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GENETIC VARIATION IN *SALVIA OFFICINALIS* L. AND *THYMUS VULGARIS* L.

A.A.M. HUSSEIN* and A.J.T. AL-TAMIMI

Department of Biology, Faculty of Science, University of Kufa, Najaf, Iraq
*Corresponding author's email: alia.alkhafaji@student.uokufa.edu.iq
Email address of co-author: atyaf.altameemi@uokufa.edu.iq

SUMMARY

The concerned study sought to determine the variations based on the DNA fingerprint in two important medicinal plants, sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.), belonging to the family Labiatae, using three different types of DNA markers—random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and start codon targeted (SCoT). The comparison of these three types of markers revealed the recording of the highest molecular size of amplified bands (988 bp) was with SCoT markers, while the lowest molecular size (156 bp) resulted in RAPD markers. By comparing the RAPD and SCoT markers, the latter produced the highest main, amplified, monomorphic, and discriminatory values. The SCoT markers also gave the most polymorphic bands. The ISSR markers outperformed the other markers in polymorphism, efficiency, and the number of unique fingerprints.

Keywords: Sage (*S. officinalis* L.), thyme (*T. vulgaris* L.), RAPD, ISSR, SCoT, DNA fingerprints

Key findings: The SCoT, RAPD, and ISSR markers considerably distinguished the two species of medicinal plants, sage (*S. officinalis* L.) and thyme (*T. vulgaris* L.), besides variations in their phytochemical content.

INTRODUCTION

Sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.) are medicinal plants belonging to the mint family Labiatae (Lamiaceae), which is one of the most important and largest families, including 236 genera and more than 7000 species (Al-Rawi and Chakravarty, 1988; Muttalib and Naqishbandi, 2012). These medicinal plants included several classes of secondary metabolites and thus have antioxidant, cytotoxic, anti-inflammatory,

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antibacterial, antifungal, antiviral, analgesic, cardiovascular, hypoglycemic, hypolipidemic, antispasmodic, antiepileptic, anti-anxiety, and anti-angiogenic activities (Abdelaty *et al*., 2021).

Medicinal plants classify as sources of antibiotics with possible activity on multidrug-resistant microorganisms, and the plant extracts work as alternative sources of novel antibiotics against various pathogens (Owusu *et al*., 2021), as well as natural sources of antioxidants (Trevizan *et al.,* 2020). One of the major challenges in the use of herbal medicines is the lack of authentication and their identification. Therefore, it is necessary to develop sensitive and effective skills to characterize, identify, authenticate, and conserve numerous medicinal herbs with their diverse content of phytochemicals. Proper identification and recognition of many plant species is crucial to improve the novel medicinal crops (Ganie *et al.,* 2015).

Typically, identifying plants can occur by the flora of different regions based on morphological traits under field conditions. It has been difficult to distinguish the plants, especially at the early stage and in different geographical locations and regional distributions, to ensure quality control and traceability of plant materials for several industries. Therefore, it is necessary to develop authentic and undisputable plant identification methods through DNA fingerprinting (Moraes *et al*., 2015; Ahmad *et al*., 2017).

DNA-based molecular fingerprints played a pivotal role in various fields, such as classification, phylogeny, physiology, embryology, plant breeding, population mapping, ecology, and genetic engineering of crop plants (Chung *et al.,* 2017). As DNA markers, ISSRs (inter-simple sequence repeats), RAPDs (randomly amplified polymorphic DNA), and SCoT (start codon targeted) are simple, inexpensive, easy to apply, and need no knowledge of the target sequence in their data analysis (Gorji *et al.,* 2011). Thus, the following study aimed to detect the RAPD, ISSR, and SCoT markers' ability to fingerprint both the sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.) plants.

MATERIALS AND METHODS

Primers

The studied primers came from the Bioneer Corporation in lyophilized form, dissolved in a TE buffer to obtain 100 pmol/µl as a final concentration (stock solution). Working solutions of 10 pmol/µl entailed preparation by diluting the stock solutions, with 10 SCoT markers used in the application that included 10 each of the SCoT (Al-Haidari and Al-Tamimi, 2023), RAPD (Al-Musawi and Al-Tamimi, 2023), and ISSR markers (Al-Shujairi and Thamir, 2023).

DNA extraction

Dried leaves of sage (*S. officinalis* L.) and thyme (*T. vulgaris* L.), used for genomic DNA extraction, employed the Genomic DNA Mini Kit provided by Geneaid Biotech., Ltd.

PCR content and amplification program

PCR reactions performed used AccuPower® TLA PCR PreMix (Bioneer, USA) in 0.2 ml thin-wall 8-strip tubes using attached cup/96 tubes, Top DNA polymerase (1U), (dATP, dCTP, dGTP, and dTTP, each at 250 µM), reaction buffer with 1.5 mM $Mgcl_2$ (1X), along with a tracking dye and 100 bp DNA ladder. According to the experimental protocol of AccuPower® TLA PCR PreMix, the preparation for the PCR reaction mixture was as follows: adding 5 µl template DNA and 5 µl of primer (10 pmol/µl) to each AccuPower® TLA PCR PreMix tube; then, adding sterilized deionized distilled water to AccuPower® TLA PCR PreMix containing 5 µl of mastermix mixture followed to a final volume of 20 µl.

PCR amplification protocol

PCR amplification proceeded using primer-specific annealing temperatures, following the thermal cycling conditions—initial temperature at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min for SCoT markers (SCoT 9, SCoT 60,

SCoT 30, SCoT 44, SCoT 54, SCoT 28, SCoT 61, SCoT 26, SCoT 6, and SCoT 12) (Al-Haidari and AL-Tamimi, 2023). The process of initial temperature at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 1 min, and annealing (48 °C–55 °C) ensued for ISSR markers (UBC808, UBC820, UBC814, HBS10, UBC811, UBC849, UBC810, UBC855, UBC881 [Al-Ghufaili and Al-Tamimi, 2017], and UBC852). Likewise, the same process, with an additional annealing at 37 °C–40 °C continued for RAPD markers (OPA-04, OPA-10, OPA-15, OPA-03, OPH-01, OPC-9, OPA-01, OPA-17, OPB-06, and OPD-13) (Al-Musawi and Al-Tamimi, 2023). An extension at 72 °C for 1 min and a final extension at 72 °C for 7 min occurred for both RAPD and ISSR markers.

Agarose gel electrophoresis

The application of electrophoresis methods used a 1.2% agarose at 70 volts for 2 h, according to Sambrook and Russel (2001), using a 100 bp DNA ladder and ethidium bromide for agarose staining.

Statistical analysis

Photographs of agarose gel electrophoresis helped in scoring bands, where the presence of a band has a score of '1' and the absence a '0.' The polymorphism, primer efficiency, and discriminatory value underwent calculations for each primer using the following three equations (Hunter and Gaston, 1988; Graham and McNicol, 1995):

$$\text{Polymorphism} = \frac{\text{No. of polymorphic bands of primer}}{\text{No. of main bands of primer}} \times 100$$

$$\text{Discriminatory value} = \frac{\text{No. of polymorphic bands of primer}}{\text{Total No.of polymorphic bands produced by all primers}} \times 100$$

$$\text{Primer efficiency} = \frac{\text{No. of polymorphic bands of each primer}}{\text{Total No. of amplified bands of each primer}}$$

RESULTS AND DISCUSSION

RAPD markers

The results revealed six RAPD primers—OPD-13, OPA-10, OPA-03, OPA-01, OPB-06, and OPA-17—were successful in examining both species of medicinal plants, sage (*S. officinalis* L.) and thyme (*T. vulgaris* L.), with a unique fingerprint (Table 1). The four RAPD primers, OPH-01, OPC-09, OPA-04, and OPA-15, failed to show the examined species with unique fingerprints. Past studies also authenticated that primers possess an ability to produce polymorphic and unique bands, with both polymorphic and unique alleles inside the genotypes increasing their ability to produce unique fingerprints (Al-Musawi and Al-Tamimi, 2023). Ibrahim *et al*. (2013) established oriented breeding programs with the help of DNA fingerprinting technology, which will be helpful to produce distinct genotypes with diverse genetic backgrounds and improved productivity.

Table 1. Summarized DNA fingerprints of *S. officinalis* and *Thymus vulgaris* produced by using RAPD, SCoT, and ISSR markers.

No.	RAPD		SCoT		ISSR	
	Primer	Fingerprint number	Primer	Fingerprint number	Primer	Fingerprint number
1	OPH-01	0	SCoT28	0	UBC852	2
2	OPC-09	0	SCoT60	2	HBS10	0
3	OPA-04	0	SCoT26	2	UBC810	0
4	OPA-15	0	SCoT12	0	UBC855	2
5	OPD-13	2	SCoT61	0	UBC814	2
6	OPA-10	2	SCoT30	0	UBC820	2
7	OPA-03	2	SCoT44	2	UBC849	2
8	OPA-01	2	SCoT9	2	UBC881	0
9	OPB-06	2	SCoT54	2	UBC811	2
10	OPA-17	2	SCoT6	2	UBC808	2

Table 2. Summarized results of RAPD amplification product**.**

Primers	Main bands	Amplified bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)	Efficiency	Discriminatory Value (%)
OPH-01	4	8	4	0	0	0	0
OPC-09	2	4	2	0	0	0	0
OPA-04	4	8	4	0	0	0	0
OPA-15	7	14	7	0	0	0	0
OPD-13	17	17	0	17	100	1	34.69
OPA-10	6	6	0	6	100	1	12.24
OPA-03	9	10	1	8	88.88	0.8	16.32
OPA-01	8	9	1	7	87.5	0.7	14.28
OPB-06	5	6	1	4	80	0.66	8.16
OPA-17	8	9	1	7	87.5	0.77	14.28

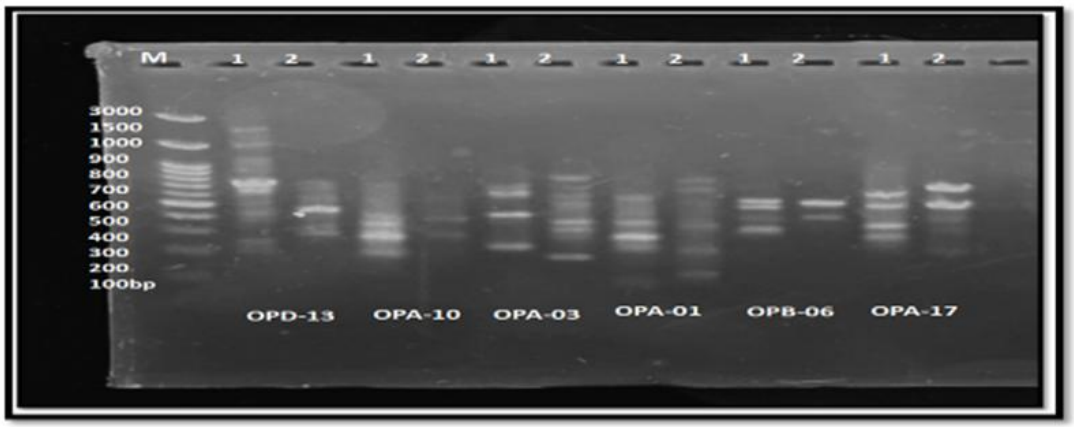

Figure 1. Amplification product of primers OPA-D-13, OPA-10, OPA-03, OPA-01, OPB-06, and OPA-17; M: DNA ladder for 1) *S. officinalis* and 2) *T. vulgaris.*

The RAPD primer OPA-15 produced higher molecular size bands (1198 bp), with the lowest molecular size bands (100 bp) produced by the RAPD primer OPA-01 (Table 2, Figure 1). Molecular size variations among generated bands showed a relation to change in primer annealing sites, which resulted in variations in the distance between two annealing sites of the primer on the DNA template. These variations may be due to an alteration in the DNA sequence resulting from diverse types of mutations such as insertion and deletion (Fadoul *et al*., 2013). The appearance of unique and polymorphic bands indicated these cultivars possess one or more novel sequences, with the same not found in others. Their importance arises from their ability to identify these cultivars and thus can tend to be valuable genetic markers (Vishwanath *et al.,* 2010).

The highest number of main, polymorphic, and amplified bands was 17, produced by the RAPD primer OPD-13 (Table 2, Figure 1). Diverse plant species result in various DNA sequences, and different primer annealing sites result in varied amplicon molecular sizes (Prakash *et al.,* 2011). Recognition of more annealing sites by the primer usually results in the maximum number of main bands, and the same results also emerged in maize (Al-Saadi and Al-Tamimi, 2018) and wheat (Al-Ghufaili, 2017). Primer efficiency and discriminatory values are mostly relevant to each other, since increased polymorphic bands might be due to enhanced discriminatory values of primers (Hunter and Gaston, 1988; Graham and McNicol, 1995).

The highest value produced for polymorphism and efficiency (100% and 1, respectively) came from the RAPD primers OPD-13 and OPA-10 (Table 2, Figure 1). Polymorphism and polymorphic bands connected with each other, since an increase in polymorphic bands could refer to an escalation of polymorphism of primers (Hunter and Gaston, 1988; Graham and McNicol, 1995). An increased binding site of primers consequently boosts the number of amplified bands, resulting in more chances of detecting polymorphism among the genotypes (Roy *et al.,* 1992). One more advantage of the RAPD method is that it has arbitrarily designed primers that can potentially anneal to homologous sequences in the entire genome, providing greater opportunities to uncover various regions (Williams *et al.,* 1990).

The RAPD primer OPA-15 produced the highest number of monomorphic bands (7) (Table 2, Figure 1). The presence of monomorphic bands usually refers to sharing similar sequences between genomes, which are constant and conserved in the genomes (Al-Badeiry, 2013). The appearance of monomorphic bands may refer to the common characters between the studied plant species (Al-Tamimi, 2014). The highest discriminatory value (34.69) surfaced from the RAPD primer OPD-13, and this value could be considerably significant with the primer's ability to produce polymorphic bands (Hunter and Gaston, 1988; Graham and McNicol, 1995).

The RAPD primer OPC-09 produced the lowest numbers of main and amplified bands, OPD-13 and OPA-10 showed no monomorphic bands, and the primers OPA-15, OPA-04, OPC-09, and OPH-01 failed to provide any polymorphic bands. Thus, their polymorphism, efficiency, and discriminatory values were zero (0.0) (Table 2, Figure 1). Despite the low polymorphism shown by the rest of the RAPD primers, these primers were able to amplify more than one band per accession; the residual heterogeneity within the accessions was apparent. Past studies enunciated the RAPD primers reached validation as the best tools to study the genetic diversity in crop plants (Ogunbayo *et al.,* 2005).

SCoT markers

The results exhibited six SCoT primers—SCoT60, SCoT26, SCoT44, SCoT9, SCoT54, and SCoT6—that gave both examined species unique fingerprints, while four SCoT primers (SCoT28, SCoT12, SCoT61, and SCoT30) failed to give the two examined species a unique fingerprint (Table 1). Primers' ability to give unique fingerprints reflects their discriminatory values and capacity to provide polymorphic bands, as shown by SCoT primers SCoT44 and SCOT 54, with an increasing number of amplified bands. The highest discriminatory values might also be in connection with the primers' ability to produce polymorphic bands (Williams *et al.,* 1990; Reddy *et al*., 2002; Tahir, 2014; Al-Haidari and Al-Tamimi, 2023).

Moreover, polymorphic bands typically occurred due to several variations in the structural DNA, such as splits, transpositions, and deletions, resulting in modification of amino acids and hence shaping the proteins (Mondini *et al.,* 2009). A prediction disclosed that the SCoT markers show a connection to functional genes and corresponding characteristics and therefore enable the amplicons' translation to gene target marker systems (Xiong *et al.,* 2011; El-Shaer and Ibrahim, 2021). Higher molecular size bands (2969 bp) materialized from the SCoT primer SCoT 30, while lower molecular size bands (161 bp) resulted from the primer SCoT 60. Variations in primer annealing sites consequently alter the size of the amplified fragment because it could provide the varied distance between two annealing sites of the primer on target DNA (Fadoul *et al*., 2013; Al-Saadi and Al-Tamimi, 2018).

The highest number of main and polymorphic bands and discriminatory value (14, 14, and 27.45, respectively) emerged with the primers SCoT44 and SCoT54 (Table 3, Figures 2 and 3). The higher number of amplified bands (14) was an outcome from the three SCoT primers, SCoT30, SCoT44, and SCoT54. Past studies demonstrated it is also a fact that when a primer showed high amplification, then this may be due to a higher homology between the primer series and plant

genotypes (Verma and Agarwal, 2005). The recognition of more annealing sites by a primer usually results in numerous main bands. The same results have also occurred in previous studies' reports on maize (Al-Saadi and Al-Tamimi, 2018) and wheat (Tahir, 2014; Al-Ghufaili, 2017).

Table 3. Summarized results of SCoT amplification product.

Primers	Main bands	Amplified bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)	Efficiency	Discriminatory Value (%)
SCoT28	3	6	3	0	0	0	0
SCoT60	7	7	0	7	100	1	13.72
SCoT26	6	10	4	2	33.33	0.2	3.92
SCoT12	4	8	4	0	0	0	0
SCoT61	2	4	2	0	0	0	0
SCoT30	7	14	7	0	0	0	0
SCoT44	14	14	0	14	100	1	27.45
SCoT9	9	9	0	9	100	1	17.64
SCoT54	14	14	0	14	100	1	27.45
SCoT6	5	5	0	5	100	1	9.80

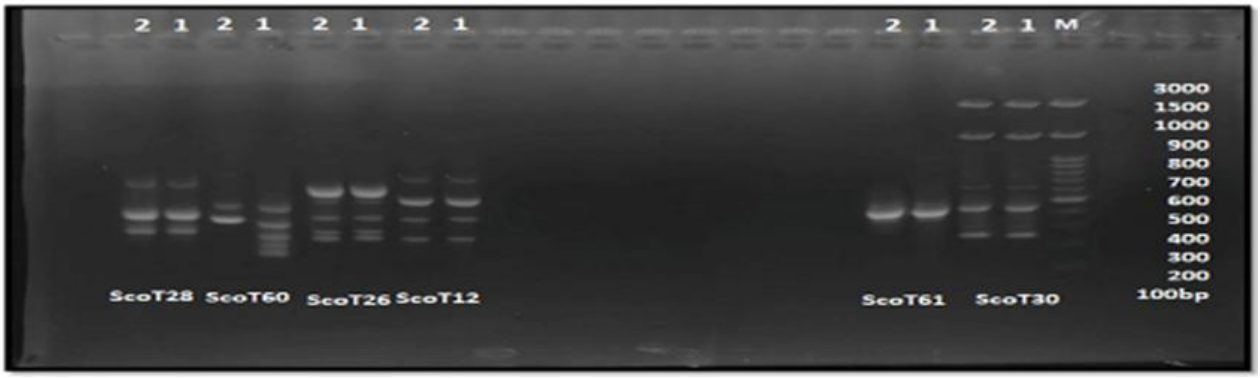

Figure 2. Amplification product of primers ScoT28, ScoT60, ScoT26, ScoT12, ScoT61, and ScoT30; M: DNA ladder for 1) *S. officinalis* and 2) *T. vulgaris*.

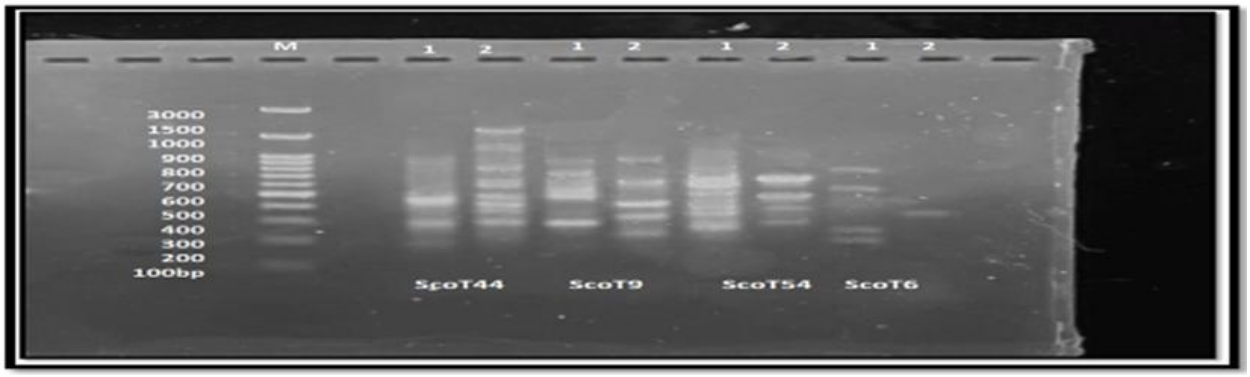

Figure 3. Amplification product of primers ScoT44, ScoT9, ScoT54, and ScoT6; M: DNA ladder for 1) *S. officinalis* and 2) *T. vulgaris.*

The primer SCoT30 produced the maximum monomorphic bands (7). The presence of a monomorphic band usually refers to a genotype that belongs to one species and shares their relatives in some genome sequences that are conserved in the genome (Al-Badeiry, 2013). The appearance of monomorphic bands may refer to a common character among the studied genotypes (Al-Tamimi, 2014). The five SCoT primers, SCoT60, SCoT44, SCoT9, SCoT54, and SCoT6, produced the highest polymorphism and efficiency (100% and 1, respectively). Increased binding sites of primers consequently boost the number of amplified bands, which results in an increased scope of detecting polymorphism among genotypes (Williams *et al*., 1990; Roy *et al.,* 1992). Polymorphism values have considerable connections to the primer's ability to provide polymorphic bands (Hunter and Gaston, 1988; Graham and McNicol, 1995).

ISSR markers

All the ISSR primers (except HBS10, UBC810, and UBC881) showed success in providing unique fingerprints in *Salvia officinalis* L. and *Thymus vulgaris* L. (Table 1). The highest value of molecular size (1225 bp) was evident in the primer UBC820, while the lowest value for molecular size (100 bp) appeared in the three primers UBC810, UBC814, and UBC849. The primers with the highest values of polymorphism generally showed an increasing number of polymorphic bands and increased chances of producing unique fingerprints (Hunter and Gaston, 1988; Graham and McNichol, 1995). Primers that produced the highest polymorphic bands can further serve as polymorphic markers for proving promising identification and genetic purity in crop plants (Pal and Singh, 2013). Polymorphic and unique alleles inside genotypes increase the chances of producing unique fingerprints (Idris *et al.,* 2012).

In ISSR primers, the highest values recorded were for main (8) and polymorphic (7) bands, with a discriminatory value of 17.49 (Table 4). The higher and lower molecular sizes of primers were relevant to the primer sequences annealed with the DNA template (Mahpara *et al.,* 2012). Insertions and deletions could change the size of the amplified products by changing the distance between the annealing sites of primers. The ISSR primer UBC810 produced the most monomorphic bands (5) and amplified bands (10). Monomorphic bands are those sequences that reveal genotypes belonging to one species sharing some genome sequences and differ in others.

The ISSR primers, UBC852, UBC814, UBC820, UBC811, and UBC808, produced the highest polymorphism (100%) and efficiency (1). Most occurrences of higher numbers of main and amplified bands were due to the primer structure, with some primers recognized with more annealing sites, which become much more useful than primers with fewer annealing sites. In this case, the number of amplified bands will be higher, thus giving a

Table 4. Summarized results of ISSR amplification product.

Primers	Main bands	Amplified bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)	Efficiency	Discriminatory Value (%)
UBC852	6	6	0	6	100	1	15.38
HBS10	2	4	2	0	0	0	0
UBC810	5	10	5	0	0	0	0
UBC855	7	9	2	5	71.42	0.55	12.82
UBC814	6	6	0	6	100	1	15.38
UBC820	6	6	0	6	100	1	15.38
UBC849	8	9	1	7	87.5	0.77	17.49
UBC881	3	6	3	0	0	0	0
UBC811	4	4	0	4	100	1	10.25
UBC808	5	5	0	5	100	1	12.82

better chance for detecting DNA polymorphisms among the genotypes (Tahir, 2014). Discriminatory values of primers indicated their ability to provide unique fingerprints (Al-Ghufaili and Al-Tamimi, 2017).

The polymorphism level of the DNA marker is the production power of the DNA band from the PCR reaction using a particular marker. The degree of polymorphism gains influence from various genotypes used and appears to be highly dependent on primers that recognize their complementary DNA sequence in the DNA template. Markers used for PCR reactions and electrophoresis showed the same banding pattern between each DNA sample of different genetic monomorphic markers. However, if the electrophoresis shows different banding patterns between each DNA sample, then it is a polymorphic marker (Dalimunthe *et al.,* 2020).

CONCLUSIONS

With different DNA marker systems targeting different regions, mixed multiple marker systems should provide more complete genome information, which is in agreement with the results obtained using the combined data set in this study based on sage (*S. officinalis* L.) and thyme (*T. vulgaris* L.). Variations in RAPD, SCoT, and ISSR markers implied a common relation to variations in their specificity and distribution along the genome. The distribution of RAPD and ISSR sequences along the genome reflects an increasing number of amplified and main DNA bands.

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