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MOLECULAR TAXONOMY AND GENETIC DIVERSITY EVALUATION AMONG SEVEN CULTIVARS OF *KALANCHOE BLOSSFELDINA* POELLN USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

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ABSTRACT

The study aimed to detect molecular intraspecific markers to identify seven cultivars of Kalanchoe blossfeldina Poeln. (*K. blossfeldiana* Calandiva Bardot; *K. blossfeldiana* Calandiva Hayworth; *K. blossfeldiana* Calandiva Monro; *K. blossfeldiana* Calandiva Fonda; *K. blossfeldiana* Calandiva Middler; *K. blossfeldiana* Calandiva Leonardo; *K. blossfeldiana* Calandiva Rosalina) have been genetically analyzed, using Randomly Amplified Polymorphic DNA (RAPD PCR) technique, ten randomize primers used for this purpose yielded 390 reproducible bands with an average of 39 bands or fragments per primer, contain 259 monomorphic bands and 145 were polymorphic bands, primers produce 12 unique bands and 8 bands were absent. The primers (80-14) yielded high number of DNA fragments (53). The results showed 48 polymorphic sites and 32 monomorphic sites, the heist polymorphism percentage reached 100% with primer (OPB-01) and the lowest polymorphism percentage with the primer (80-15) reached 25%, UPGMA analysis depending on similarity matrix, studied cultivars showed the highest genetic similarity reached (0.908) between (Calandiva Monro, Calandiva Fonda), while lowest genetic distance was (0.426) between (Calandiva Leonardo, Calandiva Hayworth) cultivars as well as genetic difference was (0.347) between (Calandiva Leonardo, Calandiva Hayworth) also. Phylogenetic dendrogram showed two main clusters, the first cluster composed of two secondary clusters comprised of 6 cultivars, while the second main cluster contain only one cultivar (Calandiva Leonardo) which showed the highest molecular differences and the purple flowers .
Keywords : *Kalanchoe blossfeldina* cultivars ; RAPD PCR ; Molecular Taxonomy ; Crassulaceae

Introduction

Kalanchoe blossfeldiana cultivars are result of interspecific hybridization, so the characterization depend on qualitative and quantitative features. The polygenic control and additive inheritance can explain the intermediacy of hybrids. (Engles *et al.*, 1975; Habu *et al.*, 1998) concluded thatgenes with partial dominant control the flower color of most interspecific hybrids have intermediate features and appear like a mixture of parental flower color. RAPD PCR play an important role in study of biochemical and physiological of crassulacean acid metabolism (Gehrig *et al.*, 1997). Genetic diversity of *Kalanchoe* genus have been detected by using RAPD PCR to study polymorphism and concluded that genus *Kalanchoe* is a monophyletic clad which have three main clusters (Gehrig *et al.*, 2001). Dwarf genotype of *Kalanchoe blossfeldiana* improved on a wide range through molecular breeding using PCR technique proved some morphological character such as plant height, number of lateral shoots, leaves number and size (Christensen, 2008), another molecular study which is published by (Sanikhani *et al.*, 2008) they could make genetic transformation and proved it through RT-PCR and produce plant have flowers not sensitive to ethylene gas through mutation. (Topp *et al.*, 2009) displayed through gene sequence four sets of mRNA of different phenotypes of *Kalanchoe blossfeldiana* to isolate and identify genes related with elongation of the plant and explain that there are two hybridizing fragments which have tetraploid nature, Gracia–

Sogo *et al*, succeeded in production a sterile plant without pollen grain which cause sensitivity for some peoples from decoration plants through genetic transformation (Gracia-Sogo *et al.*, 2010). (Kuligowsska *et al.*, 2015) showed that interspecific hybridization produce many of *Kalanchoe blossfeldiana* cultivars and the hybridization increase attractiveness of commercial plants. Yang *et al.* (2017) explained that CAM pathway is an efficient mechanism in photosynthesis in *Kalanchoe* plants which have relatively small genome.

Materials and Methods

Kalanchoe blossfeldiana were seven cultivars samples were collected from local markets. during 2019-2020 seasons. The cultivars differ in floral features, especially in petals color and number.

DNA extraction

Kalanchoe blossfeldiana DNA extracted from leaves (400) mg of fresh leaves paces of each cultivar sample using Plant Genomic DNA Extraction Mini Kit from FAVORGEN (Favorprep. Plant genomic DNA Extraction Mini Kit) from Korea.

Polymerase Chain Reaction PCR

Reactions of RAPD-PCR were performed in thermal cycler (model: BIO-RAD, USA) according to Williams *et al.* (1990) with 10-mer oligonucleotides Table1 (2) synthesized by (Integrated DNA Technologies, Jordan.

Table 1 : Primers used in RAPD PCR names and sequences (Singab *et al.*, 2012), (Gehrig *et al.*, 1997).

Primer	Sequence	GC Content %	Tm ºC
80-11	GCA GCA GCC G	80.0	44.6
80-12	CGA CGC GTG C	80.0	45.4
80-13	ACC CGT CCC C	80.0	43.4
80-14	GCA GCT CCG G	80.0	42.9
80-15	CGA GAC GGG C	80.0	42.4
OPB-12	CCT TGA CGC A	60.0	35.7
OPB-18	CCA CAG CAG T	60.0	34.8
OPB-09	TGG GGG ACT C	70.0	37.0
OPB-15	GGA GGG TGT T	60.0	33.2
OPB-01	GTT TCG CTC C	60.0	33.4

Random amplified polymorphic DNA-PCR (RAPD-PCR) was used for the molecular characterization of *Kalanchoe blossfeldiana* cultivars, PCR reaction was set at final volume of 20µl contained 10µl of 2X PCR premix (HS Prim Taq Premix, GeNet Bio, Korea), 3 µl of the primer, 4 µl of PCR grade water and 3 µl of 50 ng of extracted DNA template. The PCR reaction was performed by T100 Thermocycler (BioRad, USA) and the program included an initial denaturation step at 95°C for 10 min followed by 35 cycles with 95°C for 45 sec for DNA denaturation, 34°C for 30 sec for primer annealing and 72°C for 1 min for primer extension. Final extension was at 72°C for 5 min. After that the reactions were cooled at 4°C. The amplified products were separated using electrophoresis in 1.5% agarose gel (Promega, USA), and 5 µl of each PCR product was loaded into the well of agarose gel. The electrophoresis was carried out using 1X TBE buffer (GeNetBio, Korea) at 80 V for 1 hour using power supply (BioRad, USA). A 100 bp DNA marker, 4 µl (Promega, USA) was used as standard molecular weight marker. The gel was examined using gel documentation system (Gel Doc EZ Gel Documentation System, BioRad, USA).

Result and Discussion

Ten randomize primer used to detect *Kalanchoe blossfeldiana* cultivars genotype targeted and genetic variation, finger print and genetic distance to determine molecular similarities and differences and phylogenetic relationships. That all tested primers give reproducible patterns and generated monomrphic and polymorphic bands collectively 390 DNA fragments (band) represented by 80 position. 295 band were monomorphic, represented by (32) site and 145 band were polymorphic represented by (48) site (DNA fragments lines), 12 of the band were unique bands and (8) of them were absent bands. Each of the primers (OPB-18; 80-15; 80-14) have the upper number of band (50; 51; 53), while the primer (OPB-15) had the lower number of bands (18). One of the most basics of RAPD markers is the number of bands which are depend on DNA fragments lines. The number of bands affected by the size of gene and primer sequence (Al-Sugmiany *et al.*, 2018).

Borovkova *et al.* (1997); Williams *et al.* (1990) said that short sequence that have 9 or 10 base pair may find (2-10) position on higher plants DNA. The data obtained from RAPD PCR to detect finger prints for studied cultivars depending on DNA differences, showed by variations in bands numbers and molecular size in addition to presence and absence of some amplified DNA fragments, the following shapes showed the results obtained from using random primers:

Using the primer (80–11) cultivars DNA showed (33) bands represented by (7) sites (4)of these positions were polymorphic sites and (3) were monomorphic sites, polymorphism percentage reached 57%), molecular size of fragments ranged from 450–1550 bp, the primer reached Ability discriminatory (8.2) while the primer efficiency was (8.4) this primer diagnose the cultivar (*K. blossfeldiana* Calandiva Leonardo) with the complementary site for the band (1550 bp), and the cultivar (*K. blossfeldiana* Calandiva Monro) with the band (520 bp), these bands represent a molecular taxonomic markers and a finger prints as a unique bands for cultivars, no absent bands found. Table (2) and figure (1, A).

Also the table (2) and figure (1,B) reveals that the primer (80-12) yielded (16) bands represented by (7) sites (6) of them were polymorphic sites and one site was monomrphic; this primer characterized the cultivar (*Kalanchoe blossfeldiana* Calandiva Middler) with two unique bands have molecular size (500, 320 bp), thus can be used as finger prints and molecular taxonomic markers for this cultivar. The primer show efficiency (4.1) and Ability discriminatory (6.2).

(As showed in the table (2) and figure (1, C). the primer (80- 13) produce (8) sites (4) polymorphic sites, and (4) sites were monomorphic; the cultivar (*K. blossfeldiana* Calandiva Bardot) distinguished from other cultivar having the unique band of molecular size (1480 bp), this can be as a taxonomic marker and finger print for this cultivar the of the primer Ability discriminatory (8.2) and efficiency (11.2).

The primer (80-14) in the table (2) and figure (1, D). showed (10) sites, (4) polymorphic sites and (6) sites were monomorphic; the primer identify the cultivar (*K. blossfeldiana Calandiva* Rosalina) with the band (1230 bp) as a unique band which can be a taxonomic marker and finger print for this cultivar; this primer show Ability discriminatory of the primer (4.8) and the highest efficiency among the studied primers (13.5).

The primer (80-15) in the table (2, E) and figure (1) yielded the lowest polymorphic sites (2), and (6) sites were monomorphic, this primer showed one absent band with the cultivar (*K. blossfeldiana* Calandiva Middler) of molecular size (650 bp), Ability discriminatory of the primer reached (1.3) and the efficiency was (12.8).

From the result in the table (2) and figure (1, F). we can observe that the primer (OPB-12) yielded (8) sites; (4) sites were monomorphic, and (4) polymorphic sites, one band were absent in the cultivar (*K. blossfeldiana* Calandiva Rosalina) because it didn't have a complementary site in the

band (270 bp), the primer efficiency was (11.7), while Ability discriminatory of the primer reached (9.6).

The primer (OPB-18) showed in the table (2) and figure (2G). high number of DNA sites reached (11) sites, three of them were monomorphic, and (8) polymorphic sites, one band which have molecular size (250 bp) was unique band in the cultivar (*K. blossfeldiana* Calandiva Bardot) this can be considered as a taxonomic marker and finger print for this cultivar; also the primer showed (2) absent band in DNA the cultivar (*K. blossfeldiana* Calandiva Hayworth) with the molecular size (890; 970 bp). the primer efficiency was (13), while Ability discriminatory of the primer reached (12.4).

In contrast the primer (OPB–O9) showed the lowest number of DNA sites (4) the table (2) and figure (2,H). two of the sites were monomorphic and two of the sites were polymorphic sites, the primer produce one band as a unique band in DNA the cultivar (*Kalanchoe blossfeldiana* Calandiva Monro) with molecular size of about (460 bp) this can be considered as a taxonomic marker and finger print for this cultivar ; the primer efficiency was (4.6), while Ability discriminatory of the primer reached (6.8).

The number of DNA sites yielded with the primer (OPB -15) were (6) sites table (2) and figure (2.I) of the sites were monomorphic and (3) of the sites were polymorphic, one of them was unique band in DNA of the cultivar (*K. blossfeldiana* Calandiva Leonardo) with molecular size of about (600) and an absent band with molecular size (700) in DNA the same cultivar ,thus can be a taxonomic marker and finger print for this cultivar; the primer efficiency was (7.9), Ability discriminatory of the primer reached (8.9).

The primer (OPB -01) characterized by the highest polymorphism percentage (100%) table (2) and figure (2,J), all (11) DNA sites were polymorphic, one of them was unique band in DNA of the cultivar (p6) because it have a complementary site for the band (1620 bp) molecular size thus can be a taxonomic marker and finger print for this cultivar; whereas the primer showed two absent bands in DNA the cultivar (*K. blossfeldiana* Calandiva Bardot) have the molecular size (1080; 400 bp), the primer efficiency was (12.3), while Ability discriminatory of the primer reached (33.1).
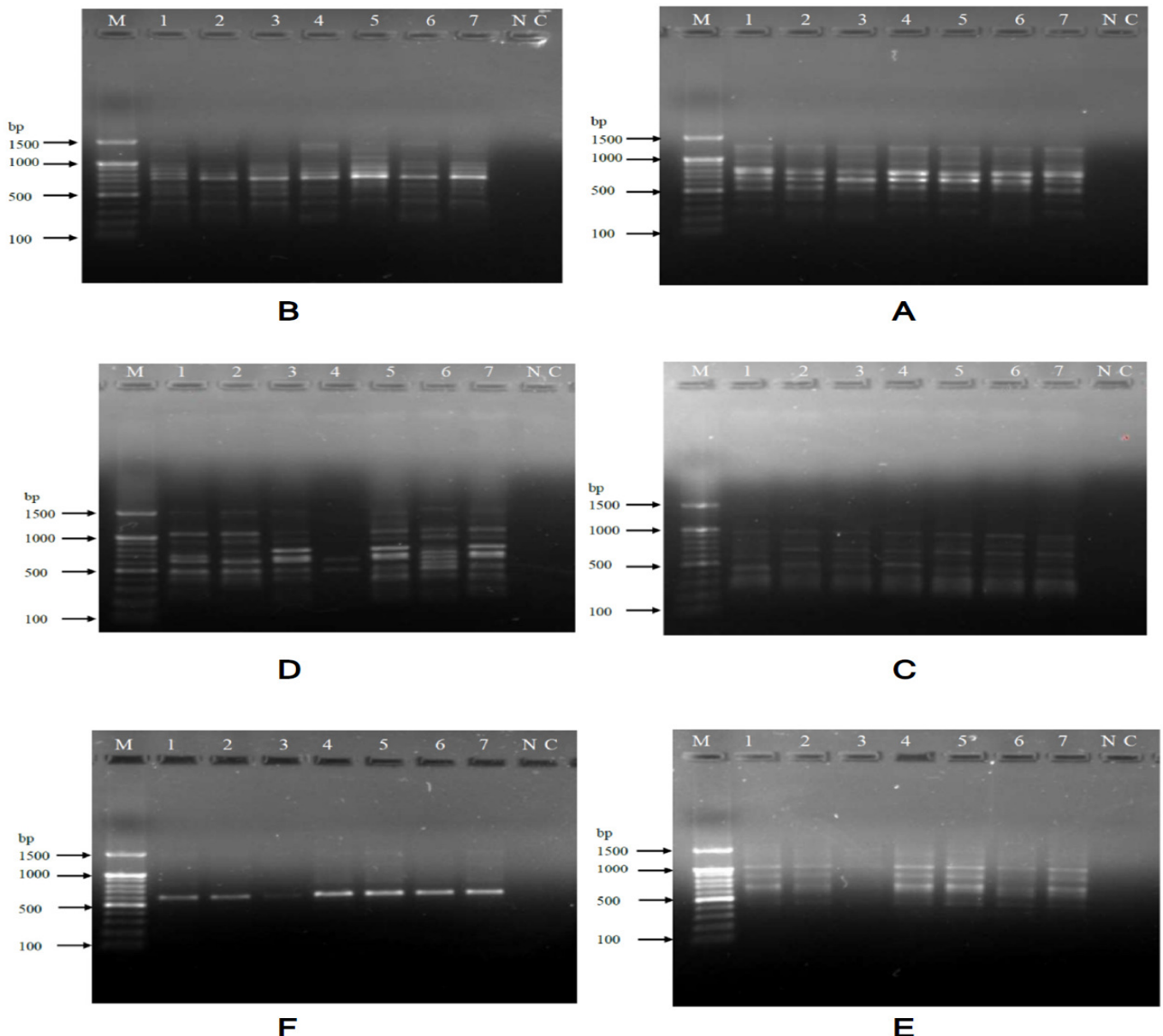
Fig. 1 : RAPD- PCR profile of seven *K. blossfeldiana* cultivars plate A using primer (80–11); plate B using primer (80–12); plate C using primer (80–13); plate D using primer (80–14); plate E using primer (80–15) plate F using primer (OPB – 12).
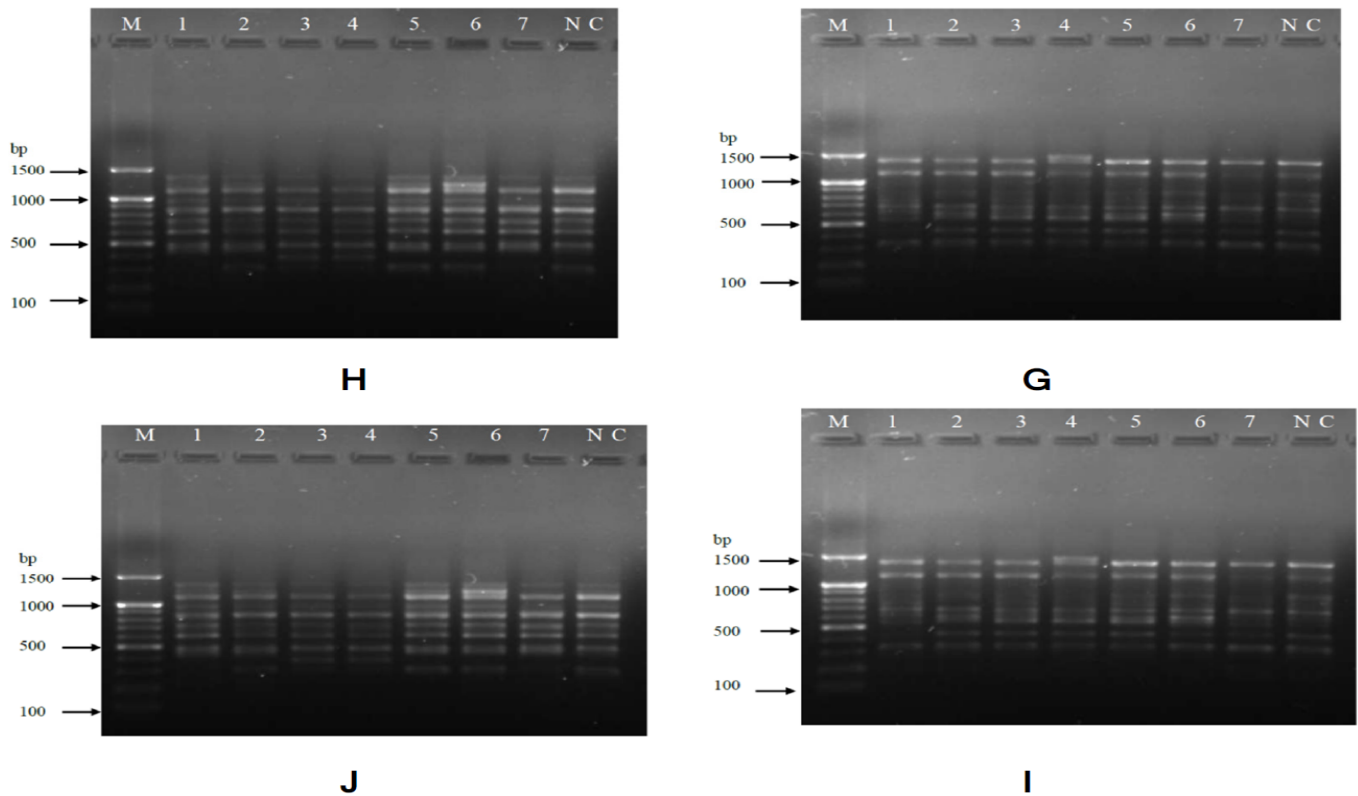
Fig. 2 : RAPD- PCR profile of seven *K. blossfeldiana* cultivars plate G using primer (OPB-18); plate H using primer (OPB – O9); plate I using primer (OPB - 15); plate J using primer (OPB – 01).

As showed in table (2), unique. and absent bands appeared, the meaning of absent is the absence of monomorphic band in DNA certain cultivar and presence this band in other cultivars this can be considered as a taxonomic marker and finger print for this cultivar (Borovkova *et al.*, 1997) that we observed in the cultivars (*K. blossfeldiana* Calandiva Bardot, *Kalanchoe blossfeldiana* Calandiva Leonardo, *K. blossfeldiana* Calandiva Hayworth, *K. blossfeldiana* Calandiva Rosalina, *K. blossfeldiana* Calandiva Middler) with six primers (80- 3; 80–15; OPB–12; OPB–15;

OPB–01), while all used primers with the except of two primers (80-5 ; OPB-12) produce unique bands. The primer (80-12) showed the highest number on unique bands (4) all of them in DNA (*K. blossfeldiana* Calandiva Middler) which have bicolored flowers pink and white, that can be considered as a strong molecular taxonomic marker and finger print for this cultivar. Primers efficiency varied from (4.8) for the primer (80–12) to (13.5) for the primer (80 – 14), as well as in the Ability discriminatory from (1.3) for the primer (80 – 15) to (33.1) for the primer (OPB-01).

Table 2 : Primers names with total bands, number of polymorphic bands and percent of polymorphyism, bands sizes, primers efficiency and ability discriminatory, unique and absent bands, per primer of polymorphic RAPD primers used for *Kalanchoe blossfeldiana* cultivars.

N.	Primer name	Total sites	Monomorphic sitess	Polymorphic sitess	Number of Total band	Number of monomorphic band	Number of polymorphic band	Unique band	Absent band	Primer efficiency %	Ability discriminatory %	Percentage polymorphism %	Band size bp
1	80-11	7	3	4	33	21	12	2	-	8.4	8.2	57	450-1550
2	80-12	7	1	6	16	7	9	4	-	4.1	6.2	85	320-1500
3	80-13	8	4	4	44	32	12	1	1	11.2	8.2	50	340-1480
4	80-14	10	6	4	53	60	7	1	-	13.5	4.8	40	330-1250
5	80-15	8	6	2	50	48	2	-	1	12.8	1.3	25	300-1000
6	OPB-12	8	4	4	46	32	14	-	1	11.7	9.6	50	200-1240
7	OPB-18	11	3	8	51	33	18	1	2	13	12.4	72	250-1440
8	OPB-09	4	2	2	18	8	10	1	-	4.6	6.8	50	460-1090
9	OPB-15	6	3	3	31	18	13	1	1	7.9	8.9	50	300-1000
10	OPB-01	11	-	11	48	-	48	1	2	12.3	33.1	100	400-1620
	Total	80	32	48	390	259	145	12	8				

The similarity matrix table (3) for studied *Kalanchoe blossfeldiana* cultivars (Nei and Li, 1979) showed that similarity ranged from (0.653) between (*K. blossfeldiana* Calandiva Leonardo, *K. blossfeldiana* Calandiva Hayworth) to (0.908) between (*K. blossfeldiana* Calandiva Fonda, *K. blossfeldiana* Calandiva Monro) the genetic similarity yielded by RAPD PCR associated with morphology and anatomy similarity (Al Hayali, 2013).

Table 3 : Genetic similarity resulting from the UPGMA cluster analysis for (Nei & Li) among studied *Kalanchoe blossfeldiana* cultivars.

	Calandiva Leonardo	Calandiva Hayworth	Calandiva Middler	Calandiva Bardot	Calandiva Fonda	Calandiva Rosalina	Calandiva Monro
Calandiva Leonardo	1						
Calandiva Hayworth	0.653	1					
Calandiva Middler	0.750	0.819	1				
Calandiva Bardot	0.765	0.838	0.793	1			
Calandiva Fonda	0.814	0.867	0.824	0.807	1		
Calandiva Rosalina	0.815	0.778	0.807	0.806	0.803	1	
Calandiva Monro	0.818	0.836	0.794	0.775	0.908	0.825	1

As showed in the genetic distance matrix table (4) the genetic distance ranged from (0.142) between (*K. blossfeldiana* Calandiva Hayworth, *K. blossfeldiana* Calandiva Fonda) to (0.425) between (*Kalanchoe blossfeldiana* Calandiva Leonardo ,*K. blossfeldiana* Calandiva Hayworth)

Table 4 : Genetic distance resulting from the UPGMA cluster analysis for (Nei & Li) among studied *Kalanchoe blossfeldiana* cultivars.

	Calandiva Leonardo	Calandiva Hayworth	Calandiva Middler	Calandiva Bardot	Calandiva Fonda	Calandiva Rosalina	Calandiva Monro
Calandiva Leonardo	0						
Calandiva Hayworth	0.426	0					
Calandiva Middler	0.287	0.199	0				
Calandiva Bardot	0.267	0.176	0.232	0			
Calandiva Fonda	0.206	0.142	0.193	0.214	0		
Calandiva Rosalina	0.204	0.251	0.214	0.215	0.219	0	
Calandiva Monro	0.200	0.179	0.230	0.255	0.096	0.192	0

The result in the table (5) showed the genetic differences studied *Kalanchoe blossfeldiana* cultivars (Nei and Li, 1979) ranged from (0.092) between (*K. blossfeldiana* Calandiva Fonda, *K. blossfeldiana* Calandiva Monro) to (0.347) between (*K. blossfeldiana* Calandiva Leonardo, *K. blossfeldiana* Calandiva Hayworth).

Table 5 : Genetic differences resulting from the UPGMA cluster analysis for (Nei & Li) among studied *Kalanchoe blossfeldiana* cultivars.

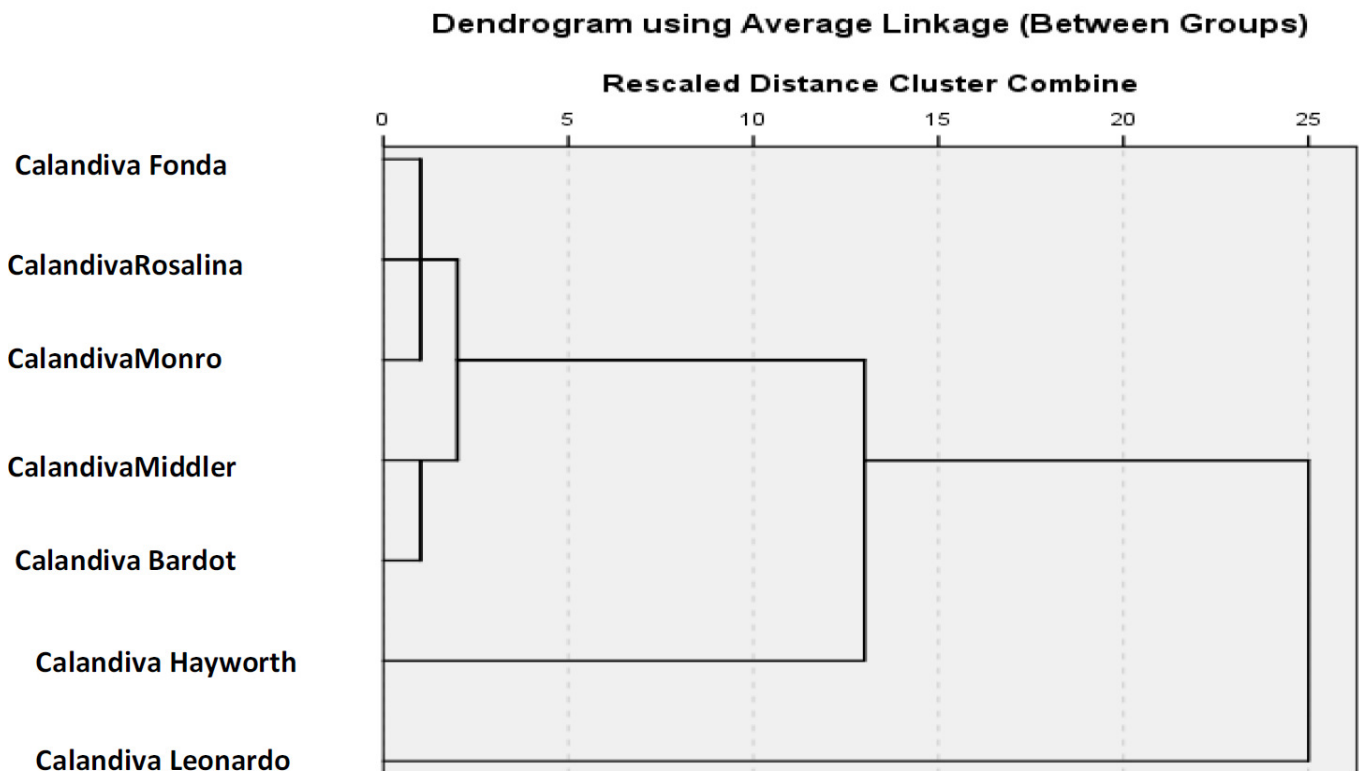|	Calandiva Leonardo	Calandiva Hayworth	Calandiva Middler	Calandiva Bardot	Calandiva Fonda	Calandiva Rosalina	Calandiva Monro
Calandiva Leonardo	0						
Calandiva Hayworth	0.347	0					
Calandiva Middler	0.250	0.181	0				
Calandiva Bardot	0.235	0.162	0.207	0			
Calandiva Fonda	0.186	0.133	0.176	0.193	0		
Calandiva Rosalina	0.185	0.222	0.193	0.194	0.197	0	
Calandiva Monro	0.182	0.164	0.206	0.225	0.092	0.175	0

From the results above depending on the genetic similarity, differences and distance showed that the cultivar (*K. blossfeldiana* Calandiva Leonardo) was the lowest genetic similarities and the highest genetic distance and differences .

Cluster Analysis

Dendrogram or cluster analysis was created depending on genetic similarity matrix (Li & Nei, 1979) table (3) and genetic distance that connect main groups for studied cultivars, by using unweighted paired group method of arithmetic UPGMA.

The Dendrogram for seven studied cultivars composed of two main groups, the first main group is divided into two secondary groups, the first secondary group composed of the cultivars (*K. blossfeldiana Calandiva Middler*, *K. blossfeldiana Calandiva Bardot*, *K. blossfeldiana Calandiva Rosalina*, *K. blossfeldiana Calandiva Fonda*, *K. blossfeldiana Calandiva Monro*) depending on the degree of proximity, high genetic similarity among cultivars morphological differences can be traced to presence of non–coding sites on the genes (Abood *et al.*, 2014) (Al-Zaidy *et al.*, 2016) explained that of some cultivars in one group to similar quantum genetic extent of these group.

Fig. 3 : The dendrogarms resulting from the UPGMA cluster analysis for (Nei & Li) among studied *Kalanchoe blossfeldiana* cultivars.

The second secondary group comprised of one cultivar (*K. blossfeldiana Calandiva Hayworth*) only, this cultivar showed (2) absent bands with the primer (OPB–18) and showed low similarity with other cultivars figure (3).

The second main group contain one cultivar (*K. blossfeldiana Calandiva Leonardo*) alone , that's due to lowest genetic similarity with other cultivars and higher genetic distance and differences and have three unique bands and (2) of absent bands all of these molecular markers put this cultivar alone in main group. Results showed by constructed phylogenetic tree revealed that *Kalanchoe blossfeldiana* cultivars are not monophelic since they grouped on two main clusters. This results agree with (Hussein *et al.*, 2005; Haider *et al.*, 2012) on Date Palm cultivars. Thus molecular analysis create distinct variation in molecular properties within the studied cultivars, so it is possible to classify *K. blossfeldiana* cultivars using RAPD PCR.

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