Molecular characterization of five *Ocimum basilium* cultivars using RAPD markers

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Abstract

Current ivestigation was accomplished at biology department/faculty of science/kufa university during 2021-2022 to asses geneticvariation 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 usin ten RAPD markers.results showed that RAPD markers were effective in generating polymorphism in basile germplasm reached to 85.7% by primer OPD-13 and givingunique fingerprint to all cultivars by primers OPA-03 and OPC-09. Phyllogenetic tree and genetic distance for studied cultivars are not stronglly related to cultivar origin or morphology.

Keywords: RAPD, O.basilicum, genetic distance, fingerprint , phyllogenetictree

DOI: 10.48047/ecb/2023.12.8.543

Introduction

Ocimum is one of the most important genera of the family Lamiaceae commonly known as basil or sweet basil (Bravo et al.,2021).The name basil is derived from the Greek word “Basileus” meaning “Royal” or “King”(Bilal etbal.,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), in addition, secondary metabolites including polyphenols, flavonoids, , essential oil , terpenic compounds,monoterpene ,sesquiterpenes, (Kisa et al.,2021). 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). 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) Both are simple,inexpensive, need noknowledge of the target sequence, and are easy to apply and in data analysis (Bahadur et al. 2015). Antioxidant activity ,antibacterial and antifungal activity are all related to plant constitutent of bioactive compounds , high performans liquid chromatography (HPLC) , is a versatile, robust, and widely used technique for the isolation of natural products , it is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify , quantify and purify the individual components of the mixture (Boligon and Athayde., 2014). 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) , 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 application of ISSR markers .Seeds and leaves illustrates in figure (1).

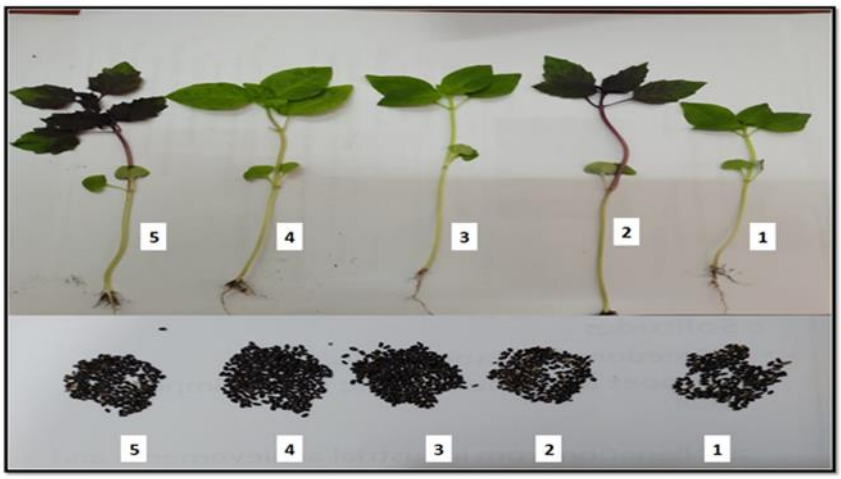

Figure (1) Leaves and seeds of Ocimum basilicum L. cultivars (1-Iranian (green) 2-Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

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, eleven primers were used in application of RAPD markers (Carelli *et al*., 2006; Abd El-Hady *et al.*, 2010; Ezekiel *et al.,* 2011 and El-Assal and Gaber,2012) in tables (1) with their nucleotide sequences and names of each primer.

Table (1) Primers used as RAPD markers.

No.	Primer name	Sequence 5' ⟶ 3'	Temperature
1	OPA-04	AATCGGGCTG	40 C°
2	OPA-10	GTGATCGCAG	40 C°
3	OPA-02	TGCCGAGCTG	40 C°
4	OPA-03	AGTCAGCCAC	40 C°
5	OPW-04	CAGAAGCGGA	40 C°
6	OPC-09	CTCACCGTCC	37 C°
7	OPA-01	CAGGCCCTTC	40 C°
8	OPX-03	TGGCGCAGTG	40 C°
9	OPX-17	GACACGGACC	40 C°
10	OPD-13	GGGGTGACGA	40 C°
11	OPA-14	TCTGTGCTGG	37 C°
Carelli *et al*., 2006; Abd El-Hady *et al.*, 2010; Ezekiel *et al.,* 2011 and El-Assal and Gaber,2012			

PCR content and amplification programe

PCR Pre Mix master mix. Bioneer Corporation USA, (0.2ml) 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 1min , annealing :variable , extension at 72 C˚ for 1min and final extension at 72 C˚ for 5min .

Agarose gel electrophoresis

The gel electrophoresis methods were done according to Sambrook and Russel (2001) 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) and analyzed using SIMQUAL (Similarity for Qualitative Data) routine to generate genetic similarity index (Nei and Li, 1979): 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) and by Graham and McNicol (1995).

Results and discussion

Genomic DNA agarose gel electrophoresis

Results in figure (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).

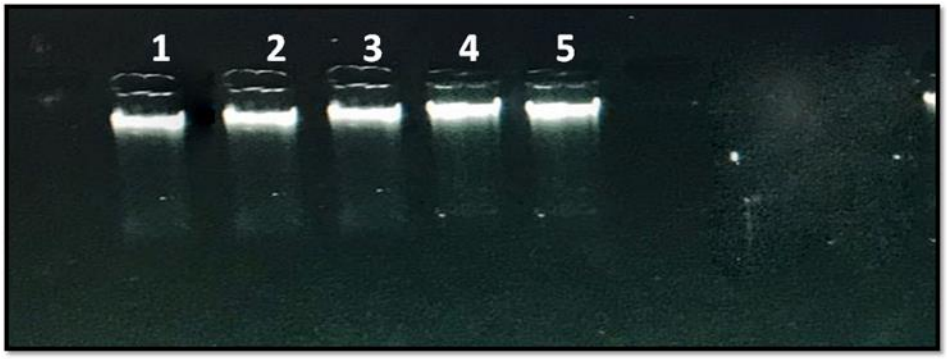

Figure (2) Genomic DNA agarose gel electrophoresis for Ocimum basilicum L. cultivars (1-Iranian (green) 2- Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

DNA fingerprint detected by RAPD markers

Results in table (2) shwed that primers OPA-03 and OPC-09 success in giving all cultivars a unique fingerprint while primer OPA-02 and OPA-01 gave only on cultivar a unique fingerprint. When primer possesses an ability to produce polymorphic and unique bands , both polymorphic and unique alleles inside genotypes increase their ability for producing unique fingerprint (AL-Haidari .,2023).

Ibrahim et al.,(2013) established that oriented breeding programs with the help of DNA fingerprinting technology will be helpful to produce distinct cultivar/genotypes with diverse genetic background and improved productivity.

Appearance of unique bands indicate that these cultivars possess one or more novel sequence not found in others . There important arises from their ability in identification of these cultivars, thus they can be considered as genetic marker. (Vishwanath, 2010). RAPD fingerprint has been can eficiently support chemotypicin many medicinal plant species .(Shasany et al.,2003). Morphological, chemical and genetic differences using RAPD markers among 12 basil (*O. gratissimun* L.) accessions were studied and established their ability as taxonomical markers.(Vieria et al.,2001).

Table (2) five *Ocimum basilium* cultivars 1-Turkish 2-Syrian 3- Turkish 4-Egyptian fingerprinting (DNA profile) using RAPD markers

No.	Primer	Cultivars	No. of fingerprint
1	OPA-04	1,3,4	3
2	OPA-10	1,3,5	3
3	OPA-02	4	1
4	OPA-03	1,2,3,4,5	5
5	OPW-04	1,2,5	3
6	OPC-09	1,2,3,4,5	5
7	OPA-01	3	1
8	OPX-03	1,2,3	3
9	OPX-17	2,4,5	3
10	OPD-13	2,4,5	3

Total RAPD marker analysis

Results in Table(3) illustrate that that higher molecular size was 1914bp produced by primer OPA-03 while lowest molecular size was 138 bp produced in primer OPA-02. Molecular size variation among generated is related to change in primer annealing sites which result in changing in distance between two annealing sites of primer on DNA template, these changes may due to change in DNA sequence result from diverse types of mutation (insertion, deletion ….etc) (Fadoul, 2013). Diverse genotypes result in diverse DNA sequence and diverse primers annealing sites result in change amplicon molecur size (Prakash et al., 2011). Highest number of main (20 band) , polymorphic(17) bands ,effeciency 0.404 and discriminatory value was 20% were produced in primer OPC-09. Primer efficiency and discriminatory value concerned with each other ,since increase polymorphic bands increase discriminatory value of primer (Hunter and Gaston (1988) and by Graham and McNicol (1995). Recognition of high number of annealing site by primer usually result in high number of main band this establish by many authors AL-Saadi (2018) in maize and AL-Ghufaili (2017) in wheat . Highest value for polymorphism was 85% and 85.715 produced by primers OPC-09 and OPD-13 respectively . Polmorphism concerned with each other ,since increase polymorphic bands increase Polmorphism of primer (Hunter and Gaston (1988) and by Graham and McNicol (1995).

Highest value for amplified band number was 49 band in primer OPX-03 . Increase binding site of primer conscuently increase number of amplified band which result in increasing chance for detecting polymorphism among individual (Roy et al. 1992).

One of the advantages of the RAPD method is that the arbitrarily designed primers can potentially anneal to homologous sequences in the entire genome providing greater opportunities to uncover regions (Williams et al., 1990). Primers OPX-17 and OPA-10 produced highest number of monomorphic bands(six bands).

Presence of monomorphic band usually refer to that genotypes belong to one species and sharing their relatives in some genome sequences, they are constant and conserved in genome (AL-Badeiry, 2013). The appearance of monomorphic band may refer to common character between studied genotype (AL-Tamimi, 2014).

Primer OPA-01 gave lowest vlaue for main ,band , amplified bands , polymorphic bands ,polymorphism , Efficiency and Discriminatory value while primer OPD-13 gave lowest value for number of monomorphic bands .

Despite low polymorphism showed by the rest RAPD primers, theses primers even when able to amplify more than one band per accession, residual heterogeneity within the accessions is apparent. It still a good technique to reveal genetic diversity (Ogunbayo et al., 2005).Figures (2),(3) and (4) illustrate agarose gel electrophoresis of previous primers.

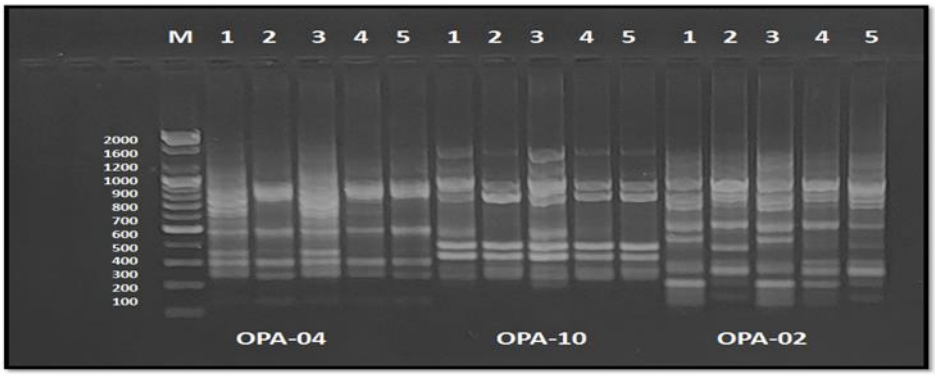

Figure (2) Amplification product of primers OPA-04 , OPA-10 ,OPA-02 ,OPA-03 and PW-04 , M: DNA ladder , Ocimum basilicum L. cultivars (1-Iranian (green) 2- Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

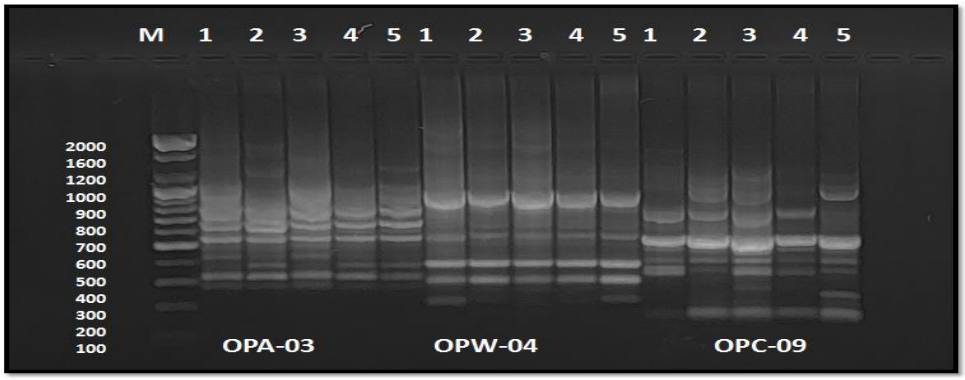

Figure (3) Amplification product of primers OPA-03 , OPW-04 ,OPC-09, M: DNA ladder , Ocimum basilicum L. cultivars (1-Iranian (green) 2- Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

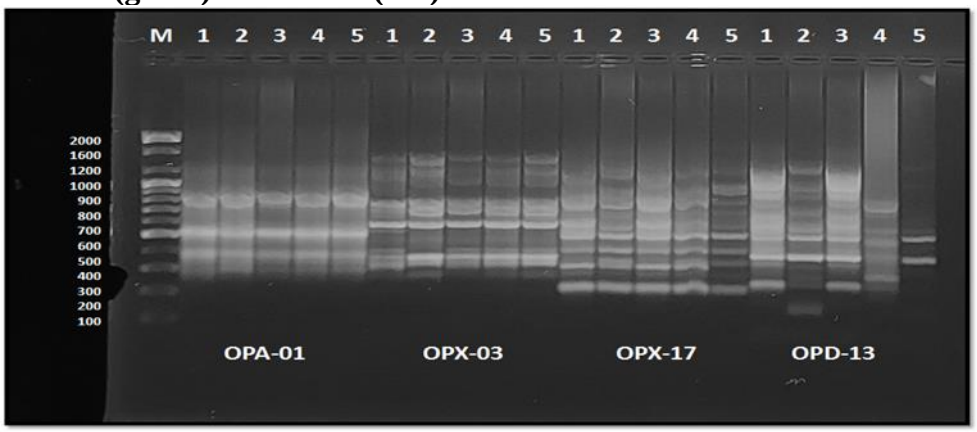

Figure (4) Amplification product of primers OPA-01 , OPX-03 ,OPX-17 and OPD-13 , M: DNA ladder , Ocimum basilicum L. cultivars (1-Iranian (green) 2- Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red)

Table(3) Summarized results of RAPDs 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 (%) .

Genetic relationships

Results in table (4) showed that highest genetic distance was 0.50492 produced between Eygptian (green) and Local (green) cultivars while lowest genetic distance was 0.31748 produced between Iranian (green) and Eygptian (green) cultivars. These relations between cultivars which independent to geographical origin may related to that they share a common parent or ancestor, this influence the similarity or dissimilarity among cultivars (Morale *et al*., 2011), it was established by Uddin and Boenor , (2008) that most closely related two genotypes originated from different collection site, thus no relation between geographical origin and genetic distance, this can interpretat that varieties may introduced from one locality to other and assigned new name (AL Adele *et al*., 2008) . Genetic similarity also concerned with presence of some common morphological characters (AL- Ghufaili ,2017). AL-Tamimi,(2014) estabilshed that geographical origin or indigenous names (Idris et al.,2012), cannot be considered good guide to the presence of diversity (Chakauya et al., 2006) ,since determination of genetic distance among genotypes is important to develop plants possessing high resistance to pathogens and unfavorable environmental conditions (Weeden et al., 1992).

Table (4) The genetic distance values among Ocimum basilicum L. cultivars (1-Iranian (green) 2- Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red) using RAPD marker

Primers	Molecular size	Main bands	Amplified bands	Monomorphic band	Polymorphic band	Polymorphism (%)	Efficiency	Discriminatory Value (%)
OPA-04	863-141	12	37	4	8	66.66	0.216	9.411
OPA-10	1497-193	10	35	6	4	40	0.114	4.705
OPA-02	937-138	14	42	4	10	71.428	0.222	11.764
OPA-03	1914-272	15	40	3	12	70.588	0.3	14.117
OPW-04	888-209	8	24	4	4	50	0.166	4.705
OPC-09	1222-165	20	42	3	17	85	0.404	20
OPA-01	769-274	5	21	4	1	20	0.0476	1.176
OPX-03	1367-264	15	49	5	10	66.66	0.204	11.764
OPX-17	1136-211	13	45	6	7	53.846	0.155	8.235
OPD-13	1200-211	14	40	2	12	85.714	0.3	14.117

Cultivars	1-ranian (green)	2-iranian (red)	3-Eygptian (green)	4-Local (green)	5-Turkish (red)
1-Iranian (green)	0				
2- Iranian(red)	0.45554	0			
3-Eygptian (green)	0.31748	0.39262	0		
4-Local (green)	0.36116	0.44233	0.50492	0	
5-Turkish (red)	0.48699	0.41466	0.49304	0.37722	0

Phylogenetic tree

Genetic relationship drowen in figure (5) illustrate that *Ocimum basilicum* cultivars distributed between two major clusters, the first large one included Iranian (green) , Iranian(red) and Eygptian (green) cultivar, both Iranian (green) and 3-Eygptian (green) seperated in one subcluster(both are green) , other large one included) Local (green) and Turkish (red) cultivars .Cultivars in divided in clusters regardless to theirgeographical origin and slightly according to their colour . When different genotypes of same or different origin cluster together , or separate inidividually despite having the same origin with rest studies genotypes, this interpretated that genotypes could be collected at different location and time.The tendency of genotypes to cluster together despite their different origin , this of great important for breeder , depending on geographical origin is not accurate indicator of genetic diversity (Celka et al.,2010 ;AL Adele et al., 2008) . similar results produced by AL-Saadi,(2018) and AL-Tamimi and AL-Janabi,(2019).

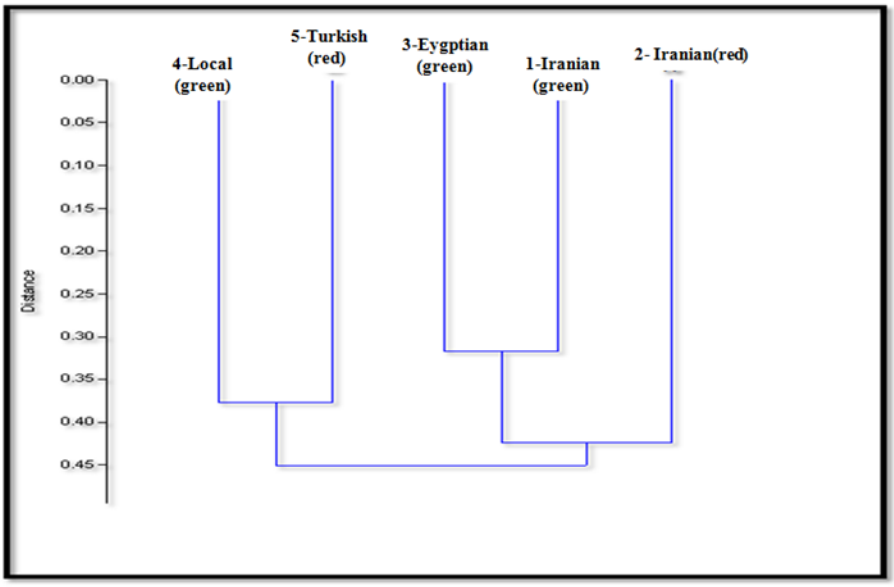

Figure (5) UPGMA dendrogram illustrating the trees of genetic relationship between Ocimum basilicum L. cultivars 1-Iranian (green) 2- Iranian(red) 3-Eygptian (green) 4-Local (green) 5-Turkish (red) using RAPD markers.

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