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Genetic Diversity and Relationships among *Verbascum* Species in Iraq by RAPD-PCR Technique

Al-Hadeethy Muazaz[1], Jawad Mohammed M[1],Nantawan Kanawapee[2], AL-Jewari Hazim[1] AL-Mshhdani Athiya[1], AL-Khesraji Talib[3], Barusrux Sahapat[4] and Piyada Theerakulpisut[2].

[1]Department of Biology, College of Education - Ebn Al- Haitham, University of Baghdad, Baghdad, Iraq.
[2]Applied Taxonomic Research Center, Department of Biology, Faculty of Science.KhonKaen University, Thailand
[3]Department of Biology, College of Education, University of Tikrit, Tikrit, Iraq.
[4]Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, KhonKaen University, KhonKaen, 40002, Thailand.

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This study was aimed to determine the genetic diversity and relationships among 20 local *Verbascum* species by using the Random Amplified Polymorphic DNA (RAPD) technique. To achieve this, Total genomic DNA of *Verbascum* plant was extracted from leaves using two different methods, by CTAB method using 2-3 g of leaves, with purity of 1.5-1.9, and also by using commercial kit, PCR reactions were carried out using 17 random decamere-nucleotide primers. Percentage of monomorphic and polymorphic bands varies for each primer used with each species and the lowest numbers of polymorphic bands were 2 with OPX16, which showed the polymorphism (50%), produced 4 bands which only 2 were polymorphic. OPA5 and OPW13 showed the highest number of bands (9) and all are polymorphic (100%). From total of 119 alleles that detected, 103 were polymorphic (86.55%) and 16 monomorphic Moreover. A total of 119 polymorphic bands were scored and used for the analysis of genetic distances. Band sizes varied between 100 and 1900 bp. According to the results of genetic distance and relationships illustrated, the ability to resolve genetic variation among different *Verbascum* species may relate to the number of polymorphic bands detected with marker technique. Dendrogram was constructed using UPGMA cluster analysis and depicted genetic relationships among 20*Verbascum* species, showed the 2 major clusters depending on their ancestor and their morphological traits. The information generated from this study can be used in the future for *Verbascum* breeding and improvement programs.

Keywords: Molecular analysis, *Verbascum*, RAPD markers, Iraq, phylogenetic tree

INTRODUCTION

The genus *Verbascum* belonging to the tribe *Verbascum*(*Scrophulariaceae*) (Valdes, 1987) with 27 species in Iraq (Al-Bermani, 1981) and 360 species worldwide (Judd *et al*. 2002) is the largest genus within the large family *Scrophulariaceae*. The main centers of its diversity are Turkey, Iraq and Pakistan (Zohary, 1973 and Huber-Morath, 1978). Its species are adapted to the various habitats and different regions including Rocky

Corresponding author Email: lubni_a75@yahoo.com

Mountains, open forests, roadsides and the bank of the rivers (Huber-Morath, 1978).

The DNA analysis has offers many useful markers for genetic fingerprinting to study genetic diversity and to characterize unknown samples. In the range of DNA-profiling methods, the RAPD (Random Amplified Polymorphic DNA) technique has been widely used because of its simplicity procedure, use of random primers and the requirement of minute quantities of DNA (Principato *et al*., 2006). The conservation of genetic diversity in endangered species is a main goal in protection planning, since long-term species survival depends on the maintenance of sufficient genetic variability within and among populations to accommodate new selection stress brought about by environmental change (Barrett and Kohn, 1991). Although some authors have questionedthe importance of genetic studies with regard to demographic approaches (Lande, 1988 and Schemske *et al*., 1994), many others think that estimate genetic diversity and understanding how diversity is structured is not only necessary in designing suitable conservation strategies (Falk and Holsinger, 1991and Avise, 1995) but furthermore, this knowledge helps to resolve taxonomic (Van Buren *et al*., 1994 and Cole and Kuchenreuther, 2001), phylogenetic (Smith and Pham, 1996), demographic and ecological problems (Bachmann, 1994 and Cruzan, 1998) .

In contrast to morphological traits, molecular markers can reveal differences among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation and management. Several types of molecular markers are available today, including those based on restriction fragment length polymorphism (RFLP) (Botstein, *et al.* 1980), random amplified polymorphic DNA (RAPD) (Welsh, *et al*. 1992 and Williams *et. al*. 1990), amplified fragment length polymorphism (AFLP) (Vos, *et al*. 1995) and simple sequence repeats (SSRs) (Singh, 1999). It is particularly useful for characterizing individual genotypes and selection of the parents for successful hybridization.

Among all molecular markers mentioned above, RAPD technique of DNA fingerprinting have been used in the present study, which is widely used in conservation biology because of quick results, cost-effectiveness, easy analyzed results and reproducibility. The PCR-based RAPD approach by using arbitrary primers requires only little quantities of DNA template, no radioactive probes, and is relatively simple compared to other techniques (Williams *et. al*. 1990).

Williams *et al*. (1990) confirmed that the RAPD markers have been increasingly employed for population studies, especially in endangered plants and developed, as an important technique and the fastest and simplest method that can detect genetic differences at DNA molecular level directly by the Polymerase Chain Reaction (PCR). RAPD technique has the advantage of assaying a greater number of potential polymorphic loci and a more random sample of genome (Fritsch and Rieseberg, 1996). In addition, when compared to other DNA-based markers, the procedure is cheaper and technically simpler and it does not require any prior knowledge of the target genome (Lynch and Milligan, 1994; Isabel *et al.*, 1995; Szmidt and Lu, 1996).

This technique allows the reproducibly of pieces DNA that distributed within the genome of the individual using primers consisting of short fragment limited nucleotides (10 nucleotides) (Williams *et al*., 1990 and Quiros *et al.*, 1993).

Pezhmanmehr *et al*.(2009) and Thormann *et al*. (1994) explaining that the RAPD markers are commonly used as an indicator for determining the genetic diversity of many plants, as Kapila *et al*. (1997) and Mittal *et al*. (2006) who study the morphological markers that were similarly used to evaluate genetic diversity of *Parse cumin*.

Moller (1999) refers that RAPD markers are commonly used to enable analysis of genetic diversity of plant populations in breeding programs and germplasm collections.

RAPD analysis is widely used for detecting genetic polymorphism between genotypes at molecular level in many plants species (Karp *et al.* 1997; Vos *et al*. 1995).

Random amplified polymorphic DNA (RAPD) technology via the polymerase chain reaction (PCR) become a means of investigating genetic diversity within and between populations and has been applied to many plant species including *Digitalis* (Nebauer *et al*. 1999, 2000).

The Mediterranean flora has been of interest to biologists because of their high level of endemic species richness and complicated patterns of community organization (Cardona and Contandriopoulos, 1979). In spite of this, only limited information is available on the genetic structure of endemic Mediterranean plant species (Thompson, 1999). This is unfortunate because such information is crucial for devising strategies to protect and preserve the genetic resources of the Mediterranean flora.

The genus *Verbascum* SPP. exhibits a remarkable range of morphological and ecological variation and poses numerous problems for the taxonomist and evolutionary biologist, therefore the aim of this study is to look for molecular indicators allow the discrimination among all species of the genus *Verbascum* in order to use them to determine the relationships among those species depending on the indicators of RAPD and then compared to tree relationships which established depending on the RAPD results, with those established based on other standards and explain the efficiency of RAPD technique in determining of genetic relationships among species.

No.	Species	Location
1	*V. agrimoniifolium*	Suleimaniyah, Dohuk, Kirkuk
2	*V. alceoides*	Suleimaniyah
3	*V. alepense*	Nineuah, Kirkuk
4	*V. andrusii*	Nineuah
5	*V. assurense*	Diyala
6	*V. calvum*	Suleimaniyah
7	*V. carduchorum*	Suleimaniyah
8	*V. cheiranthifolium*	Suleimaniyah
9	*V. damascenum*	Anbar
10	*V. geminiflorum*	Suleimaniyah
11	*V. laetum*	Suleimaniyah
12	*V.macrocarpum*	Suleimaniyah
13	*V. oreophilum*	Suleimaniyah
14	*V. palmyrense*	Nineuah
15	*V. pseudo-digitalis*	Suleimaniyah
16	*V.sinaiticum*	Nineuah
17	*V. sinuatum*L.	Baghdad, Salahuddin, Nineuah, Kirkuk
18	*V. songaricum*	Suleimaniyah
19	*V. speciosum*	Suleimaniyah
20	*V. thapsus*L.	Suleimaniyah

Table 1: List of *Verbascum* species used in this study and locations of sampling.

We have used RAPD technique to determine the genetic relationships using genotypes of 20 species of *Verbascum*, by the means of the rapid progress in the field of biotechnology to open large areas to study the genetic variations and to distinguish between species that close related to each other genetically.

Plant Usefulness

Four common medicinal species *V. sinuatum* use as a toxic to Fish, the powder of *V. assurense* used to kill external parasites on cattle while the leaves used for painting the legs in Sinjar region,northern Iraq. *V. pseudo – digitalis* used to get rid of Dermatology abscesses (Al-Bermani, 1981) and *V. thapsus is* a new record in Iraq used as medical plant aromatherapy and other health uses: The honey-scented flowers flavor liqueurs and yield skin-softening mucilage, the expectorant, soothing, and spasm-sedating properties of the leaf and flowers are used to treat raspy coughs and are added to herbal tobacco, other uses as in India, mullein is regarded as the most potent safeguard against evil spirits and magic, and it hanged over doors, in windows and carried in sachets, it is also used to banish demons and negativity, Protection, Divination and Health (Halvorson, 2003).

MATERIALS AND METHODS

Plant Collection

20 Species, whole plant of *Verbascum*, were collected in April – May 2012/2013 from different location in the north and middle regions of Iraq (Figure1 and Table 1). The collected plants at flowering stage were identified and dried in shadow at room temperature, and deposited at national Herbarium, Baghdad, Iraq.

Genomic DNA Isolation

Using Commercial Kit

Total genomic DNA was extracted and purified from 100 mg of dry leaves by squashing with liquid nitrogen and mechanical destruction, following the Vivantis GF-1 Nucleic Acid Extraction kit protocol (Vivantis Technologies, USA). The 10 mg/µlRNase treatment at 37 ^{o}C for 30 min used to remove RNA.

Electrophoresis done in 1.5% agarose gel at 100V for at least 60 min in 1X TBE buffer and the isolated DNA was visualized by staining with Ethidium bromide and using of ultraviolet trans illuminator, then documented for publishing by gel documentation system, the suitable concentrations were adjusted to 50 ng/µl and stored at -20^{o} C to -80 ^{o} C.

DNA Extraction by CTAB Method

DNA was extracted from leaf tissues according to the method described by Saghai - Maroof, *et al*. (1984) as below:

Approximately (2-3) g of leaf tissue from plants were harvested, plant leaves cut into pieces by sterilized scissors and placed into a cold mortar.

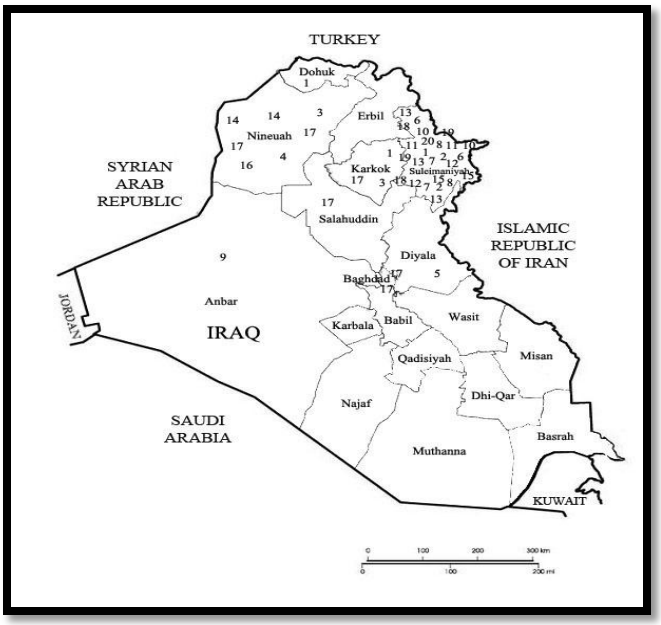

Figure 1: Map of Iraq showing sampling areas of *Verbascum* species; Numbers 1 -20 refer to the list of *Verbascum* species 1-20 (Table 1).

- Liquid nitrogen was added to the mortar to freeze the tissue. The temperature of liquid nitrogen was (-196°C) or (-321° F).
- Leaf tissue was crushed by using the pestle in a circular motion. Grinding was continued till the tissue becomes a powder.
- The powder was transferred to a 100ml flask.
- added 12 ml of the extraction buffer at 68°C, the flask covered and mixed gently by rotating and shaking.
- The flask was incubated at the 68°C for 60 minutes; mixed several times while incubation, then cooled for a couple of minutes to relieve the pressure.
- In the fume hood, 10ml of chloroform/isoamyl alcohol (24:1) was added to each flask.
- The samples were shaken for 5-10 minutes at room temperature (inside the fume hood).
- Samples were centrifuged at (4000 rpm) for 15 minutes.
- The upper phase was transferred into a fresh 50ml tube.
- Aliquot of 10ml of the supernatant (Buffer containing DNA) was placed in a test tube. Six milliliters of cold isopropanol (-20°C) was added and covered tightly before mixing gently by inverting the tube several times. A white, stringy precipitate consisting of DNA and RNA was visible at this point.
- Nucleic acids were spooled out with a glass hook. (The hook is a Pasteur pipette that was bent at the end) and transferred to 4ml of CTAB washing buffer for 10-20 minutes.
- Nucleic acids were spooled out again with a glass hook from the washing buffer and dried at room temperature.
- Nucleic acids were re-suspended pellet in 300µl of TE buffer, and incubated at 65°C for ~20-30 minutes or until the pellet was dissolved and stored in -20°C until using.

Estimation of the DNA Concentration by the Spectrophotometer:

The amount and quality of the DNA were tested by using the result of optical density at 260/ 280 nm wavelength formula with spectrophotometer (miltonroy spectrophotometer, USA), Five microliters (µl) of each sample were added to 495µl of TE (Tris-EDTA) as a blank for DNA that resulted from CTAB method and 495µl of D.W. as a blank for DNA that resulted from kit method then mixed well to determine the DNA concentration and its purity. A spectrophotometer was used to measure the optical density (O.D.) at wave length of 260nm and 280nm. An O.D of 1 corresponds to approximately 50µg/ml for double stranded DNA (Sambrook *et al.,* 1989).
The concentration of DNA was calculated according to the formula:-

DNA Concentration (µg/ml) = O.D 260nm×50×Dilution Factor

The spectrophotometer was used also to estimate the DNA purity ratio according to this formula:-

DNA Purity Ratio = OD260/OD280

Table 2: The codes and sequences of seventy RAPD primers used for PCR amplification of genomic DNA from 20 *Verbascum* genotypes. Total number and size range of amplified bands and the number of polymorphic and monomorphic bands obtained for each primer.

Primers	Primer sequence (5'-3')	NT	Size of bands (bp)	NP	%	NM	%
OPA5	AGGGGTCTTG	9	200-1000	9	100	0	0
OPA9	GGGTAACGCC	8	300 – >1500	8	100	0	0
OPA13	CAGCACCCAC	7	400-1000	7	100	0	0
OPJ1	CCCGGCATAA	7	200-800	7	100	0	0
OPJ7	ACGCCAGTTC	6	300-1000	5	83.33	1	16.66
OPJ10	AAGCCCGAGG	8	100-1000	7	87.5	1	12.5
OPJ15	TGTAGCAGGG	5	400-1200	4	80	1	20
OPW6	AGGCCCGATG	8	100-900	8	100	0	0
OPW13	CACAGCGACA	9	200-1000	9	100	0	0
OPW19	CAAAGCGCTC	3	400-600	0	0	3	100
OPX1	CTGGGCACGA	9	100-1400	7	77.77	2	22.22
OPX3	TGGCGCAGTG	5	200-1500	4	80	1	20
OPX4	CCGCTACCGA	7	400-1500	6	85.72	1	14.28
OPX15	CAGACAAGCC	7	100-1500	5	71.42	2	28.57
OPX16	CTCTGTTCGG	4	500-1600	2	50	2	50
OPX17	GACACGGACC	8	300-1000	7	87.5	1	12.5
OPX20	CCCAGCTAGA	9	200-1200	8	88.88	1	11.11
Total		119	100 –>1500	103	86.55	16	13.44

NT = Number of total bands; NP = Number of Polymorphic bands; NM = Number of Monomorphic bands.

Polymorphic = $\mathrm{NP}/\mathrm{NT} \times 100$

Monomorphic = $\mathrm{NM}/\mathrm{NT} \times 100$

This ratio was used to detect nucleic acid contamination in protein preparations. DNA quality was also assessed by simply analyzing the DNA by agarose gel electrophoresis (Sambrook *et al*., 1989).

Agarose Gel Electrophoresis

To separate DNA fragments, agarose gels were used for DNA extraction screening, concentration of 1.5% of prepared Agarose gel was used for visual checking of RAPD product. Gels were run horizontally in 1X TBE buffer. Samples of DNA were mixed with Bromophenol blue loading buffer (loading buffer/ DNA) 1/3 (v/v) and loaded into wells of the gel to increases sample density, and function as loading dyes which made the DNA visible and the end of electrophoresis distinguishable toward the positive pole when reaction products are analyzed by agarose gel electrophoresis.

Electrophoresis buffer TBE solution added to cover the gel and run for 1hour for RAPD-PCR analysis at 5V/cm. Agarose gels were stained with ethidium bromide 0.5µg/ml for 30 minutes. DNA bands were visualized

under U.V transilluminator. A gel documentation system was used to document the observed bands

Estimation of Molecular Weight

Molecular weight was estimated by using a computer software M.W. Detection program, Photo-Capture M.W. program from Consort, based on comparing the PCR products with the known size of DNA fragments of a DNA ladder (from 100 to 1500 bp, from Sib Enzyme Ltd., Russia) for RAPD-PCR products.

Estimation of Genetic Polymorphism (%) for Random Primers

Data generated from the detection of polymorphic fragments were analyzed. The amplification profile of all the used species for any given primer was scored as “1” with the presence of each band and was scored as “0” with the absence of the same band of the same size in other varieties (Bibi *et al*., 2009). Only major bands consistently amplified were scored. Polymorphism of each primer was calculated based on the following formula:

Polymorphism % = (Np/Nt) ×100

Where Np= the number of polymorphic bands of random primer.
Nt= the total number of bands of the same primer.

Genetic Distance and Relationship Estimation

Data generated from the detection of polymorphic fragments were analyzed. Only clear and reproducible amplified fragments were considered for genetic relationship analysis. Estimates of genetic distance (G.D) were calculated between all pairs of the varieties according to Nei and Li (1979) based on the data matrix based on the following formula:
G.D = 1-{2Nab/(Na + Nb)}
Where Na = the total number of fragments detected in individual 'a';
Nb = the total number of fragments shown by individual 'b'
Nab = the number of fragments shared by individuals 'a' and 'b'.

Cluster analysis was performed to construct genetic relationship tree diagrams among studied *Verbascum* species using an Unweighted Pair-Group Method with Arithmetic Average (UPGMA) (Sneath and Sokal, 1973).All computations were carried out using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) Version 2.1 package (Rohlf, 2004).

Cluster Analysis

Dendrogram was constructed based on Nei and Li's (1979) genetic distance using UPGMA cluster analysis and depicted genetic relationships among 20*Verbascum* species, Figure (3) showed the 2 major clusters **I**, **II**.

The First Group

Included **(3)** species *V. agrimoniifolium, V. alepense, V. assurense*.

The Second Group

Included the remain species under study, the second group included subgroups, the first subgroup(IIA) contains the species V. alceoides, V. pseudo-digitalis, V. andrusii, V. carduchorum, V. damascenum and the second subgroup(IIB) contains the remain species, the subgroups (IIB) included subgroups the subgroups (IIB_1) contains the species *V. calvum, V. cheiranthifolium, V. speciosum, V. oreophilum, V. geminiflorum, V. sinaiticum, V. songaricum, V. sinuatum* , *V. thapsus V. palmyrense,* the subgroups (IIB_2) contains the species *V. laetum, V. macrocarpum.*

RAPD Amplification

PCR reactions were carried out using 17 random decamere-nucleotide primers, OPA5, OPA9, OPA13, OPJ1, OPJ7, OPJ10, OPJ15, OPW6, OPW13, OPW19, OPX1, OPX3,OPX4, OPX15,OPX16, OPX17 and OPX20 (Operon Technologies Inc., USA). Each polymerase chain reaction was carried out in a 25 µl volume containing 12.5 µl of PCR mix consists of:
1X PCR buffer, 1.5 mM $MgCl_2$, 0.32 mM dNTP,0.16 unit/µl*Taq* DNA polymerase, with 2 µl of 1.0 µM primer and 2.5 µl of 8 µg/µl template DNA, (Faculty of Associated Medicine, KhonKaen University) volume completed up to 25 µl using ddH2O . Amplification was performed in thermal cycler (Corbett Research, Australia) using the following conditions:
95 C° for 3 min; 40 cycles at 94 C° for 1 min, 40 C° for 1 min and 72 C° for 2 min; final extension at 72 C° for 7 min.
Amplification was performed on ice under aseptic conditions inside a laminar air flow cabinet using 200 µl tight cap Eppendorf tubes.
In order to achieve homogeneity of reagents and reduce the risk of contamination a master mix for all samples was prepared containing all the components of the reaction except of genomic DNA (template DNA), mixed

Table 3. List of 20 species of *Verbascum* used for the present phylogenetic analyses based on RAPD sequences.

Clusters	Sub - Clusters	Minor sub-clusters	Species	Taxonomic characters		Anatomy characters	
				Shape of leaves	Shape Inflorescence	Cross section of blade	Cross section of margin
I	-	-	*V. agrimoniifolium*	Oblong-lanceolate	A	IB-P(2U-2L)	Semi-lanceolate
			V. alepense	Oblong-lanceolate	A	IB-P(2U-2L)	Semi-lanceolate
II	IIA	-	*V. assurense*	Oblong-	A	IB-P(2U-2L)	Semi-
			V. alceoides	Orbicular	B	IB-P(3U-2L)	Orbicular
			V. pseudo-digitalis	Orbicular	B	IB-P(2U-2L)	Orbicular
			V. andrusii	Orbicular	B	Bifacil	Orbicular
			V. carduochorme	Orbicular	B	IB-P(2U-2L)	Orbicular
			V. damascenum	Orbicular	B	IB-P(3U-2L)	Orbicular
	IIB	IIB_1	*V. calvum*	Lanceolate	C	IB-P(3U-2L)	Lanceolate
			V. cheiranthifolium	Lanceolate	B	IB-P(3U-2L)	Lanceolate
			V. speciosum	Lanceolate	C	IB-P(2U-2L)	Lanceolate
			V. oreophilum	Lanceolate	A	IB-P(3U-2L)	Lanceolate
			V. geminiflorum	Lanceolate	B	IB-P(2U-2L)	Lanceolate
			V. sinaiticum	Lanceolate	C	Bifacil	Lanceolate
			V. songaricum	Lanceolate	C	Bifacil	Lanceolate
			V. sinuatum	Lanceolate	C	Bifacil	Lanceolate
			V. Thapsus	Lanceolate	B	IB-P(2U-1L)	Lanceolate
			V. palmyrense	Lanceolate	B	IB-P(2U-1L)	Lanceolate
		IIB_2	*V. laetum*	Lanceolate	A	IB-P(3U-2L)	Lanceolate
			V. macrocarpum	Lanceolate	A	IB-P(3U-2L)	Lanceolate

A = spread and single flowers, B = grouped of flowers>10 per node and C = grouped of flowers<10 per node, IB = isobilateral, P = palsied layer, U = upper, L = lower.

gently with sterile distilled water to achieve the appropriate volume.

A negative control reaction in each PCR experiment was containing all components of the reaction without template DNA so that any contaminating DNA present in the reaction would be amplified and detected on agarose gel.

Data Analysis

The amplified bands were scored for each RAPD primer based on as either present (1) or absent (0), on the basis of size. RAPD matrix (table 2), was then analyzed using the NTSYS computer statistical package version 2.1 The data matrix was used to calculate genetic similarity within and among species based on Jaccard's similarity coefficients and a dendrogram displaying relationships among 20 species of *Verbascum* were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath and Sokal, 1973).

RESULTS

The genetic distance (RAPD-GD) among 20 *Verbascum* species was calculated using genetic program (Numerical Taxonomy and Multivariate Analysis System Version 1.80

package) depending on shared bands between each species when increasing bands number that lead to decreasing of genetic distance and vice versa.

All of the 17 RAPD primers were effectively used to amplify genomic DNA for each of the 20 *Verbascum* species. By using PCR, these primers have produced multiple band profiles with a number of amplified DNA fragment varying from 3 to 9 (Table 2), with mean alleles per locus of 7. The sizes of bands ranged from 100 to > 1500 bp. Two representative agarose gels are shown in Figure 3. The primer OPW19 gave the minimum number of fragments (3), whiles the highest number of fragments (9), were amplified with primers OPA5, OPW13, OPX1 and OPX20. (13.44%). The lowest number of polymorphic bands was 2 with OPX16, which showed the polymorphism (50%), produced 4 bands which only 2 were polymorphic. OPA5 and OPW13 showed the highest number of bands (9) and all are polymorphic (100%). From total of 119 alleles that detected, 103 were polymorphic (86.55%) and 16 monomorphic Moreover, the primers OPA9, OPA13, OPJ1 and OPW6 were also showed all polymorphic bands (100%) (Table 2).

The genetic similarity coefficients among 20 *Verbascum* species based on the RAPD fragments were used to construct a dendrogram (Figure 3) by UPGMA analysis. The 20 *Verbascum* species were divided into 2 clusters and genetic similarity among the 20 species ranged from 0.51 to 0.98. The highest genetic similarities (0.98) revealed were between *V. carduchorum* and *V. andrusii*, *V. cheiranthifolium* and *V. calvum,* and *V. pseudo-digitalis and V. alceoides*. Cluster I contained 3 species which all of species were oblong-lanceolate (shape of leaves), spread and single flowers (shape inflorescence) and Semi-lanceolate (Cross section of margin) also the cross section of blade is isobilateral and having two palsied parenchyma from upper and lower blade for taxonomic and anatomy characters (Table3) and showed genetic similarity coefficients 97.6%. *V. agrimoniifolium* and *V. alepense* were most closely related. Cluster II could be further divided into two subgroups, IIA and IIB at 0.72 of similarity. Sub-cluster IIA contained 5 species which all shape of leaves, shape of inflorescence and shape of cross section of margin were orbicular, grouped of flowers >10 per node and ovate to lunar (Table 3), respectively. Twelve *Verbascum* were contained in sub-cluster IIB and showed all shape of leaves as lanceolate(Table 3). This sub-cluster was divided into two branches, IIB_1 and IIB_2 at genetic similarity coefficients 75%. The IIB_2 branch consists of two species; *V. laetum* and *V. macrocarpum* at 0.85 of genetic similarity and showed only spread and single flowers for shape inflorescence while ten species (IIB_1branch) showed three types of shape inflorescence (spread and single flowers, grouped of flowers >10 per node and grouped of flowers<10 per node) and showed the cross section of margin were lanceolate (Table 3). Moreover, minor sub-cluster IIB_1, *V. palmyrense* was separated from nine *Verbascum* species at approximately of genetic similarity coefficient of 0.755. Thus RAPD analysis was able to differentiate the 20 *Verbascum* species into groups according to taxonomic characters like shape of leaves. Group I contains three species and shape of leaves was oblong – lanceolate while group II corresponded to shape of leaves were orbicular and lanceolate respectively.

DISCUSSION

Molecular methods were used to enrich this study and testify molecular methods as a tool to find the genetic bases of the differences and similarities among species under studied , find the genetic relationships among them and establish genetic database for the Iraqi verities that may use for other medicinal plants , and because of lacking of genetic information about the genus and its species in global network and the internet specific sites ,especially from Iraq ,we used RAPD –PCR technique, because it didn't need previous genetic information about the organism as the sequencing of nucleotide bases which leads to design specific primers and use of other techniques as genetic markers ,although that we test many oligonucleotide primers for RAPD (about 100) , only 17 primers that chosen gave differences among samples when screened in gel and the abandoned primers gave the same molecular weight bands in the gel , we found that the high efficiency exhibited by this method by studying the genetic variations among species, and that make it useful to be adopted in conservation programs. Primers have identified the characteristic of each species and can be used as quick indicators to see the success of hybridization between species; it can also link to some important qualities and follow isolation in successive generations.

Although a small number of primers was enough to get molecular characteristic indicators of each of the twenty species. However, the use of a relatively large number of prefixes was necessary to study the relationships between different species, because it gave a powerful tool to discriminate the species based on their genetic component and therefore allows obtaining more accurate results about the genetic similarities and differences among the studied species.

DNA extraction from plant tissue can vary, essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation of DNA which means result failure, DNA samples extracted from any source could be stored at (-20 to -80)°C for long term storage without degradation, .so there are different DNA extraction methods suitable

for different parts of the organism under study, for most of studies use leaves as a DNA source ,many use CTAB method which is a complicated one for materials and may need a time for preparation and implementation so a commercial extraction kit had been used to gain the purity and save time, but some samples didn't gave the amount of DNA needed for RAPD method ,and that might belong to the methods or time of sample preservation or the age of leaves used, so we use CTAB method for that reason as an optimum way to gain DNA as described in methodology.

The extracted DNA was from leaves not from seeds because the plant species were collected from the natural places, most of the samples not in flowering period, and we may need other samples for further studies.

Once the tissue has been sufficiently ground. Liquid nitrogen, useful to prevent samples from thawing and prevent nuclear enzymes activation such as DNase and nuclease that released when breaking cell walls. Some reports illustrated some methods using liquid nitrogen, even though the used material is considered not to be safe (Sharma *et al*., 2002). Anyhow, most laboratories prefer a simple and fast procedure for obtaining plant genomic DNA for PCR technique (Ahmadikhah, 2009) followed by suitable genetic marker that benefit the study as RAPD method.

In CTAB method the used materials were ground with liquid nitrogen to break down cell wall materials and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated.

A suitable buffer with (CTAB) can be used to purify DNA and assists cell lysis, and the CTAB would form the Nucleic acid/CTAB complex which does not dissolve in solvents. The presence of EDTA in the extraction buffer acts to withdraw the Mg+2 ions required for nucleases activity (Weigand *et al.,* 1993). The extraction buffer also contains mercaptoethanol used for lysis of DNase and removes the brown pigment from plant extraction (Palmer, 1986). Then materials were separated through mixing with chloroform: isoamyl alcohol with centrifugation that leads to the formation of aqueous and organic phases would become clearly separate with plant tissues remains between two phases was collected and precipitated. The upper aqueous phase contains, DNA transferred to micro tube with care to prevent mixing with tissues remains, and then washed thoroughly to remove contaminating salts (Ahmed *et al*., 2009). The use of chloroform in the extraction yielded more DNA. Nucleic acids were separated from other cell components by isopropanol precipitation with presence of high NaCl concentration, the DNA formed a stringy white precipitate transferred by glass hook, then DNA was washed using washing solution contains ammonium acetate +absolute alcohol (ethanol) to get super white DNA. The purified DNA resuspended and stored in Tris-EDTA (TE) buffer. This method shown to give intact genomic DNA from plant tissue (Weising *et al.,* 1998).

Many DNA extraction protocols result with high polysaccharide contamination, the potential inhibitors of enzyme reactions (Fang *et al*.,1992; Schlink and Reski2002), in order to avoid acidic polysaccharides that inhibit PCR amplification (Demeke and Adams, 1992; Pandey *et al*., 1996) by inhibiting *Taq*DNA polymerase activity, whereas, neutral polysaccharides are non-inhibitory (Do and Adams, 1991) .The polysaccharide was removed by increasing the period and speed of centrifuge (Al- Asei, 2002).

The RAPD method was employed in the past successfully for the detection of genetic relationship among individuals (Goswami and Tripathi, 2010). In this study, RAPD-PCR technique was used for identification of DNA polymorphism, genotypes and genetic diversity of DNA between studied varieties to determine DNA markers in *Verbascum* varieties.

Optimization of PCR conditions is necessary to get the highest specificity and product yield (Williams *et al.,* 1990; McPherson and Moller, 2001; Bakr, 2006 and Al-Hashimi, 2008). Therefore, optimization of PCR conditions, that include reagents, temperature, number of cycles and other parameters are very necessary to get a successful RAPD-PCR reaction. Some primers were bind with DNA since it has the same complementary sequence for template DNA while some primers didn't bind with DNA because it generates poor amplification products and/or non-repeatable banding patterns (Abdellaoui *et al*., 2010). So we test about a hundred oligonucleotide primers, but we choose only 17, for the reasons above and because we didn't see any genetic polymorphism among the species or maybe there were no bands at all.

The first principles of RAPD-PCR analysis were depending on existence or nonexistence bands, the number of bands and the molecular weight of resulted bands (Mayer *et al.,* 2000). The absence or presence of main band from any variety is considered a Marker for that variety. The other principle of RAPD markers is the band numbers which appeared in variety genome that illustrated the number of sites which that a primer detect and bind with them. The number of these sites affected by two factors, genome size and primer sequences, (Al-Asei, 2002). Polymorphism results generated in this study among *Verbascum* species could be used as a DNA marker to distinguish among species. In addition, some primers produced monomorphic bands, while other primers produced polymorphic bands that provided chances to find specific unique bands for varieties. The polymorphism occurred as a result of spontaneous mutations or induced mutations that influenced on the distance between sites (Newbury and Ford-Lloyd, 1993; Tingey and Del-Tufo, 1993) which may occur naturally in the means of the evolution. Polymorphism may be

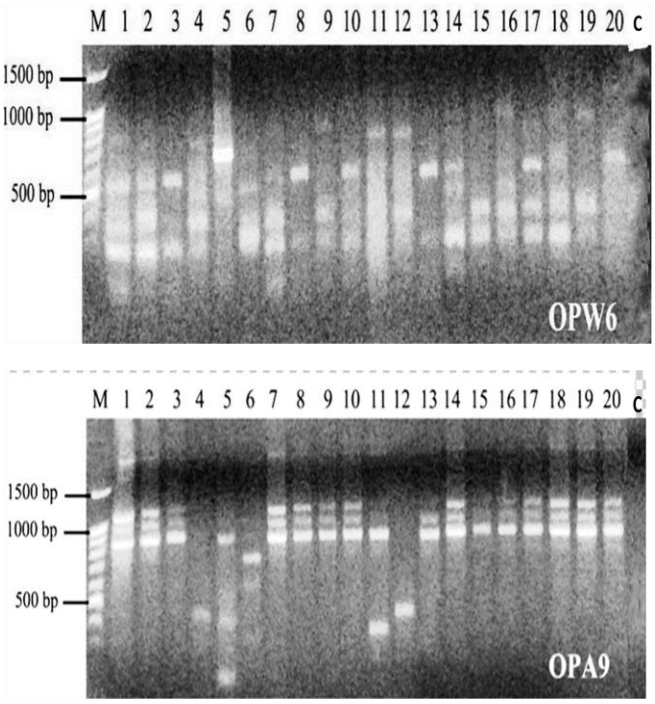

Figure 2: RAPD profiles amplified from genomic DNA of 17 *Verbascum* species using primers OPA-09 and OPW-06. M = 100-bp DNA ladder, 1-20 = *Verbascum* taxa described in Table 1, C = negative control without template DNA.

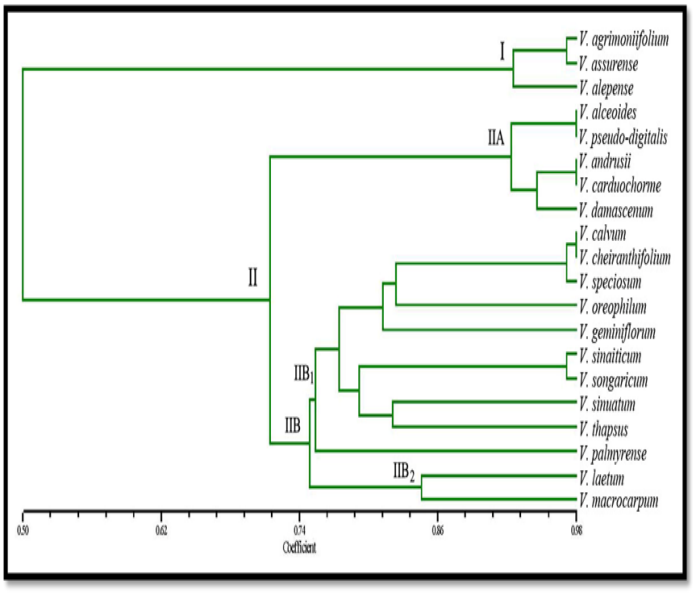

Figure 3. Dendrogram showing genetic relationship among 20Verbascum species revealed by UPGMA cluster analysis of Jaccard's similarity coefficients based on RAPD markers.

occurred by deletions or insertions at the priming site (Williams *et al.,* 1990).

We put only OPA9 and OPW6 primers gel results as an example for our study, and to show the way that work takes, because it is the same way for the others and no need to repeat them respectively,The RAPD assay generated polymorphic and monomorphic bands produced in some genotypes of *Verbascum* species. These bunds can be used as DNA fingerprints for *Verbascum* species identification. In this study (17) primers that used, some of them were generated several bands, while others generated only a few bands. Some primers gave smear DNA bands that sometimes occurred by lowest in denaturation temperature and MgCl2 or primer concentration (Newton and Graham, 1997).

Twenty *Verbascum* individuals that used in this study, representative of accessions as showed in the table 1, were analyzed with 17 primers (Table 2). A total of 119 polymorphic bands were scored and used for the analysis of genetic distances. Band sizes varied between 100 and 1900 bp (Fig. 1). The number of polymorphic bands ranged from 0 to 9 with range of polymorphisms 0% (OPW19) to 100% (OPA5, OPA9, OPA13, OPW6, OPW13, OPJ1) (Table 2).

The variation in the number of bands amplified by different primers is influenced by variable factors such as primer structure and less number of annealing sites in the genome (Kernodle *et al*., 1993).

The dendrogram showed the genetic relationships among species of *Verbascum* as between *V. agrimoniifolium* and V*. alepense* and they seem very closely related together, this similarity between them may due to their common geographical localities, On the other hand, the species *V. assurance* is similar to the two other species by comparing the number of stamens they have which are 4-5 stamens and the 5th is Sterile (Post, 1933 and bentham, 1946).

Also the *V. alceoides* is very close related to the *V. pseudo-digitalis* and this due to the same morphological and taxonomical characters they have, as the stems are standing and cylindrical, the hairs of stamens are purple – violet and the species mentioned above are similar significantly interfere in the proliferation (Huber-Morath, 1978).

On the other hand the *V. carduchorum* similar to *V. damascenum* in the shape of inflorescences and the number of the flowers per node (1-8) and the species *V. andrusii* similar to the species mentioned in the number of the flowers per node (1-9), *V. calvum* is similar to *V. cheiranthifolium* in morphological and taxonomical characters like the branching of leaves from the upper side, the number of stamens which are 5 ,the hairs around the stamens which Yellowish white and the ovate capsule, so the *V. sinaiticum* and *V. songaricum* seem closely related together in the tree relationship because they have the same morphological and taxonomic characters as the color of trichomes(white to gray) , the

number of stamens are 5 and the hair around the stamens are Yellowish white (Huber-Morath, 1978).

The similarity between *V. sinuatum* and *V. thapsus* included their uses because of the fact that both used as medical Herbs, and their seeds used for poisoning Fish (dorthy, 1977).

The *V. laetum* and *V. macrocarpum* similar in the geographical localities. In addition to the number of stamens which are 5, and the hairs around the stamens which are purple-violet (Brands, 1989).

According to the results of genetic distance and relationships illustrated, the ability to resolve genetic variation among different *Verbascum* species may relate to the number of polymorphic bands detected with marker technique that employed in this study.

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