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Genetic Diversity and Relationships Among Species of *Iris* L. (*Iridaceae*) Growing Wildly in Iraq by Using RAPD–PCR Technique

Abstract- *This study shows the diversity and genetic relations among 8 species belong to the genus Iris L. growing wildly in Iraq by using RAPD-PCR technique. This study included Genomic DNA extraction from dry leaves of plants by useing 5 random primers that produced several polymorphic bands among the 8 tested species, in addition the genetic fingerprint were identified for all species by emergence many of unique bands . The genetic distance between species was determined as it ranged from 0.10 to 0.97, as well as the use of cluster analysis to build the genetic diversity tree which formed two main groups based on their ancestors, morphological and anatomical specifications, which depending on the results of this study and the results of other studies as anatomy and pollen were recorded a new species in Iraq within this genus is Iris madonna Dykes.*

Keywords- *Iris, DNA, RAPD*

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1. Introduction

Iris L. is the biggest genus of the family Iridaceae of Monocotyledon plants, this family of 70 genera spread globally, particularly in the northern and southern temperate zones, some races only grow in Iraq, one of them was the genus studied [1], the Morphology remains widely used to determine Phylogeny, but the molecular data are used today to build the tree of life in all its branches not for specific groups only, such as those that can be easily distinguished [2], as well as the great changes that has occurred in taxonomy, which began when the molecular taxonomists introduced the knowledge of the molecular classification important information through what they have done from the data analysis of the DNA sequences [3], the rapid development that occurred in the fields of molecular biology, which allowed the right tools for the study of the genetic material and molecular analysis which greatly helped in the emergence of molecular Taxonomy. As this science work to solve a lot of taxonomic problems by inference of the existence of the relationships among organisms as DNA markers that are used to study the genetic relationships among individuals and reflect differences in the genetic information stored in their bodies.

The species of this genus did not studied like this study before, so it is the first study to find diversity and genetic relationships among the eight Iris L. species that grown in Iraq, by using the randomly amplified polymophic DNA technique (RAPD-DNA) that based on polymerization reactions of PCR to amplify any part of the DNA, however small, in vitro the presence of the DNA polymerase and random primers.

2. Material and Methods

Fresh samples were collected from different regions of Iraq; (Table 1), through field trips conducted an area where the species of the genus studied grow, then was dried and placed in plastic bags until use. Plant genomic DNA was extracted from grinded plant dry leaves, by using extraction DNA protocol of plant Genomic DNA mini kit (plant), from Geneaid, Taiwan, by weighting of 25 mg of plant leaves powder and put in Micro centrifuge tubes, centrifuged and followed the protocol steps. The purity and concentration of DNA were measured using a Spectrophotometer (Nanodrop) in which you use a small amount of DNA (0.5 µl) for the purpose of testing a programmer to be used for measuring the concentration of DNA equation depending on the

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absorbency of the solution containing the DNA and associated proteins with optical density OD 260/280 Aº respectively as follow-:

dsDNA concentration = 50 µg/mL × OD260 or 280 × dilution factor

The values obtained ranged upon the purity was between 1.7 to 1.8

Five random primers were used with PCR, (Table 2) of the eight species of the genus studied, according to the instructions that came with tubes after dissolved with double distillated sterile water ddH_2O to obtain the concentration of 10% (10 pmol / µl) The appropriate volume of 25µl PCR reaction mixture preparation obtained by adding 12.5µl of the master mix Go Taq ®Green master mix ,Bioneer, Korea, that contains Taq DNA-polymerase and MgCl2 and dNTPs (dTTP, dGTP, dCTP, dATP), then the appropriate quantity of primer 1µl was added, after the distribution of the mixture on a small PCR tubes (0.2µl), 5µl of DNA extracted was added for each tube and then volume completed by double distilled water up to 25µl to each tube. Tubes putted in Thermal cycler machine that has been programmed by tempratures listed in (Table 1) :

Table 1: PCR Program for this study

Initial denaturation	**Temp.95ºC**	**Time: 5min.**	
Denaturation	Temp.95ºC	Time: 30 sec.	٣٥cycles
Annealing	Temp.36ºC	Time: 40 sec.	
Extension	Temp.72ºC	Time: 2 min.	
Final extension	Temp.72ºC	Time: 5 min.	

Table 2: The names of the rules and sequences of random primers used in the study With their sources

No.	**Primers**	**Sequence 5'…….3'**	**references**
1	OPC-9	CTCACCGTCC	[١٦]
2	RAPD-2	TGCGCCCTTC	[١٦]
3	RAPD-4	GTAGACCCGT	[١٦]
4	OPB-10	CTGCTGGGAC	[١٧]
5	OPD-11	AGCGCCATTG	[١٧]

Agarose gels prepared for 1.5% concentration dying with Ethidium bromide and loading of 10µl from each sample took from PCR amplification in gel wells, in addition of loading 5µl of the molecular marker (ladder) after putting the gel in TBE-1x solution in gel electrophoresis system with, 50 volt / cm for 2 hours, and then examine in ultraviolet UV device equipped with a camera to photograph the gel containing the DNA bunds that studied and compared with each other and with the DNA ladder separated bands. The genetic polymorphism counted (%) random primers using results and data analyzed that obtained from fragments of polymorphed DNA, PCR information recorded by giving "1" to the presence of each band in the gel and "0" to the absence of the same band for the same size in other species [5], using the following formula to count the polymorphism that results from using of each primer:

Polymorphisms% = (NP / NT) × 100

NP = number of polymorphic bands of amplified DNA using one random primer، NT = total number of bands resulting from the use of the the same primer Genetic relationships and measurments of the genetic distance were analyzed through results obtaing from the analysis of polymorphic DNA fragments that appeared on specific locations on the gel after elecrophoresis, depending on good looking bands only. Genetic distance (GD) estimated between each pair of items according to [6] based on the data matrix and the following formula:

G.D = 1-{2Nab/ (Na + Nb)}

Where: Na = total number of DNA bands seen in the individual A ،Nb = total number of bands shown by DNA to the individual B, Nab = number of bands of common DNA between the two individuals A and B.

method (UPGMA) (Unweighted Pair-Group Method with Average arithmetic) method used [7], and calculations required performed by using computer program (NTSYS) numerical classification and multivariate analysis system Version 2.1 (Numerical Taxonomy and Multivariate Analysis System) [8], for the purpose of cluster analysis to drow the phylogenetic tree and to reveal the genetic relationships between species of the genus Iris.

3. Results and Discussion

Five primers had been used (Table 2) with eight species of the genus: OPC-9: The total number of bands that are produced by the primer were 16 bands, all of them were Polymorphic and 100% were located at molecular weights 400 bp (base pair) and 2000 bp, no Monomorphic band seen, and this primer produced three Unique bands and one at the molecular weight 400 bp from the species I.barnumae and two at 800 bp and more than 2000 bp recorded by the species I.persica, (Table 3) and (figure1), RAPD-2: number of bands produced is 11, all polymorphic and 100% of these bands is located at the molecular weights 450 bp and 1600 bp and no unilateral band shows, one unique band produced by the primer was when molecular weight 1600 bp recorded the species I.pseudocaucasica, (Table 3) and (Figure 2).

Table 3: Primers and the number of unique bands produced for the species of genus *Iris*

Primers	unique bands	species	M.W.(bp)
OPC-9	3	*I.barnumae*	400
		I.persica	800
			2000
RAPD-2	1	*I.pseudocaucasica*	1600
RAPD-4	1	*I.germanica*	2000
OPB-10	1	*I.persica*	2000
OPD-11	7	*I.pseudocaucasica*	400
		I.barnumae	1200
		I.persica	250
			700
			800
			1600
			2000

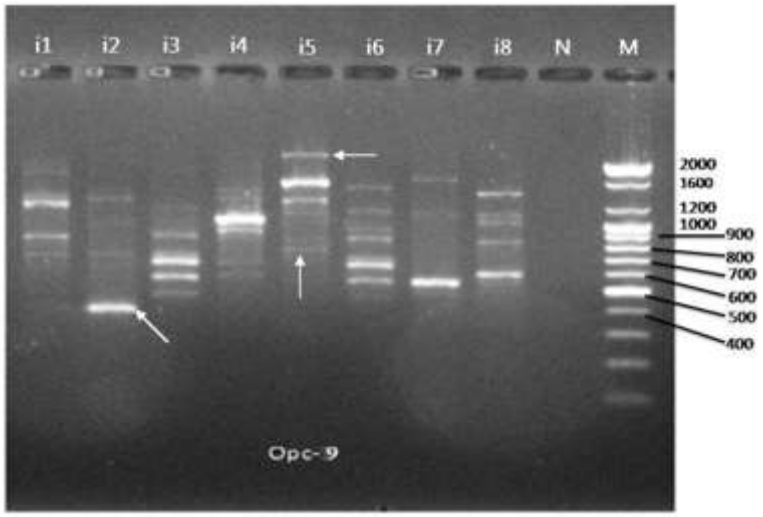

Figure (1): 1.5% agarose gel after electrophoresis of RAPD-PCR with primer OPC-9 and DNA samples of eight species of the genus *Iris* photographed under UV radiation after dying with ethidium bromide. I1 (*I.aucheri*), i2 (*I.barnumae*), i3 (*I.caucasica*), i4 (*I.germanica*), i5 (*I.persica*), i6 (*I.pseudocaucasica*), i7 (*I.reticulata*), i8 (*I.madonna*), M = 100bp DNA ladder, N = negative sample. White arrows = Unique bands.

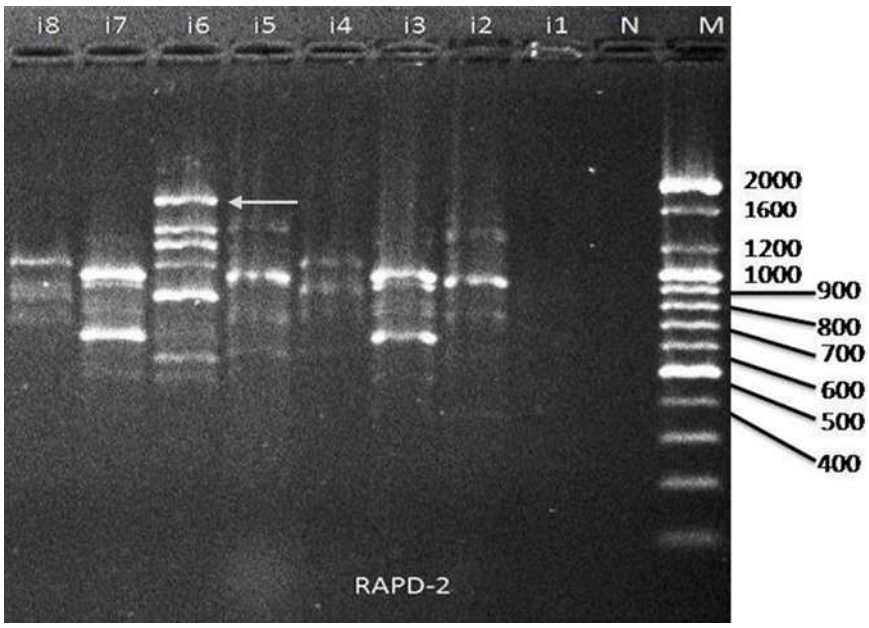

Figure (2): 1.5% agarose gel after electrophoresis of RAPD-PCR with primer RAPD-2 and DNA samples of eight species of the genus Iris photographed under UV radiation after dying with ethidium bromide. I1 (*I.aucheri*), i2 (*I.barnumae*), i3 *(I.caucasica*), i4 (*I.germanica*), i5 (*I.persica*), i6 (*I.pseudocaucasica*), i7 (*I.reticulata*), i8 (*I.madonna*), M = 100bp DNA ladder, N = negative sample. White arrows = Unique bands.

RAPD-4: The total number of bands was 11, all were polymorphic, these bands were located at molecular weights 250 bp and 2000 bp, no single band showed, one unique band at molecular weight 2000 bp with species I. germanica , (table 3) and the (Figure 3) OPB-10: this primer produced 8 bands all polymorphic (100%), all located at molecular weights between 400 bp and 2000 bp, no single band appeared, and one unique band produced recorded for the species I.persica at molecular weight 2000 bp, (Table 3) and (Figure 3).

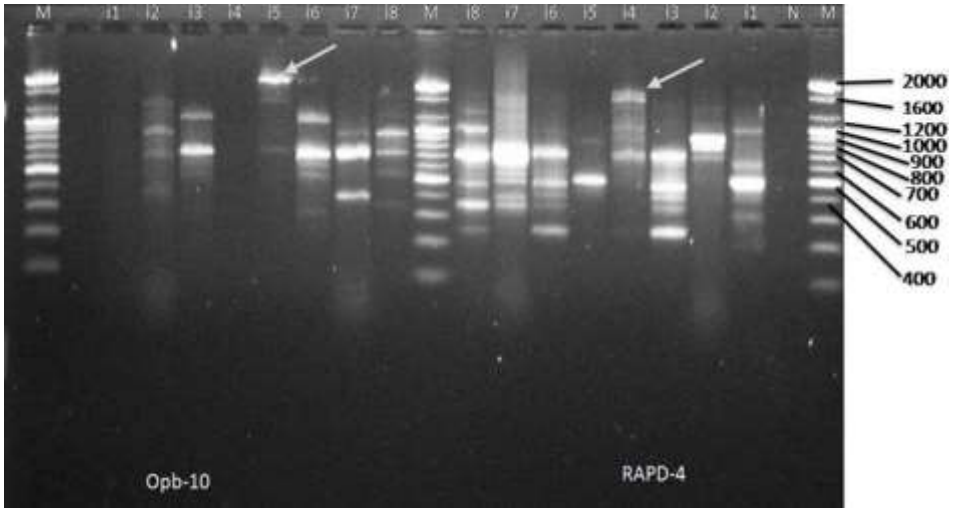

Figure 3: 1.5% agarose gel after electrophoresis of RAPD-PCR with primers RAPD-4 and OPB-10 and DNA samples of eight species of the genus Iris photographed under UV radiation after dying with ethidium bromide. I1 (*I.aucheri*), i2 (*I.barnumae*), i3 (*I.caucasica*), i4 (*I.germanica*), i5 (*I.persica*), i6 (*I.pseudocaucasica*), i7 (*I.reticulata*), i8 (*I.madonna*), M = 100bp DNA ladder, N = negative sample. White arrows = Unique bands.

OPD-11: produced 9 polymorphic bands (100%), these bands appeared at molecular weights 250 bp and 2000 bp, and no single band shown, this primer was the most primer producing of unique bands as produced seven, one at the molecular weight 1200bp was in species I.barnumae and one at 400bp recorded in the species I.pseudocaucasica and five bands at molecular weights bp (250, 700, 800 and 1600 and 2000) recorded in the species I.persica, (Table 3) and (Figure 4).

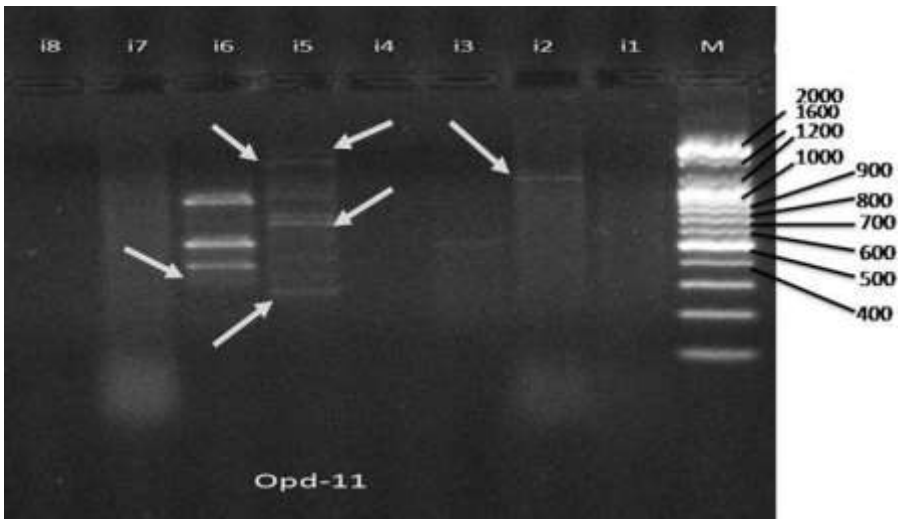

Figure 4: 1.5% agarose gel after electrophoresis of RAPD-PCR with primer OPD-11 and DNA samples of eight species of the genus Iris photographed under UV radiation after dying with ethidium bromide. I1 (*I.aucheri*), i2 (*I.barnumae*), i3 (*I.caucasica*), i4 (*I.germanica*), i5 (*I.persica*), i6 (*I.pseudocaucasica*), i7 (*I.reticulata*), i8 (*I.madonna*), M = 100bp DNA ladder, N = negative sample. White arrows = Unique band

The five primers used for the genus Iris which have proved effective in amplifying fragments of DNA for each of the eight species by using PCR technique, produced several bands when electrophoresed in gel representing amplified DNA fragments, (Table 4), which ranged between 8-16 bands according to primer used with bands average for equal to 11 each locus, the band sizes ranged from 250 to 2,000 bp as shown in gel Figures 1, 2, 3 and 4.

The five primer of the genus Iris which have proved effective in amplifying fragments of DNA for each of the eight species using technology PCR, produced several bands when paged gel representing cut DNA bloated, (Table 4), ranged between 8-16 band according primer it user with average bands each locus is equal to 11, the beam sizes ranged from 250 to 2,000 base pairs of images by observing the random gels used for starters. Figures 1, 2, 3 and 4.

The primer (OPB-10) gave the minimum number of bands which was (8) and showed polymorphism in rate (14.5%), while the most number of bands is (16) has emerged using primer (OPC-9) and its percentage compared to the number of total primers equal to (29%), of the total 55 bands that revealed. Genetic similarity coefficient had been used among the eight species of the genus Iris based on the number of DNA fragments that appeared in the gel after using RAPD technique to draw dendrogram (Figure 1) by using UPGMA analysis. The eight species of the genus Iris had been divided into groups, and found that the genetic similarity between them ranged between 0.10-0.97 (Table 5), and the shortest genetic distance is (0.10) between the two species I.aucheri and I.reticulata and between species I.aucheri and I.madonna, and also between the two species I.barnumae and I.germanica (Table 5).

Table 4: Primers used and the number of polymorphic bands for each of eight species of the genus *Iris*, NT = total number of bands per primer, NP = the number of bands

Primer name	NT	Band size(bp)	NP
OPC-9	16	400-2000	16
RAPD-2	11	450-1600	11
RAPD-4	11	250-2000	11
OPB-10	8	400-2000	8
OPD-11	9	250-2000	9
Total	55	250-2000	55

Table 5: The values of GD (genetic distance) among the eight species of the genus *Iris*

	I.aucheri	*I.barnumae*	*I.caucasica*	*I.germanica*	*I.persica*	*I.psudocaucasica*	*I.reticulata*	*I.madonna*
I.aucheri	0.00							
I.barnumae	0.12	0.00						
I.caucasica	0.97	0.12	0.00					
I.germanica	0.12	0.10	0.14	0.00				
I.persica	0.11	0.82	0.12	0.21	0.00			
I.psudocaucasica	0.15	0.16	0.36	0.15	0.11	0.00		
I.reticulata	0.10	0.91	0.59	0.19	0.12	0.92	0.00	
I.madonna	0.10	0.11	0.68	0.36	0.22	0.79	0.10	0.00

Cluster Analysis

Dendrogram analysis constructed of a set for the eight species belonging to the genus Iris using cluster analysis (Figure 5) , it showed that the four species of genus Iris growing in Iraq, has two main groups which are I and II, Group I contained most of the species included two secondary branches, IA and IB. The first subgroup included two sets subgroups IA, IA1 and IA2, the subgroup IA1 include only one species is I.aucheri while the subgroup IA2 included two species I.germanica and I.madonna, the subgroup IB included two subgroups too, IB1 which included the species I.caucasica , I.pseudocaucasica, and IB2 that included only one species I.reticulata. Second group (II), which included the fewer number of species, included only two branches, the first branch contains the species I.barnumae, while Section II included the species I.persica indicated to the presence of a genetic affinity between them. The group (IA1) which represented by I.aucheri which broke away from the other groups and differed from those species of groups by possess this species good developing stem and Oblonged vascular bundle. The species I.germanica and I.madonna (IA2) has the similarities in most of the phenotypic characteristics and the anatomical and illustrating the broad genetic closeness between them, the two species I.caucasica and I.pseudocaucasica (IB1) the similarity between them in all characteristics is a sign of high genetic relationship , either species I.reticulata (IB2) differed from other species by owning a floral scape and Erect inner perianth segments and the form of its vascular bundle is Ovate, while the second group II, a group that also separated represented two species, I.barnumae and I.persica differs from the other species in most characteristics but they similar to each other in many characteristics indicate to the genetic relationship between them.

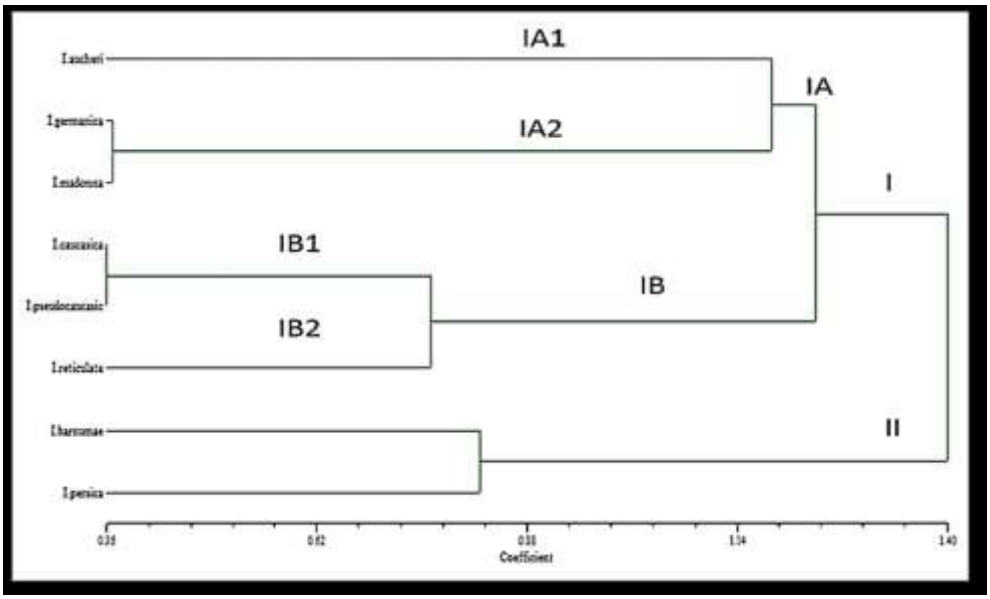

Figure 5: Dendrogram and genetic distance of the eight species of the genus *Iris* by using cluster analysis UPGMA

RAPD-PCR technique

This method used because it do not require prior genetic information about the organism studied, such as the sequence of nucleotide bases that leads to design the specialized primers, or use of other techniques with specific genetic markers, even though we tested many oligonucleotide RAPD random primers because of lack of studies like this about the species of genus Iris in Iraq. Only 5 primers chosen because there are differences in the number of bands between samples when PCR products migrate in gels as a result of differences in the molecular weight of the bands and this shows that the species I.madonna recorded as a new species in Iraq.

This method is characterized by high efficiency when used in the study of genetic diversity among species, which makes it useful to be adopted in biodiversity preservation programs in our environment .The extraction of DNA from plant tissue varies from one plant to other and that has different ways in some species of plants because of their content of polyphenols and other secondary products of metabolic processes [9]. As the DNA were extracted in the study from plant dried leaves, irrespective of being young or not young and this agree with [10], as the young are preferred in DNA extraction process because they containing less amount of polyphenols and/or other components that prevent gaining to pure DNA, therefore, different methods or modifications in DNA extraction that suitable for plants studied were used , as crush tissue of the samples used by using liquid nitrogen and laboratory mortar, which is a useful way to prevent samples corruption when conservation and inhibit the enzymes such as DNase and Nuclaese which being launched when cell walls broken, some reports indicated the importance of adding liquid nitrogen, although it is unsafe material (11). The degree of purity, quality and quantity of DNA is possible to vary because of the plant or protocols in isolation this agreed with the results of the purity and quantity of the DNA obtained in this study, as there are many different methods and techniques available for genomic DNA extraction and they depend on the choice of many factors available to obtain high purity of DNA which is important to shorten the time and results. RAPD technique are used successfully as it was revealed genetic relationships among the 8 species belonging to the genus studied, and this method used successfully before to detect the genetic relationships among individuals [12], RAPD-PCR technique was used for the appointment of genetic polymorphism and diversity by using DNA extracted from the species studied. The work on PCR had been improved by some steps , which included chemicals and the number of cycles used in the program and machine conditions, particularly annealing stage as it has been tuned to suit the samples of DNA which gave the desired results, and that led to magnify the fragments of DNA that appeared after the use of appropriate primers, to improve the work circumstances of PCR technique (Optimization) is necessary to get the highest privacy and the best desired results [13], that the principle of using RAPD technique is the number of bands that appeared in the genome of the species studied, which shows the number of sites that primer revealed it after its association with the amplification mediated by PCR device as it has been get the number of bands for each primer that been used with DNA samples and those were compared with the DNA ladder, and the number of these sites is influenced by two factors, namely, the size of the genome and primers nucleotide sequencing [14], The results of genetic polymorphism results that have emerged in this study as a genetic indicator among species of the genus studied, as well as the unique bands that produced by some of the primers, as primer OPD-11 that produced seven unique bands with some species of the Iris, which is the largest number unique bands in this study, as it can be used as a private genetic fingerprint use for definition of those species especially new species I.madonna which has been recorded in this study by using of molecular methods. Polymorphism occurs as a result of self-mutations or induced mutations that affected the distance between sites which may occur naturally in the course of evolutionary process of living organisms, and may be happened as a result of deleting or adding in the location associated with primer [15] which agreed with the study. Band sizes ranged between 250 and 2000 bp, polymorphic bands number ranging from 8-16 with a percentage of 100% as with the primers OPC-9, RAPD-2, RAPD- 4, OPB- 10 and OPD-11 in the eight species of the genus Iris that studied. dendrogram showed the nature of the genetic relationships among the species studied, as is evident in the species I.caucasica and I.pseudocaucasica, and it seems that they are linked very closely by morphological and anatomical methods as well as closely linkage of the two species I.germanica and I.madonna because of their similarity in some phenotypic and anatomical traits, either species I.barnumae and I.persica are less genetic linkage than the species mentioned above, as there are some phenotypic and anatomical differences between

them, the similarities may be due also to the common geographical areas. Depending on the results of this study, we can prove that species I.madonna is a new species in Iraq as it differed from other species belonging to this genus, in the number and type of bands resulting from amplification of different primers used in the study and this was evident in the gel photocaptures, analyzes and results obtained.

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