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Molecular Identification of Genetic Diversity and Population Structure in Moroccan Male Date Palm (*Phoenix dactylifera* L.) Using Inter-Simple Sequence Repeat, Direct Amplification of Minisatellite DNA, and Simple Sequence Repeat Markers

Maha Ibrahimi [1,2], Najiba Brhadda [2], Rabea Ziri [2], Mohamed Fokar [3], Ilham Amghar [1], Fatima Gaboun [1], Aicha Habach [4], Reda Meziani [5], Jamal Elfadile [5], Rabha Abdelwahd [1] and Ghizlane Diria [1,*]

1 Biotechnology Unit, Regional Center of Agricultural Research of Rabat, National Institute of Agricultural Research (INRA), P 6570, Rabat 10101, Morocco; maha.ibrahimi@uit.ac.ma (M.I.); amghar.ilham@gmail.com (I.A.); fatima.gaboun@inra.ma (F.G.); rabha.abdelwahd@inra.ma (R.A.)
2 Laboratory of Plant, Animal and Agro-Industry Productions, Faculty of Sciences, University of Ibn Tofail, BP 133, Kenitra 14000, Morocco; najiba.brhadda@uit.ac.ma (N.B.); rabea.ziri@uit.ac.ma (R.Z.)
3 Center for Biotechnology and Genomics, Texas Tech University, Room 101, Lubbock, TX 79409, USA; m.fokar@ttu.edu
4 Laboratory of Microbiology and Molecular Biology, Faculty of Science, Mohammed V University, B.P: 8007.N.U, Rabat 10000, Morocco; aicha.habach.fsr@gmail.com
5 UR Oasis Systems, National Laboratory of Date Palm Tissues Culture, Regional Center of Agricultural Research of Errachidia, National Institute of Agricultural Research (INRA), BP 2, Errachidia 52000, Morocco; reda.meziani@inra.ma (R.M.); jamal.elfadile@inra.ma (J.E.)
* Correspondence: ghizlane.diria@inra.ma; Tel.: +212-662-451-755

Abstract: Understanding genetic diversity and population structure plays a vital role in the efficient use of available material in plant-breeding programs and in germplasm conservation strategies. In the present study, we aim to evaluate the genetic variations and population structure of male date palms from Morocco. The genetic diversity of 100 date palm (*Phoenix dactylifera* L.) genotypes was investigated using the performance of three types of molecular markers: inter-simple sequence repeats (ISSRs), direct amplification of minisatellite DNA (DAMD), and simple sequence repeats (SSRs). On the basis of their polymorphic information content (PIC) (ISSRs = 0.38; DAMD = 0.4; SSRs = 0.33), effective multiplex ratio (EMR) (ISSRs = 27.34; DAMD = 52.31; SSRs = 22.20), Resolving power Rp (ISSR = 13.81; DAMD = 28.73; SSR = 14.6), and marker index (MI) (ISSRs = 9.22; DAMD = 20.23; SSRs = 7.54) values, all markers used in our study are considered informative markers. Among them, DAMD markers demonstrated slightly higher informativeness compared to ISSR and SSR markers. A total of 216, 438, and 248 bands were, respectively, detected using ISSRs, DAMD, and SSRs, with 95%, 98% and 94% of polymorphism, respectively. The AMOVA results revealed considerable diversity within date palms. The PCOa results showed that males of Tinghir and Errachidia were regrouped into the same cluster, while males of Goulmima were separated into another group. A cluster and structure analysis separated the studied genotypes into three groups. One group comprises genotypes of males from Zagora with some female varieties scattered in this group. The second group includes male genotypes from Goulmima along with accessions of female and male varieties. The third group contains males of Errachidia, Tata and Tinghir populations. The cluster and structure analysis separated the studied genotypes according to their origin.

Keywords: genetic diversity; *Phoenix dactylifera* L.; ISSR; DAMD; SSR; structure

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1. Introduction

The date palm (*Phoenix dactylifera* L.) is one of the world's major crops belonging to the Arecaceae family [1]. It is an iconic crop of great cultural and economic importance, espe-

cially in arid and semiarid regions. In Morocco, date palms are cultivated on 63,215 hectares of land with a production of about 143,160 tons [2].

Despite the availability of several cultivars, local date palm cultivation is susceptible to pests and diseases, which is further worsened by the severe impacts of abiotic stresses induced by climate change and global warming [3]. These factors can potentially lead to the genetic degradation of this important resource. Consequently, it becomes crucial to thoroughly evaluate the genetic diversity of this species.

The date palm is dioecious, with male and female trees requiring cross-pollination for fruit production. The genetic diversity of female date palms has received substantial attention worldwide, but comprehensive research on the genetic diversity of male date palms remains limited. A genetic diversity analysis of male date palms (Pollinizers) offers multifaceted benefits for the advancement of date palm cultivation [4]. The genetic variability present in male palms contributes to the quantity and quality of pollen production, ultimately influencing fruit yield and quality in female palms [5]. Furthermore, understanding the genetic diversity of male date palms enables the development of targeted breeding programs and facilitates the identification and preservation of valuable traits.

To assess the genetic diversity of date palms, a range of techniques have been utilized. Initially, phenotypic [6–8] and biochemical [9,10] markers were used for this purpose. However, these markers are influenced by environmental factors and the developmental stage of the date palm [11]. In order to overcome this limitation, molecular markers have been employed to effectively reveal and characterize the genetic diversity differentiation present in date palms. Various molecular markers, including random amplified polymorphism DNA (RAPD) [12–14], amplified fragment length polymorphism (AFLP) [15–17], simple sequence repeats (SSRs) [11,18–20], inter-simple sequence repeats (ISSRs) [14,21,22] sequence-related amplified polymorphism (SRAP) [23,24], and single nucleotide polymorphism (SNP) [25], have been employed to assess the genetic diversity of date palms.

Indeed, an ideal molecular marker for genetic analysis must be polymorphic, be reproducible, ideally possess significant heritability, be applicable to any part of the genome, exhibit sufficient polymorphism to distinguish closely related genotypes, and preferably be a co-dominant marker. In this study, we have utilized three markers, namely inter-simple sequence repeats (ISSRs), direct amplification of minisatellite DNA (DAMD), and simple sequence repeats (SSRs), which meet these criteria. These markers are not only easy to implement but also demonstrate remarkable reproducibility. By employing these three markers, we aim to gain a more comprehensive understanding of the genetic organization of the Moroccan male date palm. The use of SSR markers is considered one of the most effective strategies for assessing date palm genetic diversity [19,20,22,24], while DAMD has been proven to be a powerful tool for studying genetic diversity [11,21,26]. Inter-simple sequence repeat (ISSR) markers are known for their exceptional discriminatory power, simplicity, rapidity, cost-efficiency, stability, and reliability in marker identification [21]. The combination of ISSR, DAMD, and SSR markers allows us to gather complementary information, facilitating a more accurate measurement of date palm genetic diversity and population structure.

Research on the genetic diversity of date palms (*Phoenix dactylifera* L.) has been conducted in various regions. Some of this research has investigated the genetic diversity among date palm pollinizers in southern Tunisia, revealing high genetic diversity, and accessions were not grouped according to their geographical origin [27]. In Iran, a moderate level of genetic diversity has been observed among the studied cultivars [28]. In Ethiopia, genetic variations of date palms have been identified as crucial for crop improvement and conservation programs [29]. A study conducted on 102 Algerian date palm samples from the oases of the Biskra region found a high degree of genetic diversity [30]. Another study assessed the genetic diversity among thirty-nine date palm cultivars from Tunisia and India using six amplified fragment length polymorphism (AFLP) markers. The findings indicated a clear separation between Tunisian and Indian cultivars with accessions being grouped according to their geographical origin [17]. In Tunisia, a genetic analysis of wild

African date palms revealed strong genetic differentiation between Western and Eastern date palms, demonstrating high diversity among North African accessions [31]. Indeed, the assessment of genetic diversity provides valuable insights crucial for breeding programs across different regions worldwide.

This article aims to evaluate the genetic diversity of male date palm trees in Morocco using inter-simple sequence repeat (ISSR), directed amplification of minisatellite DNA (DAMD), and simple sequence repeat (SSR) markers. It also highlights the importance of such assessments in understanding the genetic composition, relatedness, and conservation of Moroccan male date palm populations.

2. Materials and Methods

2.1. Plant Genetic Resources

Fresh leaf samples of date palm germplasm were collected from five regions of Morocco, namely Zagora, Errachidia, Goulmima, Tata, and Tinghir. The plant material consisted of 100 date palm genotypes, comprising 76 males sampled from the five different locations in southern Morocco (Figure 1) and 24 varieties which include 11 varieties from the National Institute of Agricultural Research Collection in the experimental station in Zagora in southeastern Morocco. These varieties consist of 8 female cultivars—Majhoul, Boufgouss, Najda, Bouskri, Aziza, Gharass, Sedrat, Deglet Nour—and 3 male cultivars NP3, Nebch-Boufeggous NP4, and GS, beside 13 female varieties from the Zagora oasis (Table of the coordinate GPS in Appendix A). The plants were selected to capture a wide range of pollinizers' genetic diversity across these traditional regions. Only young leaves from healthy adult plants were collected and carefully stored at −20 °C for subsequent analysis.

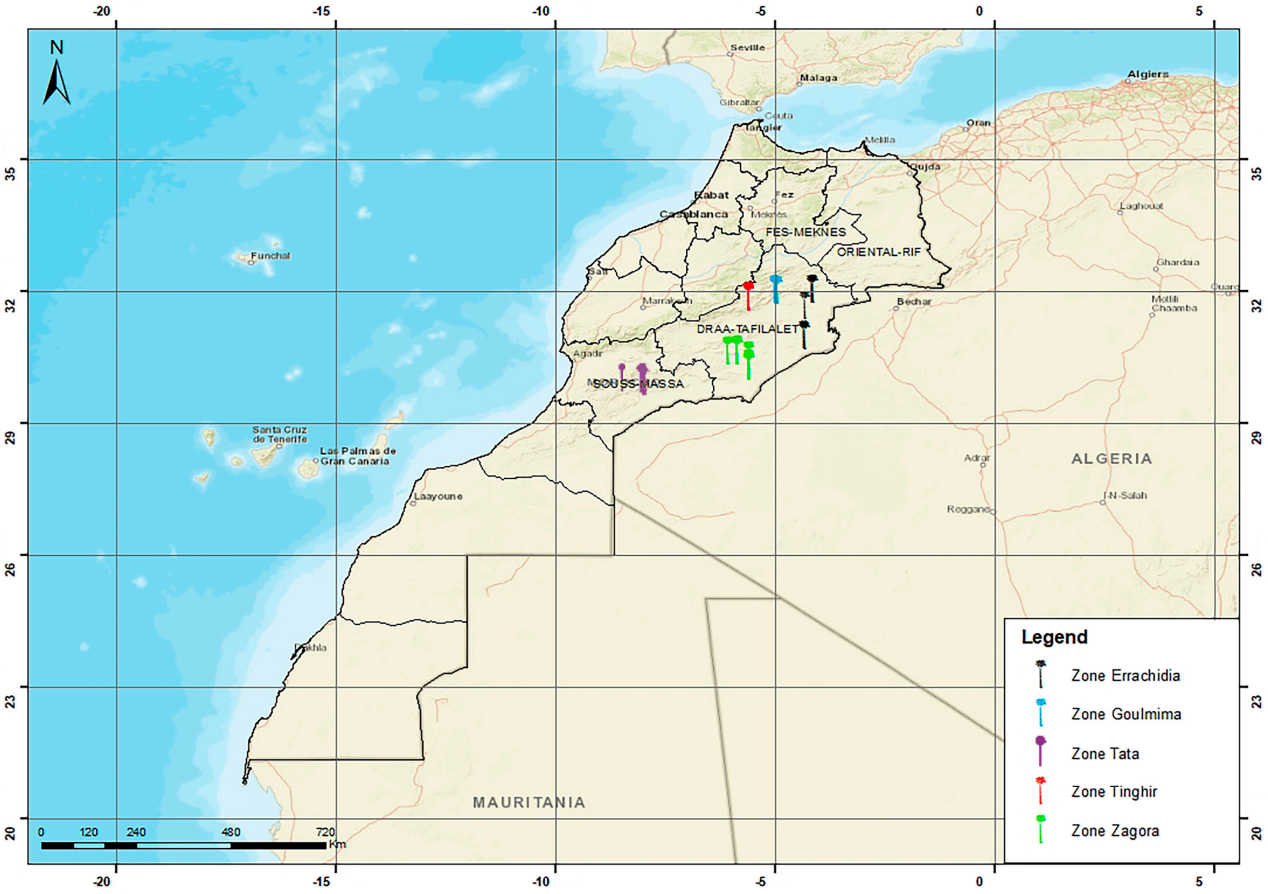

Figure 1. Geographic coordinate (via GPS) of the plant material collection location.

2.2. DNA Extraction

The procedure began by grinding approximately 60 mg of lyophilized leaves into a fine powder using a ball mill (Mill: SPEX 100 Sample Prep). The CTAB (Cetyl Trimethyl Ammonium Bromide) method was then employed to extract DNA from the ground tissues, with minor adjustments based on Saghai–Maroof's protocol [32]. The concentration and quality of the DNA were evaluated using a NanoDrop spectrophotometer. DNA purity was determined by assessing the A260/A280 ratio, which fell within the range of 1.80 to 2.0. Additionally, the quality of the DNA was assessed using electrophoresis on a 1% (*w*/*v*) agarose gel. To preserve the quality of DNA extracts, they were stored at −20 °C for future use.

2.3. ISSR Amplification and Genotyping

A set of 9 ISSR primers were selected for PCR amplification of the DNA templates (Table 1). PCR reactions were conducted in a total volume of 10 µL containing of 25 ng of genomic DNA, 2.5 µL of 1X buffer (Bioline, London, UK), 0.5 U of TaqDNA polymerase (BIOLINE, London, UK), 0.5 µM of primer, and 5.4 µL DNase-free water. Two different protocols were employed for PCR amplifications, depending on the specific requirements of the primer PCR conditions (Table 1). The first protocol, P1, involved an initial denaturation step at 94 °C for 5 min, which was followed by 40 cycles of each cycle with 10 s denaturation at 94 °C, 10 s annealing at Tm, and 10 s extension at 72 °C. The final extension was performed at 72 °C for 7 min. The second cycling profile P2 began with an initial denaturation step at 95 °C for 5 min, which was followed by 35 cycles of denaturing at 95 °C for 30 s, annealing temperatures for 1 min, and primer elongation at 72 °C for 2 min, with a final extension step at 72 °C for 8 min. The reactions were subsequently stored at 4 °C for future use in gel electrophoresis.

Table 1. ISSR primers selected and PCR programs used for genetic diversity assessment of date palm.

ISSR Primers	Sequence (5′-3′)	Tm (°C)	PCR Program
I1	GAGAGAGAGAGAGAGAGAC	52	P1
I3	GAGAGAGAGAGAGAGAGAA	52	P1
UBC8 11	GAGAGAGAGAGAGAG AC	52	P2
UBC8 34	AGAGAGAGAGAGAGAGCT	52	P1
UBC8 41	GAGAGAGAGAGAGAGACTC	52	P2
UBC8 42	GAGAGAGAGAGAGAGACG	52	P1
UBC8 80	GGAGAGGAGAGGAGA	58.5	P1
UBC8 89	DBDACACACACACACAC	52	P1
UBC8 25	ACACACACACACACACT	52	P1

A, T, C and G: nitrogenous bases, Tm: temperature annealing, B: C, G or T, D: A, G or T.

2.4. DAMD Amplification

Nine DAMD primers were carefully opted for the analysis (Table 2). The PCR composition used for DAMD analysis was identical to that employed for ISSR analysis. The PCR reactions were carried out by adhering to the following program: initial denaturation at 94 °C for 3 min, followed by 35 cycles consisting of denaturation at 92 °C for 45 s, annealing at the primer specific temperature for 2 min, and primer elongation at 72 °C for 2 min. Then, there was a final extension at 72 °C for 5 min followed by cooling at 4 °C to preserve the reactions for further steps of electrophoresis.

2.5. SSR Amplification

Ten SSR markers were chosen (Table 3) based on previous date palm research studies and used for the characterization of 100 date palm genotypes. The PCR reactions for SSR markers were conducted in a 10 µL reaction volume using the same composition as ISSR and DAMD analyses. The PCR protocol for SSR markers was set as follows: after initial denaturation of DNA at 94 °C for 1 min, 35 cycles occurred, each cycle with DNA

denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 2 min. A final elongation step was performed at 72 °C for 8 min. The reactions were then held at 4 °C for later use.

Table 2. DAMD primers selected and PCR annealing temperature used for genetic diversity assessment of date palm.

DAMD Primers	Primer Sequences (5′-3′)	Tm (°C)
M13	GAG GGT GGC GGC TCT	60
HBV3	GGT GAA GCA CAG GTG	50
HBV5	GGT GTA GAG AGG GGT	49
HVR	CCT CCT CCC TCC T	40
INS	ACA GGG GTG GGG	40
URP2R	CCC AGC AAC TGA TCG CAC AC	70
URP9F	ATG TGT GCG ATC AGT TGC TG	60
URP25F	GAT GTG TTC TTG GAG CCT GT	59

Tm (°C): PCR annealing temperature.

Table 3. SSR forward and reverse primers selected for genetic diversity assessment of date palm.

SSR Primers	Sequences (5′-3′)	Sequences (5′-3′)
	Forward	Reverse
mPdCIR025	F: GCACGAGAAGGCTTATAGT	R: CCCCTCATTAGGATTCTAC
mPdCIR015	F: AGCTGGCTCCTCCCTTCTTA	R: GCTCGGTTGGACTTGTTCT
mPdCIR035	F: ACAAACGGCGATGGGATTAC	R: CCGCAGCTCACCTCTTCTAT
mPdCIR048	F: CGAGACCTACCTTCAACAAA	R: CCACCAACCAAATCAAACAC
mPdCIR050	F: ACAAACGGCGATGGGATTAC	R: CCGCAGCTCACCTCTTCTAT
mPdCIR057	F: AAGCAGCAGCCCTTCCGTAG	R: GTTCTCACTCGCCCAAAAATAC
mPdCIR070	F: CAAGACCCAAGGCTAAC	R: G GAGGTGGCTTTGTAGTAT
mPdCIR085	F: GAGAGAGGGTGGTGTTATT	R: TTCATCCAGAACCACAGTA
mPdCIR090	F: GCAGTCAGTCCCTCATA	R: GCAGTCAGTCCCTCATA
mPdCIR093	F: CCATTTATCATTCCCTCTCTTG	R: CTTGGTAGCTGCGTTTCTTG

2.6. Gel Electrophoresis

To identify genetic variations among the genotypes, the PCR products were electrophoresed in 6% polyacrylamide gel. The amplified bands were stained with a 5-min incubation in an ethidium bromide solution (10 mg/mL). The gel was then visualized under ultraviolet light using the Molecular Imager® Gel DocTM XR System sourced from Bio-Rad company, headquartered in Hercules, California, USA. The gel profiles were captured and recorded as digital images using a Gel Documentation System for subsequent scoring and analysis.

2.7. Statistical Analyses

The binary matrices were generated by assigning a value of 1 for the presence and 0 for the absence of amplified fragments in the samples under study from each ISSR, DAMD and SSR profile. Then, various parameters were assessed.

The efficacy of a molecular marker technique relies on a specific set of parameters. To evaluate this, we calculated the polymorphic information content (PIC) using Nei's and Botstein methods [33,34]. We determined the resolving power (RP) to assess the ability of each primer to differentiate between individual genotypes, following Prevost et al.'s approach [35]. Furthermore, we computed the effective marker ratio (EMR) using the formula EMR = np(np/n), where "np" denotes the number of polymorphic loci and "n" represents the total number of loci [36]. Finally, we determined the marker index (MI) with the formula MI = EMR × PIC, which quantifies each primer's capability to identify polymorphic loci among the various genotypes.

The genetic diversity analysis of *Phoenix dactylifera* L. was conducted using GenAlex ver. 6.5 software [37] to calculate a set of parameters, including Nei's genetic diversity

index (H), the percentage of polymorphic loci (PPL), the number of effective alleles (Ne), expected heterozygosity (He), and Shannon's information index (I). Additionally, with the same software, we performed a molecular variance analysis (AMOVA) to assess the distribution of genetic variability both between and within populations.

Principal coordinate analysis (PCoA) was carried out among all paired genotypes. The significance of these genetic differences was examined using 999 permutations. Using the XLSTAT 5.14 program, a dendrogram was produced based on the Jaccard dissimilarity coefficient and the UPGMA (Unweighted Pair Group approach of Arithmetic Averages) clustering approach. The population structure was inferred through the utilization of the STRUCTURE 2.3.4 software [38]. For each value of K, we conducted three independent replications. In each run, we employed 100,000 Monte Carlo Markov Chain replicates with a burn-in period consisting of 50,000 steps. Then, the Evanno method was utilized to identify the most probable structure with the optimal K value using the HARVESTER online tool [39].

3. Results

3.1. Characterization and Efficiency of ISSR Markers

The eight ISSR primers used produced a total of 216 bands, 208 of which were polymorphic with 95.47% polymorphism. PIC, EMR, RP, and MI had mean values of 0.38, 27.34, 13.81, and 9.22, respectively. Among the ISSR primers tested, I1 had the highest PIC value of 0.47, UBC8 25 had the highest RP and MI values of 16.42 and 12.77, respectively, while UBC8 42 had the highest EMR value of 45 (Table 4). Figure 2 shows an example of the ISSR profile of certain samples generated using the I3 primer.

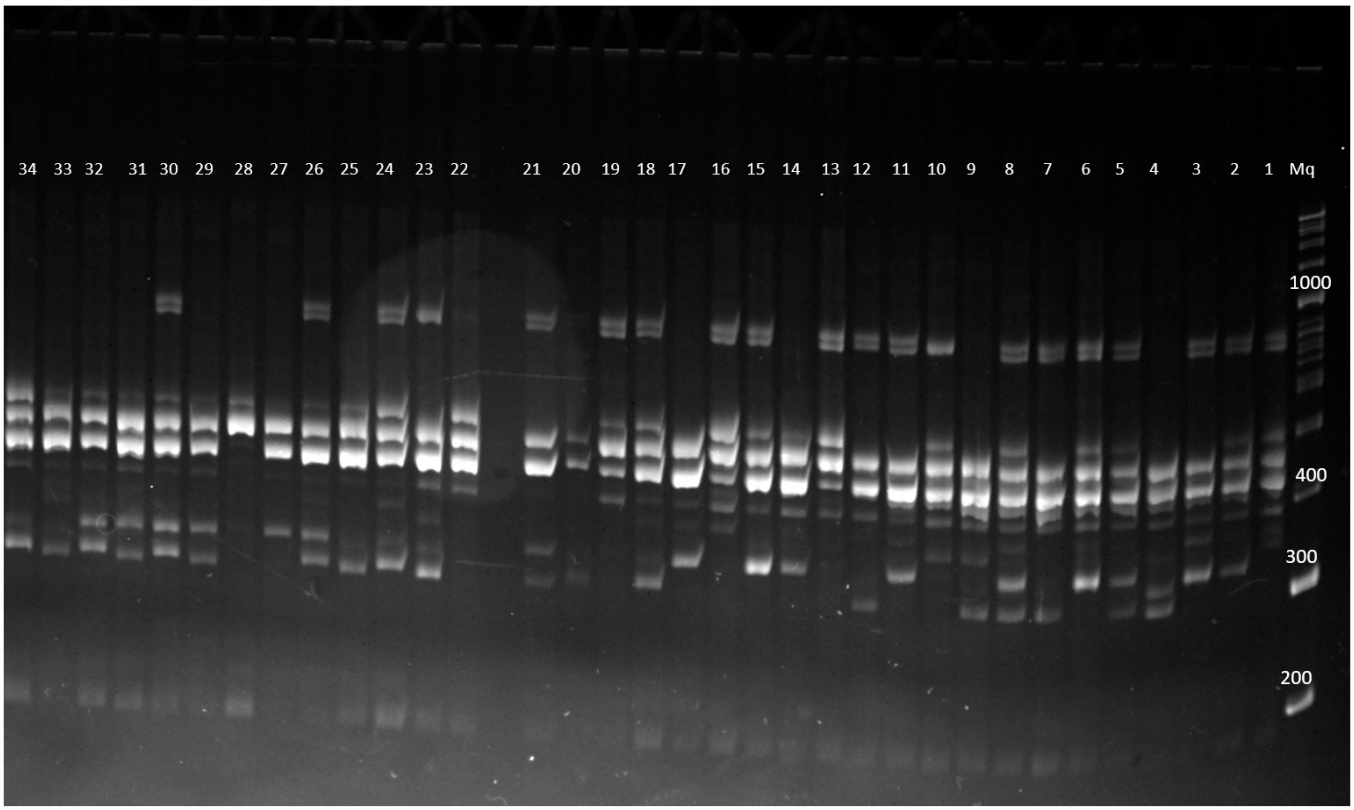

Figure 2. The polyacrylamide gel image depicts the amplification profile of I3 primer for some male samples from the Zagora population.

Table 4. Characterization and efficiency of ISSR markers.

ISSR Primers	Amplicons (bp)	TF	PB	PP	PIC	EMR	RP	MI
UBC8 11	200–1400	25	25	100	0.4235	25	15.22	10.587
I1	240–800	20	20	100	0.471	20	15.8	9.4192
I3	290–1400	17	15	88.235	0.4689	13.2353	12.76	6.206
UBC8 34	170–500	19	18	94.736	0.3807	17.0526	9.72	6.4923
UBC8 41	220–1300	28	26	92.8571	0.3728	42.1429	13.88	9.0016
UBC8 42	60–2000	45	45	100	0.2697	45	14.46	12.1368
UBC8 89	180–1000	25	22	88	0.36	19.36	12.22	7.1504
UBC8 25	230–1300	37	37	100	0.345	37	16.42	12.7765
Total		216	208					
Mean		27	26	95.4786	0.386	27.348	13.81	9.221225

TF = Total number of fragments. PB = Number of polymorphic bands. PP = Percent polymorphism. PICv = Polymorphic information content values. EMR = Effective multiplex ratio. RP = Resolving power. MI = Marker index.

3.2. Characterization and Efficiency of DAMD Markers

DAMD primers yielded a total of 438 bands, among which 428 were polymorphic, reflecting a polymorphism rate of 97.99%. The average values for PIC, EMR, RP, and MI were recorded at 0.4, 52.31, 28.73, and 20.23, respectively. Among the range of DAMD primers utilized, M13 had the highest PIC value of 0.496, and HBV5 had the highest EMR, RP, and MI values of 74, 37.7, and 27.6, respectively (Table 5). An example of the DAMD profile of some samples produced by URP9F is documented in Figure 3.

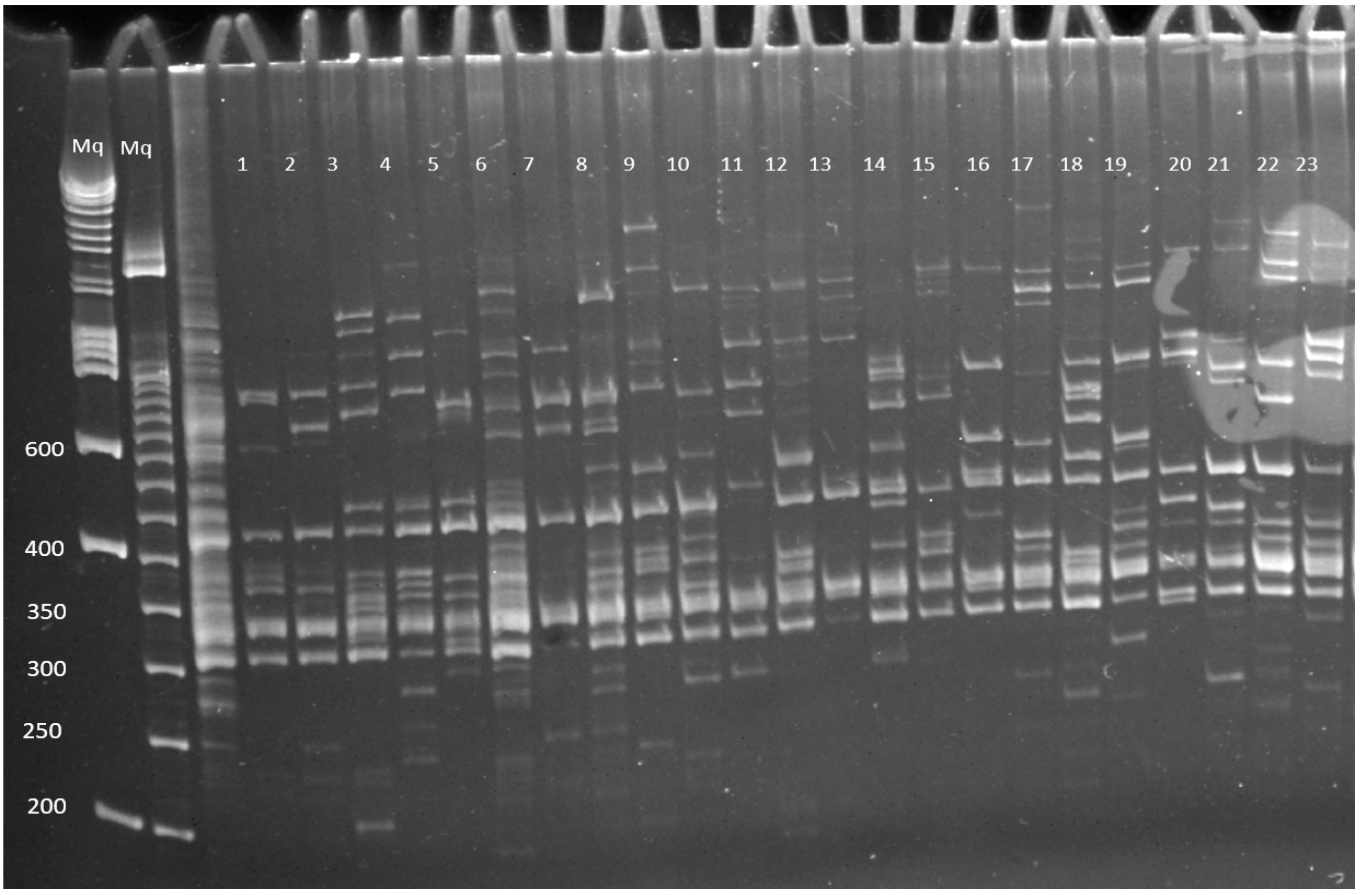

Figure 3. The polyacrylamide gel image shows the amplification profile of URP9F primer for some male samples from the Zagora population.

Table 5. Characterization and efficiency of DAMD markers.

DAMD								
Primers	**Amplicons (bp)**	**TF**	**PB**	**PP**	**PIC**	**EMR**	**RP**	**MI**
M13	190–2000	34	34	100	0.4968	34	31.26	16.88
HBV3	200–7000	55	55	100	0.3502	55	24.9	19.2635
HBV5	100–8000	76	75	98.68421	0.37	74	37.7	27.6
HVR	300–2500	45	45	100	0.4806	45	36.14	21.6278
INS	150–4100	62	59	95.16129	0.4	56.14	34.38	22.5
URP2R	80–550	28	27	96.42857	0.4382	26.0357	18.16	11.4101
URP9F	120–(+1 Kb)	59	59	100	0.37	59	19.12	21.83
URP25F	130–3000	79	74	93.6708	0.3	69.31	28.24	20.79
Total		438	428					
Mean		54.75	53.5	97.99311	0.400725	52.31071	28.7375	20.23767

TF = Total number of fragments. PB = Number of polymorphic bands. PP = Percent polymorphism. PICv = Polymorphic information content values. EMR = Effective multiplex ratio. RP = Resolving power. MI = Marker index.

3.3. Characterization and Efficiency of SSR Markers

SSR primers resulted in the production of 248 bands, with 234 of them displaying polymorphism, signifying a polymorphism rate of 93.99%. The mean values for PIC, EMR, RP, and MI were 0.33, 22.2, 14.60, and 7.54, respectively. Among the employed SSR primers, Cir90 exhibited the highest PIC value of 0.37, and Cir 50 had the greatest EMR and MI values of 41 and 15, respectively, while Cir85 showcased the maximum RP value of 17.98 (Table 6). Figure 4 shows an example of the SSR profile of certain samples generated using the Cir 25 primer.

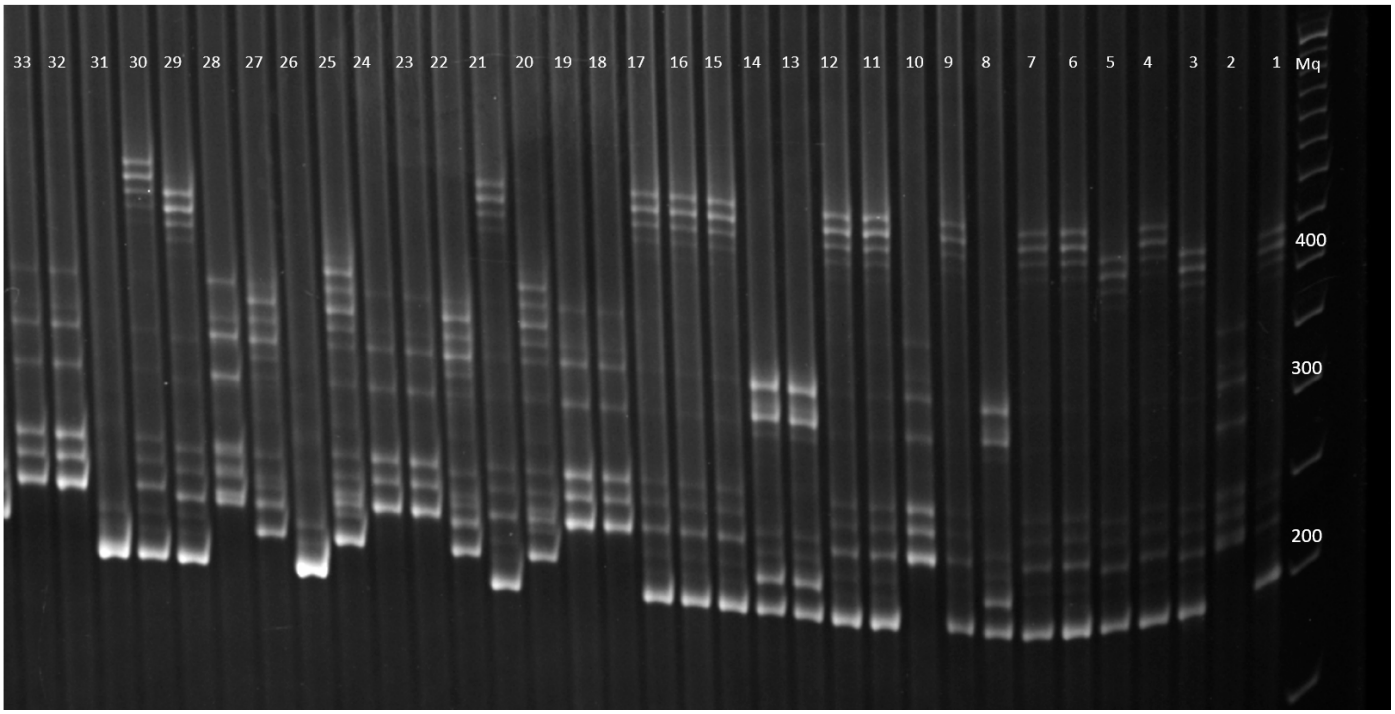

Figure 4. The polyacrylamide gel image shows the amplification profile of Cir 25 primer for some male samples from the Zagora population.

Table 6. Characterization and efficiency of SSR markers.

SSR								
Primers	**Amplicons (bp)**	**TF**	**PB**	**PP**	**PIC**	**EMR**	**RP**	**MI**
Cir 25	150–310	26	26	100	0.358	26	12.04	9.31
Cir 15	118–182	19	18	94.736	0.325	17.05	12.26	5.55
Cir 35	179–300	17	17	100	0.302	17	14.36	5.14
Cir 48	138–270	18	16	88.888	0.299	14.22	16.18	4.25
Cir 50	160–500	41	41	100	0.366	41	17.28	15.01
Cir 57	258–500	19	19	100	0.310	19	14.42	5.90
Cir 70	180–390	29	23	79.31	0.335	18.24	16.96	6.12
Cir 85	160–475	33	33	100	0.342	33	17.98	11.31
Cir 90	130–460	31	28	90.328	0.373	25.29	11.88	9.44
Cir 93	170–320	15	13	86.666	0.302	11.26	12.72	3.40
Total		248	234					
Mean		24.8	23.4	93.992	0.3316	22,206	14.608	7.5468

TF = Total number of fragments. PB = Number of polymorphic bands. PP = Percent polymorphism. PICv = Polymorphic information content values. EMR = Effective multiplex ratio. RP = Resolving power. MI = Marker index.

3.4. Genetic Diversity

In this study, we observed high genetic diversity in male date palm genotypes. The values obtained for the observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), expected heterozygosity (He), and unbiased expected heterozygosity (uHe) for male date palm genotypes were as follows: 1.820, 1.30, 0.322, 0.199, and 0.200, respectively. In contrast, females exhibited comparatively lower values with respective figures of 1.318, 1.272, 0.271, 0.172, and 0.176 (Table 7).

Table 7. Genetic diversity indices and polymorphic features of *Phoenix dactylifera* L. populations revealed by combined data of SSR, ISSR and DAMD markers.

Pop	**N**	**Na (±SE)**	**Ne (±SE)**	**I (±SE)**	**He (±SE)**	**uHe (±SE)**	**%P**
Females	21.000	1.318(0.030)	1.272(0.010)	0.271(0.008)	0.172(0.006)	0.176(0.006)	64.66%
Males	79.000	1.820(0.018)	1.30(0.010)	0.322(0.007)	0.199(0.005)	0.200(0.005)	90.91%
Mean	50.000	1.569(0.018)	1.289(0.007)	0.296(0.005)	0.185(0.004)	0.188(0.004)	77.78%

N = number of individuals. Na = number of different alleles. Ne = number of effective alleles. I = Shannon's information index. He = expected heterozygosity. uHe = unbiased expected heterozygosity. Np = number of private bands. PPL = percentage of polymorphic loci.

3.5. AMOVA Analysis of Phoenix dactylifera L.

AMOVA results using combined ISSR, DAMD, and SSR data showed that variance within the population was higher than that among populations with values of 94% and 6%, respectively. The PhiPT for 100 date palm accessions was 0.060 ($p < 0.001$) (Table 8). The pairwise population PhiPT value was 0.060 (Table 9).

Table 8. Analysis of molecular variance (AMOVA) of *Phoenix dactylifera* L. from seven regions based on data from combined SSR, ISSR and DAMD markers.

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	1	387.840	387.840	7.919	6%
Within Pops	98	12,259.690	125.099	125.099	94%
Total	99	12,647.530		133.018	100%
Stat	Value	*p*			
PhiPT	0.060	<0.001			

Note. Df = degree of freedom. SS = sums of squares. MS = mean squares. Est. Var = estimate of variance. % = percentage of total variation. PhiPT = Phi-statistics probability level after 1000 permutations. P = is based on 1000 permutation.

Table 9. Population PhiPT values based on 999 permutations from AMOVA.

	F	M
Females	0.000	
Males	0.060	0.000

3.6. Genetic Distance

Using the Nei method, the genetic distance and identity coefficient were evaluated between male and female date palms. The obtained values were 0.018 for genetic distance and 0.983 for the identity coefficient (Table 10).

Table 10. Pairwise comparison matrix of Nei genetic identity and Nei genetic distance for males and females *Phoenix dactylifera* L. populations, using ISSR, DAMD and SSR data.

	Pairwise Population Matrix of Nei Genetic Distance		Pairwise Population Matrix of Nei Genetic Identity	
	Females	Males	Females	Males
Females	0.000		1.000	
Males	0.018	0.000	0.983	1.000

3.7. Principal Coordinate Analysis (PCoA)

The Principal Coordinate Analysis (PCoA) of 100 date palm samples was conducted to examine the relationships among studied genotypes. The results showed that the total variation explained by PCoA was 19.66% with the first three axes (1, 2, and 3) corresponding to 8.38%, 6.26%, and 5.02%, respectively (Figure 5).

PCoA biplots based on ISSR, DAMD and SSR markers separate the studied date palm accessions into three groups. One group comprises genotypes of males from Zagora with some female varieties scattered in this group, whereas the second group includes male genotypes from Goulmima along with accessions of female and male varieties. The third group contains males of Errachidia, Tata and Tinghir populations.

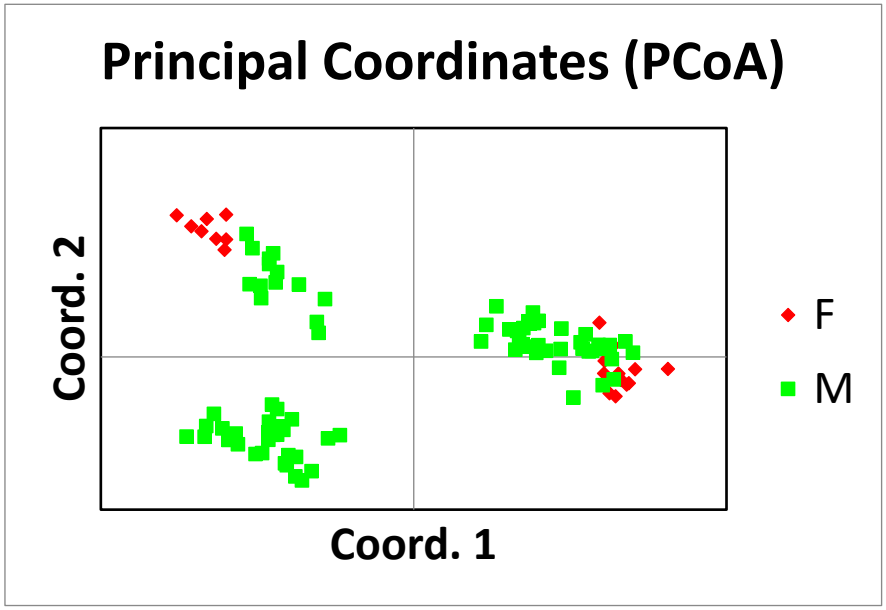

Figure 5. *Cont.*

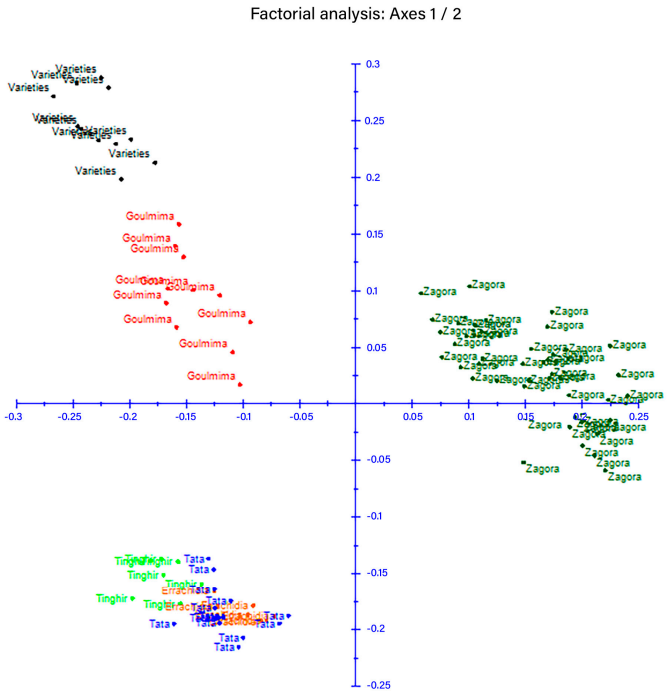

Figure 5. Principal Coordinate Analysis (PCoA) of the 100 *Phoenix dactylifera* L. genotypes based on combined data from ISSR, DAMD and SSR markers.

3.8. Cluster Analysis

The results of the clustering analysis revealed that the dendrogram divided the investigated genotypes into five major groups. The first group included all genotypes from Zagora, the second group comprised males of the Tata population, the third group consisted of males from Tinghir and Errachidia, the fourth group contained males from Goulmima, and the last group included the varieties males and females (Figure 6).

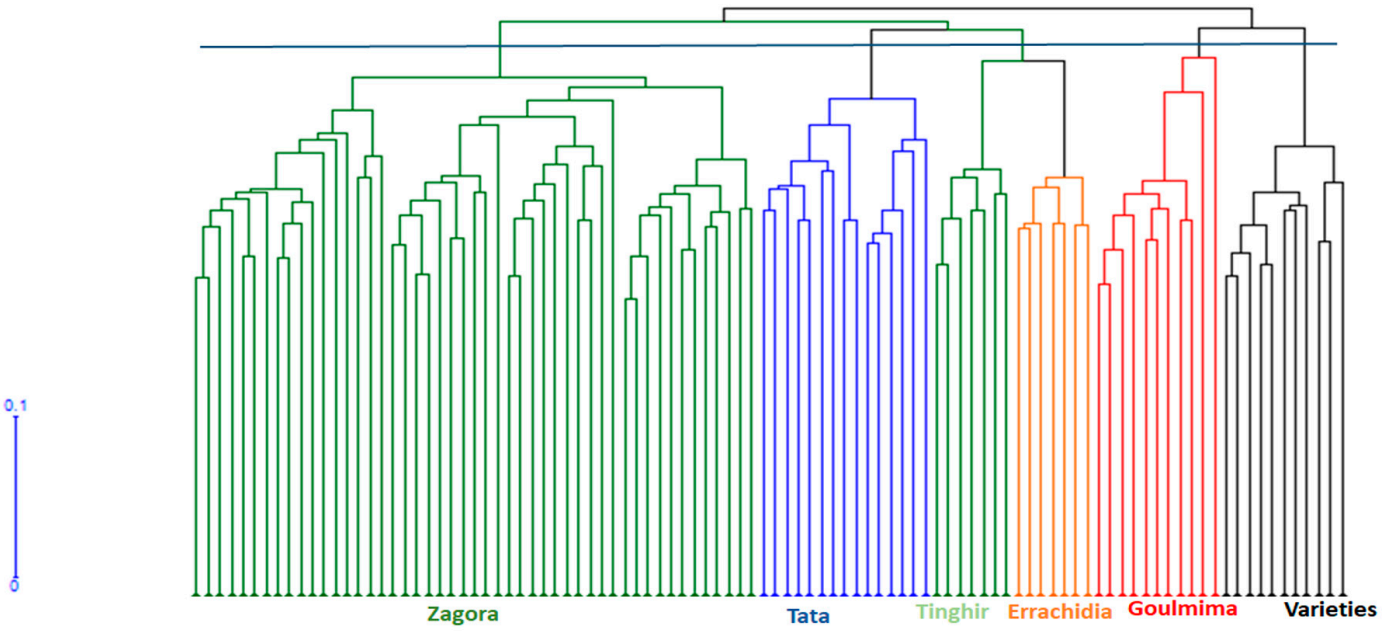

Figure 6. Integration of ISSR, DAMD and SSR Data in UPGMA Cluster Analysis for all Sampled Individuals of *Phoenix dactylifera* L.

These results indicated that there is no overlapping between populations. The accessions were closely related to their geographical distribution.

3.9. Population Genetic Structure

The Bayesian clustering approach was employed to assess the overall genetic structure using the STRUCTURE 2.3.4 software. The output data were extracted with Structure Harvester, and the method described by [39] was used to estimate the most probable number of genetic clusters (K) within the range of K = 1 to K = 10. The maximum Δk value was observed at K = 6, suggesting that the tested samples are likely to be distributed into six principal clusters (Figure 7A).

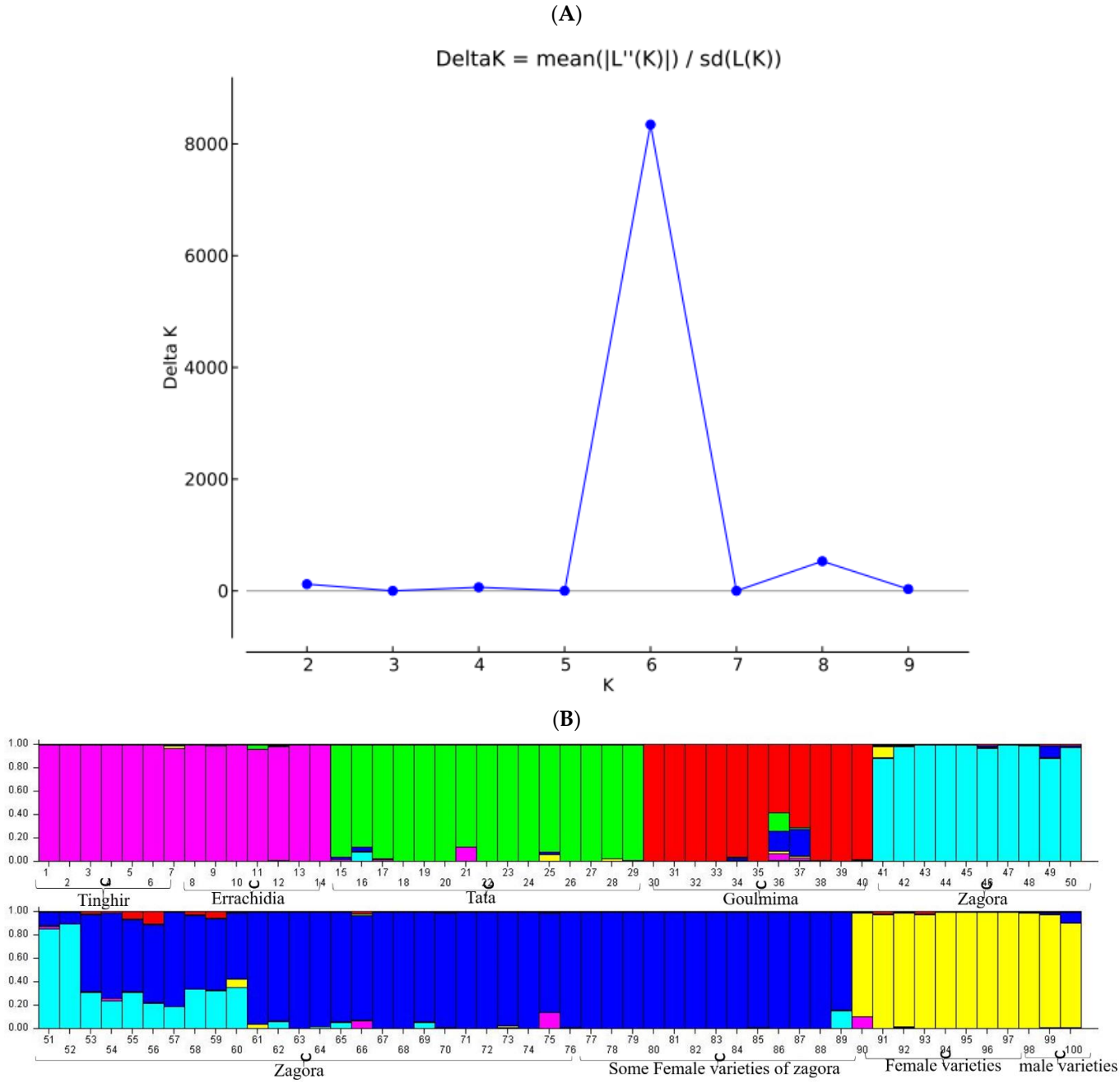

Figure 7. Results of the STRUCTURE analysis of 100 *Phoenix dactylifera* L. genotypes based on ISSR, DAMD, and SSR markers. (**A**) DeltaK curve indicating the most likely K, in the largest change in magnitude at K = 6. (**B**) Population genetic structure at K = 6. The same color assigned to different individuals indicates their membership in the same genetic cluster.

In the visual representation, individuals are organized into vertical columns and differentiated by colors (Figure 7B). The same color assigned to different individuals indicates their membership in the same genetic cluster, while different colors within the same individual suggest potential membership in distinct genetic groups.

The analysis revealed that the genetic structure of 100 *Phoenix dactylifera* L. genotypes could be classified into six distinct populations. The first cluster primarily included males of the Tinghir and Errachidia populations. The second cluster consisted of males of the Tata population, and the third cluster consisted of males from the Goulmima population. The major group of the Zagora population was divided into two clusters, and the last group encompassed varieties.

4. Discussion

Genetic variability plays a pivotal role in species evolution and the adaptation of plants to various environmental stresses. Consequently, assessing genetic diversity is essential for the success of breeding programs. The genetic diversity of female date palms has received substantial attention worldwide, but comprehensive research on the genetic diversity of male date palms remains limited. This study aims to evaluate the genetic diversity of Moroccan male date palms.

Understanding the genetic diversity of male date palms is critical for informing effective pollination strategies, ultimately leading to optimized fruit production and improved overall date palm cultivation practices. Furthermore, this research underscores the importance of such assessments in gaining insights into the genetic composition, relatedness, and conservation of male date palm populations.

The choice of molecular marker for assessing the genetic diversity is a crucial step. In this study, we combined the performance of three molecular markers: ISSR, DAMD and SSR markers.

Numerous studies have employed SSR and ISSR markers to evaluate the genetic diversity and relatedness of date palm genotypes, either individually [20,22,40–45] or in combination of the both molecular markers [18,46].

The DAMD marker has been applied to assess the genetic diversity of *Phoenix dactylifera* L. in the United Arab Emirates in combination with the ISSR marker [21] and in Morocco in association with the SSR marker [11]. This study is the first to combine ISSR, DAMD, and SSR molecular markers to assess the level of genetic variation in Moroccan male date palms.

It is notable that the effectiveness of a molecular marker technique depends on a set of parameters. On the basis of polymorphism, the present investigation revealed that the used molecular markers (ISSRs, DAMD and SSRs) generated high polymorphism levels with 95%, 98% and 94%, respectively. These values were higher than several previous studies such as [47], showing an 84% polymorphism level using ISSRs markers and [48], reporting 39.77% of PP using RAPD. The three markers were informative with regard to the amount of polymorphism detected [47]. However, on the basis of polymorphic information content (PIC), effective multiplex ratio (EMR), resolving power (Rp), and marker index (MI), all markers used in our study are considered informative markers. Among them, DAMD markers demonstrated slightly higher informativeness compared to ISSR and SSR markers.

Overall, the used markers were good enough for evaluating the genetic diversity of the 100 date palm accessions with regard to the amount of polymorphism detected [47] beside the high values found of PIC, EMR, Rp, and MI [33–36].

In our analysis, we detected a total of 216 bands generated by eight ISSR primers, resulting in an average of 27 bands per primer. These results closely mirrored those obtained with SSRs, where we identified 248 bands using ten SSR primers with an average of 24 bands per primer. Notably, the number of amplified bands was slightly higher with DAMD primers, reaching 438 bands with an average of 54.75 per primer. These results consistently highlight the substantial presence of allelic diversity within date palm, which is a conclusion that aligns with previous research [49,50]. A significant number of alleles were also detected in the study conducted by Ibrahimi et al. during their assessment of

Moroccan date palms [11]. They found 249 bands from 10 SSR primers, with an average of 25 bands, and using DAMD markers, they detected 471 bands with an average of 59 bands. In the assessment of date palm cultivars from Australia and the Middle East using SSR markers, Al Najm et al. [51] identified 313 bands. A substantial number of alleles were reported also on date palms from Sudan, with a total of 343 alleles across 16 primers, with an average of 21.4 alleles per locus [52].

In this study, we observed high genetic diversity in male date palm genotypes. The observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), expected heterozygosity (He), and unbiased expected heterozygosity (uHe). correspond to 1.820, 1.30, 0.322, 0.199, and 0.200, respectively. In contrast, females exhibited notably lower values, with corresponding figures of 1.318, 1.272, 0.271, 0.172, and 0.176, respectively.

These results can be explained by several factors. Most female date palm cultivars are recognized by vegetative reproduction, which involves cloning and results in less genetic diversity. Additionally, breeding programs for female cultivars often focus on selecting for the most desirable traits, which can further reduce genetic diversity. In contrast, most male palm trees are seed propagated, which results in greater genetic diversity.

The results of the AMOVA analyses indicated that the genetic diversity within Moroccan date palms is predominantly represented within populations, accounting for 94%, rather than among populations, which accounts for 6%. This result is in consonance with earlier observations made by Ibrahimi et al. [11] while assessing Moroccan date palms using SSRs and DAMD markers. Additionally, comparable results were observed in Iran using SSR and ISSR markers [46] as well as SCOT markers [53], and in Tunisia [54], Sudan [52], and Qatar [55] using SSR markers. The higher molecular variance within the population of date palms may be attributed to its dioecious nature and the reproduction that is ensured by cross-pollination. The Nei analysis revealed relatively small distance values between male and female populations, aligning with the AMOVA result that indicates a low level of diversity among populations, at 6%.

UPGMA and structure analyses showed that date palm genotypes were clustered according to their origin (Oasis). Similar results were observed by [56] but oppositely found by [27] in the genetic diversity of the Tunisian male date palm (*Phoenix dactylifera* L.) genotypes using morphological descriptors and molecular markers which found that the accessions do not group according to geographical origin.

In addition, UPGMA dendrogram and structure analysis pointed out that the male of Zagora was divided into two groups that may be explained by the high number of Zagora samples and the breeding programs adopted in the region. The intriguing division within the male population of Zagora into two groups suggests inherent genetic variations that could be explored further for their implications in breeding. By investigating the specific genetic markers responsible for this differentiation, breeders can gain insights into traits related to male reproductive success, potentially improving pollination efficiency and fruit yield. Also, UPGMA results showed that males of Tinghir and Errachidia were regrouped in the same cluster, which was confirmed by PCoA.

In this study, PCoA revealed that Errachida, Tinghir and Tata were clustered in the same group, which was likely indicative of historical exchanges of plant material and seeds among different regions. This finding holds significant implications for breeding programs, as they can strategically replicate historical practices to facilitate successful genetic exchanges. By doing so, breeding efforts can introduce novel traits into populations, enhancing the overall genetic diversity and adaptability of date palms within breeding programs. The PCoA also revealed that Goulmima form a distinct group despite its close proximity to Errachidia, suggesting genetic separation likely influenced by historical human relationships among regions. This outcome aligns with previous findings reported by [11]. This insight prompts considerations for conservation efforts, emphasizing the need to preserve the unique genetic heritage of each population. Conservation strategies can be

tailored to protect genetically distinct groups, mitigating the risk of genetic erosion and ensuring the long-term sustainability of date palm populations.

These findings underscore the dual importance of leveraging historical practices for breeding programs to enhance genetic diversity and implementing targeted conservation strategies to preserve the distinct genetic identities of date palm populations.

The genetic diversity of female date palms has received substantial attention worldwide, but comprehensive research on the genetic diversity of male date palms is still lacking, especially in Morocco. This research underscores the importance of such assessments in gaining insights into the genetic composition, relatedness, and conservation of the male date palm populations as well as in formulating effective conservation and breeding strategies to ensure the resilience and diversity of these vital plant populations.

In conclusion, our results offer practical implications for the development of breeding strategies specific to male date palms, considering both geographic and historical factors. These insights can contribute to the formulation of effective conservation plans, ensuring the preservation and sustainable utilization of date palm genetic resources.

5. Conclusions

The current study has established a database of Moroccan male date palm genotypes utilizing molecular markers. ISSR, DAMD and SSR markers used revealed high genetic diversity within date palm genotypes. This high genetic diversity provides a good potential for the further selection of male genotypes to optimize fruit quality when used for pollination. However, a clear relationship was found between geographical origin and genetic composition, suggesting there was no exchange of seeds and offshoots among different regions. These findings contribute important information regarding genetic relationships among male date palms for improvement and conservation programs. Generally, this study will provide eminent evidence and be a source of information on the genetic diversity of date palms in Morocco.

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Appendix A

Table A1. Table of the plant material coordinate GPS.

Populations	Samples	Sexe	Geographic Positions		Populations	Samples	Sexe	Geographic Positions	
			x_cg	y_cg				x_cg	y_cg
Zagora	Za1	M	005°50.766′ W	30°18.694′ N	Tinghir	Tin1	M	005°34′5.20″ W	31°32′8.85″ N
	Za2	M	005°50.796″ W	30°18.814′ N		Tin2	M	005°34′4.94″ W	31°32′9.18″ N
	Za3	M	005°50.768″ W	30°18.697′ N		Tin3	M	5°33′44.467″ W	31°32′47.971″ N
	Za4	M	005°50.780″ W	30°18.807′ N		Tin4	M	5°33′43.460″ W	31°32′36.165″ N

Table A1. *Cont.*

Populations	Samples	Sexe	Geographic Positions		Populations	Samples	Sexe	Geographic Positions	
			x_cg	y_cg				x_cg	y_cg
	Za5	M	005°50.768″ W	30°18.696′ N		Tin5	M	5°33′43.816″ W	31°32′35.960″ N
	Za6	M	005°50.780″ W	30°18.811′ N		Tin6	M	5°33′44.023″ W	31°32′43.495″ N
	Za7	M	005°50.704″ W	30°18.803′ N		Tin7	M	5°33′43.709″ W	31°32′43.905″ N
	Za8	M	005°50.705″ W	30°18.819′ N	Errachidia	Er1	M	004°06′0.17″ W	31°42′9.82″ N
	Za9	M	005°50.709″ W	30°18.807′ N		Er2	M	004°08′0.90″ W	31°42′6.21″ N
	Za10	M	005°50.780″ W	30°18.807′ N		Er3	M	004°08′2.28″ W	31°42′5.80″ N
	Za11	M	005°50.778″ W	30°18.701′ N		Er4	M	004°15′7.97″ W	31°48′3.24″ N
	Za12	M	005°50.710″ W	30°18.805′ N		Er5	M	004°17′5.87″ W	31°21′1.26″ N
	Za13	M	005°50.771′ W	30°18.991′ N		Er6	M	004°18′3.67″ W	31°21′1.26″ N
	Za14	M	005°50.766′ W	30°18.987′ N		Er7	M	004°19′0.04″ W	31°20′9.13″ N
	Za15	M	005°50.648′ W	30°18.884′ N	Tata	Ta1	M	7°56′52.765″ W	29°38′57.226″ N
	Za16	M	005°50.647′ W	30°18.880′ N		Ta2	M	8°24′55.959″ W	29°39′58.605″ N
	Za17	M	005°50.650′ W	30°18.876′ N		Ta3	M	8°0′5.637″ W	29°40′27.003″ N
	Za18	M	005°50.660′ W	30°18.872′ N		Ta4	M	7°55′50.620″ W	29°38′25.466″ N
	Za19	M	005°50.663′ W	30°18.869′ N		Ta5	M	7°55′45.142″ W	29°38′25.083″ N
	Za20	M	005°50.662′ W	30°18.868′ N		Ta6	M	8°0′5.214″ W	29°40′27.921″ N
	Za21	M	005°50.670′ W	30°18.861′ N		Ta7	M	7°58′49.409″ W	29°40′43.898″ N
	Za22	M	005°50.670′ W	30°18.852′ N		Ta8	M	7°55′46.123″ W	29°38′25.617″ N
	Za23	M	5°34′50.412″ W	29°56′47.165″ N		Ta9	M	7°58′59.064″ W	29°40′57.619″ N
	Za24	M	5°34′52.572″ W	29°56′50.687″ N		Ta10	M	7°54′49.761″ W	29°0′31.999″ N
	Za25	M	5°33′59.426″ W	29°59′57.018″ N		Ta11	M	7°58′44.754″ W	29°40′40.869″ N
	Za26	M	5°33′59.566″ W	30°1′3.736″ N		Ta12	M	8°0′8.009″ W	29°40′29.524″ N
	Za27	M	5°33′59.566″ W	30°1′3.736″ N		Ta13	M	7°58′48.695″ W	29°40′45.082″ N
	Za28	M	5°34′12.089″ W	30°11′10.633″ N		Ta14	M	7°58′46.174″ W	29°40′42.532″ N
	Za29	M	5°34′14.065″ W	30°11′6.894″ N		Ta15	M	7°58′47.134″ W	29°40′43.715″ N
	Za30	M	5°34′55.782″ W	29°57′2.843″ N	Goulmima	GM1	M	4°56′58.565″ W	31°40′52.155″ N
	Za31	M	5°35′1.78″ W	29°57′11.45″ N		GM2	M	4°56′17.222″ W	31°40′44.306″ N
	Za32	M	5°49′53.352″ W	30°19′14.875″ N		GM3	M	4°56′17.586″ W	31°40′47.391″ N
	Za33	M	5°49′55.159″ W	30°19′17.273″ N		GM4	M	4°56′54.861″ W	31°40′48.829″ N
	Za34	M	5°49′53.543″ W	30°19′15.914″ N		GM5	M	4°56′24.843″ W	31°40′31.478″ N
	Za35	M	5°49′54.068″ W	30°19′15.977″ N		GM6	M	4°56′30.100″ W	31°40′35.426″ N
	Za36	M	5°49′56.609″ W	30°19′14.669″ N		GM7	M	4°56′58.831″ W	31°40′52.026″ N
Varieties	JIHL	F	5°34′50.028″ W	29°56′45.077″ N		GM8	M	4°56′28.993″ W	31°40′43.895″ N
	JIHL	F	5°34′27.798″ W	29°59′57.071″ N		GM9	M	4°56′17.300″ W	31°40′43.851″ N
	BOUFGOUSS	F	5°34′28.272″ W	29°59′57.761″ N		GM10	M	4°59′4.560″ W	31°43′22.208″ N
	BOUFGOUSS	F	5°33′58.368″ W	30°1′2.016″ N		GM11	M	4°56′57.766″ W	31°40′52.379″ N
	BOUSTHAMI	F	5°34′14.252″ W	30°11′6.894″ N	Varieties	Deglet Nour	F	5°50′56.4′′ W	30°18′59.569′′ N
	JEAFRI	F	5°34′50.526″ W	29°56′45.407″ N		Boufgouss	F	I NRA	I NRA
	BOUZEKRI	F	5°34′27.630″ W	29°59′56.699″ N		Nejda	F	I NRA	I NRA
	AHARDAN	F	5°34′14.251″ W	30°11′6.829″ N		Bouskri	F	I NRA	I NRA
	IKLAN	F	5°34′49.920″ W	29°56′45.521″ N		Aziza	F	I NRA	I NRA
	IKLAN	F	5°34′27.672″ W	29°59′57.008″ N		Gharass	F	I NRA	I NRA
	TANMOUTRET	F	5°34′50.382″ W	29°56′45.743″ N		Sedrat	F	I NRA	I NRA
	AGLID	F	5°34′50.658″ W	29°56′45.587″ N		Majhoul	F	I NRA	I NRA
	BOUSLIKHEN	F	5°34′49.980″ W	29°56′45.299″ N		NP3	M	I NRA	I NRA
						Nebch-Boufeggouss	M	INRA	INRA
						NP4	M	INRA	INRA

INRA: National Institute of Agricultural Research.

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