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Detection of genetic variation among five basil species by using ISSR, SCOT and SRAP markers

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Abstract---Background: Eight inter-simple sequence repeats (ISSR), 14 Start codon-targeted (SCoT), and eleven sequence-related amplified polymorphisms were used to determine genetic diversity among five basil species (*Ocimum tenuiflorum*, *Ocimum kilimandscharicum* Guerke, *Ocimum basilicum* L., *Ocimum americanum*, and *Ocimum basilicum* var. *minimum*). Results: All of the markers' PCR products can be used to discriminate between the different basil species. The ISSR has the highest polymorphism percentage (73.1%), followed by SCoT (57.2%) and SRAP (55.6%). According to the combined data from the three markers, the basil species used were clustered into four groups, each with extremely excellent coordination between the similarity matrix and dendrograms. Species-specific markers have been identified. The SCoT analysis generates the highest number of species-specific marker (17) compared with ISSR and SRAP analysis which generated 14 and 13 markers respectively. Conclusions: These markers can be considered as a useful marker to improve *Ocimum* characteristics.

Keywords---Basil, molecular markers, ISSR, SCoT, SRAP, cluster analysis.

Background

Ocimum basilicum L., known as common basil, is a yearly savory herb belonging to the Lamiaceae family. It is renowned for a wide range of remedial and fragrant species of assorted financial significance. *Ocimum* was depicted in 1753 by Linneaus, who recorded five species. (Paton *et al.* 1999). The variety *Ocimum* is profoundly assorted and comprises a wide extend of morphology, chemical, and hereditary cosmetics variability (Chowdhury *et al.*, 2017). due to tremendous levels of both morphological and chemical variability that exist within the genus as a result to interspecific hybridization, polyploidy and the intensive of chemotypes or chemical races which do not differ substantially in morphology (Simon *et al.*, 1990). *Ocimum* spp. are commercially implanted for their profoundly esteemed vital oils that found its applications in culinary, perfumery, flavoring, and pharmaceutical industry (Chalchat and Özcan 2008). On the other hand, basil taxonomy is complex because of the occurrence of inter-/intra-specific hybridization and morphological similitude (De Masiet *et al.*, 2006). Occasionally, taxonomists defined different classes as synonymous and confused different varieties as homonyms.

The characterization through distinct molecular markers is prominent for protection and sustainable utilization of important therapeutic and fragrant plants (Kumar *et al.*, 2014). As molecular markers are neutral, not related to age and tissue type, and not influenced by the environmental conditions, it can be used to estimate hereditary variation in plants (Marshall, 1995). DNA markers have the most intensive use due to their availability in great numbers in general, DNA fingerprinting is being widely used to analyze the variability that occurs in the genus *Ocimum* (Kumar *et al.*, 2016).

Therefore, the present study discusses the genetic diversity and relationship of five *Ocimum* species, by molecular markers based on ISSR (Inter sequence repeats) and SCoT (Start codon-targeted) and SRAP (Sequence-related amplified polymorphism) together. The inter simple sequence repeats (ISSR) technique is a PCR-based method that involves amplification of DNA fragments that present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposing directions, ISSR is a reproducible technique, highly polymorphic marker .It's generated by microsatellite sequences, which are highly variable and distributed over the genome, so it has a wide application in studies of genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al.*, 2002). (Start codon-targeted polymorphisms) SCoT markers are reproducible and known as start codon, are being as a short-conserved region in plant genes nearby the ATG sequences (Thakur, 2016).

SCoT markers are used to estimate genetic heterogeneity, classify cultivars, and map quantitative trait loci (QTL) in many crops (Etminan 2018).SRAP Sequence-related amplified polymorphism (system was developed by Li and Qui 2001)to target overlapping coding and non-coding regions of the genome. It concerned as a useful PCR based molecular marker system for diversity study and linkage mapping in various crop(Li *et al.*, 2007). The major goal of this research was to assess the genetic diversity of basil species using several marker systems like as

ISSR, SCoT, and SRAP. In addition, the optimal marker system for genetic diversity study in basil was established by comparing marker systems.

Methods

Plant material

Five *Ocimum* species/genotypes were used in the present investigation, the common and scientific names of the basil species used along with their origin are presented in Table (1).the *Ocimum* species/genotypes used were obtained from the Faculty of Pharmacy experiment station, Cairo University.

Table 1
List of the *Ocimum* species used in the analysis

Genotypes	Scientific name	Source
Soudi basil	<i>Ocimum tenuiflorum</i>	India
African blue basil	<i>O. kilimandscharicum</i> <i>Guerk.</i>	Nasik, Maharashtra (India)
Sweet basil	<i>O. basilicum</i> L.	Chennai, A.P., (India)
American Basil	<i>Ocimum americanum</i>	Africa
French basil	<i>O. basilicum</i> var. <i>minimum</i>	Bangalore, Karnataka (India)

DNA Extraction

Total DNA was extracted from fresh-leaf tissues (0.2 g) of five *O. basillium* species using CTAB (Cetyl trimethyl ammonium bromide) method according to (Khanuja *et al.*,1999). The quality and quantity of the extracted DNA were visualized under UV light after electrophoresis on 0.8% agarose. DNA samples were stored at -20°C.

Molecular markers analyses

PCR amplifications were carried out using a total of 8 ISSR, 14 SCoT, and 11 SRAP primers. Their sequences and annealing temperatures were presented in Tables (2-4)

ISSR analysis

All PCR amplifications were performed in a thermo cycler UNO II, Biometra (Whatman company, Göttingen, Germany). In the case of ISSR, the reaction volume of 20 µL containing 1 µL DNA template (20 ng/µL), 10 µL master mix (Biotecke Corporation), 1 µL primer (100 ng/µL) and 8 µL of distilled water. The PCR condition was one cycle of 5 min. at 94°C (initial denaturation) followed by 37 cycles of denaturation at 94 °C for 1 min., annealing at 52–56 °C for 1 min. and extension at 72 °C for 2 min. followed by a final extension at 72 °C for 10 min.

Table 2
Standardization of PCR condition of ISSR marker

Primer's names	Sequence 5'--3'	Code	Annealing temperature
UBC 824	(TC)8G	ISSR-24	54 °C
UBC 808	(AG)8C	ISSR-I3	54 °C
UBC 810	(AG)8T	ISSR-I4	54 °C
UBC 811	(GA)8C	ISSR-I1	54 °C
UBC 817	(CA)8A	ISSR-I7	57 °C
UBC 825	(AC)8T	ISSR-I2	57 °C
UBC 834	(AG)8YT	ISSR-I6	54 °C
UBC 841	(GA)8YC	ISSR-I5	54 °C

SCoT analysis

For SCoT analysis the reactions mixture contained (1 µL DNA template (40 ng/µL), 10 µl master mix (Biotecke Corporation), 2µl scot primer and complete volume to 20 µl with 7 µl nuclease-free water). The PCR reactions were amplified with the following program: initial denaturation at 94 °C for 5 min; 35 cycle of denaturation at 94 °C for 1 min, annealing at 49–60 °C for 1 min.

Table 3
The primer's name and its annealing temperature of the ScoT Markers used for *O. basillium* genotyping

Primer	Sequence (5'-3')	Annealing temperature (°C)
SCOT-13	ACGACATGGCGACCATCG	50
SCOT-14	ACGACATGGCGACACGC	50
SCOT-24	CACCATGGCTACCACCAT	50
SCOT-26	ACCATGGCTACCACCGTC	50
SCOT-31	CCATGGCTACCACCGCCT	52
SCOT-33	CCATGGCTACCACCGCAG	50
SCOT-34	ACCATGGCTACCACCGCA	54
SCOT-36	GCAACAATGGCTACCACC	50
SCOT-52	ACAATGGCTACCACTGCA	50
SCOT-60	ACAATGGCTACCACCACA	49.70
SCOT-66	ACCATGGCTACCAGCGAG	49.70
SCOT-70	CCATGGCTACCAGCGCG	50
SCOT-71	CCATGGCTACCACCGCCG	50
SCOT-77	CCATGGCTACCACTACCC	54

SRAP analysis

The SRAP analyses were performed using 20 µl reaction volume which comprised: (1 µL DNA template (40 ng/µL), 10 µl master mix (Biotecke Corporation), 1µl forward primer, 1µl reverse primer forming complete volume to 20 µl with 7 µl

distilled water), PCR reactions were performed as follows: the initial step of 5 min at 94°C, then 5 cycles of 1 min at 94°C, 1 min at 35°C, 1 min at 72°C followed by 35 cycles comprised 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and a final extension for 10 min at 72°C then cooling down to 15°C (Li and Quirons 2001). Amplified PCR products were separated on 1.2% agarose gel and further visualized under gel documentation system (Bio-Rad® Gel Doc 2000). Using 1000 bp DNA ladder as standard (Promega, USA). Amplified products were scored across the lane concerning their molecular weight. The presence of the band was scored as (1) and the absence of band scored as (0).

Table 4
Primer combinations sequence designed for SRAP analysis

Primers combination	Forward primer (5'-3')	Reverse primer (5'-3')
ME1-EM3	TGAGTCCAAACCGGATA	GACTGCGTACGAATTGAC
ME1-EM4	TGAGTCCAAACCGGATA	GACTGCGTACGAATTTGA
ME1-EM6	TGAGTCCAAACCGGATA	GACTGCGTACGAATTGCA
ME2-EM3	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTGAC
ME2-EM4	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGA
ME3-EM1	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTAAT
ME3-EM6	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTGCA
ME4-EM1	TGAGTCCAAACCGGACC	GACTGCGTACGAATTAAT
ME4-EM3	TGAGTCCAAACCGGACC	GACTGCGTACGAATTGAC
ME4-EM6	TGAGTCCAAACCGGACC	GACTGCGTACGAATTGCA
ME5-EM6	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTGCA

Data Scoring and Statistical Analysis

The polymorphism percentages were calculated by measuring the sum of the polymorphic bands from binary data. To estimate the similarity between all the samples analyzed, the ISSR, SRAP and SCoT bands were classified as present "1" or absent "0". Quantify the informative value of these markers to distinguish *Ocimum* species. The pairwise comparison between the species was made using the Dice coefficient (Sneath and Sokal, 1973). The tree diagram was produced by clustering the similarity data with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method using Systat ver. 7 (SPSS Inc. 1997 SPSS Inc.3/97 standard version) (Yang and Quiros,1993).

Results

The genetic variability of five basil species was estimated using ISSR, SCoT, and SRAP analyses. According to the results obtained, the ISSR analysis generates a total of 67 amplified fragments (Table 5 and Fig.1), 49 of which are polymorphic markers with a percentage polymorphism band (PPB) of 73.1 percent. The primers used differed in the number of bands they produced; UBC825 produced the most (11 bands), whereas UBC817 produced the least (six bands). Nonetheless, the UBC834 primer had the highest polymorphism percentage (88.8%), whereas the UBC810 primer had the lowest (62.5%). (Table 5).

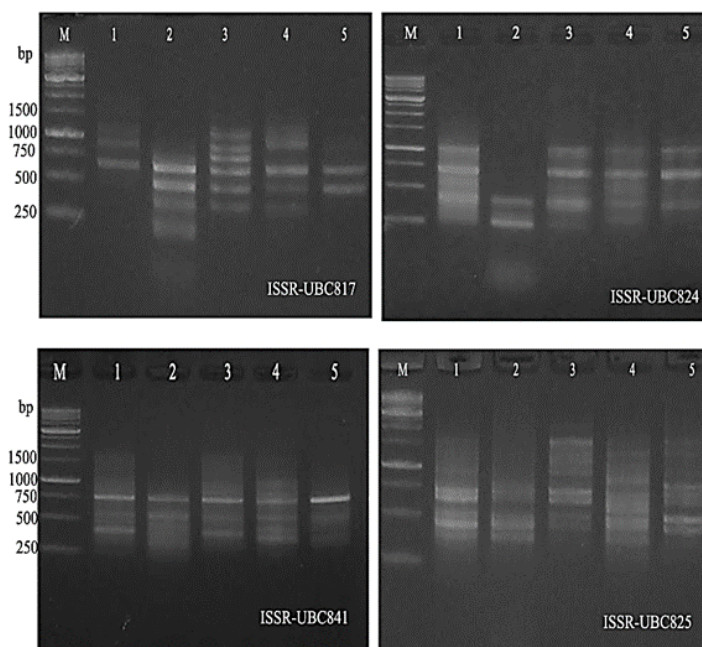


Fig 1. ISSR profile of the *O. basilium* species. M refers to DNA marker of 1Kb ladder, 1: Soudi basil, 2: African blue basil, 3: Sweet Basil, 4: American Basil, 5: French Basil

The SCoT profile of the five *O. basilium* species was illustrated in Figure 2. (1: Soudi basil, 2: African blue basil, 3: Sweet Basil, 4: American Basil, 5: French Basil). The data revealed that 14 SCoT primers generated 110 amplified bands (Table 5), with 63 polymorphic bands being detected. PPB (percentage polymorphic band) was 57.2 percent on average. The primers employed produce a variety of band numbers, ranging from two bands for SCoT 61 to eight bands for SCoT 31, for a total of 7.85 polymorphic bands per primer. The primer SCoT 31 had the largest polymorphism percentage (88.8%), whereas the SCoT 77 primer had the lowest polymorphism percentage (20%). (Table 5).

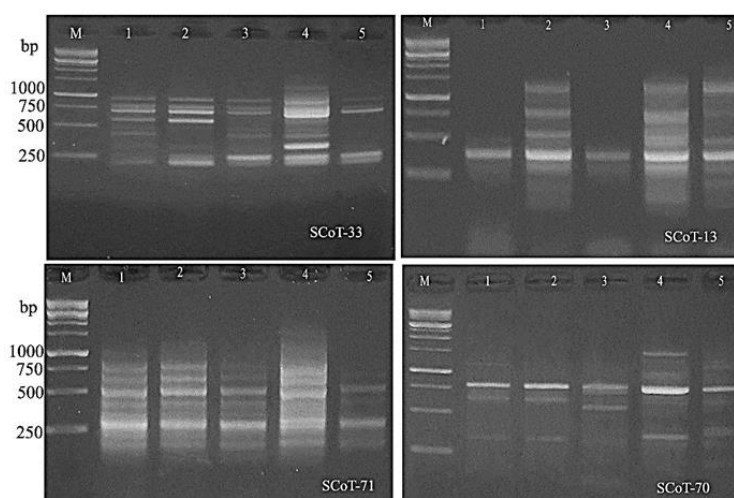


Fig 2. SCoT profile of the *O. basilicum* species. M refers to DNA marker of 1Kb ladder; 1: Soudi basil, 2: African blue basil, 3: Sweet Basil, 4: American Basil, 5: French Basil

The SRAP analysis, revealed that 79 bands were amplified with 11 primer combinations out of 25 primers (Table 5 and Fig.3). There were 44 polymorphic bands, with a 55.6 % percentage polymorphic band (PPB). The number of markers produced by the different primers ranged from 1 (ME3-EM6) to 7 (ME4-EM3), with an average of 7.18 bands per primer, according to the data. The ME4-EM3 primer recorded the highest polymorphism percentages (85.7%), while the ME3-EM6 primer recorded the lowest polymorphic percentages (16.6%). (Table 5).

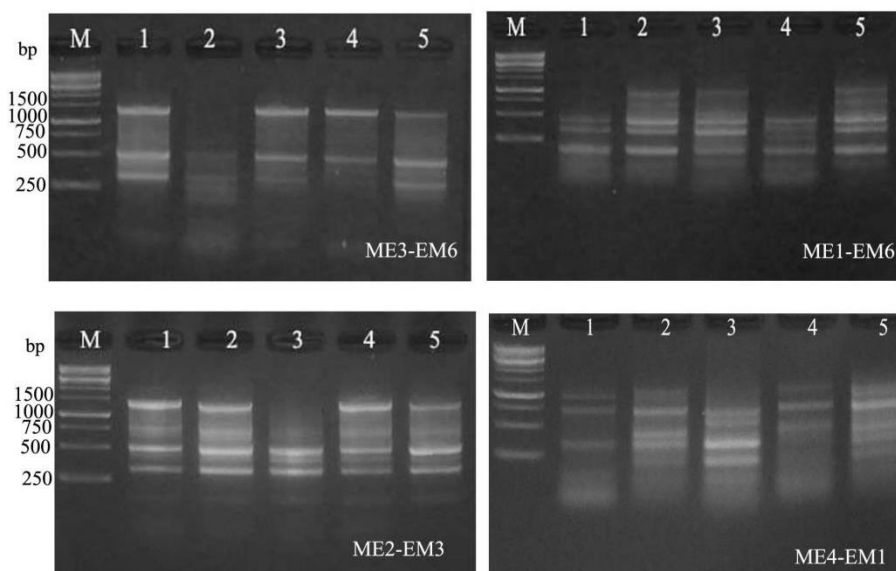


Fig 3. SRAP profile of the *O. basilicum* species. M refers to DNA marker of 1Kb ladder; 1: Soudi basil, 2: African blue basil, 3: Sweet Basil, 4: American Basil, 5: French Basil

Table 5
Total band numbers, number of polymorphic bands and polymorphism percentages as revealed by ISSR, SCoT and SRAP analyses

Marker type	Primer name	Total No. of bands	Polymorphic Bands	% of polymorphism	Band Size range (bp)
ISSR	UBC824	7	6	85.7	240-1000
	808	8	6	75.0	230-2000
	810	8	5	62.5	220-920
	811	9	7	77.7	220-1750
	817	6	4	66.6	200-780
	825	11	7	63.6	350-1600
	834	9	8	88.8	350-1150
	841	9	6	66.6	300-1600
	Total	67	49		
	Aver.	8.3	6.1	73.1	
SCoT	SCOT-13	8	7	87.5	200-1300
	SCOT-14	6	5	83.3	300-1250
	SCOT-24	8	3	37.5	250-1700
	SCOT-26	7	3	42.8	250-900
	SCOT-31	9	8	88.8	225-1250
	SCOT-33	9	4	44.4	200-950
	SCOT-34	7	4	57.1	250-1200
	SCOT-36	6	3	50.0	200-1500
	SCOT-52	9	6	66.6	200-1150
	SCOT-60	6	2	33.3	310-1300
	SCOT-66	7	4	57.1	220-1200
	SCOT-70	9	6	66.6	200-1400
	SCOT-71	9	6	66.6	200-800
	SCOT-77	10	2	20.0	200-1350
	Total	110	63		
	Average	7.8	4.5	57.2	
SRAP	ME1-EM3	10	6	60.0	250-1500
	ME1-EM4	6	2	33.3	220-920
	ME1-EM6	7	4	57.1	200-1000
	ME2-EM3	7	2	28.5	200-1200
	ME2-EM4	6	1	16.6	200-1300
	ME3-EM1	6	2	33.3	280-1300
	ME3-EM6	6	4	66.6	200-1250
	ME4-EM1	8	6	75.0	210-1200
	ME4-EM3	7	6	85.7	200-1450
	ME4-EM6	7	4	57.1	200-1150
	ME5-EM6	9	7	77.7	200-1200
	Total	79	44		
	Average	6.2	4.0	55.6	

The genotype specific of ISSR, SCoT and SRAP markers were determined. Table (6) shows that 15 markers distinguish Soudi Basil from the other genotypes studied, while African blue basil has 11 markers and American basil has 10. According to the results, 5 and 3 markers for French and sweet basil, respectively, might be considered genotype specific markers (Table 6). SCoT analyses produced the highest numbers of genotype specific markers (17), followed by ISSR (14) and SRAP, which produced 13 genotype specific marks (Table 6).

Table 6
Genotype specific markers for the five basil species used in this investigation

Species	ISSR	SCoT	SRAP	Total
Soudi basil	UBC811-550,1500,1750 UBC808-650,1130,1600,2000 UBC834-400,850,1080 UBC841-1350	SCoT52-200,300,400	ME4EM3-1050	15
African blue basil	UBC817-200 UBC825-700	SCoT31-225,620,850,1000	ME1EM3-850 ME2EM3-590 ME3EM6-200 ME4EM1-400 ME5EM6-850	11
Sweet basil			ME1EM3-250,350 ME4EM1-550	3
American Basil		SCoT26-700,820 SCoT24-550,1400,1700 SCoT70-1400 SCoT77-1350	ME4EM3-1450 ME1EM6-360 ME5EM6-1200	10
French basil	UBC834-1150	SCoT34-600 SCoT70-200 SCoT60-1300	ME1EM6-650	5
Total	14	17	13	44

Based on the results of ISSR, SCoT, and SRAP analyses, the genetic similarity of the five *Ocimum* species (1: Soudi basil, 2: African blue basil, 3: Sweet Basil, 4: American Basil, 5: French Basil) was determined. The data in Table (7) showed that the tested *Ocimum* species had varying percentages of similarity. Soudi basil and French basil had the highest similarity of 0.76, while African blue basil and American basil had the lowest similarity of 0.56.

Table 7
Similarity matrix based on the combined data analyses

Species	Soudi basil	African blue basil	Sweet basil	American Basil	French basil
Soudi basil	1.00				
African blue basil	0.59	1.00			
Sweet basil	0.58	0.62	1.00	1.00	
American Basil	0.58	0.56	0.62	0.75	1.00
French basil	0.76	0.74	0.75		

The scoring data from each marker type assay were used to build similarity matrices, which were then used in cluster analysis to generate dendrograms using UPGMA analysis to determine the genetic relationships among the *O.basilium* species. Fig.4 . The combined data from ISSR, SCoT, and SRAP analyses revealed that the first cluster was split into two sub-clusters, the first of which was split into two groups, the first of which contained French basil and Saudi basil species. The second group consists entirely of African blue basil. The second sub-cluster, on the other hand, includes just sweet basil. Furthermore, the second cluster only contained American basil (Fig.4).

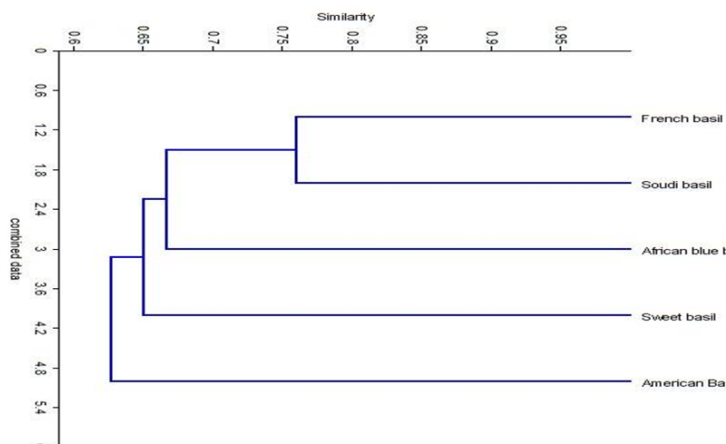


Fig 4. Cluster analysis of the basil species as revealed by combined data across ISSR, SCoT

Discussion

The genus *Ocimum* is very diverse and encompasses a wide range of morphological, chemical and genetic variability (Chowdhury *et al.*, 2017). DNA markers such as RFLP, AFLP, RAPD, SSR, SNP developed many systems based on different polymorphism-detecting techniques (southern blotting – nuclear acid hybridization, PCR – polymerase chain reaction, and DNA sequencing) (Collard *et al.*, 2005), (Xu, 2010). Due to insufficient information on genetic heterogeneity

between *O. basillium* species based on molecular markers, the present investigation used ISSR, SCoT and SRAP analyses to estimate the genetic variability between five *Ocimum* species. It is well documented that ISSR has randomly amplified markers, while SCoT is a gene-targeted marker, and SRAP is a relatively new PCR marker that has recently been widely used for germplasm characterization, cultivar classification, molecular mapping, and gene cloning (Bhattet *et al.*, 2017). The ISSR exhibited a higher polymorphic percentage (73.1%) and was more informative, followed by SCoT with 57.2%, then by SRAP with 55.6% among the five studied species of *O. basillium*.

In comparison to SCoT and SRAP markers, these findings demonstrated that the ISSR marker system is useful for distinguishing distinct *Ocimum* species and assessing their genetic diversity. These findings support the findings of (Budak *et al.*, 2004), who found that ISSR markers are more reliable than other markers since they had the highest average discriminatory power among AFLP, ISSR, and RAPD. ISSR markers also have the ability to amplify DNA fragments that are present at an amplifiable distance between two identical microsatellite repeat regions orientated in opposite direction (Reddy *et al.* 2002). High polymorphism of ISSR was also reported in many previous studies, as in the case of *Mentha arvensis*, *Ocimum* (Malav *et al* 2015; Dhawan *et al.*, 2016; Chaturvedi *et al* 2018; Alves *et al* 2019; Gupta *et al* 2021). Previous studies have also recommended that SCoT markers work better performance than other molecular marker systems in other plants (Luo *et al.*, 2011). The ISSR marker system is useful for differentiating *Ocimum* species and in examining their genetic diversity in comparison to SCoT and SRAP markers. The efficacy of the ISSR was demonstrated in the present study and in several other studies on genetic diversity, for example in which species of the genus *Ocimum* were analyzed (Aghaei *et al.*, 2012; Patel *et al.*, 2015, Kumar *et al.*, 2016; Alves *et al.*, 2019; and Gupta *et al.*, 2021), and other medicinal and aromatic species e.g., *Croton tetradenius* (Almeida-Pereira *et al.*, 2016).

The genotype-specific ISSR, SCoT and SRAP markers have been determined and these markers can be considered useful markers to enhance the properties of *Ocimum*. The data showed an overall 44 genotype-specific markers which were generated from the three molecular markers system used. The SCoT analyses generate the highest number of genotype-specific markers (17) which constituted 27 % of the polymorphic bands generated (Tables 5 and 6) while the ISSR and SRAP analyses generated 14 and 13 genotype-specific markers which represent 28.6 and 29.5 % out of the polymorphic band respectively. Genetic similarity between the five *Ocimum* species was determined using data from ISSR, SCoT and SRAP analyses. The data showed that the closest similarity between Souidi and French basil was 0.76, while the lowest similarity between African blue basil and American basil was 0.56. Cluster analysis of combined data from ISSR, SCoT and SRAP analyses revealed that, the first cluster is divided into two sub-cluster, the first is divided into two groups, and the first group contain species of French basil and Saudi basil species. While the second group only included African blue basil. However, the second sub-cluster contains only sweet basil. Moreover, the second cluster included only American basil.

Conclusion

ISSR, SCoT, and SRAP, alone and in combination, were found to be effective techniques for studying genetic diversity in basil species in this study. The ISSR marker analysis produced results that were more similar to the pooled data set, indicating that ISSR markers were better tools for clustering basil species than SCoT and SRAP. This research was also useful in determining the potential applicability of these three marker systems in basil molecular breeding. The development of new markers can help not only with diversity and conservation analysis, but also with feature identification and planned breeding studies.

Abbreviations

SCoT (Start codon-targeted)

SRAP (Sequence-related amplified polymorphism)

ISSR (The inter simple sequence repeats)

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