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DISCRIMINATING LAMIACEAE SPECIES FROM SAUDI ARABIA USING ALLOZYME AND SPECIFIC DNA MARKERS

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Abstract

For preliminary characterization and discrimination among four wild and three cultivated economic species of family Lamiaceae scattered in Taif governorate of Saudi Arabia, different molecular approaches were used. Nineteen loci from five enzyme systems were determined: twelve were monomorphic and the other (ADH-1, MDH-2, α-EST-4, α-EST-5, α-EST-6, α-EST-7 and PRX-2) were polymorphic in at least one species. The estimated observed heterozygosities were higher than those of the expected in 6 species. The negative values of the inbreeding coefficient referred to an excess in heterozygosity in the 6 species indicating some tendency to outcrossing selection against homozygosity. The UPGMA dendrogram grouped all individuals of the same species together revealing lower genetic diversity within each species. The seven DNA primers generated 16 bands of which 14 were polymorphic with polymorphism percentage ranging between 0.00 to 100% indicating a high level of polymorphism. The eleven unique bands identified were stable and specific for the four species of the two genera; *Mentha* and *Lavendula.* Results can be used for the DNA barcoding approaches and subsequently the conservation of these Saudi Arabian plant resources.

Key words: Lamiaceae, Allozyme, Heterozygosity, DNA barcoding, SSR.

Introduction

Lamiaceae (the mint family) is represented in the Saudi Arabia flora by 76 species; of them 23 are medicinal plants (Collenette, 1999). These species are used for medicinal, flavoring and food preservation purposes. Due to the presence of many secondary metabolites, they show anticancer, antioxidant, antiviral and antibacterial activities (Carović-Stanko *et al*., 2016). Limited work has been done on the characterization and discrimination among Lamiaceae species found in Saudi Arabia using allozyme and other molecular markers.

Allozyme electrophoresis was conducted to assess the genetic diversity and the population structure in *Ocimum* (Mustafa *et al*., 2006), *Thymus* (Lopez-Pujol *et al*., 2004; Ali *et al*., 2012), *Lavandula multifida* (Chograni *et al*., 2012), *Rosmarinus officinalis* (Zaouali *et al*., 2012). Also, several molecular markers were used in different researches related to Lamiaceae taxa: RAPD (Chograni & Boussaid, 2010; Shinwari *et al*., 2011), ISSR (Rodrigues *et al*., 2013; Safaei *et al*., 2016), SSR (Radosavljević *et al*., 2012), ESTs (Karaca *et al*., 2013), DNA barcode genes (*rbcL, matK* and *trnH-psbA*) (De Mattia *et al*., 2011; Zahra *et al*., 2016; Zahra & Shinwari, 2016), regions of chloroplast DNA (Jabeen *et al*., 2012) and nrDNA ITS sequences (Özcan *et al*., 2015).

Generally, molecular markers play an important role in identification, detecting variability, discriminating among wild and cultivated species and populations of family Lamiaceae around the world. As a preliminary study, our present work aims to characterize and discriminate among wild and cultivated species of family Lamiaceae scattered in Taif governorate of Saudi Arabia depending upon different allozyme loci, one RAPD primer, three SSR primers and three DNA specific barcode primers (NY, *matK*-KIM and *matK*-pK).

Materials and Methods

Plant material: Fresh leaves of 42 individuals of 3 cultivars and 4 wild species, varying from 4 to 7 per species, belonging to family Lamiaceae were collected from Taif highlands of Saudi Arabia. The collected plant material was indentified and named according to Collenette (1999). The principal characteristics of their sites are summarized in Table 1.

Table 1. Names and sources of seven species of Lamiaceae.

No.	Species	Type	Geographic origin	Coordinates	
				Latitude (N)	Longitude (E)
1.	*Lavandula dentate* L.	Wild	Ash Shafa	21 05 55	40 20 34
2.	*Lavandula pubescens* Decne.	Wild	Ash Shafa	21 05 55	40 20 34
3.	*Mentha longifolia* L.	Wild	Ash Shafa	21 04 47	40 23 03
4.	*Mentha viridis* L.	Cultivated	Ta'if city	21 16 00	40 25 00
5.	*Plectaranthus comosus* Sims.	Cultivated	Al-Hawiyya	21 16 00	40 25 00
6.	*Rosmarinus officinalis* L.	Cultivated	Al-Hawiyya	21 16 00	40 25 00
7.	*Otostegia fruticosa* Forssk.	Wild	Ash Shafa	21 05 54	40 20 35

Isozyme electrophoresis: The investigated isozymes were: alcohol dehydrogenase (ADH); (E.C. 1.1.1.1), α- and β-esterases (EST); (E.C.3.1.1.1), malate dehydrogenase (MDH); (E.C.1.1.1.37) and peroxidase (PRX); (E.C.1.11.1.7). For their extraction, 1 g of fresh leaves was homogenized in 1 ml extraction buffer (1 M Tris-HCl, pH 8.8). The homogenate was centrifuged at 10000 rpm for 5 min and the clear supernatant was kept at –20ºC. According to Stegemann *et al*., (1985), the supernatant of each sample was separated by 10% Native-polyacrylamide gel electrophoresis method. ADH, MDH, PRX and α-& β-EST gels were stained according to protocols of Weeden & Wendel (1990), Jonathan & Wendell (1990), Heldt (1997) and Scandalios (1964), respectively.

DNA isolation and amplification: Fresh leaves (250 mg) belonging to four species; *Larandula dentate* L. *pubescens, Mentha longifolia* and *M. viridis* were used for DNA isolation using CTAB method (Doyle & Doyle, 1987). To obtain different DNA markers, each species was analyzed with one random primer (RAPD), three specific SSR primers and three coding DNA genomic regions belonging to *matK* (*matK*-KIM, *matK*-pK and NY) (Table 4). These well-known universal primers were produced by Macrogen Inc. (Seoul, Korea). A PCR amplification for each sample was done with a 25 µL total reaction/sample that included 10 µL Taq Master Mix, 1 µL each, forward and reverse primers, and 1 µL DNA. Thermal cycling was done on a Techne TC-3000 (Barloworld Scientific, Ltd. Staffordshire, UK) with the following program: 105°C heated lid, initial denaturation of 94°C for 5 min, and 35 cycles (1 min at 94°C), annealing (30 s at different temperatures) and extension (1 min at 72°C) and a final extension at 72°C for 7 min. Primers details and annealing temperatures are provided in Table 4.

Statistical analysis: The genetic variability within the seven species of Lamiaceae and the inbreeding coefficient; Wright's *F* [$F = (1 - H_o/H_e)$] were analyzed by the parameters that described by Hamrick & Godt (1989) and NEI (1973) and illustrated in Table 3. Levels of statistical significance for each parameter were determined by t-test (Varghese *et al*., 1999). For further clarification of within species genetic variability, each band coded as 1 or 0 for its presence or absence, respectively. Cluster analysis was performed using UPGMA procedure and represented in a phenogram by using SAHN and TREE modules, respectively depending on NTSYS-pc 2.2 program (Rohlf, 1998). For DNA analysis, The characteristic bands of RAPD, SSR and *matK* patterns were estimated by comparing with 100bp DNA ladder (Cleaver Scientific Ltd, UK) using gel analyzer program (version 3).

Results and Discussion

Results in this research represented the first use of different molecular markers to characterize the interspecific genetic variability and discriminating among some species of Lamiaceae in Saudi Arabian flora.

A total of 26 alleles were scored among the 19 loci from 5 isozymes investigated (Fig. 1). Their frequencies are illustrated in Table 2. Twelve loci (MDH-1, PRX-1, α-EST-1, α-EST-2, α-EST-3, β-EST-1, β-EST-2, β-EST-3, β-EST-4, β-EST-5, β-EST-6 and β-EST-7) were monomorphic, having one allele, in the seven species, whereas seven loci having two alleles; ADH-1, MDH-2, α-EST-4, α-EST-5, α-EST-6, α-EST-7 and PRX-2, were considered as polymorphic in at least one species. The locus MDH-1 was detected in all species. Loci α-EST-2, α-EST-3, β-EST-2 and β-EST-4 distinguished *L. dentata* from *L. pubescens* that characterized by only two loci; MDH-2 and PRX-1. On the other hand, 6 loci; MDH-2, α-EST-2, α-EST-3, β-EST-1, β-EST-2 and β-EST-3; discriminated between *M. longifolia* and *M. viridis* (Table 5).

The parameters of genetic variation within species are shown in Table 3. *A* ranged from 1.00 in *L. dentata* to 1.42 in *P. comosus* with a mean of 1.24 alleles per locus, whereas the $A_p$mean was 1.81, ranging from 1.00 in *L. dentata* to 2.00 in 5 species. Plectranthus ranged from 0.00% in *L. dentata* to 41.7% in *P. comosus* with a mean of 23.9%. H_o ranged from 0.00 in *L. dentata* to 1.00 in 5 species with a mean of 0.797, whereas the mean of H_e was 0.412. Obviously, the observed heterozygosities were higher than those of the expected in 6 species. Similar results were exactly obtained in *Thymus loscosii* and *Lavandula multifida* populations (Lopez-pujol *et al*., 2004; Chograni *et al*., 2008).

The inbreeding coefficient (*F)* values in all species were lower than zero except *L. dentata* (Table 3). These values ranged from –1.00 to 0.00 with a mean of –0.787. The negative values referred to an excess in heterozygosity in the 6 species that could be due to some tendency to out crossing selection against homozygosity enhancing higher heterozygosity or random events in their environment. The increased heterozygosity was also reported in *Rosmarinus officinalis, Lavandula stoechas* and *Lavandula multifida* (Zaoual & Boussaid, 2008; Chograni *et al*., 2008, 2013). The levels of inbreeding coefficient (*F*) have a tendency to decrease in self-compatible species (Leimu *et al*., 2006).

The UPGMA dendrogram obtained depending on the 29 isozyme bands scored in the 42 individuals showed little variability within species examined (Fig. 2). At coefficient 0.70, seven groups were formed and corresponded to the seven species. All individuals of the same species grouped together presenting higher similarity values among individuals revealing the lower genetic diversity within each species. The decrease in genetic variation within species may be due to two reasons: investigating small number of individuals for each species and the self-compatibility of Lamiaceae species. This result was in accordance with those of Leimu *et al*., (2006) and Owens & Ubera-Jiménez (1992). Hamrick & Godt (1989) concluded that the genetic variation stayed largely among populations not within them in self-compatible species.

Table 2. Allele frequencies of different gene loci influencing isozyme patterns detected in seven species of Lamiaceae, (N) represents the number of individuals examined.

Locus	Allele	*L. dentata*		*L. pubescens*		*M. longifolia*		*M. viridis*		*P. comosus*		*R. officinalis*		*O. fruticosa*	
		N	Freq.	N	Freq.	N	Freq.	N	Freq.	N	Freq.	N	Freq.	N	Freq.
ADH-1	a	7	1.00	7	1.00	7	0.5	7	0.5	0	0.00	0	0.00	0	0.00
	b		0.00		0.00		0.5		0.5		0.00		0.00		0.00
MDH-1	a	7	1.00	7	1.00	7	1.00	7	1.00	4	1.00	4	1.00	6	1.00
MDH-2	a	0	0.00	7	0.5	7	0.5	0	0.00	0	0.00	4	0.5	0	0.00
	b		0.00		0.5		0.5		0.00		0.00		0.5		0.00
PRX-1	a	0	0.00	7	1.00	0	0.00	0	0.00	2	1.00	0	0.00	6	1.00
α-EST-1	a	7	1.00	7	1.00	0	0.00	7	1.00	4	1.00	0	0.00	0	0.00
α-EST-2	a	7	1.00	0	0.00	5	1.00	0	0.00	4	1.00	4	1.00	6	1.00
α-EST-3	a	2	1.00	0	0.00	3	1.00	0	0.00	0	0.00	0	0.00	0	0.00
α-EST-4	a	0	0.00	0	0.00	0	0.00	0	0.00	4	0.5	0	0.00	0	0.00
	b		0.00		0.00		0.00		0.00		0.5		0.00		0.00
α-EST-5	a	0	0.00	0	0.00	0	0.00	0	0.00	4	0.5	4	1.00	0	0.00
	b		0.00		0.00		0.00		0.00		0.5		0.00		0.00
α-EST-6	a	0	0.00	0	0.00	0	0.00	0	0.00	4	0.5	4	1.00	0	0.00
	b		0.00		0.00		0.00		0.00		0.5		0.00		0.00
α-EST-7	a	0	0.00	0	0.00	0	0.00	0	0.00	4	0.5	4	0.5	6	0.17
	b		0.00		0.00		0.00		0.00		0.5		0.5		0.83
β-EST-1	a	7	1.00	7	1.00	7	1.00	0	0.00	4	1.00	4	1.00	0	0.00
β-EST-2	a	7	1.00	0	0.00	4	1.00	0	0.00	0	0.00	0	0.00	6	1.00
β-EST-3	a	0	0.00	0	0.00	3	1.00	0	0.00	0	0.00	0	0.00	0	0.00
β-EST-4	a	3	1.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
β-EST-5	a	0	0.00	0	0.00	0	0.00	0	0.00	4	1.00	4	1.00	0	0.00
β-EST-6	a	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	2	1.00
β-EST-7	a	0	0.00	0	0.00	0	0.00	0	0.00	4	1.00	4	1.00	0	0.00
β-EST-8	a	0	0.00	0	0.00	0	0.00	0	0.00	4	0.5	0	0.00	5	0.42
	b		0.00		0.00		0.00		0.00		0.5		0.00		0.58

Table 3. Estimates of genetic variability and the fixation index (inbreeding coefficient) *F* in seven species of Lamiaceae.

Species	P	A	A_e	H_o	H_e	F
L. dentata	0.00	1.000	1.000	0.000	0.000	0.000
L. pubescens	16.7	1.167	2.000	1.000	0.500	-1.000
M. longifolia	25.0	1.250	2.000	1.000	0.500	-1.000
M. viridis	33.3	1.333	2.000	1.000	0.500	-1.000
P. comosus	41.7	1.417	2.000	1.000	0.500	-1.000
R. officinalis	22.2	1.222	2.000	1.000	0.500	-1.000
O. fruticosa	28.6	1.286	1.673	0.580	0.385	-0.506
Mean	23.9	1.24	1.81	0.797	0.412	-0.787
SE	13.26	0.133	0.378	0.385	0.187	0.393
t-value	4.776***	24.73***	12.69***	5.481***	5.84***	-5.30***

p (<0.99) percentage of polymorphic loci; A average number of alleles per locus; A_e average effective number of alleles per locus; H_o observed heterozygosity; H_e expected heterozygosity under Hardy–Weinberg equilibrium; F Fixation Index (Inbreeding Coefficient); SE the standard error. *** $p < 0.005$.

Table 4. DNA primers used in the study.

Primercode	Sequence (5'-3')	Anneal temp.	Bands no.	Polymorphic bands	Polymorphism (%)
RAPD	TGCCGAGCTG	36˚C	5	5	100
SSR 1-F	GAAACACTCACAGCGAGAGC	50˚C	1	0	0
SSR 1-R	CCTCCATTCACACTCCCCTA				
SSR 2-F	TGTGGGCTGGTGATAGATGT	50˚C	4	4	100
SSR 2-R	GCTTCATCCCACGGACTA				
SSR 3-F	AGACGTTATTTGGAGCAGCA	50˚C	1	0	0
SSR 3-R	TCTCGGATCAACATGAGCTG				
NY-552-F	CTGGATYCAAGATGCTCCTT	52˚C	1	1	100
NY-1150-R	GGTCTTTGAGAAGAACGGAGA				
matK-KIM3-F	CGTACAGTACTTTTGTGTTTACGAG	52˚C	2	2	100
matK-KIM1-R	ACCCAGTCCATCTGGAAATCTTGGTTC				
matK-pk4-F	CCCTATTCTATTCAYCCNGA	52˚C	2	2	100
matK-pk1-R	CGTATCGTGCTTTTRTGYTT				
Total			16	14	87.5

Table 5. Allozyme loci and molecular size (bp) of characteristic DNA bands discriminating among *L. dentata, L. pubescens, M. longifolia* and *M. viridis*.

Marker system		*L. dentata*	*L. pubescens*	*M. lonifolia*	*M. viridis*
Allozyme	MDH	-	MDH-2	MDH-2	-
	PRX	-	PRX-1	-	-
	α-EST	α-EST-2, α-EST-3	-	α-EST-2, α-EST-3	-
	β-EST	β-EST-2, β-EST-4	-	β-EST-1, β-EST-2, β-EST-3	-
DNA	RAPD	-	-	408.8, 300.6	596.0, 354.1, 242.9
	SSR 2	-	147.0	391.5	-
	NY	-	-	-	579.1
	matK-KIM	863.6	-	230.3	-
	matK-pk	-	-	-	283.3 ,179.2

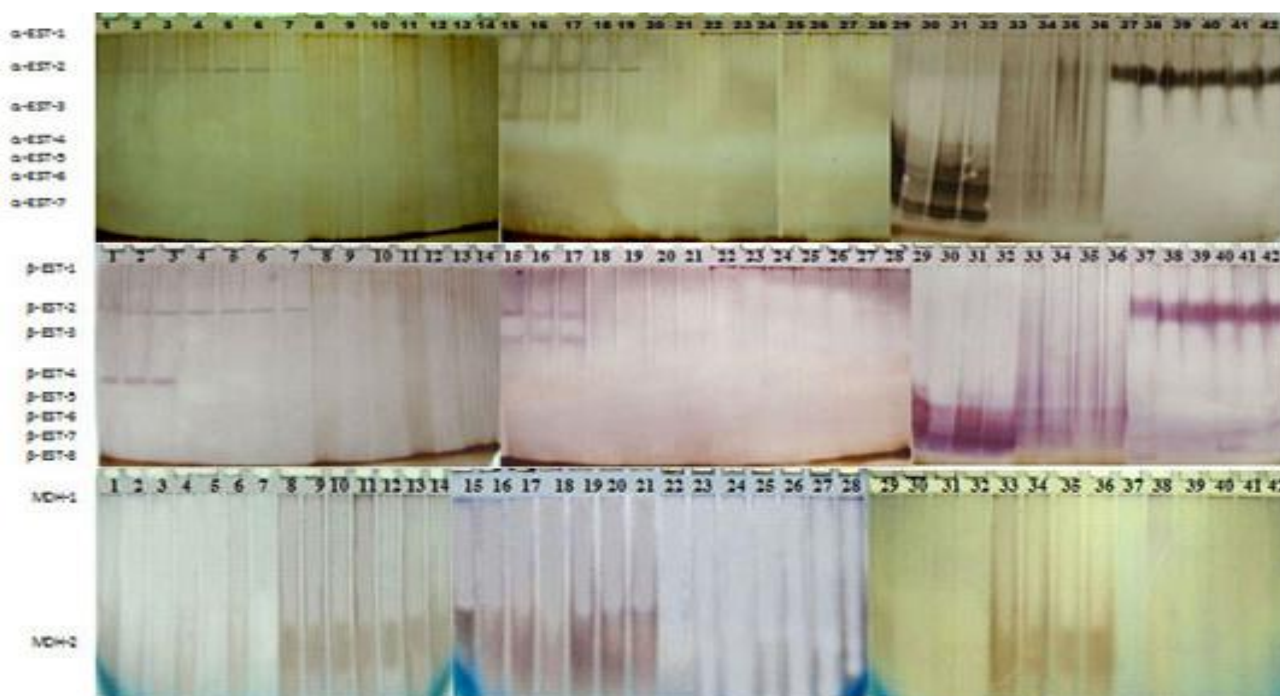

Fig. 1. Zymograms of 42 individuals belonging to seven Lamiaceae species using three isozymes. (1-7) *L. dentata,* (8-14) *L. pubescens,* (15-21*) M. longifolia,* (22-28) *M. viridis,* (29-32) *P. comosus,* (33-36) *R. officinalis,* (37-42) *O. fruticosa.*

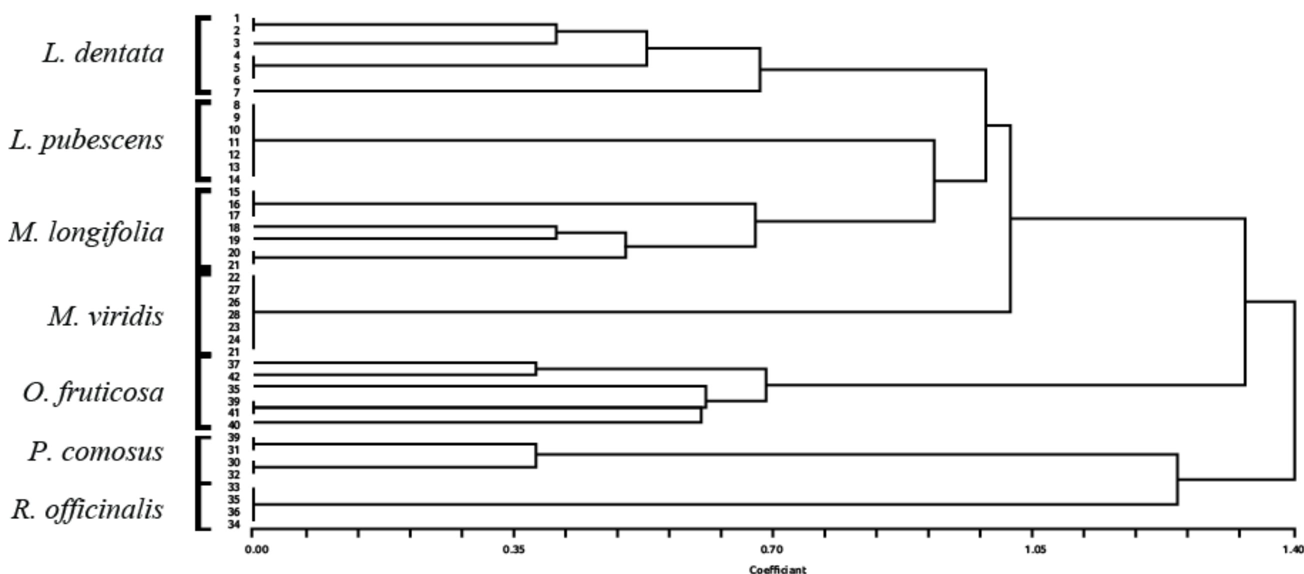

Fig. 2. UPGMA phenogram showing genetic relationships among seven Lamiaceae species depending on allozyme data.

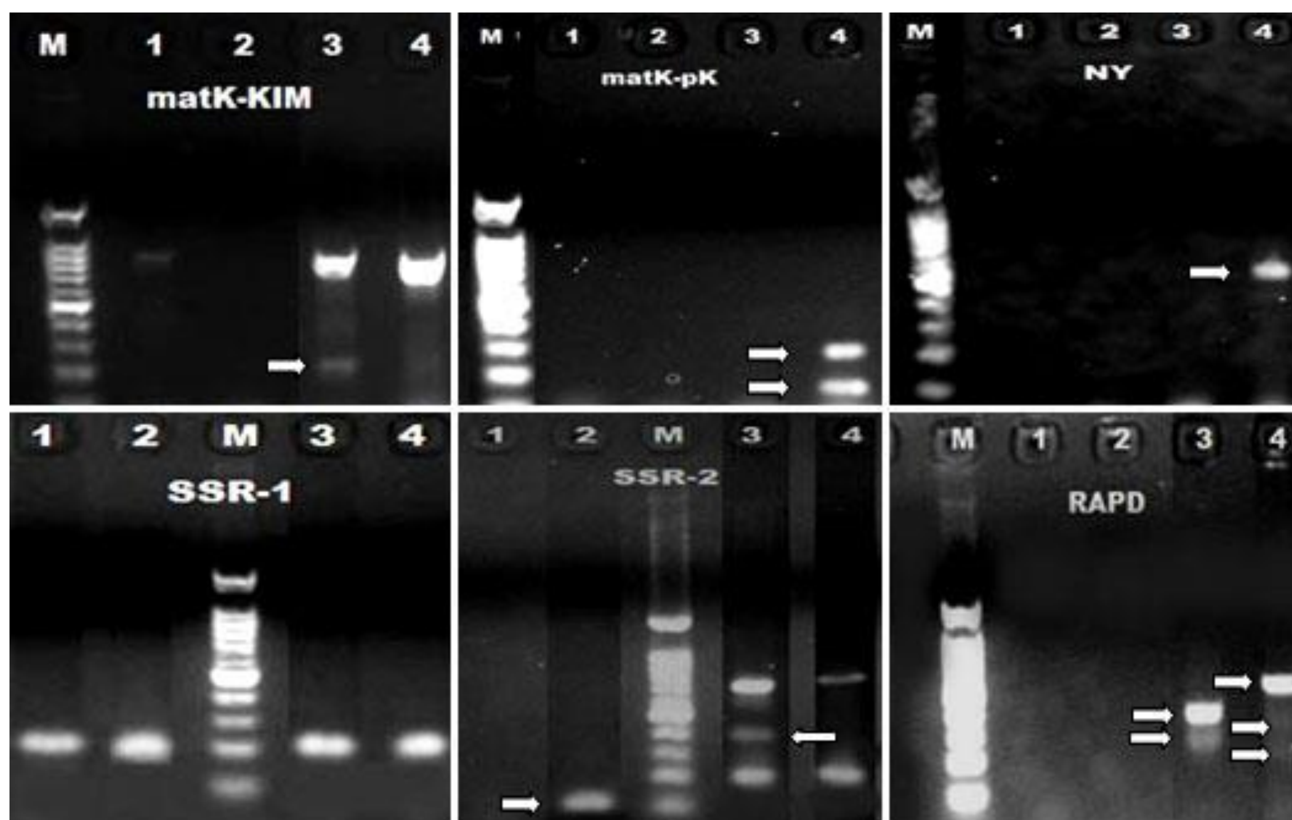

Fig. 3. DNA patterns of four Lamiaceae species. (M) marker, (1) *L. dentata,* (2) *L. pubescens,* (3) *M. longifolia,* (4) *M. viridis.* Arrows indicate characteristic bands.

A diverse level of polymorphism with DNA markers in different Lamiaceae taxa and their populations has been reported earlier such as *Teucrium polium* (Boulila *et al*., 2010), *Rosmarinus officinalis* (Zaouali *et al*., 2012), two *Thymus* species (Ali *et al*., 2012), *Mentha cervina* (Rodrigues *et al*., 2013), *Salvia officinalis* (Hao *et al*., 2015), *Micromeria* (Puppo *et al*., 2016) through using various molecular techniques. For that, DNA from the 4 species; *L. dentata, L. pubescens, M. longifolia* and *M.viridis* was studied with one oligonucleotide RAPD primer, three microsatellite SSR primer pairs and three coding DNA genomic regions (*matK*-KIM, *matK*-pK and NY) for the plastid gene Maturase K. The primers sequences, number of bands and molecular size of characteristic DNA bands discriminating among the two species belonging to each of genera *Lavendula* and *Mentha* are shown in Tables 4 and 5 and Fig. 2. Only two common bands were observed in the 4 species by two SSR primers. The 7 primers generated 16 bands of which 14 were polymorphic with polymorphism percentage ranging between 0.00 to 100% indicating a high level of polymorphism (Table 4). Our results were similar to those of Al-Rawashdeh (2011) who reported a unique DNA sequence for *Mentha spicata, Mentha longifolia* and *Ziziphora tenuior* using RAPD markers and differed from those scored by Jabeen *et al*., (2012) and Gobert *et al*., (2002) who showed high similarity coefficient between *M. spicata* and *M. longifolia* using three chloroplast genes (*rbs*).

In addition to polymorphism, 11 unique bands were identified in *L. dentata, M. longifolia and M. viridis* (Table 5). Bands of RAPD, SSR-2, *matK*-pk and NY distinguish the two species of genus *Mentha* from those of genus *Lavendula*. Ten unique bands discriminated between *M. longifolia* and *M. viridis*. On the other hand, only two bands; in *matK*-KIM and SSR-2, could differentiate between *L. dentata* and *L. pubescens* (Table 5 and Fig. 3). De Mattia *et al*., (2011) identified and discriminated six Lamiaceae genera by four barcoding loci (*rpoB, rbcL, matK* and *trnH-psbA*) and their results suggested that *matK* was suitable marker for the identification of species. Similar study was conducted by Zahra *et al*., (2016) for 32 herbal medicinal products of Lamiaceae suggesting *matK* as the best barcode. The unique bands were stable and specific for these species and thus could be considered as markers and used for their characterization. Except polymorphic bands, no reports of unique bands were available for the three species (*L. dentata, M. longifolia and M. viridis*) (Table 5), therefore, this is the first report about unique bands for these species from Saudi Arabia.

Conclusion

In our preliminary investigation, high differentiation among seven Lamiaceae taxa was revealed by all parameters of markers reflecting the efficiency of them in the identification and discrimination among and within species of Lamiaceae. The purpose of our future research is to evaluate the possibility of DNA barcoding approaches to reach an unambiguous identification of these important aromatic Lamiaceae species. This will give us new prospects for more characterization that will be useful not only for taxonomic purposes within family Lamiaceae, but also for the evolution and conservation of these Saudi Arabian plant resources.

Conflict of interests

The authors have not declared any conflict of interests.

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References

Ali, I.B., A. Guetat and M. Boussaid. 2012. Inter-specific relationships among two Tunisian *Thymus* taxa: *Thymus capitatus* Hoffm. et Link. and *Thymus algeriensis* Boiss. et Reut. using molecular markers. *Afr. J. Biotech*., 11(36): 8810-8819.

Al-Rawashdeh, I.M. 2011. Molecular taxonomy among *Mentha spicata, Mentha longifolia* and *Ziziphora tenuior* populations using the RAPD technique. *J. Biol. Sci.,* 4(2): 63- 70.

Boulila, A., A. Be´jaoui, C. Messaoud and M. Boussaid. 2010. Genetic diversity and population structure of *Teucrium polium* (Lamiaceae) in Tunisia. *Biochem. Genet.*, 48: 57-70.

Carović-Stanko, K., M. Petek, M. Grdiša, J. Pintar, D. Bedeković, H. Ćustić and Z. Satovic. 2016. Medicinal plants of the family Lamiaceae as functional foods – a Review. *Czech J. Food Sci.*, 34(5): 377-390.

Chograni, H. and M. Boussaid. 2010. Genetic diversity of *Lavandula multifida* L. (Lamiaceae) in Tunisia: implication for conservation. *Afr. J. Ecol*., 49: 10-20.

Chograni, H., C. Messaoud and M. Boussaid. 2008. Genetic diversity and population structure in Tunisian *Lavandula stoechas* L. and *Lavandula multifida* L. (Lamiaceae). *Biochem. Syst. Ecol*., 36: 349-359.

Chograni, H., C. Messaoud and M. Boussaid. 2013. Genetic diversity of natural Tunisian *Lavandula multifida* L. (Lamiaceae) populations assessed by allozymes and random amplification of polymorphic DNA (RAPD). *Afr. J. Biotech*., 12(7): 648-657.

Chograni, H., Y. Zaouali and M. Boussaid. 2012. Genetic diversity and chemical polymorphism of Tunisian *Lavandula multifida* L. (Lamiaceae) populations. *Afr. J. Biotechnol.,* 11(83): 14858-14867.

Collenette, S. 1999. *A Checklist of Botanical Species in Saudi Arabia*. International Asclepiad Society, Burgess Hill, England.

De Mattia, F., I. Bruni, A. Galimberti, F. Cattaneo, M. Casiraghi and M. Labra. 2011. A comparative study of different DNA barcoding markers for the identification of some members of Lamiacaea. *Food Res. Inter.,* 44: 693-702.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull*., 19: 11-15.

Gobert, V., S. Moja, M. Colson and P. Taberlet. 2002. Hybridization in the section *Mentha* (Lamiaceae) inferred from AFLP markers. *Am. J. Bot*., 89(12): 2017-2023.

Hamrick, J.L. and M.J.W. Godt. 1989. Allozyme diversity in plant species. In: *Plant Population Genetics, Breeding, and Genetic Resources*. (Eds.): Brown, H.D., M.T. Clegg, A.L. Kahler and B.S. Weir. Sinauer Associates, Sunderland, Massachusetts, pp. 43-63.

Hao, D., S. Vautrin, C. Song, Y.J. Zhu, H. Berges, C. Sun and L. Shi Chen. 2015. The first insight into the S*alvia* (lamiaceae) genome via bac library construction and high-throughput sequencing of target bac clones. *Pak. J. Bot*., 47(4): 1347-1357.

Heldt, W.H. 1997. A leaf cell consists of several metabolic compartments. *Plant Biochemistry and Molecular Biology*. Oxford Univ. Press, Oxford.

Jabeen, A., B. Guo, B. Abbasi, Z. Shinwari and T. Mahmood. 2012. Phylogenetics of selected *Mentha* species on the basis of *rps8, rps11* and *rps14* chloroplast genes. *J. Med. Plants Res*., 6(1): 30-36.

Jonathan, F.W. and N.F. Wendell. 1990. Visualization and interpretation of plant allozyme. In: *Allozymes in Plant Biology*. (Eds.): D.E. Sdtis and P.S. Sottis, Champan and Hall Press, London, pp. 5-45

Karaca, M., A. Ince, A. Aydina and S. Ay. 2013. Cross-genera transferable e-microsatellite markers for 12 genera of the Lamiaceae family. *J. Sci. Food Agric*., 93: 1869-1879.

Leimu, R., P. Mutikainen, J. Koricheva and M. Fischer. 2006. How general are positive relationships between plant population size, fitness and genetic variation? *J. Ecol*., 94: 942-952.

Lopez-Pujol, J., M. Bosch, J. Simon and C. Blanche. 2004. Allozyme diversity in the tetraploid endemic *Thymus loscosii* (Lamiaceae). *Ann. Bot.*, 93: 323-332.

Mustafa, A.M.A., A. Badr, M.A. El-Galaly, A.A. Mobarak and M.G. Hassan. 2006. Genetic Diversity among *Ocimum* populations in Egypt as reflected by morphological, seed proteins and isozyme polymorphism. *Int. J. Bot*., 2 (3): 261-269.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci*., 70: 3321-3323.

Owens, S.J. and J.L. Ubera-Jiménez. 1992. Breeding systems in Labiatae. In: (Eds.): Harley, R.M. & T. Reynolds. Advances in Labiate science. Kew, Royal Botanic Gardens, pp. 257-280.

Özcan, T., T. Dirmenci, F. Coşkun, E. Akçiçek and Ö. Güner. 2015. A new species of *Teucrium* sect. scordium (Lamiaceae) from SE of Turkey. *Turk. J. Bot*., 39: 310-317.

Puppo, P., M. Curto and H. Meimberg. 2016. Genetic structure of *Micromeria* (Lamiaceae) in Tenerife, the imprint of geological history and hybridization on within-island diversification. *Ecol. Evol.*, 6(11): 3443-3460.

Radosavljević, I., Z. Satovic, J. Jakse, B. Javornik, D. Greguraš, M. Jug-Dujaković and Z. Liber. 2012. Development of new microsatellite markers for *Salvia officinalis* L. and its potential use in conservation-genetic studies of narrow endemic *Salvia brachyodon* Vandas. *Int. J. Mol. Sci*., 13: 12082-12093.

Rodrigues, L., C. Berg, O. Póvoa and A. Monteiro. 2013. Low genetic diversity and significant structuring in the endangered *Mentha cervina* populations and its implications for conservation. *Biochem. Syst. Ecol.,* 50: 51-61.

Rohlf, F.J. 1998. *NTSYSpc: Numerical Taxonomy and Multivariate Analysis System*, version 2.02. Exeter Software, New York.

Safaei, M., M. Sheidai, B. Alijanpoor and Z. Noormohammadi. 2016. Species delimitation and genetic diversity analysis in *Salvia* with the use of ISSR molecular markers. *Acta Bot. Croat*., 75(1): 45-52.

Scandalios, J.C. 1964. Tissue-specific allozyme variations in maize. *J. Hered*., 55: 281-285.

Shinwari, Z.K., S. Sultan and T. Mehmood. 2011. Molecular and morphological characterization of selected *Mentha* species. *Pak. J. Bot*., 43(3): 1433-1436.

Stegemann, H., A.M.R. Afifiy and K.R.F. Hussein. 1985. Cultivar Identification of dates (*Phoenix dectylifera*) by protein patterns. *2nd International Symposium of Biochemical Approaches to Identification of Cultivars*. Braunschweig, West Germany, pp 44.

Varghese, M., M.A. Edwards and J.L. Hamrick. 1999. Genetic variation within two subspecies of *Acacia nilotica*. *Forest Genet*., 6(4): 221-228.

Weeden, N.F. and J.F. Wendel. 1990. Genetics of plant isozymes. In: (Eds.): Soltis, D.E. and P.S. Soltis. *Isozymes in plant biology*. Chapman and Hall Press, London, pp. 46-72.

Wendel, J.F. and N.F. Weeden. 1989. Visualization and interpretation of plant allozymes. In:D.E. Soltis and P.S. Soltis (Eds.). *Allozymes in plant biology, Advances in plant sciences, series 4*. Dioscorides Press, Portland, OR, pp. 5-45.

Zahra, N.B. and Z.K. Shinwari. 2016. What is done and what has to be done in Lamiaceae, a review of phylogenetics. PeerJ Preprints 4:e2277v1 https://doi.org/10.7287/peerj.preprints.2277v1.

Zahra, N.B., Z.K. Shinwari and M. Qaiser. 2016. DNA Barcoding: a tool for standardization of herbal medicinal products (HMPs) of Lamiaceae from Pakistan. *Pak. J. Bot*., 48(5): 2167-2174.

Zaouali, Y. and M. Boussaid. 2008. Isozyme markers and volatiles in Tunisian *Rosmarinus officinalis* L. (Lamiaceae): A comparative analysis of population structure. *Biochem. Syst. Ecol*., 36: 11-21.

Zaouali, Y., H. Chograni, R. Trimech and M. Boussaid. 2012. Genetic diversity and population structure among *Rosmarinus officinalis* L. (Lamiaceae) varieties: var. *typicus* Batt. and var. *troglodytorum* Maire. based on multiple traits. *Indust. Crops Prod*., 38: 166-176.

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