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DNA Fingerprinting and Polymorphism of Five *Ocimum basilium* Cultivars Detected using ISSR Markers

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Abstract
Current investigation was accomplished at biology department/faculty of science/kufa university during 2021-2022 to asses genetic variation among five Ocimum basilicum L. cultivars 1-Iranian (green) 2- Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red) with diverse geographical origin using ten ISSR markers. Results showed that ISSR markers were effective in generating polymorphism in basile germplasm reached to 100% by primers UBC812 and UBC852 and givingunique fingerprint to three cultivars by eight primers. Phyllogenetic tree and genetic distance for studied cultivars are not strongly related to cultivar origin or morphology.

Keywords: *Ocimum basilium*, ISSR, Phyllogenetic Tree, Fingerprint, Genetic Distannce

Introduction
Ocimum is one of the most important genera of the family Lamiaceae commonly known as basil or sweet basil (Bravo *et al*., 2021) [7]. The name basil is derived from the Greek word "Basileus" meaning "Royal" or "King" (Bilal *et al*., 2012).
This species posses nutritional importance by their content of protein, carbohydrate, fats and oils, minerals, vitamins, water (Carbohydrates, lipid, fibre contents, protein, calcium, Iron, phosphorus and Sodium (Shuaib *et al*., 2015) [25], in addition, secondary metabolites including polyphenols, flavonoids, essential oil, terpenic compounds, monoterpene, sesquiterpenes, (Kisa *et al*., 2021) [16].
Study of genetic diversity (variation in genes and genotypes) using molecular markers offer numerous advantages overconventional phenotype based alternativesas they are stable and detectable in all tissues regardless of growth, differentiation, development, or defense status of the cell. (Rao and Hodgkin., 2002; Dhutmal *et al*., 2018) [22, 9].
ISSRs (Inter Simple Sequence Repeat) and RAPDs (Randomly Amplified Polymorphic DNA) are both used to evaluate genetic diversity in *Ocimum* germplasm (Khatun and Ray, 2021) [15].
Both are simple, inexpensive, need no knowledge of the target sequence, and are easy to apply and in data analysis (Bahadur *et al*. 2015) [5].
The selection of genotypes with a high genetic distance in terms of the molecular marker, along with desirable agronomic traits, can be effective in future breeding programs to produce new superior hybrids (Zafar-Pashanezhad *et al*., 2020) [24], it's a critical step in plant breeding programes for determining superior hybrid, thus this study aimed to evaluate genetic diversity among *O.basilicum* L. cultivars, examining their antibacterial, antifungal and antioxidant activity and finally determination of seed oil constitnuents.

Materials and Methods
Seeds of five Ocimum basilicum Linn L. cultivars (1-Iranian (green) 2-Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red) were provided from local market, seeds sowing was conducted at the orchid of agriculture division at the University of Kufa using plastic pots filled with beatmoss to get fresh leaves for DNA extraction and molecular markers application of RAPD markers. Seeds and leaves illustrate in Fig 1.

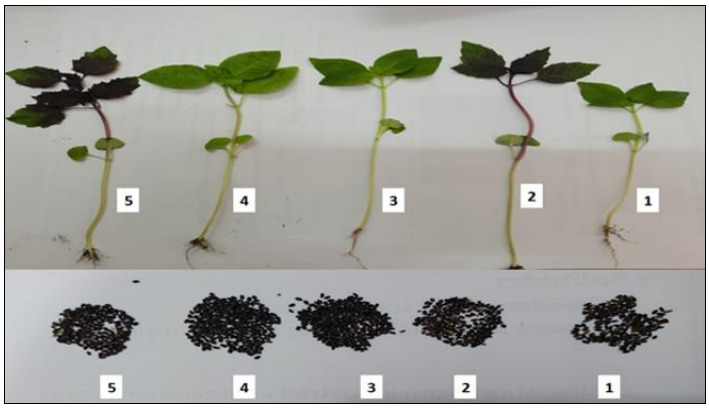

Fig 1: Leaves and seeds of Ocimum basilicum L. cultivars (1-Iranian (green) 2- Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

DNA Extraction

Fresh seedling leaves were used to take apical fresh leaves for genomic DNA extraction using Genomic DNA Mini Kit provided from Geneaid Biotech.

Primers

The Primers were provided by Bioneer Corporation in lyophilized form, dissolved in TE buffer to obtain 100 pmol/µl as a final concentration (stock solutions). Working solutions 10 pmole/µl were prepared from stock solutions, ten primers were used in application of ISSR markers (Sofalian *et al.*, 2008, Abou-Deif *et al.*, 2013; Singh and Sengar, 2015 and Muhammad *et al*., 2017) [27, 1, 26, 18] in Tables 1 with their nucleotide sequences and names of each primer.

Table 1: Primers used as ISSRs markers

Primer name	Sequence 5' ⟶ 3'	Temperature
844A	CTC TCT CTC TCT CTC TGC	48 C°
UBC820	GTGTGTGTGTGTGTGTC	45 C°
UBC816	CAC ACA CAC ACA CAC AT	52 C°
HBS10	GAG AGA GAG AGA CC	48 C°
UBC811	GAGAGAGAGAGAGAGAAC	52 C°
UBC817	CAC ACA CAC ACA CAC AA	52 C°
UBC812	GAG AGA GAG AGA GAG AA	52 C°
UBC852	GATAGATAGACAGACA	49 C°
17889A	CAC ACA CAC ACA AC	48 C°
HB12	CAG CAG CAG GC	48 C°

PCR Content and Amplification Programe

PCR Pre Mix master mix. Bioneer Corporation USA, (0.2 ml) thin-wall 8-strip tubes with attached cup/96 tubes were used, (Top DNA polymerase (1U), (dATP, dCTP, dGTP, dTTP) (Each 250 µM), Reaction Buffer with 1.5 mM Mgcl2 (1X) and Stabilizer and tracking dye, 100 bp DNA ladder used.

According to the Experimental Protocol of AccuPower® TLA PCR PreMix (at volume of 5 µl), the PCR reaction mixture was prepared as follows: 5µl template DNA and 5 µl of primer (10 pmole/µl), were added to each AccuPower® TLA PCR Pre Mix tube. Sterilized deionized distilled water was added to AccuPower® TLA PCR PreMix tubes to the final volume of 20 µl.

Performing PCR of samples: The amplified of each primer were done according to annealing temperatures and following programe of initial temperature at 94C˚ for 3 min, 40 Cycles of (denaturation at 94C˚ for 1 min, annealing: variable, extension at 72 C˚ for 1 min and final extension at 72 C˚ for 5 min.

Agarose Gel Electrophoresis

The gel electrophoresis methods were done according to Sambrook and Russel (2001) [23] using 1.2% agarose at 70volt for two hours.

Statistical Analysis

The photographs resulted from agarose gel electrophoresis was used to score data, presence of a product was identified as (1) and absence was identified as (0), data then entered into PAST statistic vital program, Version 62.1 (Hammer *et al.*, 2001) [12] and analyzed using SIMQUAL (Similarity for Qualitative Data) routine to generate genetic similarity index (Nei and Li, 1979) [19]: GS =2Nij (Ni+Nj).

Nij is the number of bands in common between genotypes I and j, and Ni and Nj are the total number of bands observed for genotypes I and j, a dendrogram was constructed based on genetic distance (GD=1-GS) using the Unweighted Pair-Group Method with Arithmetical Average (UPGMA). Polymorphism, primer efficiency, and discriminatory value were calculated for each primer using the following three equations as described by Hunter and Gaston (1988) [13] and by Graham and McNicol (1995) [11].

Results and Discussion

Genomic DNA Agarose Gel Electrophoresis

Results in Fig 2 show agarose gel electrophoresis of *Ocimum basilium* cultivar in which of concentration of isolated DNA was was 80.61µg/ml with purity 1.9, this accompanied by the locations of bands near wells and their intensity which shows their good quality and high molecular size. (Sambrook and Russell, 2001) [23].

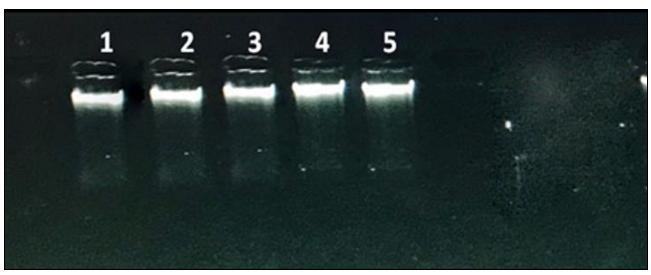

Fig 2: Genomic DNA agarose gel electrophoresis for Ocimum basilicum L. cultivars (1-Iranian (green) 2- Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

DNA Fingerprint Detected by ISSR Markers

Results in Table 2 show that primers UBC820, UBC816, HBS10, UBC811, UBC817, UBC812 and UBC852 success in giving three cultivars a unique fingerprint, while primers 844A, 17889A and HB12 gave only one cultivar a unique fingerprint.

As primer gave high value for polymorphism, this increased generally with increasing number of polymorphic bands (Hunter and Gaston, 1988 and Graham and McNichol, 1995) [13, 11], and increases chance of producing unique fingerprint. Primer which produces high polymorphic bands can be further used as polymorphic marker which will prove promising in identification and genetic purity testing of crops (Pal and Singh, 2013) [20].

Polymorphic and unique alleles inside genotypes increase chance for producing unique fingerprint (Idris, *et al*., 2012) [14].

Table 2: *Ocimum basilicum* cultivars 1-Iranian (green) 2- Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red) fingerprinting (DNA profile) using ISSR markers

Primer	Cultivars	No. of fingerprint
844A	1	1
UBC820	1,2,5	3
UBC816	1,2,5,	3
HBS10	1,2,3	3
UBC811	1,4,5	3
UBC817	1,4,5	3
UBC812	1,4,5	3
UBC852	1,4,5	3
17889A	1	1
HB12	5	1

Total ISSR Marker Analysis

In Table 3, highest value for molecular size 2447 bp in primer UBC852,while lowest value for molecular size was 130 bp in primer UBC811.

The higher molecular size and lower molecular size of primers related to primer sequence annealed with DNA template. Insertions and deletions could change the size of the amplified product by changing distance between annealing sites of primers (Powell *et al*., 1996) [21]. Highest value for main bands (17), amplified bands (42), polymorphic bands (13) and discriminatory value (17.567) in primer UBC811.

The higher number of main bands and amplified are mainly due to primer structure and that some primers recognize a high number of annealing site, which is more useful than primers recognizing lower number of annealing sites. In this case the number of amplified bands will be higher, thus giving a better chance for detecting DNA polymorphisms among individuals (Tahir, 2014) [28].

Discriminatory value of primer concerned with its ability to give uniquefingerprint (Al Ghufaili and Al-Tamimi, 2017) [2], this was clearly observed in primers UBC820, UBC816, HBS10, UBC811, UBC812, UBC817 and UBC852.

Highest number of monomorphic bands was six in primer UBC816. Monomorphic bands are type of these sequences, which reveal that genotypes that belong to one species share some genome sequences and differ in others (Al-Judy, 2004) [3].

Other lowest value for studied criteria were main, amplified and polymorphic bands, efficiency and discriminatory value in primers UBC816 and 17889A. Monomorphic bands in primers 844A and UBC817 and polymorphism in primer 844A.

Primer UBC812 produced highest value for polymorphism 100%, while primer UBC852 gave highest value for both polymorphism 100% and efficiency 0.687.

It is noteworthy that di-nucleotide repeats, anchored either at 3' or 5' end, usually reveal high polymorphism and the primers anchored at 3' end give clearer banding pattern compared to those anchored at 5' end. Since the primer is a SSR motif, the frequency and distribution of the microsatellite repeat motifs vary in different species and influence the generation of bands as well. In general, primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism than primers with other di-, tri- or tetra-nucleotide repeats, primer efficiency usually affect posotively with primer polymorphism (Tarinejad *et al*., 2015) [29].

The polymorphism level of the DNA marker is the production power of the DNA band from the PCR reaction results using a particular marker/primer. The degree of polymorphism is influenced by the variety of genotypes used and it is highly dependent on how primers recognize their complementary DNA sequence in the DNA template used. Markers used for PCR reactions and electrophoresis results show the same banding pattern between each DNA sample of different genetic monomorphic markers whereas if the electrophoresis results show different banding patterns between each DNA sample is a polymorphic marker (Dalimunthe *et al*., 2020) [8]. PCR amplification illustrated in Fig (3, 4, 5).

Table 3: Summarized results of ISSR amplification product include: Amplified bands molecular size range in bp; No. of: main, amplified, monomorphic, polymorphic and unique bands; primer polymorphism (%), efficiency and discriminatory value (%)

Primers	Molecular size	Main bands	Amplified bands	Monomorphic band	Polymorphic band	Polymorphism (%)	Efficiency	Discriminatory Value (%)
844A	887-268	9	32	2	7	77.77	0.218	9.45
UBC820	1619-280	11	40	5	6	54.54	0.15	8.108
UBC816	649-206	11	40	6	5	45.45	0.125	6.756
HBS10	1533-188	14	39	4	10	71.42	0.256	13.513
UBC811	1720-130	17	42	4	13	76.47	0.309	17.567
UBC817	848-192	6	17	2	4	66.66	0.235	5.405
UBC812	1587-239	12	29	0	12	100	0.413	16.216
UBC852	2447-263	11	16	0	11	100	0.687	14.864
17889A	745-160	7	29	5	2	28.571	0.068	2.702
HB12	677-209	8	28	4	4	50	5.405	***14.2***

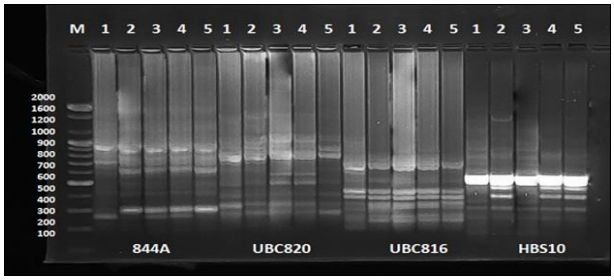

Fig 3: Amplification product of primers 844A, UBC820, UBC816 and HBS10, M: DNA ladder, Ocimum basilicum cultivars (1-Iranian (green) 2- Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

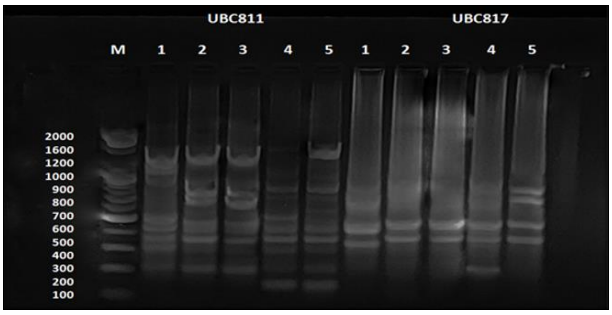

Fig 4: Amplification product of primers UBC811 and UBC817, M: DNA ladder, Ocimum basilicum L. cultivars (1-Iranian (green) 2-Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

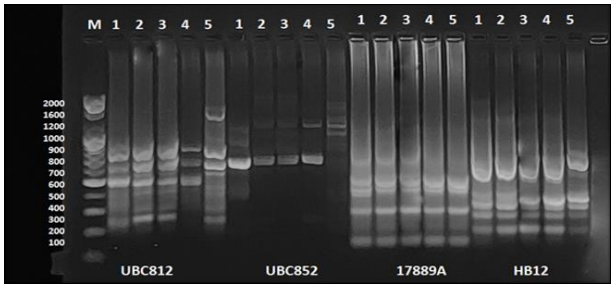

Fig 5: Amplification product of primers UBC812, UBC852, 17889A and HB12, M: DNA ladder, Ocimum basilicum cultivars (1-Iranian (green) 2- Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

Genetic Relationships

Results in Table 4 showed that highest genetic distance was 0.74667 Iranian (green) and Turkish (red) cultivars, while lowest genetic distance was 0.16705between Local (green) and Turkish (red) cultivars.

In compatiblity of genetic relationships with the pattern of geographical distribution, refer to that cultivars cultivated in various geographical regions were situated in an identical group, different studies have reported the incompatibility of genetic diversity with geographical diversity (Tarinejad *et al*., 2013; Tarinejad *et al*., 2015) [30, 29], and also inspite of their variation or similarity in morphological characters (AL-Tamimi, 2014) [4].

El Saied *et al*. (2012) [10] interpretate high and low genetic distance is associated with number of amplified bands, thus high number of common bands (monomorphic bands) decrease genetic distance because it refers to high genetic similarity, while, low number of these common bands increase genetic distance because it refer to low genetic similarity, these bands may distributed in coding or non coding region.

Table 4: The genetic distance values among *Ocimum basilicum* cultivars 1-Iranian (green) 2- Iranian (red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red) using ISSR markers

Cultivars	1-Iranian (green)	2-iranian (red)	3-Eygptian (green)	4-Local (green)	5-Turkish (red)
1-Iranian (green)	0				
2- Iranian(red)	0.60256	0			
3-Eygptian (green)	0.5041	0.3	0		
4-Local (green)	0.62038	0.60218	0.30449	0	
5-Turkish (red)	0.74667	0.60923	0.31077	0.16705	0

Phylogenetic Tree

Genetic relationship among cultivars in Fig 6 illustrates that *Ocimum basilicum* cultivars arranged among two major custers, the first small one included only Iranian (green) cultivar, the other large one included the rest four cultivarswhich divided between twosubcluster each of them contain two cultivar. The first contain Iranian (red) and Eygptian (green) cultivars and the other contain Local (green) and Turkish (red) cultivars.

Lack of clustering according to phyto-geographical areas indicates that accessions from different geographical areas were not significantly different genetically. This may be due to spread of seed and/or high rates of gene flow between the adjacent populations (Malav *et al*., 2015) [17].

Pattern of genetic diversity is not compatible with the pattern of geographical distribution, so that the cultivars cultivated in various geographical regions were situated in an identical group, different studies have reported the incompatibility of genetic diversity with geographical diversity (Tarinejad *et al*., 2013; Tarinejad *et al*., 2015) [30, 29].

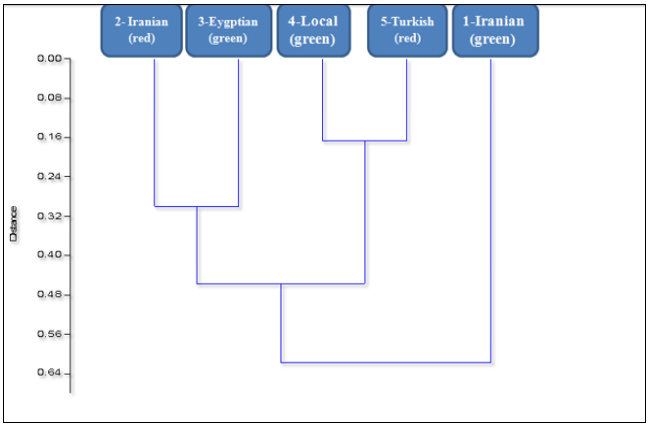

Fig 6: UPGMA dendrogram illustrating the trees of genetic relationship between *Ocimum basilicum* cultivars 1-Iranian (green) 2- Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red) using ISSR markers

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