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Full Length Research Paper

Molecular research on the genetic diversity of Tunisian date palm (*Phoenix dactylifera* L.) using the random amplified microsatellite polymorphism (RAMPO) and amplified fragment length polymorphism (AFLP) methods

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The genetic diversity organization and evaluation of relationships within Tunisian date-palm cultivars were assessed using the random amplified microsatellite polymorphism (RAMPO) and amplified fragment length polymorphism (AFLP) methods. 18 combinations of random/ISSR primers and six AFLP primers combinations were tested with DNAs isolated from 40 date-palm cultivars. Our results show that using both markers systems, the Tunisian date-palm germplasm is characterized by a large and continuous genetic diversity. In addition, due to the greater number of markers per assay, the AFLP technique seems to be more informative than the RAMPO method. In fact, 186 and 428 polymorphic bands were detected using RAMPO and AFLP primers, respectively. Moreover, AFLP markers were found most polymorphic with the highest average PIC value (0.7) and marker index (50.54). In addition, independent as well as combined analyses of the cluster analyses of the RAMPO and AFLP fragments showed that cultivars are clustered independently from the sex of trees or else their geographical origin. On the other hand, based on Pearson and Spearman correlation between RAMPO and AFLP distance, matrices were positive and highly significant. This result indicates good congruence between these two molecular markers. The opportunity of the designed methods is discussed with the molecular characterization of genotypes in order to enhance the conservation and the improvement of the local date-palm germplasm.

Key words: *Phoenix dactylifera*, genetic diversity, random amplified microsatellite polymorphism (RAMPO), amplified fragment length polymorphism (AFLP), Tunisian.

INTRODUCTION

In tropical and sub-tropical habitats, oasis cultures consist of date-palm groves (*Phoenix dactylifera* L., 2n = 36), which are major factors of social, environmental and economic stability. Probably domesticated, since more than 5000 years ago in Mesopotamia, date-palm is cultivated for fruit production and all parts of the tree are used for many industrial purposes such as timber, furniture, rope and packing material (Hodel and Johnson, 2007). In Tunisia, throughout the long history of its cultivation, date-palm constitutes a source of income to oasians and creates appropriate conditions both for men or animals habitat and for the establishment of subjacent fruit crop and many other vegetable cultures (fig, apricot, pomegranate, pepper, tomato, etc.). Its utilisation consisted of locally adapted ecotypes, selected mainly for their productivity and date qualities, which are clonally reproduced throughout offshoots (Munier, 1973; Nixon

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and Carpenter, 1978). Recently, large scales of plantlets were generated via *in vitro* culture methods and permitted to enhance modern date-palms' plantations. Current investigations have reported that the local germplasm is widely diversified since more than 250 cultivars have been identified (Rhouma, 1994, 2005). Nevertheless, the number of trees per cultivar varied from around twenty (cv Deglet Bey) to several millions (cv Deglet Nour). In fact, due to favourable economic circumstances, the predominance of the cv Deglet Nour in modern plantations has been established and seriously threatened other cultivars with medium and low fruit qualities (Rhouma, 1994). This tendency has considerably contributed in the genetic erosion of the local germplasm and accelerated its vulnerability to biotic and abiotic stresses. In addition, the local date-palm groves are currently menaced both by plagues such as the bayoud disease (a vascular fusariosis due to the imperfect fungus *Fusarium oxysporum* f.sp. *albedenis*), as well as by the brittle leaf disease (Triki et al., 2003). Therefore, it was imperative to preserve the local germplasm. For this purpose, studies have reported and described the use of either morphological traits or isozyme makers to identify the Tunisian date-palm cultivars (Rhouma, 1994; 2005; Ould Mohamed Salem et al., 2001). As a result, it has been assumed that a high level of genetic diversity characterises the local germplasm. DNA based techniques have been also designed in order to precise the observed diversity (Trifi et al., 2000; Sakka et al., 2004; Zehdi et al., 2004). These studies have proved the efficiency of the evidenced molecular markers to assess genetic diversity within date-palm genotypes. As a part of our research work, we have focused our efforts to generate additional molecular markers suitable to obtain a deeper insight of the genetic organization in the Tunisian date-palm germplasm. For this purpose, we have designed the development of the random amplified microsatellites polymorphisms (RAMPO) method and the amplified fragment length polymorphism (AFLP). The RAMPO method is a procedure that combined the random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) avoiding any radio labelling manipulation (Chatti et al., 2007; Rhouma et al., 2008). More sophisticated, AFLP markers (Vos et al., 1995) are becoming more and more a routine tool in plant genetic studies. Cultivar identification, germplasm characterisation, genetic diversity assessment and biosystematics studies are among the most frequent applications of these markers (Naohiko et al., 1999; Lubberstedt et al., 2000; Cabrita et al., 2001; Soleimani et al., 2002; Jbir et al., 2008; Rhouma et al., 2007). Both RAMPO and AFLP markers are dominants and theoretically allow the assessment of the DNA sequences all along the genome, independent of their location in the chromosomes or particularities in their nucleotide sequence. In this study, the suitability of the RAMPO and AFLP markers to distinguish among (*Phoenix dactylifera* L.) cultivars was assessed. We report a comparison of the RAMPOs and AFLPs to survey polymorphisms within 40 date-palm ecotypes from Tunisia.

MATERIALS AND METHODS

Plant material

40 accessions (30 varieties and 10 male trees) of date-palm reported in Table 1 were used in this study. These were collected at random from plantations in oases of Tozeur located in the south of Tunisia. Among these, eight varieties recently introduced into Tunisian date-palm groves ('Ghars Mettig' and 'Tantabecht' from Algeria, 'Berhi', 'Khadhraoui' and 'Zehdi' from Iraq, 'Halaoui' and 'Abou Maan' from the United Arabic Emirates and 'Khessab' from Oman) were included in this study.

DNA isolation

Total cellular DNA was purified from 10 g of frozen young leaves according to the procedure of Dellaporta et al. (1984). The DNA concentration was estimated spectrophotometrically and its integrity was checked by analytical [1% (w/v)] agarose minigel electrophoresis (Sambrook et al., 1989).

Primers and RAMPO assays

The RAMPO method consists of the combination of two PCR-DNA based procedures namely RAPD and ISSR (Chatti et al., 2007). Therefore, the two following primers were tested: universal decamer oligonucleotides purchased from Operon Technology Inc. (Alameda, USA) used to perform RAPD assays and oligonucleotides that are complementary to simple sequence repeats used to perform ISSR assays (Table 2). The experience was performed as described by Rhouma et al. (2008).

AFLP procedure

AFLP was performed using the AFLP analysis system and the AFLP starter primers kit based on Vos et al. (1995) and as described by Rhouma et al. (2007). Different primers pairs were used to generate AFLP banding patterns. A reproducibility test was performed on forty plants with all six primer combinations. For each sample, AFLP fingerprints were compared. Amplified fragments were separated and silver stained in 6% (w/v) denaturing polyacrylamide gels. Each PCR product (between 60 and 650 bp) was assumed to represent a single locus with two alleles and data were scored as the presence (1) or absence (0) of each polymorphic band.

Data analysis

Each primer combination was tested for its ability to generate RAMPO and AFLP banding pattern. The total number of bands was determined and monomorphic bands were discarded from the analysis, only the polymorphic ones were taken into account in this study to estimate the percentage of polymorphic bands (PPB). The ability of the most informative primers to discriminate among ecotypes was assessed by calculating the resolving power (Rp) which has been reported to correlate between accessions (Prevost and Wilkinson, 1999). Estimation of the Rp was performed according to the formula of Gilbert et al. (1999): $Rp=\Sigma Ib$, where, $Ib=1-(2\times|0.5-p|)$ and p is the proportion of the accessions containing

Table 1. Tunisian date palm accessions studied and their country of origin.

Cultivar	Label	Country of origin
Cheddakh	Ck	Tunisia
Lagou	Lg	Tunisia
Tantabecht	Tb	Algeria
Besser Helou	Bh	Tunisia
Gasbi	Gb	Tunisia
Halaoui	Hl	Iraq
Boufeggous	Bf	Tunisia
Hamra	Hm	Tunisia
Berhi	Br	Iraq
Tezerzit Safra	Ts	Tunisia
Tezerzit Soda	Tk	Tunisia
Chekenet el Hej	Ch	Tunisia
Kharroubi	Kb	Tunisia
Kenta	Kn	Tunisia
Goundi	Gd	Tunisia
Bidh Hmam	Bm	Tunisia
Abou Maan	Ab	UAE
Menakher	Mk	Tunisia
Aligue	Al	Tunisia
Zehdi	Zh	Iraq
Ammari	Am	Tunisia
Khessab	Kh	Oman
Deglet Nour	Dn	Tunisia
Kintichi	Kt	Tunisia
Oum Laghlez	Ol	Tunisia
Arichti	Ar	Tunisia
Guelb Jemel	GJ	Tunisia
Ghars Mettig	Gm	Algeria
Khouet Aligue	Ka	Tunisia
Khadhraoui	Kd	Iraq
Borhane1*	B1	Tunisia
Borhane2*	B2	Tunisia
Borhane3*	B3	Tunisia
CRPh1*	C1	Tunisia
CRPh2*	C2	Tunisia
CRPh3*	C3	Tunisia
CRPh4*	C4	Tunisia
CRPh5*	C5	Tunisia
CRPh6*	C6	Tunisia
Hajji*	Hj	Tunisia

*:Male trees.

the I band. Moreover, polymorphic information content (PIC), effective multiplex ratio (E), marker index (MI) were calculated as follows (Lynch and Walsh, 1998):

$$PIC = 1-\sum_{i=1}^{k} Pi^2$$

Where, k is the total number of alleles detected for a given marker locus and P_i is the frequency of the ith allele in the set of genotypes investigated; $E = n\times\beta$, where β is the fraction of polymorphic markers and is estimated after considering the polymorphic loci (n_p) and non-polymorphic loci (n_{np}) as $\beta= n_p/(n_p + n_{np})$. The multiplex ratio (n) is the average number of DNA fragments amplified/ detected per genotype using a marker system. A product of information content as measured by PIC and effective multiplex ratio called marker index may provide a suitable estimate of marker

Table 2. Primers combinations used for RAMPO and AFLP analysis.

Method	Primer combination	Band number		PPB	Rp	PIC	MI
		Total	Polymorphic				
RAMPO	OPB04 × 02	11	10	90.9	3.55	0.51	5.35
	OPB04 × 06	10	7	70	2.9	0.38	3.10
	OPB04 × 07	13	11	84.61	6.85	0.68	6.66
	OPB04 × 09	14	12	85.71	4.2	0.78	7.82
	OPB04 × 10	16	16	100	8.25	0.59	6.87
	OPB04 × 14	8	7	87.5	2.15	0.71	7.20
	OPA19 × 02	9	8	88.88	3.5	0.49	5.02
	OPA19 × 06	11	9	81.81	4.25	0.58	5.54
	OPA19 × 07	11	11	100	3.8	0.68	7.92
	OPA19 × 09	13	12	92.3	3.2	0.72	7.72
	OPA19 × 10	12	12	100	4.05	0.41	4.78
	OPA19 x 14	10	10	100	2.6	0.55	6.41
	OPA12 × 02	12	10	83.3	5.1	0.40	3.87
	OPA12 × 06	13	10	76.9	2.45	0.63	5.65
	OPA12 × 07	13	12	92.3	3.45	0.78	8.36
	OPA12 × 09	13	11	84.6	5.3	0.49	4.79
	OPA12 × 10	14	12	85.7	6.2	0.46	4.55
	OPA12 × 14	7	6	85.71	1.1	0.61	6.04
	Total	210	186	-	-	-	-
	Average	11.66	10.33	88.57	4.06	0.58	5.98
AFLP	E_{AAC}/M_{CAA}	50	50	100	25.60	0.56	41.24
	E_{AGC}/M_{CAA}	56	56	100	23.40	0.72	53.03
	E_{AAC}/M_{CAG}	65	60	92.3	21.55	0.75	50.82
	E_{ACA}/M_{CAG}	67	67	100	23.80	0.82	60.40
	E_{ACC}/M_{CTA}	100	95	95	27.00	0.66	46.18
	E_{AAC}/M_{CAT}	104	100	96.15	33.95	0.73	51.62
	Total	442	428	-	-	-	-
	Average	73.66	71.33	97.24	25.88	0.70	50.54

utility (Powell et al., 1996): MI = PIC × E.

Cluster analysis

Each polymorphic band was treated as a separate character and scored as present (1) or absent (0), to generate a binary data matrix. Data were then computed using the Genedist program (Felsenstein, 1995) to produce a genetic distance matrix using the formula of Nei and Li (1979). The matrix was then analysed with the neighbour joining programme using PHYLIP software to produce a tree file using the unweighted pair group method with arithmetic averaging (UPGMA) algorithm (Sneath and Sokal, 1973). This tree file was computed with the TreeView program to draw a phylogenic diagram. All these analyses were carried out using appropriate programs of the Felsenstein's PHYLIP software (Felsenstein, 1995) and the TreeView software of Page (1996). In addition, principal component analysis (PCA) was performed by computing the data matrix with appropriate programs of the SAS software (SAS, 1990). Also, individual data obtained with RAMPO and AFLP markers were combined to prepare identical cluster analysis hold earlier. Finally, the correlation between RAMPO and AFLP's genetic distances matrix were tested by estimating the coefficients of Pearson and Sperman using XLSTAT 2007.8.01 (http://www.xlstat.com).

RESULTS

Genetic diversity and phylogenetic relationships revealed by RAMPO markers

18 primer combinations (RAPD primers × ISSR primers) were tested for their ability to generate RAMPO markers from DNA templates corresponding to the 40 cultivars studied (Table 2). All these combinations have produced reproducible bands scored as RAMPOs (Figure 1). Among the 210 generated bands, 186 polymorphic ones were scored (88.57% of polymorphic bands). All tested primers are powerful to detect DNA polymorphisms in date-palm crop. This hypothesis is strongly supported by regards the high resolving power (Rp) rates. Moreover, as reported in Figure 2a, the polymorphism information content (PIC) values varied from 0.04 to 0.99 with a mean of 0.58. On the other hand, 78 over the 186 RAMPOs exhibited PIC values ranged from 0.8 to 1. The MI values for these markers in the examined genotypes ranged from 3.10 (OPB04 × 06) to 8.36 (OPA12 × 07)

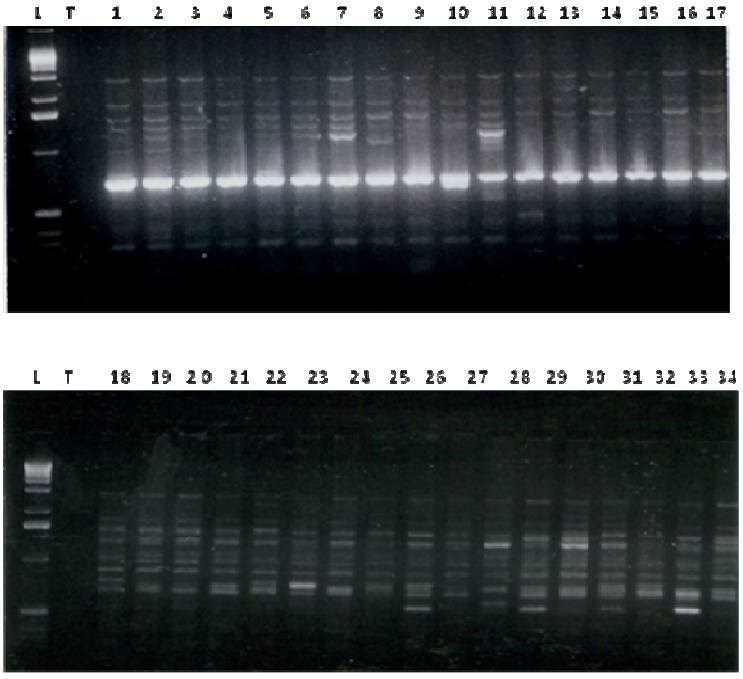

Figure 1. RAMPO DNA banding profiles generated using OPB04/02 primers' combination. (L): Molecular size marker; (T): negative control; (1-34): analysed ecotypes

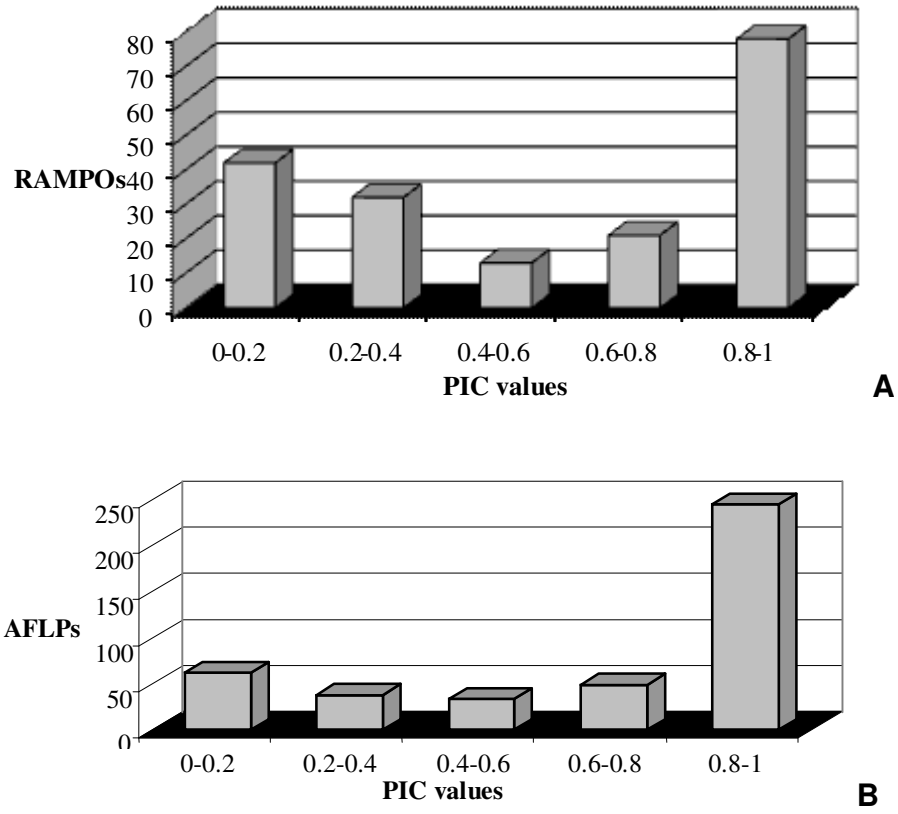

Figure 2. Distribution of the polymorphism information content (PIC) data obtained using random amplified microsatellites polymorphisms (a) and amplified fragment length polymorphisms (b).

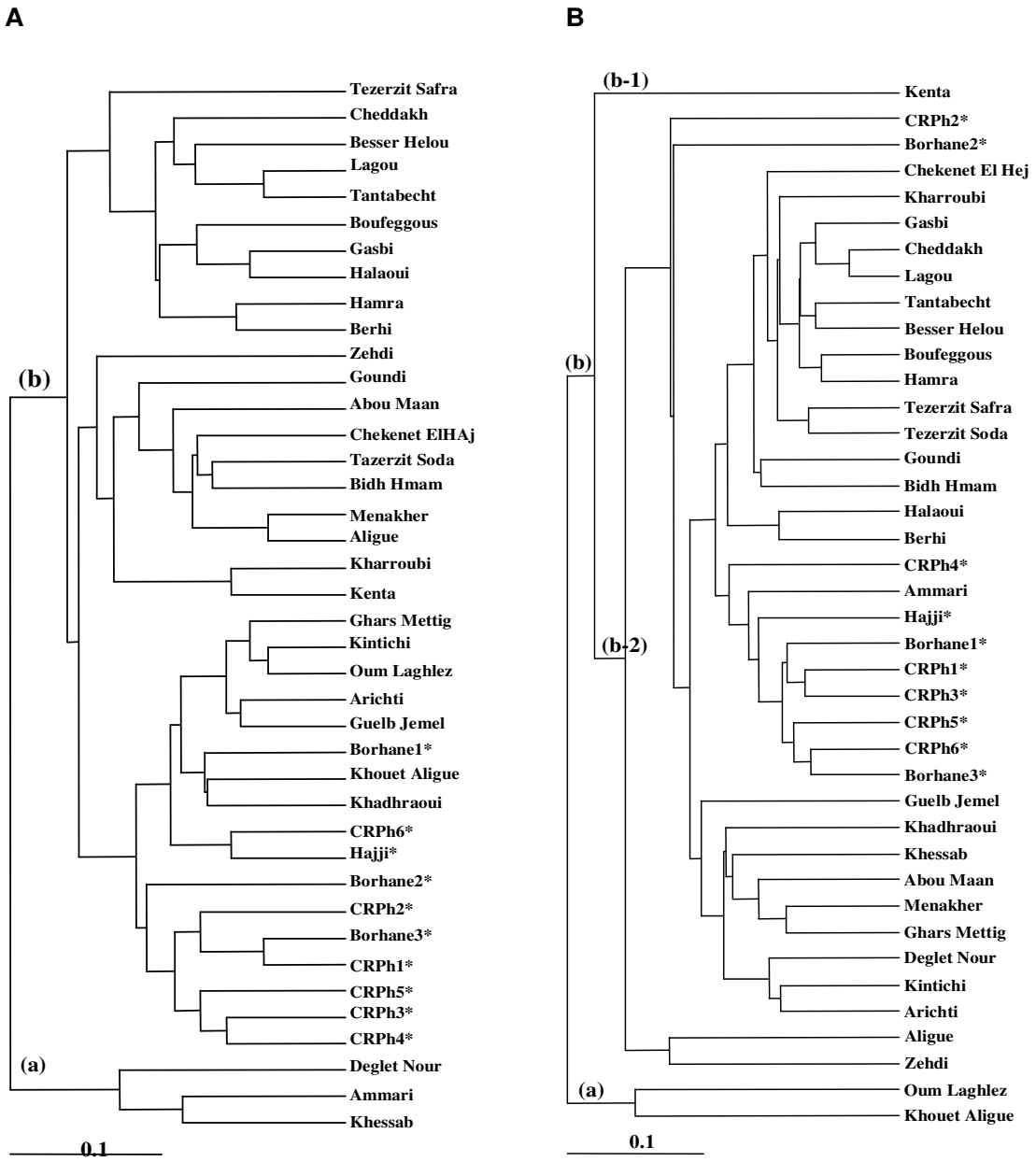

Figure 3. Dendrograms of 40 Tunisian date palm cultivars constructed by UPGMA method and based on RAMPOs (A) and AF LPs (B) (Table 1 for cultivar codes, *: male trees).

with an average of 5.98. Therefore, data suggested that RAMPO constitutes an efficient and informative procedure for evidencing genetic diversity between date-palm cultivars as well as in the discrimination of date-palm genotypes. Based on the 186 RAMPO markers, genetic distance exhibited values ranged from 0.10 to 0.76 with a mean of 0.34 suggesting that the ecotypes studied are characterised by great divergence at the DNA level. The smallest genetic distance of 0.10 was scored between Menakher [Mk] and Aligue [Al] cultivars that are characterised by great similarities. However, Bidh Hmam [Bm] and Khessab [Kh] cultivars are the most divergent since they presented the highest genetic distance value of 0.76. All the remaining genotypes displayed intermediate levels of similarity.

The derived UPGMA dendrogram illustrated the divergence among genotypes and supported their clustering into two main groups (Figure 3a). The first one labelled (a) is composed of the Deglet Nour [Dn], Ammari [Am] and Khessab [Kh] cultivars. All the remaining genotypes are ranged in the second cluster labelled (b) which exhibited different sub-clusters. It is worth noting that the derived clustering is made independently either from the geographical origin of the genotypes since the foreign

Table 3. Correlation coefficient of Pearson (higher diagonal) and Spearman (lower diagonal) estimated from distances matrix based on AFLPs, RAMPOs and AFLPs/RAMPOs (significant value = 0.05).

Method	AFLP	RAMPO	RAMPO/AFLP
AFLP	1.000	0.166	0.770
RAMPO	0.202	1.000	0.757
RAMPO/AFLP	0.784	0.728	1.000

cultivars recently introduced in the Tunisian plantations did not significantly diverge from the autochthonous ones. In order to precise this assumption, data were computed to perform a PCA analysis. Results summarized in Table 4a exhibited that the three first axes accounted for 28.18 of the total variability. Figure 4 illustrates the distribution of ecotypes according to the first two components (axis 1 to axis 2). Results show that genotypes are randomly aggregated in the plot with a little divergence of Khessab [Kh] and Kenta [Kn] cultivars. This result suggests that a typically continuous genetic diversity characterised the Tunisian date-palm germplasm.

Genetic diversity and phylogenetic relationships revealed by AFLP markers

Six primer sets were tested for their ability to generate AFLP banding patterns (Figure 5). A total of 428 polymorphic bands ranging in size from 50 to 600 bp were scored using the designed primers combination (ppb = 96.8) (Table 2). The number of yielded markers varied from 50 E_{AAC}/M_{CAA} to 100 for E_{AAC}/M_{CAT} with a mean of 71.34 bands per primer. Thus, we assume that the primers tested are powerful to evidence DNA polymorphisms in this crop. This assumption is strongly supported by regard of the high values of the percentage of polymorphic bands (ppb) scored using each primers combination. Moreover, estimation of the resolving power (Rp) exhibited high rates of collective Rp of 155.3 with a mean of 25.88. On the other hand, the majority of obtained PIC values are ranged from 0.8 to 1 (Figure 2b). Moreover, the MI values for individual primer combinations were recorded, however, the overall MI values for individual primer combinations were in the range of 41.24 (E_{AAC}/M_{CAA}) – 60.40 (E_{ACA}/M_{CAG}) with an average of 50.54 per primer combination (Table 2). Adding together, data suggested that AFLP constitutes a very attractive and informative procedure for evidencing genetic diversity between date-palm cultivars.

Genetic distances matrix based on AFLP data varied from 0.07 to 0.63 with a mean of 0.33. Thus, it is assumed that ecotypes studied are characterised by great divergence at the DNA level. The smallest genetic distance of 0.07 was scored between Cheddakh [Ck] and Lagou [Lg] varieties suggesting their great similarities. However, Khouet Aligue [Ka] accession and pollinator CRPh2 [C2] are the most divergent since they presented the highest genetic distance value of 0.63. All the remaining ones reveal diverse intermediate levels of similarity.

The derived UPGMA dendrogram illustrates the divergence among accessions and suggests their tree branching (Figure 3b). Two main clusters are identified. The first one labelled (a) is composed of Oum Laghlez [Ol] and Khouet Aligue [Ka] cultivars. All the remaining ecotypes are ranged in the second cluster labelled (b). It should be stressed that this last one exhibited sub-clusters identified as followed: the first sub-cluster labelled (b-1) is constituted by Kenta [Kn] cultivar that is significantly divergent from all the remaining individuals ranged in the second sub-cluster (b-2). In addition, this observed clustering is made independently either from the cultivar's geographic origin or from the sex of trees.

In order to confirm this conclusion, the data were analyzed by multivariate PCA. The results are summarized in Table 4. The three first axes contributed 26.3% of the total variability. Taking into account the distribution of cultivars according to the PCA (axis 1 to axis 2), three clusters were obtained that were similar to those reported in the UPGMA dendrogram and confirmed the significant divergence of 'Kenta' and Khouet Aligue' from the all remaining cultivars. Moreover, 'Oum Laghlez', 'Aligue' and 'Zehdi' accessions were clustered together (Figure 6).

Combined analysis: 'RAMPO and AFLP markers'

The combined AFLP and RAMPO data matrix count 614 polymorphic markers. The derived genetic distances varied from 0.13 to 0.55 with a mean of 0.33. The derived UPGMA dendrogram, (Figure 7), suggests tree branching among accessions. Two main clusters are identified. The first one labelled (a) is monophyletic composed only by 'Kenta' cultivar. All the remaining ecotypes are ranged in the second cluster labelled (b). We noted that the detachment of 'Kenta' cultivar was obtained using AFLP markers only. This confirmed the better discrimination power of AFLP. In fact, this cultivar is characterized by its capacity of adaptation in climatic conditions and its presence of all phoenicicultural regions in Tunisia. This conclusion is strongly supported by the results showing by multivariate principal component analysis (PCA).

Table 4. Relative contribution of each variable to the variation provided by the first three axis of the PCA.

Component	RAMPO			AFLP		
	Axe 1	**Axe 2**	**Axe 3**	**Axis 1**	**Axis 2**	**Axis 3**
% Variation	11.85	9.20	7.12	12.3	07.5	06.5
% Cumulated	11.85	21.05	28.18	12.3	19.8	26.3
Variables contributing to the definition of the PCA axis	$OPB_{04\times06}$-3 (+0.15) $OPB_{04\times14}$-6 (+0.148) $OPA_{19\times10}$-1 (+0.147) $OPA_{12\times07}$-1 (+0.14) $OPA1_{2\times14}$-4 (+0.15)	$OPB_{04\times07}$-1 (+0.183) $OPB_{04\times09}$-1 (+0.196) $OPB_{04\times09}$-3 (+0.209) $OPB_{04\times09}$-5 (+0.182) $OPB_{04\times09}$-7 (+0.171) $OPB_{04\times09}$-8 (+0.168) $OPB_{04\times10}$-3 (+0.168) $OPA_{12\times14}$-5 (-0.186)	$OPB_{04\times02}$-6 (+0.165) $OPB_{04\times02}$-6 (+0.165) $OPB_{04\times02}$-1 (+0.171) $OPB_{04\times09}$-10 (+0.141) $OPA_{19\times02}$-4 (+0.147) $OPA_{19\times09}$-5 (+0.147) $OPA_{19\times09}$-6 (-0.136) $OPA_{19\times10}$-10 +0.156) $OPA_{12\times02}$-6 (+0.200) $OPA_{12\times10}$-8 (-0.137)	$E_{AGC}/M_{CAA(13)}$(+0.11) $E_{AGC}/M_{CAA(16)}$(+0.103) $E_{AGC}/M_{CAA(29)}$(+0.103) $E_{AGC}/M_{CAA(37)}$(+0.11) $E_{AAC}/M_{CAA(27)}$(+0.11) $E_{AAC}/M_{CAA(37)}$(+0.103) $E_{ACA}/M_{CAG(37)}$(+0.11) $E_{ACA}/M_{CAG(51)}$(+0.104) $E_{ACC}/M_{CTA(71)}$(+0.103) $E_{AAC}/M_{CAT(72)}$(+0.103) $E_{AAC}/M_{CAT(83)}$(+0.115) $E_{AAC}/M_{CAG(61)}$(-0.101)	$E_{AAC}/M_{CAA(19)}$(-0.15) $E_{AAC}/M_{CAA(29)}$(-0.14) $E_{AAC}/M_{CAA(30)}$(-0.14) $E_{AAC}/M_{CAA(31)}$(+0.14) $E_{ACC}/M_{CTA(93)}$(+0.13)	$E_{AGC}/M_{CAA(5)}$(+0.123) $E_{AGC}/M_{CAA(6)}$(+0.123) $E_{AGC}/M_{CAA(26)}$(+0.123) $E_{AAC}/M_{CAA(46)}$(-0.123) $E_{ACA}/M_{CAG(18)}$(+0.123) $E_{ACA}/M_{CAG(32)}$(+0.123) $E_{ACC}/M_{CTA(1)}$(-0.123) $E_{ACC}/M_{CTA(15)}$(-0.123) $E_{ACC}/M_{CTA(16)}$(-0.123) $E_{ACC}/M_{CTA(29)}$(+0.123) $E_{ACC}/M_{CTA(72)}$(+0.123) $E_{AAC}/M_{CAT(37)}$(+0.123)

Component	AFLP and RAMPO		
	Axe 1	**Axe 2**	**Axe 3**
% Variation	9.65	7.59	6.06
% Cumulated	9.65	17.25	23.32
Variable contributing to the definition of the PCA axis	E_{AGC}/M_{CAA}13(0.0943) E_{AGC}/M_{CAA}37(0.0943) E_{AAC}/M_{CAA}27(0.0943) E_{ACA}/E_{CAG}37(0.0943) E_{AAC}/M_{CAT}80(0.0982) E_{AAC}/M_{CAT} 82(0.0923) E_{AAC}/M_{CAT}84(0.0923) E_{AAC}/M_{CAG} 56(-0.0903)	E_{AAC}/M_{CAA}19(0.1288) E_{AAC}/M_{CAA}20(0.1166) E_{AAC}/M_{CAA}29(0.1148) E_{AAC}/M_{CAA}30(0.1253) E_{AAC}/M_{CAA}31(-0.1039) E_{AAC}/M_{CTA} 88(-0.1009) OPB04×10. 7(-0.1007) OPB04×10-9(-0.1064)	E_{AAC}/M_{CTA}89(0.0992) E_{AAC}/M_{CAT}18(0.0922) OPB04×02-1(0.1062) OPB04× 07-4(0.0938) OPB04×07-10(0.1265) OPA19× 07-3(0.0934) OPA12×07-10(0.0934) OPA12× 10-2(0.1021) OPA12× 10- 8(0.0968) OPA12× 09-8(0.1224)

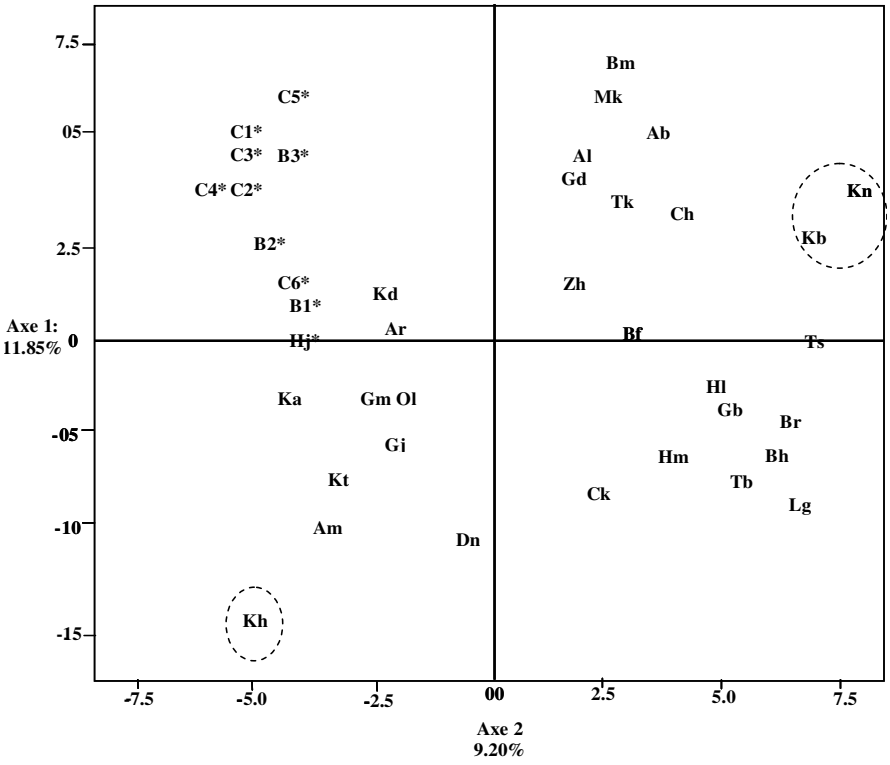

Figure 4. Plot of 40 Tunisian date palm genotypes according to the two first axes of the principal component analysis (PCA) (28.18% of the total genetic variability) based on 186 RAMPOs (Table 1 for cultivar codes; * male trees).

The three first axes contributed 23.32% of the total variability (Table 4). Taking into account the distribution of cultivars according to the PCA (axis 1 to axis 2), three clusters were obtained that were similar to those reported in the UPGMA dendrogram. As well, we noted the significant divergence of 'Kenta' and 'Khouet Aligue' from all remaining cultivars (Figure 8). On the other hand,this observed clustering is made independently from geographic origin of cultivars studied. This assumption is strongly supported since the recently introduced foreign accessions did not significantly diverge from the autochthonous ones. On the other hand, the male trees are clustering together in the same sub-cluster and they did not significantly diverge from female trees.

Correlation between genetic markers: Mantel test

To understand the degree of correspondence, if any, comparison of distances matrix was carried out based on correlation coefficient of Pearson and Sperman using XLSTAT (version:2007.8.01) (Table 3). Results show that correlation between RAMPO and AFLP genetic distance matrices was positive and highly significant (Pearson cœfficient = 0.166; Sperman cœfficient =0.202; P-value= 0.001) (Figure 9). On the other hand, positive and highly significant correlation was deduced between AFLPs and the combined distance matrix AFLP/RAMPO (Pearson cœfficient = 0.770; Sperman cœfficient = 0.784; P-value = 0.001) and between RAMPOs and the combined AFLP/RAMPO (Pearson cœfficient = 0.757; Sperman coefficient = 0.728; P-value = 0.001). This result indicates a good congruence between these two molecular markers. In fact, it can be explained by the elevated numbers of AFLPs and their capacity to overcome all studied genome (Vos et al., 1995; Donini et al., 1997; Saliba-Colombani et al., 2000; Gerber et al., 2000).

DISCUSSION

The date palm, one of the oldest domesticated fruit crops, is the most adapted tree to growing in desert areas. It has always been looked upon as a key source of stability, survival and evolution of the oasis agro-system as it constitutes the basic features of the ecological pyramid in desert regions.

All over the world, date-palm germplasms are characterised by the presence of a large number of cultivars (Rizvi and Davis, 1983; Ben Khalifa, 1996; Sedra, 1996). In early periods, germplasm was characterised using classical morphometric and vegetative criteria. Despite the usefulness of the latter methods in the establishment of phenotypic divergence in the

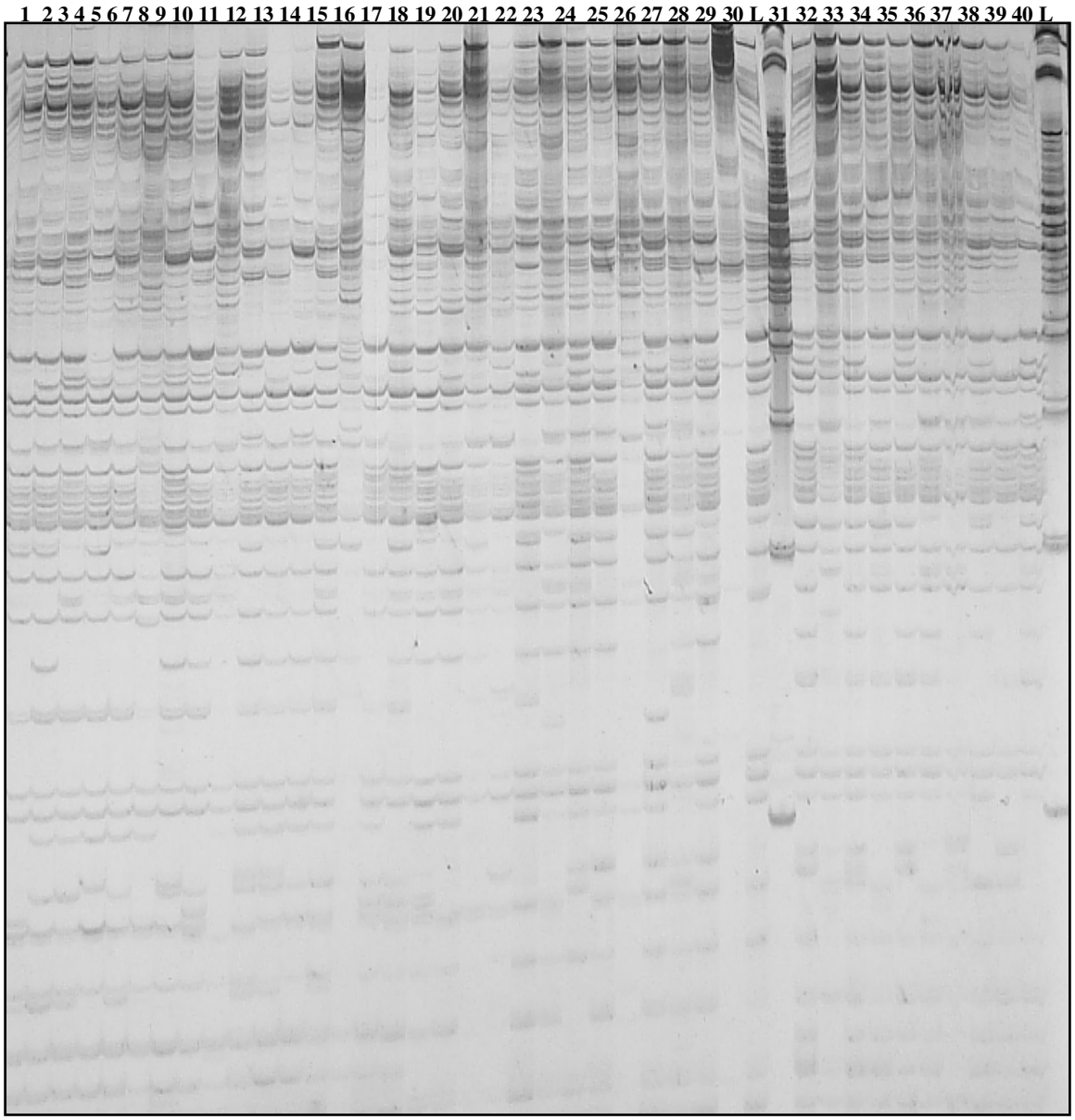

Figure 5. Typical examples of AFLP banding profiles generated by E_{AGC}/M_{CAA} primers' combination separated and silver stained in 6% (w/v) denaturing polyacrylamide gels. (L): Standard molecular size marker; (T): negative control; (1-40): analysed ecotypes**.**

cultivars studied, in order to set up a catalogue of the most important date palm cultivars, both in North African and other producing countries, analytic fruit parameters and isozyme markers were used. However, taking advantage of the large panel of DNA-based markers developed in the last two decades, investigations have focussed on identifying DNA markers suitable for fingerprinting of date palm cultivars and/or for varietal identification, as well as for the survey of the genetic organisation of this crop.

In this context, this study illustrates the development of the RAMPO and AFLP procedures to generate molecular markers suitable in the assessment of the genetic diversity within Tunisian date-palms cultivars. Starting from a set of local and introduced genotypes, the primers combinations tested have permitted to evidence a total of 614 polymorphic bands. Opportunely, these primers are characterized by the higher rates of resolving power (Rp) and of percentage of polymorphic bands (ppb). Our results evidence that both AFLP and RAMPO markers expressed a high level of polymorphism allowing the distinction of the accessions in comparison with the previously reported in Tunisian date-palm using isozyme and molecular markers such as RAPD, ISSR and CAPS

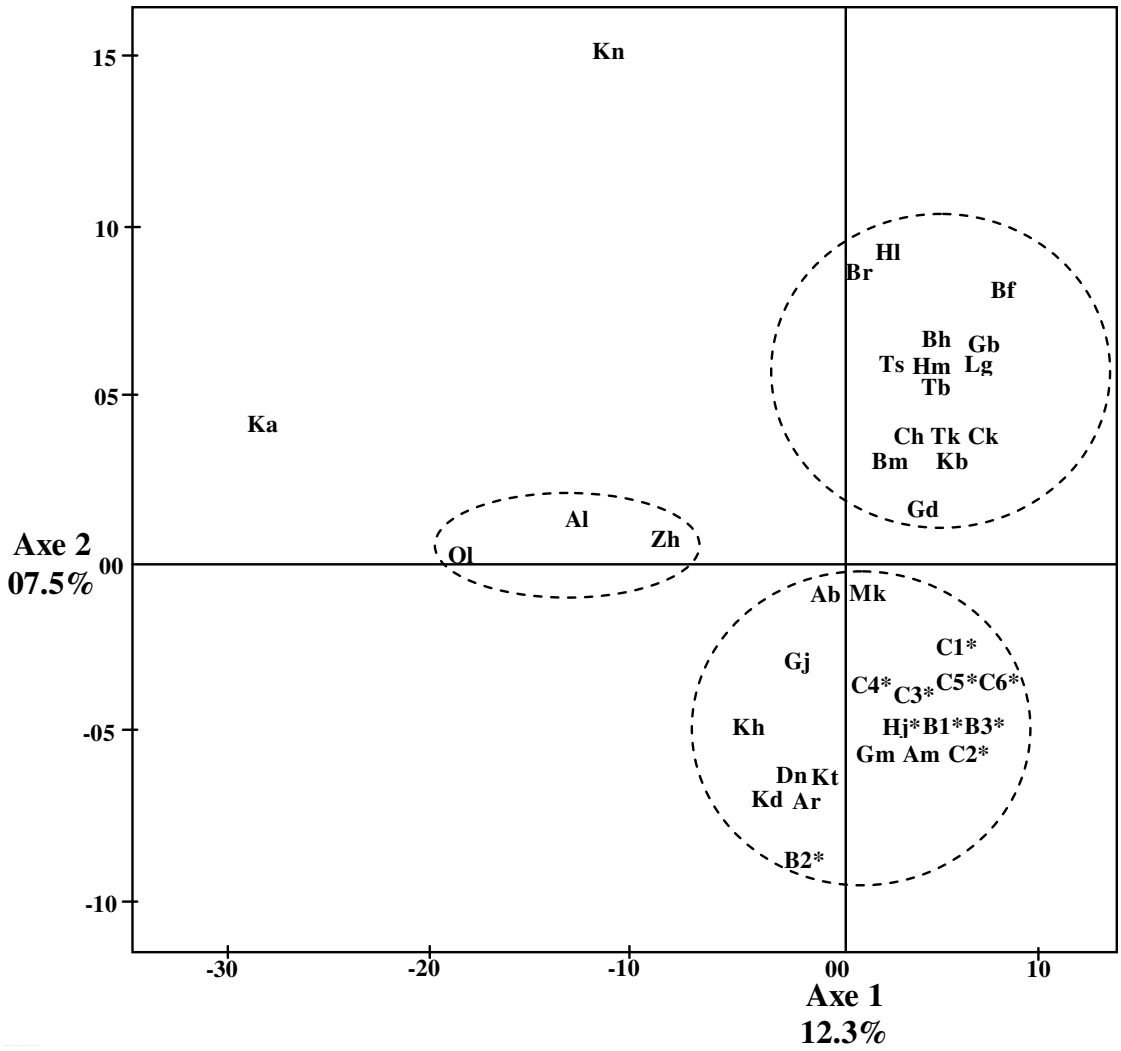

Figure 6. Plot of 40 Tunisian date palm genotypes according to the two first axes of the principal component analysis (PCA) (26.3% of the total genetic variability) based on 428 AFLPs (Table 1 for cultivar codes; * male trees).

(Trifi et al., 2000; Ould Mohamed Salem et al., 2001; Zehdi et al., 2002; Sakka et al., 2004). In addition, we perceive that the AFLP technique is more laborious and time consuming than RAMPO methods, but is also more reliable, AFLP being able to detect a large number of polymorphic bands in a single lane rather than high levels of polymorphism at each locus.

In conclusion, our study indicated that AFLPs balanced favourably with RAMPOs when only genetic relationships among accessions, evaluated at the whole-genome level were required. The important polymorphism observed for AFLP and RAMPO markers reflected a large genetic diversity in the Tunisian date palm germplasm. Several factors might affect the estimation of genetic diversity: at least the number of used markers, the distribution of markers throughout the genome and the nature of evolutionary mechanisms underlying the measured variation. The expected polymorphism on the basis of these two classes of molecular markers (AFLP and RAMPO) seems to be sufficient to explore the genetic diversity structure of Tunisian date palm germplasm.

Furthermore, utility of a molecular marker technique depends upon both the polymorphism information content and the number of markers generated by each primer. Whole RAMPO and AFLP exhibited comparable PIC values, owing to the greater number of markers per assay, the AFLP markers had a higher marker index (MI) and seems to be more informative. In this case, Gerber et al. (2000) suggest that the high numbers of polymorphic loci revealed by AFLP methods counterbalance the loss of information resulting from its inability to distinguish heterozygotes from homozygotes because of binary scored AFLPs.

On the other hand, data proved that continuous genetic diversity characterises the local date-palm germplasm. The topology of dendrograms obtained with RAMPO,

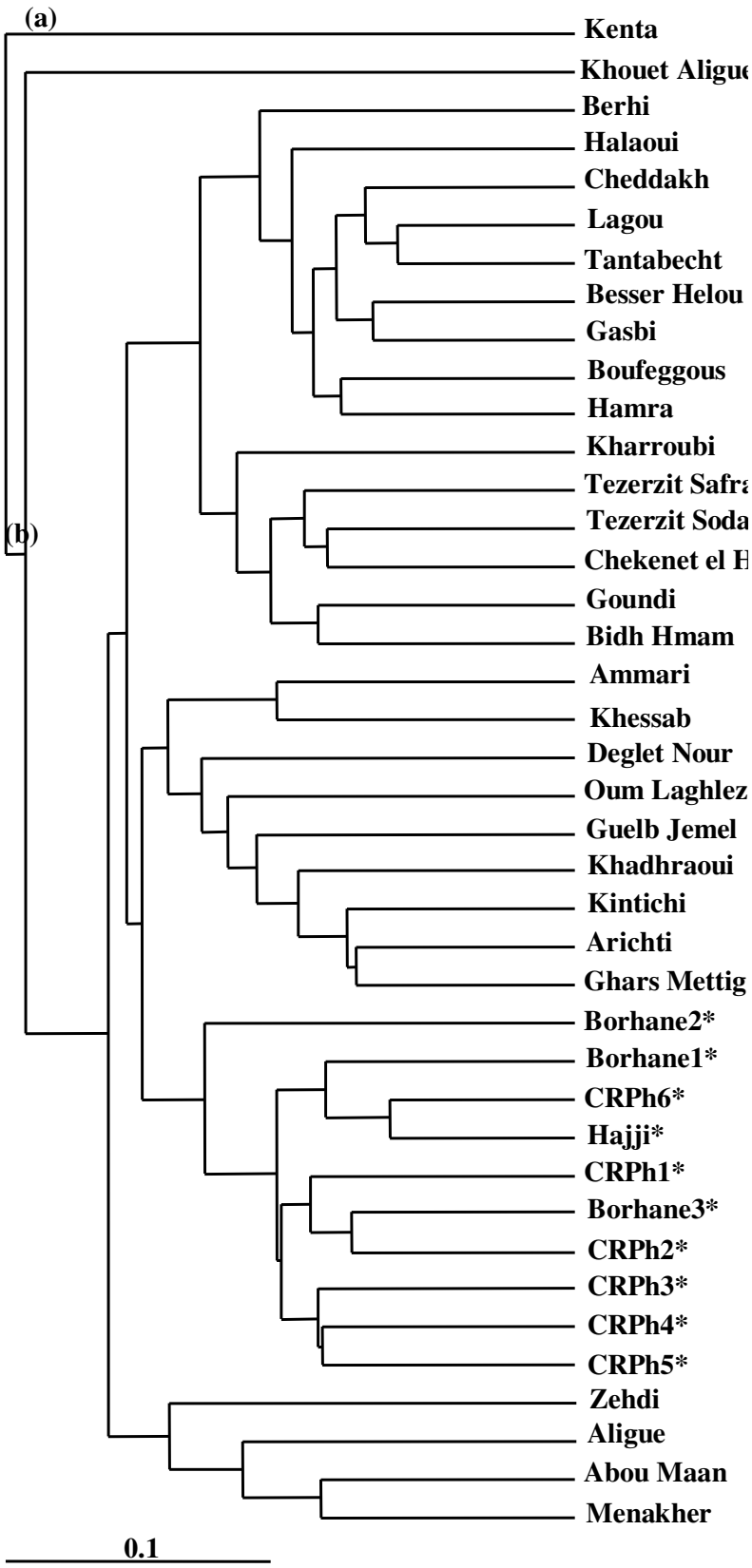

Figure 7. Dendrogram of 40 Tunisian date palm cultivars constructed by UPGMA method and based on RAMPOs and AFLPs (Table 1 for cultivar codes, *: male trees).

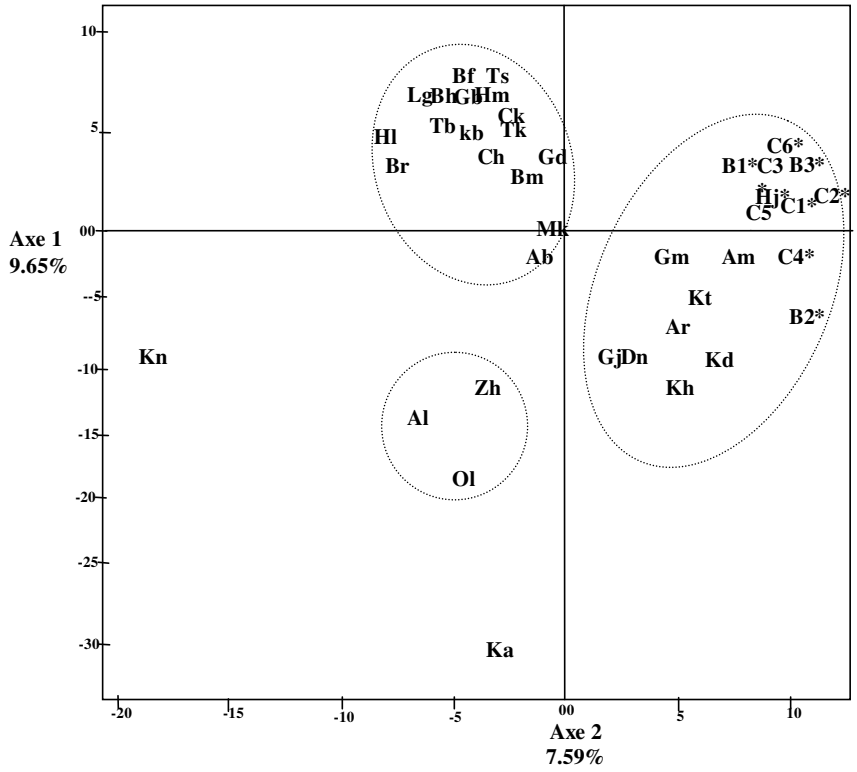

Figure 8. Plot of PCA (23.32% of the total diversity) of 40 Tunisian date palm accessions based on RAMPO and AFLP pooled markers (Table 1 for cultivar codes; * male trees).

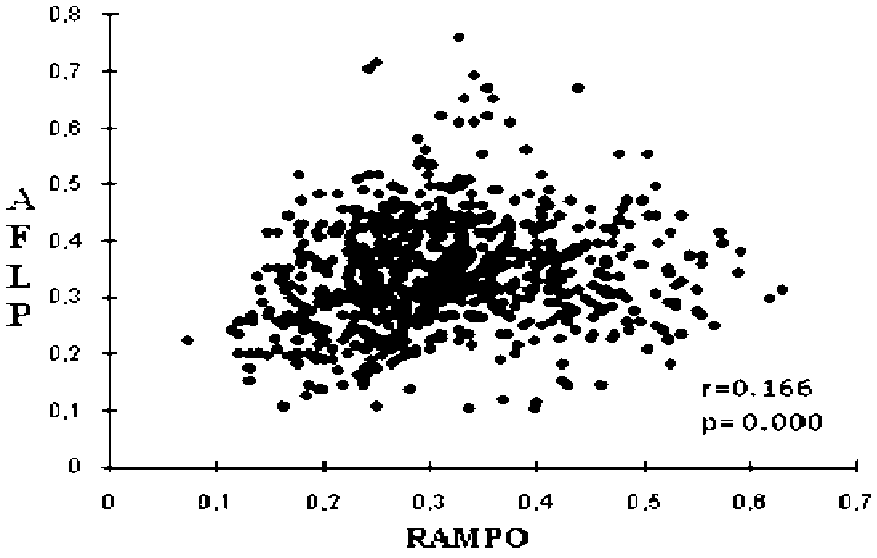

Figure 9. Correlation estimated with Mantel test between genetic distances matrix for 40 date palm cultivars as calculated using data from AFLP and RAMPO methods.

AFLP and the combined analysis strongly supported this assumption. In fact, ecotypes are clustered independently either from their geographic origin or from the sex of trees suggesting a narrow genetic basis among the cultivars studied in spite of their phenotypic distinctiveness. This is in agreement with the ancient date-palm's Mesopotamian (Fertile Crescent) domestication origin (Wrighly, 1995; Hodel and Johnson, 2007).

Taking into account the positive and highly significant correlation between results assessed with AFLPs and

RAMPOs separately and the high positive correlation with the combined AFLP-RAMPO data, constant results emerged from the genetic relationships assessment such as some very stable groups of cultivars and indicates a good congruence between these two molecular markers. Our results confirm the complementarities of these two powerful markers and their efficiency in the genetic diversity assessment when combined in the same data set.

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