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GENETIC ANALYSIS OF *PLECTRANTHUS* L. (LAMIACEAE) IN SAUDI ARABIA BASED ON RAPD AND ISSR MARKERS

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Abstract

The genetic diversity and phylogenetic analyses of seven species of the genus *Plectranthus* (Lamiaceae) from Saudi Arabia were carried out by using the Inter Simple Sequence Repeats (ISSR), Randomly Amplified Polymorphic DNA (RAPD), and combined ISSR and RAPD markers. Ten RAPD primers and five ISSR primers generated 137 polymorphic amplified fragments, which pointed a relatively high level of genetic variation in *Plectranthus*. RAPD markers revealed a higher level of polymorphism (105 bands) than ISSR (32 bands). The clustering of genotypes within groups showed difference upon comparison of RAPD and ISSR derived dendrograms. We could identify four clades within *Plectranthus*, which are largely in support, with a bit contradiction, of traditional groupings. Taxonomic and phylogenetic implications are discussed in comparison with the available gross morphological, anatomical, and phytochemical data. The results of this study present useful data for assessing the taxonomy of *Plectranthus* both at subgeneric and sectional levels. Moreover, our results indicate some level of resemblance among the species of subgenus Germanea and support the monophyly of this subgenus. The most interesting outcome of this analysis was identifying *P. arabicus* with distinguishing characters and suggesting that it should be treated as a distinct subgenus. In the same vein, distinguishing differences between the closely related endemic species *P. asirensis* and *P. hijazensis* were also noted suggesting that they should be placed in different subgenus. Similarly, *P. asirensis* and *P. cylindraceus* should be placed under a monophyletic group and this shows some closeness with *P. tenuiflorus*.

Key words: *Plectranthus*; Genetic diversity; ISSR; RAPD; Lamiaceae; Taxonomy.

Introduction

Lamiaceae is a large family, which is widely spread and adapted to nearly all habitats and altitudes. The genus *Plectranthus* is one of the largest genera of Lamiaceae, belonging to the subfamily Nepetoideae, tribe Ocimeae, and subtribe Plectranthinae. It comprises about 300 species distributed in both tropical and warm regions of the Old World (Codd, 1985; Retief, 2000). *Plectranthus* itself is taxonomically problematic due to the taxonomic similarities between the species. Several terminologies are used to refer to the same species of *Plectranthus* genus, which makes difficult the compilation of the information about the ethnobotanic use of this genus. Bentham (1832, 1848) monographed the species of *Plectranthus*, and divided *Plectranthus* into seven sections: *Germanea* (Lam.) Benth., *Coleoides* Benth., *Heterocylix* Benth., *Melissoides* Benth., *Isodon* Schrad. Ex Benth. *Pyramidium* Benth. and *Amethvstoides* Benth. However, in Bentham & Hooker (1867), Bentham revised this arrangement, recognizing two primary groups: sect. *Germanea* and sect. *Isodon*. Briquit (1897) adapted this classification treating *Germanea* and *Isodon* as subgenera. In South Africa, Codd (1975) revised the genus and divided *Plectranthus* into five subgenera: *Nodiflorus* Codd, *Burnatastrum* (Briq) Codd, *Coleus* (Lour.) Codd, *Calceolanthus* Codd and *Plectranthus* on the basis of inflorescence characters. Paton *et al*. (2004), in a phylogenetic study of the tribe Ocimeae based on plastid genes characteristic of *Plectranthus*, found that *Plectranthus* is paraphyletic. This genus includes several plants of medicinal and economic importance. Several species belonging to the *Plectranthus* genus are used in general medicine for their anti-dyspeptic, analgesic, and digestion-stimulating properties (Viganó *et al*., 2007). In Saudi Arabia, *Plectranthus* species are used economically in traditional medicines and have potential to be incorporated into the primary health care system. *Plectranthus barbatus* is the most important species of the genus, as it is used as a remedy for stomach, intestine, and liver disorders, heart problems, and respiratory diseases. Besides this, it is also resistant to insect attack (Grayer *et al*., 2010). Furthermore, *Plectranthus tenuiflorus* is cultivated as an ornamental plant, and used to treat ear infections; and the leaves of *P. asirensis* and *P. cylindraceus* are used as antiseptic and deodorant dressing for wounds (Abulfatih, 1987; Marwah *et al*., 2007).

In the flora of Saudi Arabia, Collenette (1999) reported seven species of *Plectranthus*: *P. arabicus, P. cylindraceus, P. tenuiflorus, P. comosus, P. barbatus, P. pseudomarrubioides* and *P. asirensis*, but Chaudhary (2001) accepted only six species. Despite of its commercial value, the genus *Plectranthus* has been poorly analysed genetically among all the genera of the family Lamiaceae. The taxonomy of the genus is rather unclear. Genome polymorphism of most species of *Plectranthus* in Saudi Arabia has not been studied so far. DNA technology has a recent history of being used in determining the interspecific and intraspecific genome polymorphism, and in delineating the phylogenetic and evolutionary relations among species.

Molecular markers have a great importance in identifying different parental genotypes through the evaluation of genetic variety, which is valuable in cultivar identification (Abdel Khalik *et al*., 2014). Among the different molecular markers, Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) are the simple, rapid, highly efficient, and sensitive techniques. These techniques have now been widely used for genetic diversity. These markers have been used to identify and determine relationships at the species, population, and cultivar levels in many plants (Pezhmanmehr *et al*., 2009; Zhang & Dai, 2010;), and to determine the genetic diversity (Pradeep *et al*., 2005; Xavier *et al.*, 2011; Shen *et al*., 2012; Aghaabasi & Baghizadeh, 2012; Abdel Khalik *et al.*, 2012 & 2014). The advantage of these two molecular marker techniques is that it decreases possible errors intrinsic to each of them, thus provides reliable results.

In the literature review, we could not find any phylogenetic analysis (molecular markers, ISSR, and RAPD) of *Plectranthus* species; therefore, we focused on seven species of the genus collected from different locations in Saudi Arabia and on comparing and aligning them with the help of these specific genetic markers. The aim of the present study was to assess the interspecific genetic diversity of the seven species by the means of the RAPD and ISSR techniques to obtain combined results and address the taxonomic problems of the genus.

Material and Methods

Plant materials: The leaf samples of *Plectranthus* were taken from wild populations and some herbarium specimens. The voucher specimens of the populations studied are deposited in the herbarium of the Department of Biology of Umm Al-Qura University (UQU) (Table 1).

Plant genomic DNA extraction: Total genomic DNA was extracted from leaf samples. The leaves were first ground into a fine powder in liquid nitrogen using a pestle and mortar following the CTAB protocol (Porebski *et al*., 1997; Hussien *et al*., 2003).

Random amplified polymorphic DNA (RAPD) analysis: RAPD was performed as described by Williams *et al*. (1993) with slight modifications. PCR reactions were carried out in a volume of 25 µL, containing 25 ng of total genomic DNA, 10 pmol primer, 200 µM dNTP, 2 mM $MgCl_2$, 1X PCR buffer and 2 units of AmpliTaq polymerase (RTS *Taq*DNA polymerase). Ten random oligonucleotide primers A3, A7, A13, A19, G3, G7, O2, 5, O9 and O11 were used in the experiment (Operon technologies, Alameda, USA) (Table 2). Amplification was performed in a Perkin-Elmer 9600 thermal cycler (Foster City, USA) with the following temperature profile: 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 36°C for 1 min, and extension at 72°C for 90 s. The final extension step was carried out at 72°C for 5 min.

Table 1. Taxa arranged in alphabetical order according to Collenette (1999) and Chaudhary (2001). The table compares traditional classifications of Bentham (1832–36), Bentham & Hooker (1867), Briquit (1897), Codd (1975) and Lukhoba*et al.* (2006) classifications.

No.	Taxon	Voucher	Bentham (1832-1836)	Bentham & Hooker (1866)	Briquit (1897)	Codd (1975)	Lukhoba*et al.* (2006)	Present study RAPDs + ISSRs
1.	*Plectranthusarabicus* EA Bruce	Saudi Arabia, Jizan, JabalFayfa, Garada, Abdel Khalik& Al-Ozekii s. n. (UQU)	Section: Isodon	Section: Isodon	Subgenus: Isodon	-	-	P1
2.	*Plectranthustenuiflorus* (Vatke) Agnew	Saudi Arabia, Abha, Raydah village, Abdel Khalik&Howldar s. n. (UQU).	Section: Aromaria (*Coleus*)	Section: Germanea	Subgenus: Germanea	Subgen. Coleus	1b (group 8)	P2
3.	*Plectranthusbarbatus* Andrews	Saudi Arabia, Jizan, JabalFayfa, Abdel Khalik& Al-Ozekii s. n. (UQU).	Section: Calceolus (*Coleus*)	Section: Germanea	Subgenus: Germanea	Subgen. Calceolanthus	1b (group 2)	P4
4.	*Plectranthusasirensis* JRI Wood	Saudi Arabia, Taif, Sageif, Abdel Khalik& Al-Ozekii s. n. ((UQU)	Section: Isodon	Subgenus: Isodon	Subgenus: Isodon	-	1a (group 8)	P3
5.	*Plectranthuspseudomarrubioides* RH Willemse	Saudi Arabia, Jizan, JabalFefa, Abdel Khalik& Al-Ozekii s. n. (UQU).	Section: Germanea	Section: Germanea	Subgenus: Germanea	Subgen. Burnatastrum	1b (group 7)	P4
6.	*Plectranthushijazensis* K Abdel Khalik	Saudi Arabia, Al Baha, Medhass dam, Abdel Khalik&Howldar s. n. (UQU).	-	-	-	-	-	P4
7.	*Plectranthuscylindraceus* Hochst. ex Benth	Saudi Arabia, WadiAluss, JabalSawdah, near police station, S. Collenette 6364 (E)	Section: Germanea	Section: Germanea	Subgenus: Germanea	Subgen. Burnatastrum	1b (group 7)	P3

Table 2. The characteristics of RAPD and ISSR primers' sequencing and amplification products generated by the studied taxa.

Marker type	Primer	Sequence (5' – 3')	Total no. of bands	Monomorphic bands	Polymorphic bands	% of Polymorphism	Size of DNA fragments (bp)
RAPD	A3	AGTCAGCCAC	9	0	9	100	400-2000
	A7	GAAACGGGTG	13	0	13	100	300-2000
	A13	CAGCACCCAC	13	0	13	100	250-1800
	A19	CAAACGTCGG	9	0	9	100	400-3000
	G3	GAGCCCTCCA	10	5	5	50	300-2000
	G7	GAACCTGCGG	20	7	13	65	450-2000
	O2	ACGTAGCGTC	10	6	4	40	200-600
	O5	CCCAGTCACT	11	4	7	64	200-1500
	O9	TCCCACGCAA	18	4	14	78	300-1500
	O11	GACAGGAGGT	20	3	17	85	400-2500
ISSR	ISSR7	GGAGAGGAGAGGAGA	10	4	6	60	350-1000
	ISSR8	AGAGAGAGAGAGAGT	11	5	6	55	250-750
	ISSR1	TATATATATATATAC	9	3	6	67	300-1000
	ISSR3	CTCTCTCTCTCTCTT	11	4	7	64	300-1000
	ISSR4	GTGTGTGTGTGTGTC	10	3	7	70	200-600
Total number			**185**	**48**	**137**		

Inter simple sequence repeats (ISSR) analysis: ISSR procedure was carried out as described by Dogan *et al.* (2007). ISSR scorable primers were designed and screened for PCR amplification (Table 2). The PCR reactions were prepared by using 50 ng of genomic DNA, 1x PCR buffer, 200 μM dNTP, 2 mM $MgCl_2$, and 2 units of Ampli*Taq* polymerase (RTS-*Taq* DNA polymerase) and 15 ng of ISSR primer. The following temperature profile was used for amplification: 94°C for 5 min followed by 45 PCR cycles at 94°C for 1 min, 49°C for 45 s and 72°C for 2 min, and then a final extension step for 7 min at 72°C. The PCR products were separated on 1.4% and 1.6% agarose gel for RAPD and ISSR, respectively, in 1X TAE buffer containing 0.1 μg mL^{-1} of ethidium bromide for about 2 h at 80 V. The gels were photographed under UV light with Tracktel GDS–2 gel documentation system.

Gel-electrophoretic analysis: Gel electrophoresis following a previously established protocol (Abd El-Twab & Zahran, 2008) was used to determine the presence or absence of the total genomic DNA and size of the DNA fragments after RAPD and ISSR. The samples were loaded on 1.5% agarose gel in loading buffer. DNA was stained in the gel by ethidium bromide (0.5 μg mL^{-1}), and the images were recorded using a digital system (Past software) (Figs. 1, 2).

Data analysis: RAPD and ISSR markers resulted in different DNA bands in the gel after DNA amplification, which can be interpreted in the terms of similarity or difference (Table 3). The pairwise similarity among the genotypes or genetic phenotypes characterized in the different lanes can be quantified using indexes or coefficients of similarity. These estimators describe the genetic distances that represent DNA divergence between the organisms in phenetic and cladistic analyses (Huang *et al*., 2000). The corresponding amplified products were monitored for each primer. The polymorphic fragments (RAPD and ISSR) were named by the primer code followed by the size of the amplified fragment in base pairs. For phylogenetic analysis, each amplified band was treated as a unit character, disregarding its intensity, and was scored in terms of a binary code based on the presence (1) and absence (0) in the gel. Only clear and reproducible bands were considered for scoring (Tables 4 & 5).

For phylogenetic analysis, all the members of *Plectranthu*s were taken into consideration. IBM SPSS Statistics package was used for the statistical analysis of the data obtained from the binary matrices. Three datasets were used, RAPD, ISSR, and a combined dataset of RAPD and ISSR. The statistical method employed only the presence or absence of a band as its differential feature. The binary qualitative data matrices were then used to build similarity matrices based on Jaccard similarity coefficients (Jaccard, 1908). The similarity matrices were then used to create dendrograms using unweighted pair group method with arithmetic average (UPGMA).

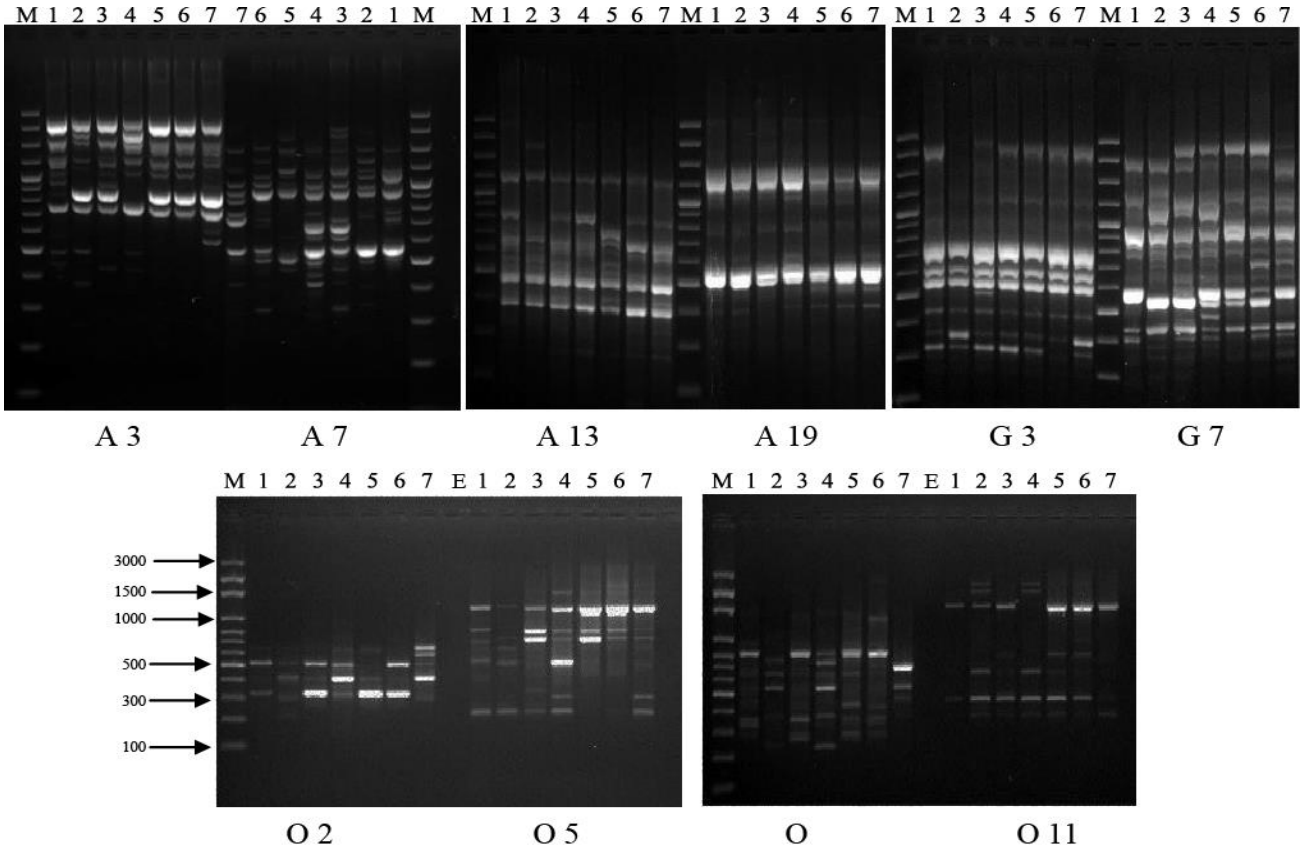

Fig. 1. DNA polymorphism obtained by the ten RAPD-PCR primers from the genomic DNA of the investigated species of *Plectranthus*. Species names are arranged and numbered as in Table 1.

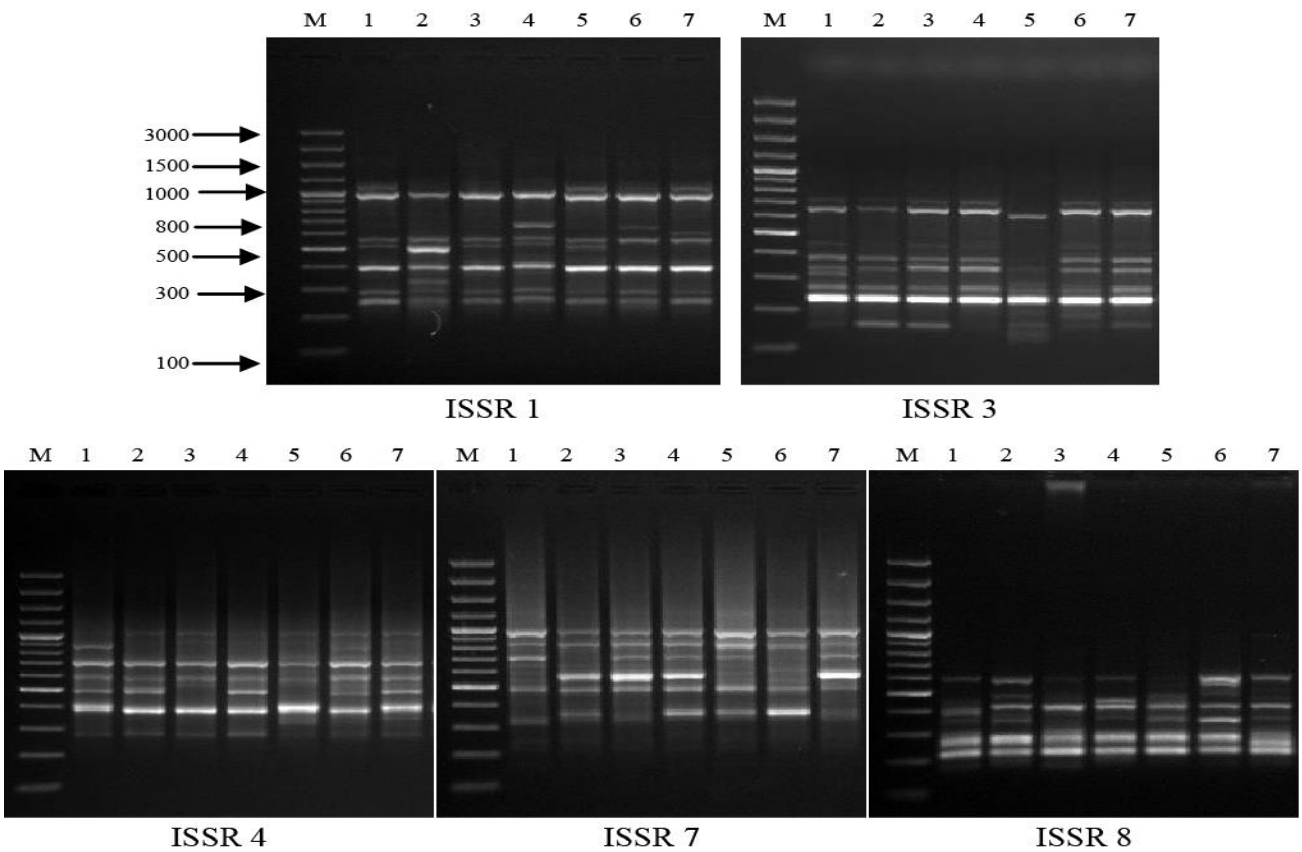

Fig. 2. DNA polymorphism generated by five ISSRs primers from the genomic DNA of the investigated species of *Plectranthus*. Species names are arranged and numbered as in Table 1.

Table 3. Similarity matrix between all pairs of studied taxa based on the RAPD +ISSR. Species names from 1–7 as in Table 1.

Sp	1	2	3	4	5	6	7
1	1	0.53	0.62	0.62	0.62	0.61	0.59
2	0.53	1	0.55	0.62	0.52	0.52	0.59
3	0.62	0.55	1	0.63	0.65	0.67	0.60
4	0.62	0.62	0.63	1	0.60	0.63	0.62
5	0.62	0.52	0.65	0.60	1	0.66	0.53
6	0.61	0.52	0.67	0.63	0.66	1	0.59
7	0.59	0.59	0.59	0.62	0.53	0.59	1

Results

RAPD analysis: Fifteen primers were used for the RAPD analysis to investigate the pattern of genetic variations among the considered species of the genus *Plectranthus* growing in Saudi Arabia. Among the primers tested, only ten revealed a polymorphism. Each primer was tested on all samples and was selected for genotype analysis if its patterns were reproducible and stable. Polymorphic bands were selected for identifying the genetic similarity for the group of species. A total of 104 reproducible polymorphic bands were produced by using 10 RAPD-PCR primers. The average similarity coefficient ranged from 0.48 to 1.00. The highest number of polymorphic amplification DNA fragments obtained per primer was 17 bands for the primer O11 with size ranging from 400 to 2500 bp. The relations between the studied taxa are presented with the help of a dendrogram built on the basis of similarity coefficients. For the ease of assessment, the 105 bands were taken together and the number of bands for each size of DNA fragments (bp) was scored for every species. four branches and one cluster with about 0.63 similarity were obtained (Fig. 3): (i) a branch including *Plectranthus cylindraceus;*(ii) a branch including *Plectranthus tenuiflorus;* (iii) a branch including *Plectranthus asirensis*; (iv) a branch including *Plectranthus arabicus*; and (v) a cluster including *Plectranthus barbatus, P. pseudomarrubioides,* and *P. hijazensi*s with about 0.62 genetic similarity.

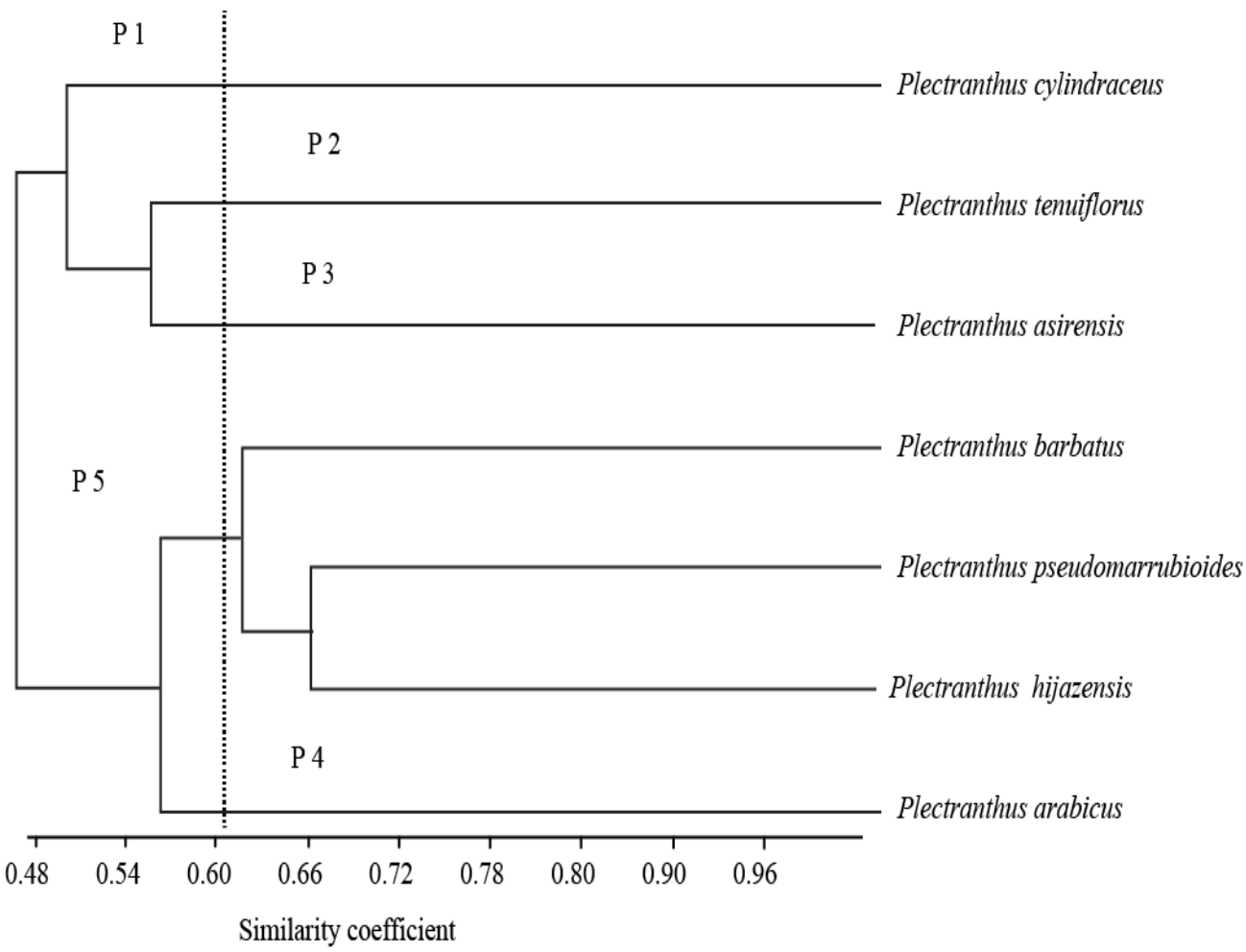

Fig. 3. UPGMA phenogram showing the genetic diversity of the seven species of *Plectranthus* based on RAPDs characters.

Table 4. Comparative analysis of polymorphic molecular bands of RAPD, 0 absent, 1 present, used in the analysis of the genus *Plectranthus*.

Primer name	*P. arabicus*	*P. tenuiflorus*	*P. barbatus*	*P. asirensis*	*P. pseudomarrubioides*	*P. hijazensis*	*P. cylindraceus*
	0	1	0	0	0	0	0
	0	0	1	0	1	1	0
	0	1	0	0	0	0	1
	1	0	1	1	1	1	0
A3	0	1	1	1	1	0	1
	0	1	1	1	1	0	1
	1	1	1	1	0	1	1
	0	0	0	0	0	0	1
	1	1	0	1	1	0	1
	1	1	1	1	0	0	1
	0	0	0	1	0	0	1
	0	0	1	0	0	0	0
	1	0	0	1	1	1	0
	1	0	0	1	1	1	0
	0	1	0	1	0	0	1
A7	1	0	0	0	0	0	0
	0	1	0	0	0	0	1
	1	0	1	1	1	1	1
	1	0	1	1	1	1	1
	0	0	0	0	1	1	1
	1	1	0	0	0	0	0
	0	0	1	1	0	0	0
	0	1	0	1	0	0	0
	0	0	1	0	1	1	0
	1	0	1	0	1	1	0
	1	0	0	1	1	1	0
	1	0	1	0	1	1	0
	1	1	0	1	0	0	0
A13	1	0	1	0	1	0	0
	1	1	0	1	0	0	1
	1	1	1	1	1	1	0
	1	0	1	0	1	1	0
	1	0	1	1	1	1	0
	1	0	1	0	1	1	0
	0	0	1	1	1	1	0
	0	1	1	1	1	1	1
	1	1	1	1	1	1	0
	0	1	1	1	1	1	0
	0	1	0	1	1	1	0
A19	0	0	1	0	1	1	0
	0	0	0	0	0	1	0
	1	1	1	0	1	1	1
	0	1	0	1	0	0	0
	0	1	0	1	0	0	0
	1	1	1	1	1	0	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	0	1	0	0	0	0
	1	1	1	1	1	1	1
G3	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	0	1	1	1	1	1
	0	1	0	0	0	0	0
	1	0	0	1	1	1	1
	0	1	1	1	0	0	1
	1	0	1	1	0	1	1
	1	1	1	1	1	1	1
	0	0	1	0	0	0	0
	0	0	0	1	0	0	0
	0	1	1	1	1	1	0
G7	1	0	1	1	1	1	1
	1	1	1	1	1	1	1
	0	1	0	1	0	1	1
	1	1	1	1	1	1	1
	0	1	1	1	1	0	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1

Table 4. (Cont'd.).

Primer name	*P. arabicus*	*P. tenuiflorus*	*P. barbatus*	*P. asirensis*	*P. pseudomarrubioides*	*P. hijazensis*	*P. cylindraceus*
	0	1	0	1	0	0	0
	0	1	1	1	0	1	1
	0	1	0	0	0	1	0
	0	0	0	0	0	0	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	0	1	1	1	1	1
	1	1	1	1	1	1	1
	0	0	0	0	1	0	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
O2	1	1	1	0	1	0	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	0	0	1	0	0
	1	0	1	1	0	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	0
	1	1	1	1	1	1	1
	1	1	1	1	0	0	1
	1	1	1	1	0	0	0
O5	1	1	1	0	1	1	1
	0	0	0	0	1	1	0
	1	0	0	1	0	0	0
	0	0	0	0	1	0	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	1	0	0	0	0	0
	0	0	1	1	0	0	0
	1	0	0	0	0	0	0
	1	1	0	1	0	0	0
	0	0	0	0	0	0	1
	0	0	0	0	0	0	1
	0	0	0	0	0	0	1
	0	0	0	0	0	1	1
	1	1	1	1	1	1	1
O9	0	1	1	0	1	1	1
	1	0	1	0	0	0	1
	1	0	1	1	1	1	0
	1	1	0	0	1	0	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	0	0	1	1	1	1
	0	1	0	1	0	0	0
	1	1	1	1	1	1	1
	0	1	0	0	1	0	0
	1	0	0	1	0	0	0
	0	0	0	1	0	0	0
	1	1	1	0	1	0	0
	1	0	0	1	1	0	0
	0	1	1	0	1	0	0
	1	1	1	1	0	1	1
	1	1	0	1	1	1	1
	0	0	0	1	1	0	0
	0	0	0	1	1	0	0
O11	1	1	0	0	1	0	1
	1	1	0	1	1	1	1
	1	0	0	0	0	0	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	1	1	1	0	1	1
	1	0	1	0	0	1	1
	0	1	0	0	0	1	0
	1	1	1	1	1	1	1
	0	0	1	0	1	0	0

Table 5. Comparative analysis of polymorphic molecular bands of ISSR, 0 absent, 1 present, used in the analysis of the genus *Plectranthus*.

Primer name	*P. arabicus*	*P. tenuiflorus*	*P. barbatus*	*P. asirensis*	*P. pseudomarrubioides*	*P. hijazensis*	*P. cylindraceus*
ISSR7	1	1	1	1	1	1	1
	0	1	0	1	1	1	0
	1	1	1	1	1	1	1
	1	0	0	0	1	1	0
	1	1	1	1	1	0	1
	1	1	0	0	0	0	0
	1	0	1	1	1	0	1
	1	0	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
ISSR8	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	1	0	0	0	0	0
	1	1	1	1	1	1	1
	0	1	0	0	0	0	0
	1	0	1	1	1	0	0
	1	1	1	1	1	1	1
	0	0	0	0	0	0	1
	0	0	0	1	0	1	0
	1	1	1	1	1	1	1
	1	0	1	1	1	1	1
ISSR1	1	1	1	1	0	1	1
	1	1	1	1	1	1	1
	1	0	0	0	1	0	1
	1	1	0	1	0	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	0	0	0	0	1	0
	0	0	1	1	1	1	0
	0	1	1	1	1	1	1
ISSR3	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	0	1	0	0	1	0
	1	1	0	1	1	1	1
	1	0	0	0	0	0	0
	0	1	1	1	1	1	1
	1	0	0	1	1	0	1
	0	1	0	0	1	0	0
	0	0	0	0	0	1	0
	1	1	1	1	1	1	1
ISSR4	0	0	0	0	1	0	0
	1	1	1	0	1	1	1
	0	0	0	0	0	1	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	0	1	1
	1	1	1	1	0	1	1
	1	1	1	1	0	1	1

ISSR analysis: In total five primers for ISSR were used to investigate the patterns of genetic variations among the species of *Plectranthus* growing in wild habitat in Saudi Arabia and few other related species. In total, 32 reproducible polymorphic bands were resulted by the five ISSR primers; and these bands were used for studying the genetic similarity among the species. The average similarity coefficient ranged from 0.59 to 1. The results showed that all primers were polymorphic. The highest number of polymorphic amplification DNA fragments obtained per primer was seven for the primer 3 and 4, with size ranging from 200 to 1000 bp, while for remaining primers, it was six bands. The results of the consensus tree from ISSR data indicated that the tree was divided into four branches and one cluster with 0.70 similarity (Fig. 4): (i) a branch including *Plectranthus pseudomarrubioides;* (ii) a branch including *Plectranthus tenuiflorus*; (iii) a branch including *Plectranthus*

arabicus; (iv) a branch including *Plectranthus hijazensis*; (v) a cluster including *Plectranthus barbatus, P. asirensis*, and *P. cylindraceus* with about 0.70 genetic similarity.

Combined RAPD and ISSR analysis: The UPGMA dendrogram obtained from the cluster analysis of RAPD and ISSR combined data offered near similar clustering pattern, with Jaccard's similarity coefficient ranging from 0.55 to 0.96. The consensus tree was divided into five major branches and clusters with a similarity score of 0.62 (Fig. 5): (i) a branch including *Plectranthus arabicus;* (ii) a branch including *Plectranthus tenuiflorus*; (iii) a cluster containing *Plectranthus asirensis* and *P. cylindraceus* with about a similarity score of 0.63; (iv) a cluster containing *Plectranthus barbatus, P. hijazensis* and *P. pseudomarrubioides* with a similarity score of 0.65.

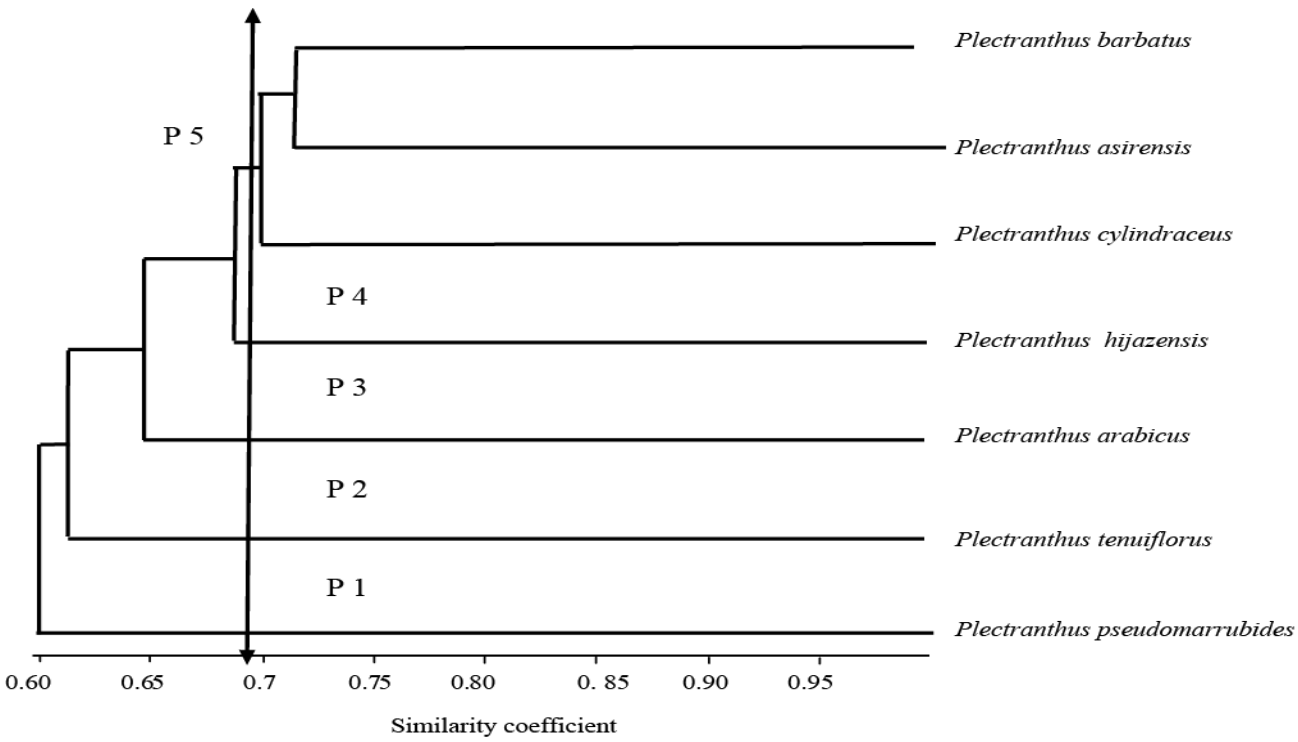

Fig. 4. UPGMA phenogram showing the genetic diversity of the seven species of *Plectranthus* based on ISSRs characters.

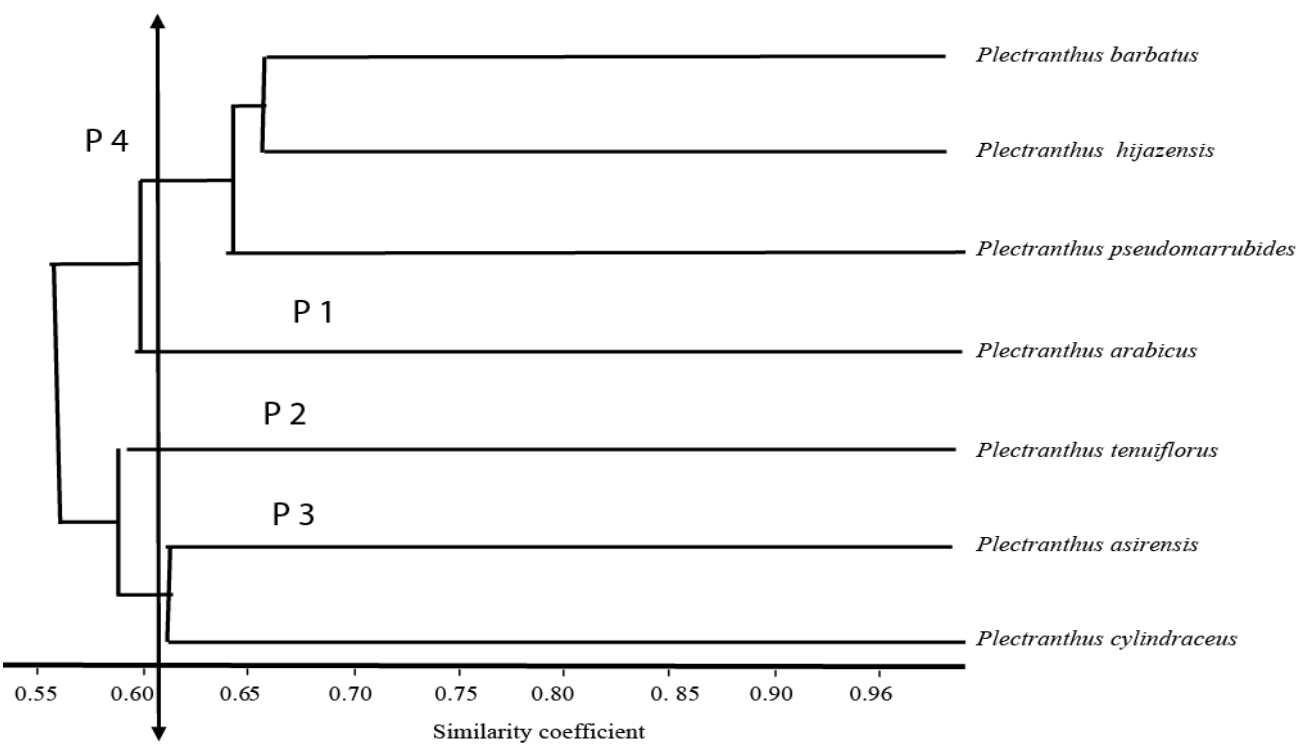

Fig. 5. UPGMA phenogram showing the genetic diversity of the seven species of *Plectranthus* based on combination of RAPDs and ISSRs characters.

Discussion

Morphological characters in plants may be affected by environmental conditions. Therefore, morphological characters cannot be taken as a reliable criterion for classification and may result in inconsistencies. Output of a molecular marker technique depends on the amount of polymorphism it can detect among the set of accessions under investigation (Abdel Khalik *et al*., 2014).

RAPD and ISSR markers have been used in several studies for DNA fingerprinting and phylogenetic analyses. Galvan *et al.,* (2003) concluded that ISSR could serve as a better tool than RAPD for phylogenetic studies. The present study, however, demonstrated that both RAPD and ISSR techniques with suitable statistical tools could be successfully applied, in parallel, to assess the genetic diversity and phylogeny of *Plectranthus*. Although, RAPD and ISSR markers showed considerable differences in detecting polymorphism and discriminating efficiency, they showed nearly similar topology in dendrograms generated on the basis of similarity matrices.

A highly significant correlation between these two dendrograms suggested that both the markers are equally effective in establishing the phylogenetic relationships among the investigated taxa. Furthermore, the genotype distribution on the consensus tree based on the combined banding patterns of RAPD and ISSR may significantly differ, because it is possible that each technique intensifies different parts of the genome. The RAPD markers cover the whole genome for amplification, while ISSR amplifies the region between two micro satellites (Abdel Khalik *et al.,* 2014). Hence, the polymorphism is indicative of the diversity of only these regions of the genome. It is therefore better to use the combination of banding patterns of both the methods in order to get more segment sites in the genome that will increase the strength of the consensus tree. As a whole, our results, obtained from the RAPD and ISSR analyses, partially established the subgenera and sectional classification of the genus *Plectranthus* proposed by most recent traditional (Bentham & Hooker 1867; Briquit, 1897; Codd, 1975) and phylogenetic taxonomies based on molecular data (Paton *et al*., 2004; Lukhoba *et al*., 2006**).**

In clade P1 (*Plectranthus arabicus*): Bentham (1832 & 1848), Bentham & Hooker (1867), and Briquit (1897) treated *P. arabicus* and *P. asirensis* as a separate subgenus *Isodon* section *Isodon*. According to the combined RAPD and ISSR tree, the results do not support the placement of *P. arabicus* with *P. asirensis* in the subgenus (section) *Isodon*. This is due to the placement of *P. arabicus* within a separate branch with high genetic similarities. This species is distinguished morphologically from the other species by having annual life form, plant length (15 cm), watery-succulent, sessile leaves, and branched and glandular multicellular hairs. Moreover, Abdel Khalik &Karakish (2016) do not support the placement of *P. arabicus* with *P. asirensis* in the subgenus (section) *Isodon* and suggest that *P. arabicus* should be treated as separate subgenus, because the anatomical characters of the stem and leaf distinguish *P. arabicus* from the rest of the species by having terete stem, narrow cortex with only parenchyma, and six vascular bundles. These data are congruent with those of Abdel Khalik & Karakish (2016), but disagree with that of Bentham (1832& 1848), Bentham & Hooker (1867), and Briquit (1897) regarding the placement of *P. arabicus* and *P. asirensis* in an enlarged concept of subgenus *Isodon* section *Isodon* and suggest that *P. arabicus* should be treated under as separate subgenus.

In cluster P4 (subgenus *Germanea*): The subgenus *Germanea* was separated from the rest of the subgenera on the basis of the species having two-lipped calyx with the upper lip consisting of a single broad tooth and the lower lip of four narrower acute or acuminate teeth: cymes are pediculate and branched (Briquit, 1897). Codd (1975) reviewed the genus and placed *P. barbatus* and *P. pseudomarrubioides*in two different subgenera.

Lukhoba *et al*. (2006) studied extensively the phylogeny of 62 species of *Plectranthus* and its ethnobotanical uses. They provided an informal classification that divided the species into two main clades. Clade 1, the *Coleus* clade of Paton *et al*. (2004), is divided into two subclades (1a and b). Clade 2 is recognized as the *Plectranthus* clade. Within subclade 1b, they proved that *P. barbatus* and *P. pseudomarrubioides* are well separated from the rest of taxa, but in two groups (groups 2, 7). Moreover, Grayer *et al*. (2010) surveyed 34 species of *Plectranthus* for exuding flavonoids to see whether the distribution of these compounds could support a recent classification of the genus based on molecular and morphological characters. They identified two major groups: the *Coleus* and *Plectranthus* clades. They found that flavones were restricted to only five species of the *Plectranthus*, and *P. pseudomarrubioides* was one of these. In a similar vein, Abdel Khalik & Karakish (2016) maintained this division, because subgenus *Germanea* (*P. barbatus, P. hijazensis* and *P. pseudomarrubioides*) has wide pith, numerous bundles, obtuse-convex leaf shape in cross section, palisade tissue covers almost a half of the mesophyll, and covered with capitate hairs. Generally, these results agree with classification of Bentham & Hooker (1867), Briquit (1897), Lukhoba *et al*. (2006), and Abdel Khalik & Karakish (2016) concerning placement of *P. barbatus, P. hijazensis* and *P. pseudomarrubioides*in an enlarged concept of subgenus *Germanea* section *Germanea*, but disagree with that of Codd (1975).

In clade P 2 (*Plectranthus tenuiflorus*): The second clade includes *P. tenuiflorus* treated as section *Aromaria* that belongs to the genus *Coleus* (Bentham 1832–1836) as subgenus *Germanea* (Briquit 1897), and subgenus *Coleus* (Codd, 1975) belongs to *Plectranthus*. Moreover, Lukhoba *et al*. (2006) treated *P. tenuiflorus* (*P. aegyptiacus*) within sub-clade 1b and placed in group 8. Shaheen *et al*. (2017) investigated phytochemical screening of the genus *Plectranthus* in Saudi Arabia, and revealed the presence of hydrolysable tannins, gallic, and rosmarinic acids in all plant samples except *P. tenuiflorus*. Moreover, Abdel Khalik (2016) showed that *P. tenuiflorus* shares certain characteristics with both *P. asirensis* and *P. cylindraceus*, such as being sub-shrubs,

erect, having woody stem at the base, terete to quadrangular stem outline, seed with isodiametric or 4–5–6 polygon epidermal cells, but also differs as the pollen grains with primary lumina are reticulate and with secondary lumina are microreticulate, while in *P. asirensis* and *P. cylindraceus*, pollen grains are bi-reticulate. Generally, these results agree with those of Codd (1975), Lukhoba *et al.* (2006) and Shaheen *et al*. (2017) asserting that *P. tenuiflorus* should be a monophyletic group and this species has close relationship with *P. asirensis* and *P. cylindraceus*.

In cluster P3 (*P. asirensis* & *P. cylindraceus*): The group of *P. asirensis* and *P. cylindraceus* showed a similarity score of 0.64. These species can be clearly defined on the basis of various structures: sub-shrubs, erect, woody stem at the base, terete to quadrangular stem outline, bract deciduous, ovoid seed, and isodiametric or 4–5–6 polygon epidermal cells. Bentham & Hooker, (1867) treated *P. asirensis* as a separate section *Isodon*. However, Briquit (1897) preserved this species as subgenus *Isodon*. Furthermore, *P. cylindraceus* corresponds to previously recognized position within subgenus *Germanea* section *Germanea* (Bentham & Hooker 1867; Briquit 1897). However, Codd (1975) reviewed the genus and put this species within subgenus *Burnatastrum*. Furthermore, Lukhoba *et al*. (2006) suggested an informal classification that divided the species into two main clades. Clade 1, the *Coleus* clade of Paton *et al*. (2004), broadly corresponding to the formally recognized genus *Coleus*, is divided into two subclades, clades 1a and b. Clade 2 is recognized as the *Plectranthus* clade. They placed *P. asirensis* and *P. cylindraceus* within clade 1 but in two separated sub-clades (1a & 1b). Moreover, Grayer *et al*. (2010) surveyed 34 species of *Plectranthus* for exudated flavonoids to see whether the distribution of these compounds would support a recent classification of the genus based on molecular and morphological characters. They identified two major groups the *Coleus* and *Plectranthus* clades. They found that flavones were restricted only to *P. cylindraceus* (*P. montanus*) and *P. tenuiflorus* (*P. aegyptiacus*). Present observations confirmed the possibility that *P. asirensis* and *P. cylindraceus* should be monophyletic groups and there are close relationships between these species and *P. tenuiflorus* subgenus *Germanea* (Briquit, 1897), and subgenus *Coleus* (Codd, 1975). These results are in partially agreement with the results of Lukhoba *et al.* (2006), Grayer *et al*. (2010), and Shaheen *et al*. (2017) but disagree with that of Bentham & Hooker (1867), Briquit (1897), and Codd (1975).

Conclusions

The genetic range and phylogenetic studies of seven species, representing the genus *Plectranthus* (Lamiaceae) in Saudi Arabia, were carried out by employing the Inter Simple Sequence Repeats (ISSR), Randomly Amplified Polymorphic DNA (RAPD), and combined ISSR and RAPD markers. We obtained useful data for assessing the taxonomy of *Plectranthus* both at subgeneric and sectional levels. The results indicated some degree of similarity among the species of subgenus Germanea and supported the monophyly of the subgenus. An outstanding result from this study was identification of *P. arabicus* with distinguishing characters, suggestion that it should be treated as a separate subgenus. In a similar vein, individual differences between closely connected endemic species *P. asirensis* and *P. hijazensis* were confirmed, recommending that they should be preserved as a different subgenus. Furthermore, *P. asirensis* and *P. cylindraceus* seem to be a monophyletic group as there are close relationships between this group and *P. tenuiflorus*.

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