width in the middle of the Branched Part at the flower stalk (WBP) and Length of the Branched Part at the flower stalk (LBP) has characterized the group G2. The great magnitude of variability was identified at the Spathe Maximum width (SMW), the Longest Spikelet Length (LSL), Shorter Spikelet Length (SSL) and Flower Number by the Shorter Spikelet (FNSS) for group G4 individuals. The group G5 had recorded the highest variation at Flower Number by the Longest Spikelet (FNLS). The violin plots illustrate a considerable variation in the distribution shape of the quantitative morphological data. In return, the higher probability observed is concentrated around the median for the majority of the examined inflorescence traits with either long-tail distribution below the first quartile or a long-tail above the third quartile.

Means of all inflorescence quantitative of the 49 male genotypes traits have been subject to the ANOVA analysis. As given in Table S4, this analysis of the variance revealed statistically significant differences between the male genotypes studies for all the morphological characters with the exception of the Longest Spikelet Length (LSL). The high discriminated power has been recorded by three of the nine quantitative traits used which are Spathe Total Length (STL) (p = 3.19e-05), Spathe Maximum Width (SMW) (p = 0.000806) and Length of the Branched part at the flower stalk (LBP) (p = 0.000734). The used morphological quantitative traits have confirmed their efficiency in the Knowledge of male date palm.

Besides, Duncan's test of mean comparisons of these significant parameters highlighted three different patterns (Table 4). The first pattern discloses two distinct morphotypes by the two significant morphological characters: Spathe Total Length (STL) and Length of the Branched part at the flower stalk (LBP). Whereas, the second pattern are defined by the four morphological variables: Spathe

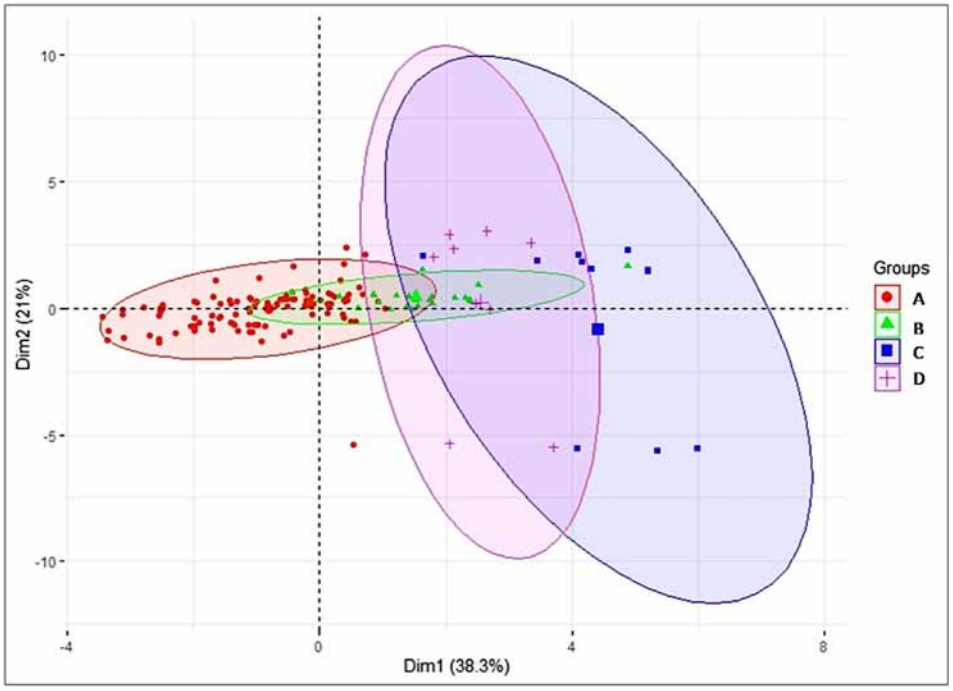

Fig. 3. Principal component analysis (PCA) graph for the studied samples generated from the flowering traits.

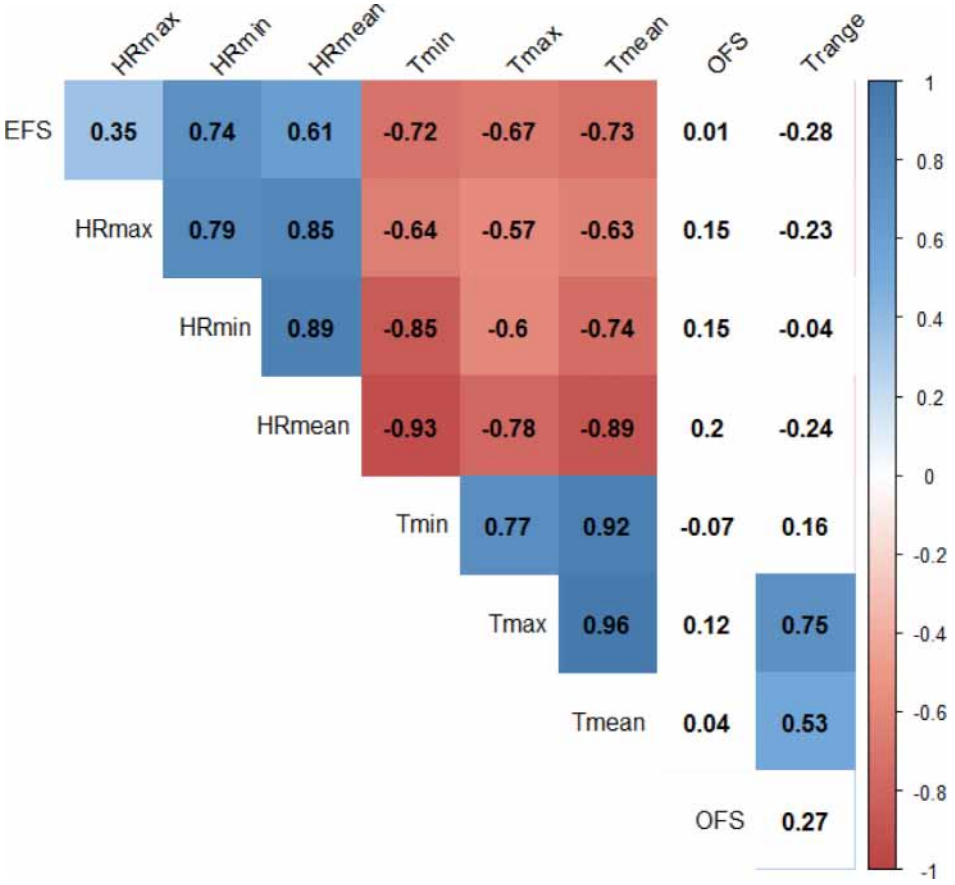

Fig. 4. Pearson's correlations test between flowering traits and climatic variables. Positive correlations are colorized in blue and negative correlations in red. The intensity of the color is proportional to the correlation coefficients according to the color legend.

Table 3
Male genotype groups used for the morphological study and pollen quality evaluation.

Group	Number of male genotypes	Flowering phenotype
G1	12	Early flowering stability
G2	19	Two successive seasons with early flowering
G3	4	Two successive full-seasons flowering
G4	11	Two successive seasons with late flowering
G5	3	Flowering fluctuation

Maximum Width (SMW), Shorter Spikelet Length (SSL), Flower Number by the Shorter Spikelet (FNSS) and Width in the middle of the Branched Part at the flower stalk (WBP). In this level, these morphological characteristics gave three morphotypes. The last one displayed four morphotypes which are defined by the two inflorescence traits Spikelet Number (SN) and Flower Number by the Longest Spikelet (FNLS).

3.2.3. Principal component analysis of inflorescence morphology

These discriminated characters have been used to project the male genotypes relationships in a principal component analysis (PCA) (Fig. 7A). For a total of 73.88% of the variation, the first axis accounts 49.89% for variance and the second one explains 23.99%. This representation showed a significant approximation of the five groups at the intersection of the two axes of the projection plan. The obtained dispersion exhibited a remarkable degree of similarity between the examined groups but it was more pronounced among the flowering groups G1, G2 and G3.

3.2.4. Pattern of the hierarchical clustering on principal components (HCPC)

To clarify the morphological relationship among the studied male genitors, a hierarchical classification clustering on factor map was performed (Fig. 7B). The two dimensions of the obtained factor map explain 58.83% of the projected inertia. This morphological pattern was categorized the studied male genotypes in three main clusters. The first cluster included 18 male genotypes. The second one gathered six male plants. The last cluster is represented by 25 male palms. This distribution indicated that there are no specific flowering behavior and inflorescence morphology relationships of the male palms collection.

3.2.5. Clustering analysis of date palm male genotypes

The morphological data were subjected to hierarchical cluster analysis using the Ward's method to evaluate the biodiversity among the male genotypes (Fig. 8). The examined collection is differentiated in four major clusters. The first cluster consisted of the five male palms M32, M33, M24, M65 and M148. Out of 14 male genotypes of the second cluster, early flowering trend was observed in 12 male genotypes during the studied years. The third one is consisted of nine male plants. The remaining's male palms clustered in the fourth group. This hierarchical classification highlighted the heterogeneous clustering of the male accessions independently of their flowering phenotype with reflect their morphological similarity.

3.3. Pollen reproductive quality

Pollen quality evaluation is done for the same 49 males genotypes used for the morphological study which are classified into five groups based on their flowering phenotypes (Table 3).

3.3.1. Pollen viability

The Pollen viability rate ranges from 51.10% to 98.75%. For the early flowering group G1, the highest rate (98.75%) was recovered from M24, while the lowest value was determined in M103 as 51.10%. In the group G2, the viability rate was between 82.20% for M53 and 98.34% for M103. For the group G3, the percentage of viability was higher for all male genotypes with a mean of 97.45%. At the group G4, the highest rate was obtained from M35 (96.50%), whereas the lowest value was observed in M44 as

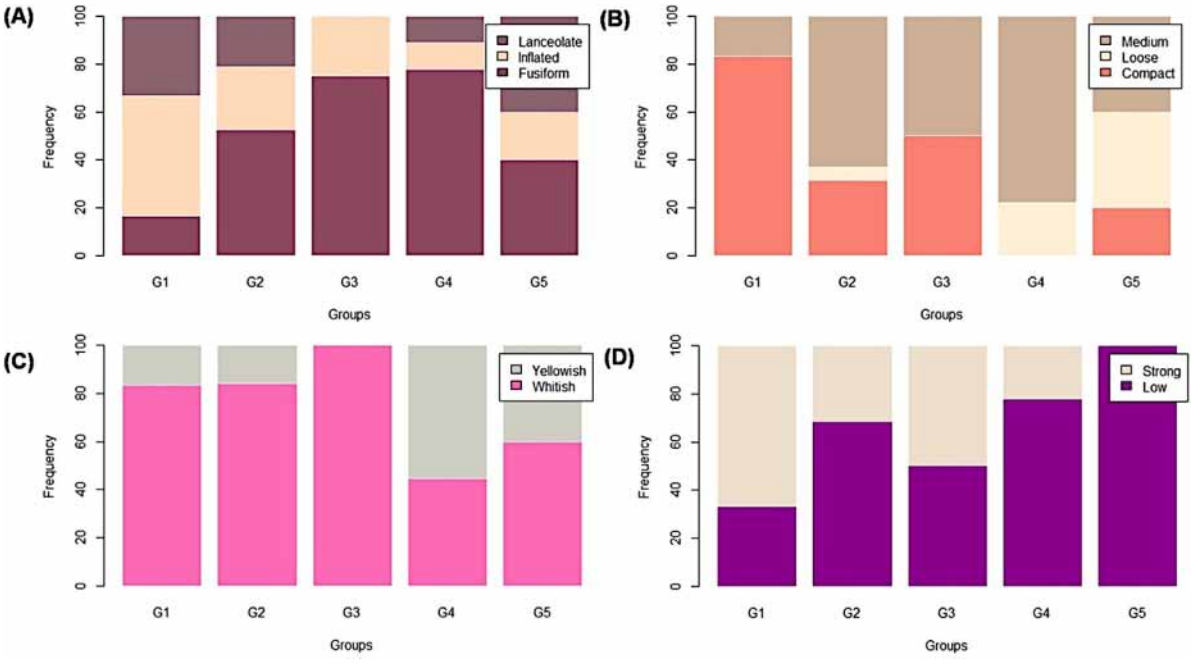

Fig. 5. Frequency distribution of qualitative morphological traits of inflorescence and pollen for the five groups of the studied male genotypes. **(A)** Inflorescence Shape; **(B)** Spikelet Density; **(C)** Pollen color; **(D)** Pollen odor.

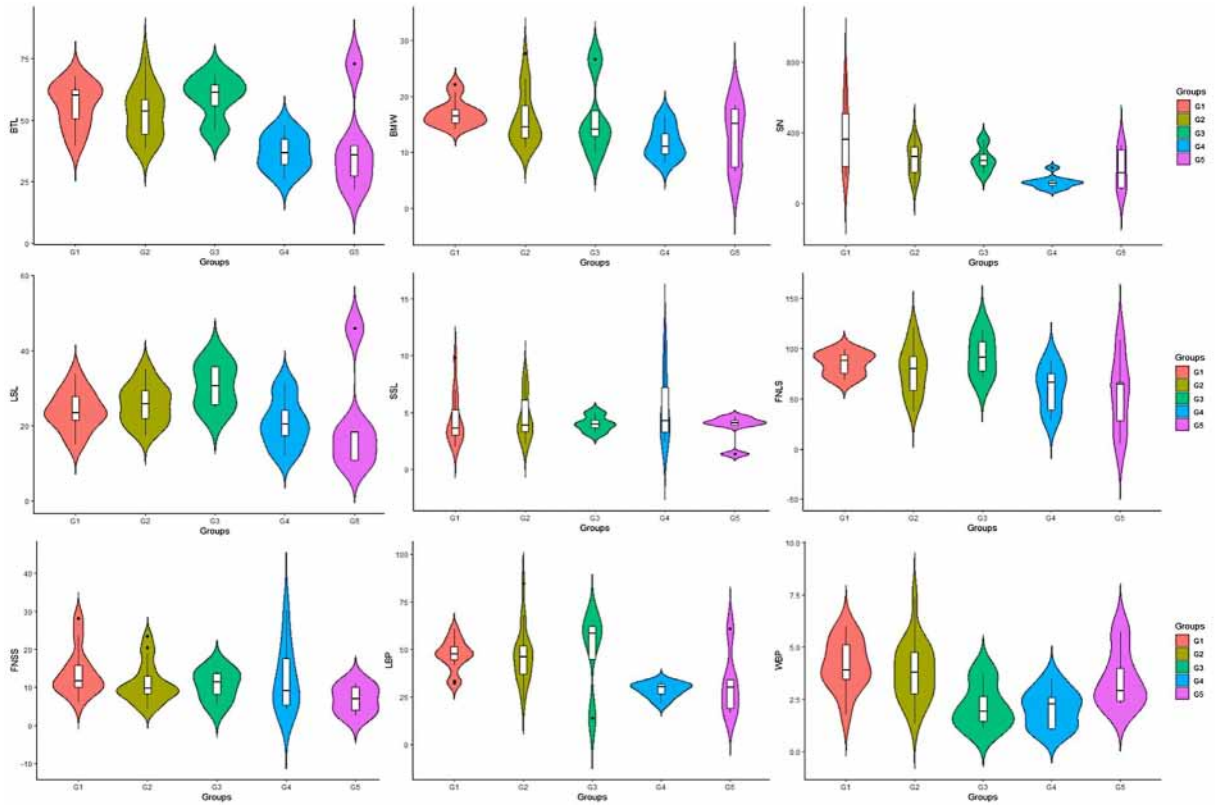

Fig. 6. Violin plots integrating box plots generated from inflorescence quantitative traits for the male plants used in this study; **STL:** Spathe Total length; **SMW:** Spathe Maximum Width; **SN:** Spikelet Number; **LSL:** Longest Spikelet Length; **SSL:** Shorter Spikelet Length; **FNLS:** Flower Number/Longest Spikelet; **FNSS:** Flower Number/Shorter Spikelet; **LBP:** Length of the Branched Part; **WBP:** Width at the middle of the Branched Part.

Table 4
Duncan test at 5% of significance level for mean comparisons of the significant traits revealed by the ANOVA test.

	STL		SMW		SN		SSL		FNLS		FNSS		LBP		WBP	
	Means	Duncan groups	Means	Duncan groups	Means	Duncan groups	Means	Duncan groups	Means	Duncan groups	Means	Duncan groups	Means	Duncan groups	Means	Duncan groups
Group 1	56.10	a	17.03	a	383.94	a	4.42	ab	85.31	a	13.95	a	47.01	a	4.16	a
Group 2	53.21	b	16.05	ab	249.59	b	4.62	ab	78.48	ab	11.10	ab	46.79	a	3.83	a
Group 3	59.17	a	16.18	ab	252.015	b	4.03	ab	93.23	a	10.59	ab	48.41	a	2.18	b
Group 4	36.89	b	11.86	b	119.49	c	5.54	a	60.85	bc	12.47	ab	28.68	b	2.07	b
Group 5	39.50	b	13.06	ab	193.55	bc	3.64	b	54.80	c	6.95	b	32.01	b	3.49	ab

STL: Spathe Total length; **SMW:** Spathe Maximum Width; **SN:** Spikelet Number; **SSL:** Shorter Spikelet Length; **FNLS:** Flower Number/Longest Spikelet; **FNSS:** Flower Number/Shorter Spikelet; **LBP:** Length of the Branched Part; **WBP:** Width at the middle of the Branched Part.

88.70%. For the last group, the percentages of viability vary from 84.90% for M126 to 98.05% for M30 (Table 5).

3.3.2. Germinative capacity evaluation

The germination rate ranges from 0.90% to 70.50%. At the group G1, the highest percentage (49.50%) was obtained from M142, while the lowest rate (1.10%) was observed in M45. For the group G2, the maximum germination was scored by M148 with 56.70% and the lower rate was found in M33 with 3.53%. The group G3 exhibited pollen germination rate between 3.29% for M123 to 46.37% for M28. The male genotypes of the group G4 showed a pollen germination rate varying from 0.90% for M33 to 70.50% for M44. At the last group G5, M93 showed the highest ratio (37%), whereas M52 showed the lowest rate (7.80%). Unlike these results, a remarkable absence of germination was recorded in the pollen of five male genotypes M1, M138, M141, M154 and M157 (Table 5).

3.4. Correlation between pollen quality and the morphological variables

The Pearson correlation coefficient performed between the pollen reproductive quality and the set of quantitative data are indicated in Table 6. The inflorescence morphological characters were in statistically significant correlation with the pollen quality. These relationships are presented with a negative correlation between four of the nine inflorescence traits and only the germination capacity of pollen. The obtained correlations are defined by the Spathe Total Length (STL) (r = -0.383, p-*value* = 0.007), the Spi-

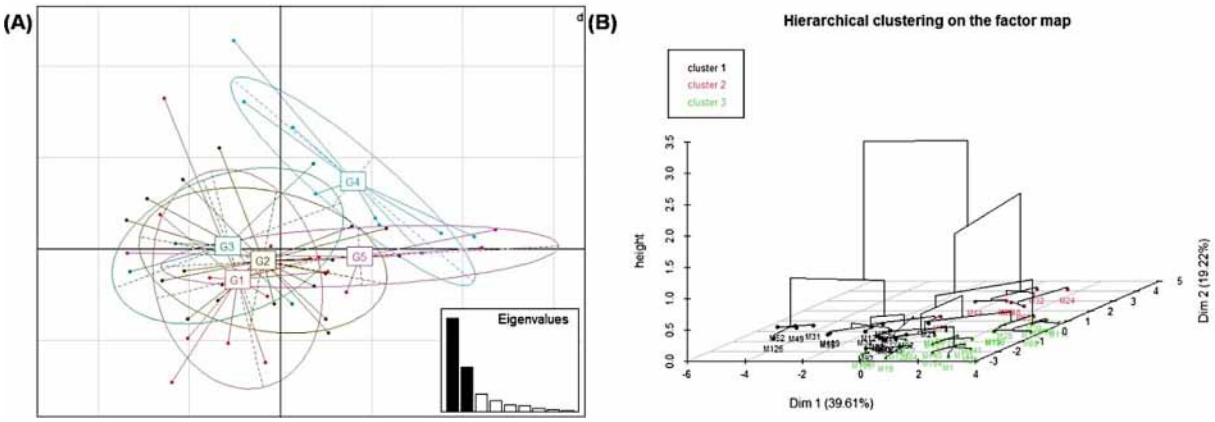

Fig. 7. Factorial analysis and clustering patterns based on the inflorescence morphological descriptors. **(A)** Principal component analysis (PCA) using the discriminated inflorescence traits for the examined male genotypes; (**B**) Hierarchical clustering on the factor map for the inflorescence morphological traits.

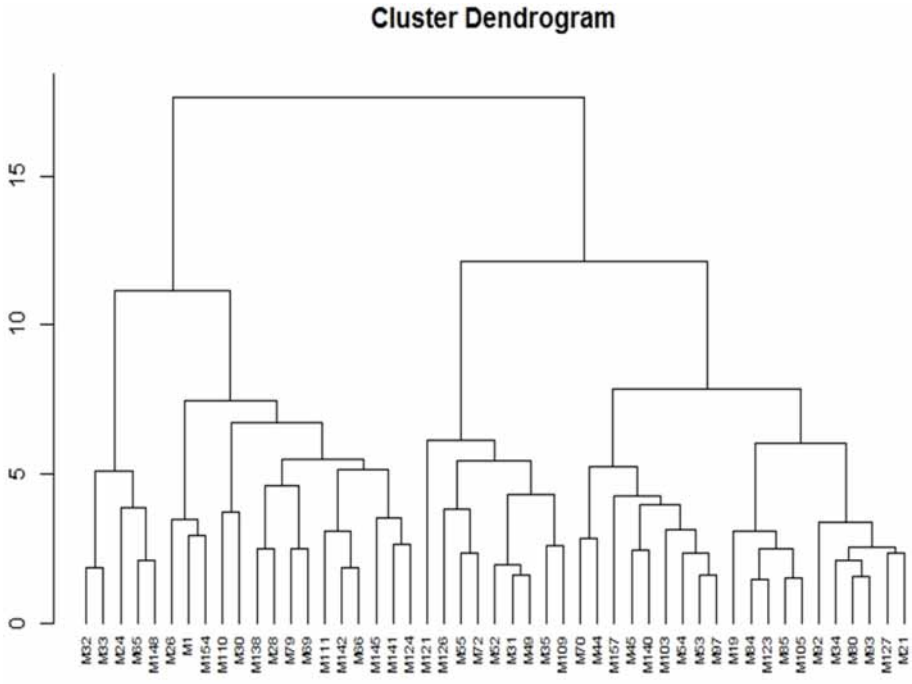

Fig. 8. Hierarchical clustering of the male genotypes based on the morphological data using Ward method and Euclidian distance.

kelet Number (SN) (r = -0.339, *p-value* = 0.017), the Branched part at the flower stalk (LBP) (r = -0.305, *p-value* = 0.033) and Width in the middle of the Branched Part at the flower stalk (WBP) (r = -0.394, *p-value* = 0.005).

3.5. Superior male date palms identification

Based on the agro-morphological characters analysis, the Tunisian collection discloses a large size of trait variability. The data resulting is an excellent autochthonous resource for selection. Stability of flowering precocity, very early flowering time, precocity and flowering time conservation, early pollen availability, late pollen availability, high pollen viability and good germination capacity are important selection criteria. Given the current situation of the oasis ecosystem, identification of male genotypes possessing these valuable traits forms a plant improvement solution.

Of this collection, 15 male plants were selected in terms of flowering features, pollen availability and pollen performance (Table 7). From this list, several individuals have confirmed their capacity to synchronize the headway or the delayed flowering period of the female accessions and to provide pollen for their pollination in the case of early or late opening spathe period. Moreover, male genotypes with a good pollen viability and or good pollen germination are qualified and characterized by the criteria "quality pollen donor" that will guarantee to the farmers the quality and the quantity of dates requested. Fascinatingly, 86 % of the selected male genotypes displayed character combinations which reflect the richness of Tunisian date palm heritage.

To conserve this collection of interesting male plants and as described by (Patent Application Publication: US2010337395A1, 2015) a molecular identification key was established to fingerprinting (Fig. 9).

The constructed identification key permitted the unambiguous discrimination of 100% of the 15 selected male date palms. Therefore, this identification key would be of great interest in the description, registration, certification and *in vitro* multiplication of these valuable resources and for improvement programs.

Table 5
Viability and germination rate of pollen for the 49 evaluated male genotypes (average of 2014–2015 years).

Flowering Groups	Male Genotypes	Reproductive quality	
		Viability (%)	Germination (%)
Group G1	**M1**	89.40	0.00
Early flowering stability	**M19**	88.25	37.56
	M24	98.75	23.35
	M26	97.90	15.40
	M45	96.10	1.10
	M103	51.10	9.04
	M127	94.23	33.23
	M140	97.55	12.08
	M141	95.00	0.00
	M142	97.30	49.50
	M154	94.20	0.00
	M157	93.80	0.00
Group G2	**M53**	82.20	44.18
Two successive seasons with	**M54**	97.05	28.26
early flowering	**M55**	91.40	45.03
	M65	98.25	37.90
	M66	97.70	17.95
	M72	98.37	31.84
	M79	98.25	54.70
	M80	93.75	28.20
	M84	97.60	17.83
	M85	92.05	33.57
	M97	93.55	11.28
	M105	98.38	45.98
	M110	96.10	5.40
	M111	88.60	7.80
	M121	94.50	8.95
	M124	92.00	3.53
	M138	96.40	0.00
	M145	92.65	13.16
	M148	97.50	56.70
Group G3	**M28**	94.50	46.37
Two successive full-seasons	**M69**	98.40	12.55
flowering	**M70**	98.15	43.95
	M123	98.75	3.29
Group G4	**M21**	94.85	36.25
Two successive seasons with	**M31**	89.07	61.83
late flowering	**M32**	93.25	10.65
	M33	93.20	0.90
	M34	94.38	41.07
	M35	96.50	36.07
	M44	88.70	70.50
	M49	94.90	48.36
	M109	92.05	51.11
	M30	98.05	12.50
	M52	90.45	7.80
Group G5	**M92**	96.70	43.45
Flowering fluctuation	**M93**	96.40	37.00
	M126	84.90	11.65

4. Discussion

Knowledge of the potential variability contributes enormously in conservation, management and breeding programs of crop resources. In date palm, the morphological and the phenotypic variability have been one of the main techniques used. The morphological markers of vegetative and reproductive organs are extensively used at a phenotyping assessment of female accessions (Rhouma, 1994, 2005; Elhoumaizi et al., 2002; Elshibli and Korpelainen, 2009; Ould Mohamed Ahmed et al., 2011; Al-Khalifah et al., 2012; Naqvi et al., 2015; Bedjaoui and Benbouza, 2018).

After studying three consecutive years, the male genotypes featured a great variability at the flowering traits. Thus, this floral characterization has recorded a tendency of precocity at the flowering onset, mid-season opening phenotype and a prolonged emergence-opening duration. Such phenotypic variation of flowering traits has also been reported in the work of Cosmulescu and Ionescu (2021) for native population of walnut genotypes in Oltenia, Romania.

Overall, the distribution of the male genitors in the PCA using the flowering traits patterns has depicted a high variability of Tunisian germplasm. In the light of these observations, it seems clear that these phenotypic traits are very efficient in understanding Tunisian male genotypes biodiversity. This result is in consonance with earlier observations found by Acosta-Quezada et al. (2016) and Martínez et al. (2019).

Basing on the data set of inflorescence morphological traits, wide morphological variation has exhibited between the five groups of the classified male genotypes according to their flowering behavior. Within this collection, the qualitative characters have revealed different proportion between groups. Also, statistically significant differentiation for these morphological variables is recorded among the investigated samples. All this information proved the great validation use of these morphological markers in assessment of male date palms variability.

Likewise, the quantitative variables taken into account in the present study exhibited a high level of variability. Eight of the nine traits showed significant differences among the examined male plants. These findings suggest the role of the reproductive organ in understanding and evaluating the male palms biodiversity. This result is in consonance with earlier observation found by Hamza et al. (2011) in study of Tunisian continental cultivars. These authors obtained the high discrimination at the bunch length without spikelet and spikelet length without fruit. Palm traits which have proved their powerful discrimination in previous studies of date palm deserves to be studied at this collection. Leaf morphological traits with these different components leaflets, pinnaes and spines have been successfully used to elucidate the phenotypic variability of female varieties in Tunisian oases (Hammadi et al., 2009; Hamza et al., 2011), Algerian germplasm (Bedjaoui and Benbouza, 2018), date palm cultivars in Moroccan oases (Elhoumaizi et al., 2002) and Mauritanian date-palm accessions (Ould Mohamed Salem et al., 2008; Ould Mohamed Ahmed et al., 2011). The morphospace projection PCA, the HCPC and Ward's classification strongly support each other. These analyses based on the inflorescence morphological variables depicted the great similarity between the defined flowering groups of the investigated collection.

Table 6
Pearson's coefficient between pollen quality and the quantitative morphological markers studied.

		Inflorescence morphological characters								
		STL	SMW	SN	LSL	SSL	FNLS	FNSS	LBP	WBP
Viability	***r***	0.178	0.161	0.106	0.212	0.082	0.091	−0.014	0.165	−0.123
	p-value	0.222	0.271	0.468	0.144	0.557	0.533	0.924	0.259	0.400
Germination	***r***	−0.383**	−0.115	−0.339*	−0.192	−0.094	−0.176	−0.165	−0.305*	−0.394**
	p-value	0.007	0.432	0.017	0.186	0.521	0.227	0.257	0.033	0.005

'' at the 0.01 level, '*' at the 0.05 level; STL:** Spathe Total length; **SMW:** Spathe Maximum Width; **SN:** Spikelet Number; **LSL:** Longest Spikelet Length; **SSL:** Shorter Spikelet Length; **FNLS:** Flower Number/Longest Spikelet; **FNSS:** Flower Number/Shorter Spikelet; **LBP:** Length of the Branched Part; **WBP:** Width at the middle of the Branched Part.

Table 7
Agronomic data relative to the selected male genotypes among the examined collection.

Male Genotypes	Interesting Agronomic Traits						
	Stability of Flowering Precocity	Very Early Flowering Time	Precocity and Flowering Time Conservation	Early Pollen Availability	Late Pollen Availability	High Pollen Viability	Good Germination Capacity
M45	■	■		■			
M4	■		■				
M19	■			■			
M24	■					■	
M148	■						■
M14		■		■			
M176			■				
M31					■		■
M44					■		■
M109					■		■
M34					■		
M49					■		
M79						■	■
M69						■	
M123						■	

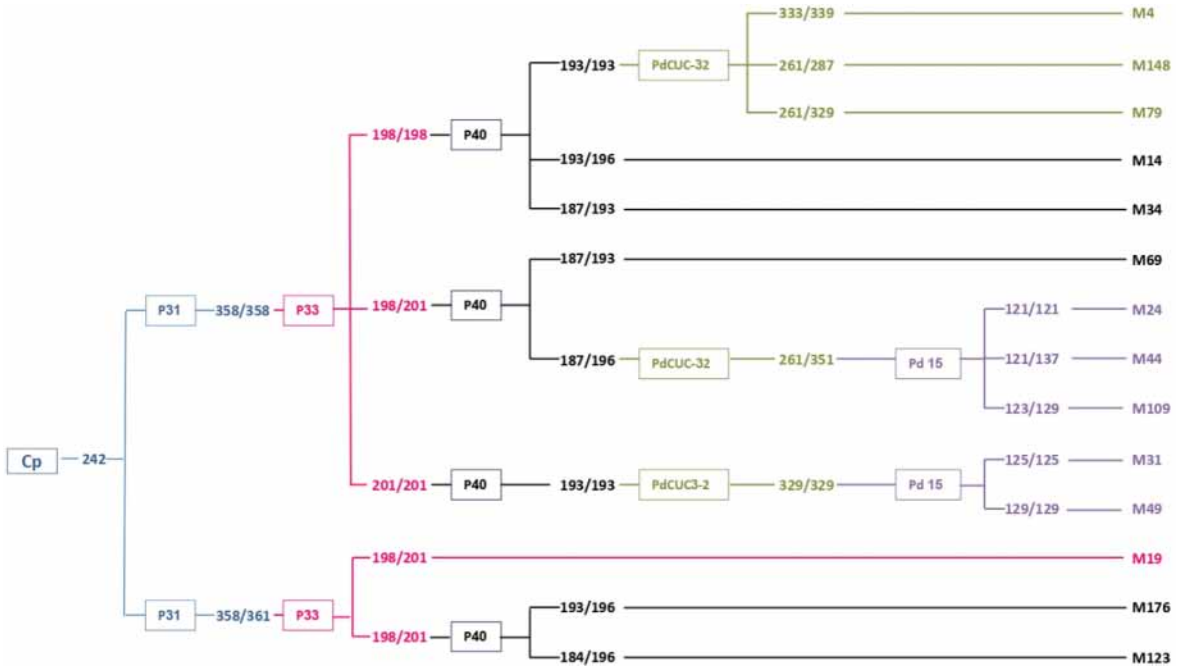

Fig. 9. Identification key of the 15 selected male genotypes based on seven patented SSR makers of date palm cultivars. Each branch corresponds to a different allelic profile which is reported in base pairs.

At pollen reproductive quality level, the viability of the pollen grains varies from 55 to 98.23 %. In the same context, Benamor et al. (2014) have obtained a viability percentage which varies from 81.33 to 99.67 % at Algerian collection. The percentage values of germination were between 0.90 and 70.5 %. These values are higher than the values in studies of El Mardi and Bakheit (1996) which vary from 32 to 39 %. In our study, low values and absence of germination were noticed in some male palms. In view of this, we can conclude that some pollen grains studies are sensitive to storage conditions which explain the decrease even the loss of their germination capacity. The results presented here support numerous publications which found the negative effect of storage conditions in viability, germination and tube growth and elongation of pollen date palm (Al-Helal, 1994; El Mardi and Bakheit, 1996; Damankeshan and Panahi, 2013; Maryam et al., 2015). While, Mesnoua et al. (2018) have discussed the effect of temperature storage in pollen germination and pollen fertility. In addition, relationships have been detected between the pollen performance and the inflorescence morphological markers. These results emphasize the importance of these agro-morphological traits used in this study for assessment of existing agrobiodiversity of date palm.

Alongside the agro-morphological characterization, the screening of the investigated collection led to the identification and selection of interesting male genotypes. These plants with stability of flowering precocity, very early flowering time, precocity and flowering time conservation considered suitable candidates for genetic research to cope the oasis agro-system perturbations under future climate change scenarios. In the same way, Neyshaburi et al. (2021) demonstrate by flowering examination of local pistachio genotypes that the selected male genotypes have a high flowering period which covers the commercial pistachio cultivars.

Focusing on the selected list, the set of the male genotypes, possessing early pollen availability and late pollen availability, provide

pollen at appropriate time for female varieties with early or late flowering to achieve pollination and fertilization. The high reproductive quality of the male genitors pollen among the selected list contributes for a good part to the success of the pollination and therefore to the obtaining of a good yield. These performers' palms can ensure the high commercial value of fruit production and the development of improvement programs. These materials are of paramount importance in establishment research in association mapping, genomic selection and tissue culture for achieving a genetic improvement program of competitive, performer and climate-resilience phœniciculture.

5. Conclusions

In this study, the male date palm germplasm shows a great wealth of morphological variability thus forming an available genetic reservoir for exploitation. The selected male progenitors; M4, M14, M19, M24, M31, M34, M44, M45, M49, M69, M79, M109, M123, M148 and 176 reflect the valuable gene pool for the local resources of date palm in Tunisia. So, further phenological and agronomic investigations on these male genotypes are highly recommended. Finally, the range of information presented here gives a suitable framework for the basic knowledge of morphological traits variability, especially those of agronomic importance. Hence, developing molecular markers associated with these traits could provide a powerful tool to shape selection programs and conservation strategies *via* MAS (Marker Assisted Selection) approach.

CRediT authorship contribution statement

Afifa Hachef: Investigation, Software, Writing-original draft. **Hédia Bourguiba:** Conceptualization, Methodology. **Emira Cherif:** Formal analysis, Visualization. **Sarah Ivorra:** Data curation. **Jean-Frédéric Terral:** Validation. **Salwa Zehdi-Azouzi:** Writing-review and editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103574.

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