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GENETIC RELATIONSHIPS AMONG WILD SPECIES OF SUBFAMILY MALVOIDEAE IN SAUDI ARABIA AS INFERRED FROM SCoT AND ISSR MARKERS

KADRY ABDEL KHALIK[1,2*] AND SUAD AL-RUZAYZA[1]

[1]*Biology Department, Faculty of Science, Umm-Al-Qura University, Mecca, Saudi Arabia*
[2]*Botany and Microbiology Department, Faculty of Science, Sohag University, 82524 Sohag, Egypt*
[*]*Correspondence: kadry3000@yahoo.com*

Abstract

Genetic investigation and phylogenetic analyses of 21 species, representing 8 genera of the subfamily Malvoideae from Saudi Arabia, were carried out by using Start Codon Targeted (SCoT), Inter Simple Sequence Repeats DNA (ISSR), and combined ISSR and SCoT markers. Ten SCoT and five ISSR primers created 138 polymorphic amplified fragments, which pointed to a comparatively high level of genetic difference in Malvoideae. SCoT markers exposed a higher level of polymorphism (89 bands) than ISSR (49 bands). The comparison of SCoT and ISSR based dendrograms revealed significantly similar grouping patterns of genotypes. Five clusters and clades were documented within Malvoideae, which generally verified traditional groupings with a few exceptions. Taxonomic and phylogenetic results were discussed in contrast to existing morphological and phylogenetic data. The results of this study provided useful data for evaluating the taxonomy of two Malvoideae tribes at infrageneric and subgeneric levels. In general, the results are consistent with the previous phylogenetic findings of the polyphyletic nature of *Abutilon, Hibiscus,* and *Malva*. The species of sections Bombicella and Malva were highly heterogeneous. The most exciting result of this analysis was identifying the *Senra incana* with unique characters suggesting that it should be preserved as a separate tribe. Similarly, distinctive genetic profiles between the closely related genera *Fioria* and *Althaea* were also noted suggesting that they should be placed in different tribes. Furthermore, the present results also indicated parallel characters among the species of *Pavonia* that supports the monophyly of this genus.

Key words: Malvaceae, Molecular markers, Phylogenetic, Taxonomy, SCoT, ISSR.

Introduction

Malvaceae is a universal family of herbs, shrubs, and small trees. This family is abundant in tropical regions consisting of 244 genera and approximately 4225 species (Christenhusz & Byng, 2016). This family is characterized by simple palmate leaves, monothecal anthers, monadelphous stamens, stellate hairy indumentum, and large echinate pollen grains. It is closely related to Bombacaceae, Sterculiaceae, and Tiliaceae but varies in containing monadelphous stamens and one-celled anthers (Hutchinson, 1967, Heywood, 1993, La Duke & Doeby, 1995, Fryxell, 1997, Mabberley, 1997). The systematic of Malvaceae at the generic, subfamily and tribal level is unclear (Bentham & Hooker, 1862, Schumann 1890, Bates, 1968). Bayer & Kubitzki (2003), and Bayer (1999) categorized Malvaceae into nine subfamilies based on the morphological characters and molecular data as Brownlowioideae, Bombacoideae Byttnerioideae, Grewioideae, Dombeyoideae, Malvoideae, Helicteroideae, Sterculioideae, and Tilioideae. Kearney (1951) divided the family Malvaceae into four tribes Malpeae, Hibisceae, Ureneae, and Malveae, which are further divided into four subtribes Malvinae, Abutilinae, Ureneae, and Siodinae. Based on the fruit characters, Hutchinson (1967) divided the Malvaceae into five tribes as Malopeae, Malveae, Abutileae, Hibisceae, and Ureneae whereas Schultze-Motel (1964) reported only three tribes. La Duke & Doebley (1995), and Krebs (1994) also separated Malvaceae into five or six tribes as Abutilieae, Malopeae, Decaschistieae, Malveae, Hibiscieae, and Ureneae. Recently, Takhtajan (2009), and Bayer & Kubitzki (2003) classified the subfamily Malvoideae (formerly Malvaceae) based on the morphological characters and molecular data into four main tribes Gossypieae, Kydieae, Hibisceae including *Pavonia*, *Fioria*, *Hibiscus*, and *Senra*) and Malveae including *Malva*, *Abutilon*, and *Althaea*. Collenette (1999) recognized 11 genera comprising of 38 species in the Saudi Arabian flora. However, Chaudhary (2001) reported 13 genera and 54 species of Malvaceae including cultivated species. Molecular markers more reliably detect different parental genotypes than assessing the genetic difference in cultivar identification (Abdel Khalik *et al*., 2014). The detection of DNA polymorphism through molecular markers is significant in the field of molecular genetics. Start Codon Targeted DNA (SCoT) and Inter Simple Sequence Repeat (ISSR) markers are a highly effective, rapid, and simple tools for genetic characterization, and they employed to classify and define the genetic range of various plants (Zietkiewicz *et al*., 1994, Bornet & Branchard, 2001, Collard & Mackill, 2009, Celka *et al*., 2010, Hamidi *et al*., 2014, Fahad Al-Qurainy *et al*., 2015, Ibrahim *et al*., 2016, Abdel Khalik & Osman, 2017, Abdel-Hak *et al*., 2019). Previously, the application of SCoT and ISSR molecular markers for the phylogenetic investigation of Malvaceae species has not been reported. Therefore, the present study was conducted to assess the interspecific genetic diversity among Malvoideae species found in Saudi Arabia by using SCoT and ISSR markers. In addition to the taxonomic difficulties of the subfamily, the study also elaborates the results that match with the systematics of the genera as reported in other Malvoideae tribe classification systems.

Material and Methods

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

Table 1. List of the studied species of Malvoideae (Chaudary 2001) sited according to traditional (Bentham & Hooker, 1862), more recent traditional (Hutchinson, 1967) and a recent phylogenetic classification based on molecular data (Bayer & Kubitzki, 2003; Jennifer et al. 2005; Reveal's system, 2012).

No.	taxa	Source and voucher	Bentham & Hooker (1862)	Hutchinson (1967)	Bayer & Kubitzki, 2003; Jennifer *et al.*, 2005	Reveal's system (2012)	Present study SCoT & ISSR
1.	*Abutilon bidentatum* Hochst.	Wadii Thalolah near AL-Baha, Suad Al-Ruzayza, 11 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
2.	*Abutilon hirtum* (Lam.) Sweet	Mahil Aseir, Suad Al-Ruzayza, 8 15 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
3.	*Abutilon figarianum* Webb	Wadii Qusai, Suad Al-Ruzayza, 5 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
4.	*Abutilon fruticosum* Guill.	Near Al-Howtah, Alfarhan and J. Thomas, 22274 (KSU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
5.	*Abutilon pannosum* (G.Forst.) Schltdl.	Al-Edabi, Suad Al-Ruzayza, 17 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
6.	*Abutilon ramosum* (Cav.) Guill. & Perr.	Shada mountiain, Suad Al-Ruzayza,2 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
7.	*Abutilon grandifolium* (Willd.) Sweet	Fifa mountain, Suad Al-Ruzayza,15 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
6.	*Abutilon muticum* Sweet	Alardah, Suad Al-Ruzayza,6 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Abutilinae	G 2 B
7.	*Abutilon pannosum* (G.Forst.) Schltdl.	Al-Edabi, Suad Al-Ruzayza, 17 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe:Sideae Subtribe: Abutilinae	G 2 B
8.	*Abutilon ramosum* (Cav.) Guill.	Shada mountiain, Suad Al-Ruzayza, 2 (UQU)	Tribe: Abutileae Subtribe: Abutilinae	Tribe: Abutileae Subtribe: Abutilinae	*Abutilon* alliance	Tribe:Sideae Subtribe: Abutilinae	G 2 B
9.	*Althaea ludwigii* L.	Qassim Road, Suad Al-Ruzayza, 22 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	*Malva* alliance	Tribe: Malveae Subtribe: Malvinae	G 2 C
10.	*Fioria vitifolia* (L.) Mattei	Al Darb, near Abha, Suad Al-Ruzayza, 21 (UQU)	Tribe: Hibiscieae	Tribe: Hibisceae	-	Tribe: Hibiscieae	G 5
11.	*Hibiscus deflersii* Schweinf.	Taif, Suad Al-Ruzayza , 37 (UQU)	Tribe: Hibiscieae	Tribe: Hibisceae	-	Tribe: Hibiscieae	G 1
12.	*Hibiscus purpureus* L.	Fifa mountain, Alfarhan and J. Thomas, 999 (KSU)	Tribe: Hibiscieae	Tribe: Hibisceae	-	Tribe: Hibiscieae	G 4
13.	*Hibiscus micranthus* L.f.	Raidah, Suad Al-Ruzayza, 34 (UQU)	Tribe: Hibiscieae	Tribe: Hibisceae	-	Tribe: Hibiscieae	G 4
14.	*Sida alba* L.,	Fifa mountain, J. Thomas and R. Basahi, 21876 (KSU)	Tribe: Malveae Subtribe: Sidinae	Tribe: Abutileae Subtribe: Sidinae	*Abutilon* alliance	Tribe: Sideae Subtribe: Sidinae	G 1
15.	*Malva parviflora* L.,	Alkarj, Suad Al-Ruzayza, 29 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	*Malva* alliance	Tribe: Malveae Subtribe: Malvinae	G 1
16.	*Malva verticillata* L.,	AL-ahsaa, Suad Al-Ruzayza, 32 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	*Malva* alliance	Tribe: Malveae Subtribe: Malvinae	G 1
17.	*Malva sylvestris* L.	Al-Qasim, Buradah, Suad Al-Ruzayza, 46 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	*Malva* alliance	Tribe:Malveae Subtribe: Malvinae	G 1
18.	*Malva neglecta* Wallr.	Syll. Ushaqur, Suad Al-Ruzayza, 2 (UQU)	Tribe: Malveae Subtribe: Eumalvinae	Tribe: Malveae Subtribe: Malvinae	*Malva* alliance	Tribe: Malveae Subtribe: Malvinae	G 1
19.	*Pavonia arabica* Hochst.	Wadi Bani Zaher, S. Chaudhary, 7087 (RAWRC)	Tribe: Ureneae	Tribe: Ureneae	-	Tribe: Hibiscieae	G 2A
20.	*Pavonia kotschyi* Hochst.	Jeddah, Suad Al-Ruzayza, 40 (UQU)	Tribe: Ureneae	Tribe: Ureneae		Tribe: Hibiscieae	G 2A
21.	*Senra incana* Cav.	Harrat Al-Shara, Suad Al-Ruzayza, 41(UQU)	Tribe: Hibiscieae	Tribe: Hibiscieae		Tribe: Gossypieae	G 3

Table 2. The characteristics of ISSR and SCoT primers sequence 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)
ISSR	ISSR- 1	5'-AGAGAGAGAGAGAGYC-3'	11	1	10	91%	170- 660
	ISSR- 2	5'-AGAGAGAGAGAGAGYG-3'	11	2	9	82 %	80- 750
	ISSR- 3	5'-ACACACACACACACYT-3'	12	2	10	83%	90- 1300
	ISSR- 4	5'-ACACACACACACACYG-3'	14	2	12	86%	60- 900
	ISSR- 5	5'-GTGTGTGTGTGTGTYG-3'	12	4	8	67%	80- 1050
SCoT	SCoT-7	ACAATGGCTACCACTGAC	15	1	14	93 %	140- 870
	SCoT-16	CCATGGCTACCACCGGCA	21	2	19	90%	90- 1200
	SCoT-18	CCATGGCTACCACTAGCA	22	2	20	91%	160- 1400
	SCoT-35	AACCATGGCTACCACCAC	23	3	20	87%	90- 1250
	SCoT-36	CACCATGGCTACCACCAT	19	3	16	84%	160- 1050
Total number			**160**	**22**	**138**		

A: Adenine, T: Thymine, G: Guanine and C: Cytosine Y: (C or T)

Plant genomic DNA extraction: Total genomic DNA was extracted by crushing the leaf samples into a fine powder in liquid nitrogen with pestle and mortar and following the CTAB protocol (Porebski *et al*., 1997, Hussein *et al*., 2003).

Estimation for the DNA concentration: DNA concentration were estimated visually in 1% agarose gel by comparing bands intensity with different bands of the DNA marker by using 10 µl of a DNA Ladder (100bp) (Solis BioDyne) and 2 µl of template DNA samples.

SCoT and ISSR- PCR amplifications: SCoT was performed as decribed by (Collard & Mackill, 2009). Five primers SCoT 7, SCoT 16, SCoT 18, SCoT 35 and SCoT 36 were used for initial evaluation (Operon Technologies, Alameda, USA). 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 1_X PCR buffer, 0.2 µM dNTPs, 1.5 mM MgCl2, 1 µ *Taq* DNA polymerase (RTS-*Taq* DNA polymerase), 1 µM primer, and 30 ng template DNA.

Thermocyling profile: PCR amplifications were carried out in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems). The amplification process consisted of an initial denaturation cycle for 5 min at 94ºC followed by35 cycles. Each cycle consisted of a denaturation step at 94ºC for 1 min, a hardening step at 50ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

Detection of the ISSR and SCoT products: Amplification products were separated on a 1 % agarose gel, stained with 0.5µg/ml ethidium bromide in 1_X TBE buffer at 95 volts. DNA ladder (1Kb) was used to estimate the product size. PCR products were photographed under UV light in the Gel Documentation System (BIO-RAD 2000) (Figs. 1 & 2).

Data analysis: The DNA bands generated by PCR amplification of ISSR and SCoT-PCR markers were compared to determine the genetic resemblance of the samples (Table 3). The pairwise similarity among genotypes characterized in different lanes was enumerated by the similarity index according to the Dice coefficient (Sneath & Sokal, 1973). To conduct the phylogenetic investigation, each amplified band in the gel was considered a unit character regardless of its intensity and scored in terms of a binary code as the presence (1), and absence (0). Only pure and reproducible bands were counted. Three datasets were used including SCoT, ISSR, and their combined dataset. The numerical method differentiated among bands based on their presence or absence. The binary qualitative data matrices were used to build similarity matrices based on the similarity coefficients. These similarity matrices were used to generate dendrograms by following the Unweighted Pair Group Method with Arithmetic Average (UPGMA).

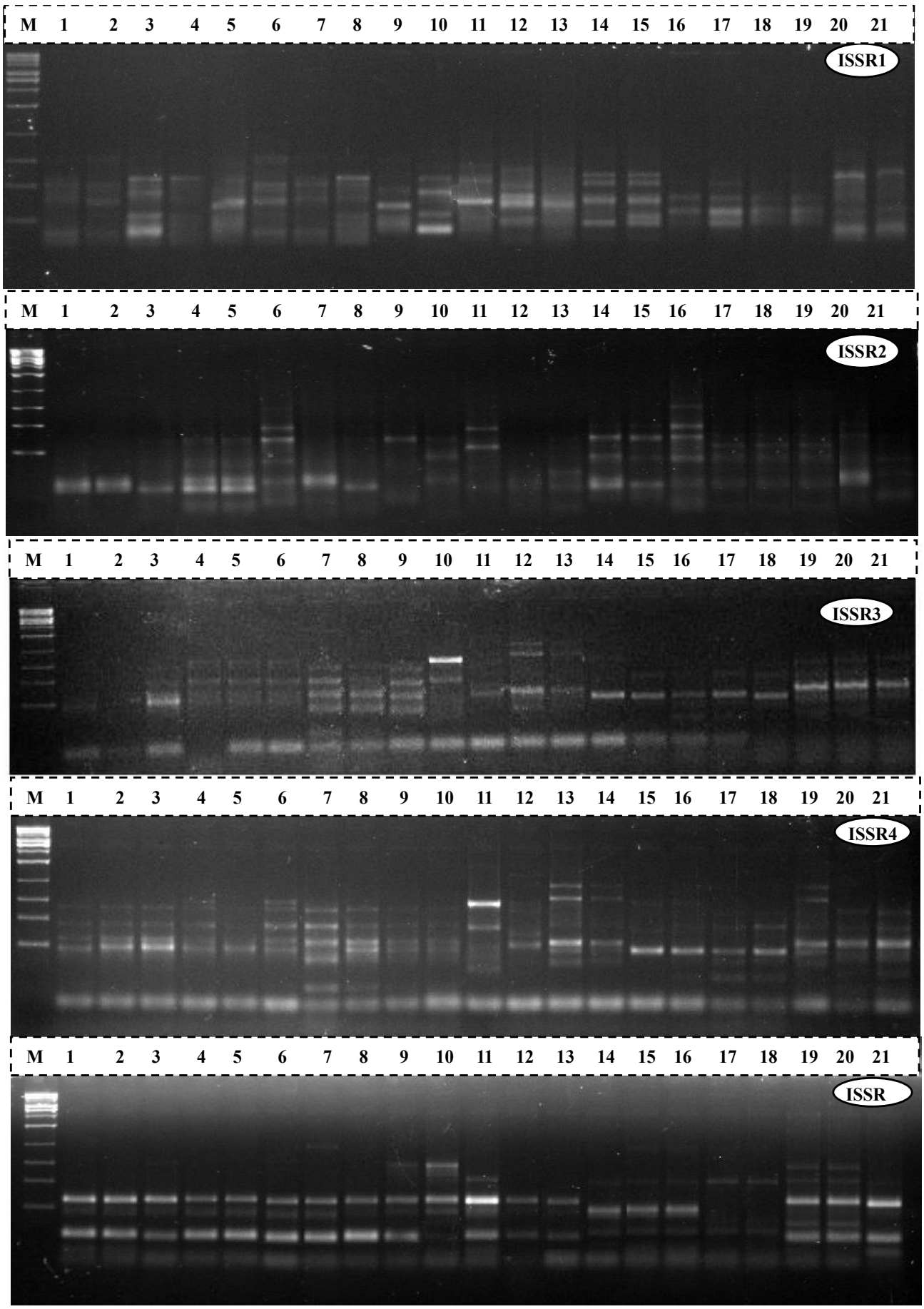

Fig. 1. DNA polymorphism generated by five ISSRs primers from the genomic DNA of the investigated species of Malvoideae. Species names are arranged and numbered as in Table 1.

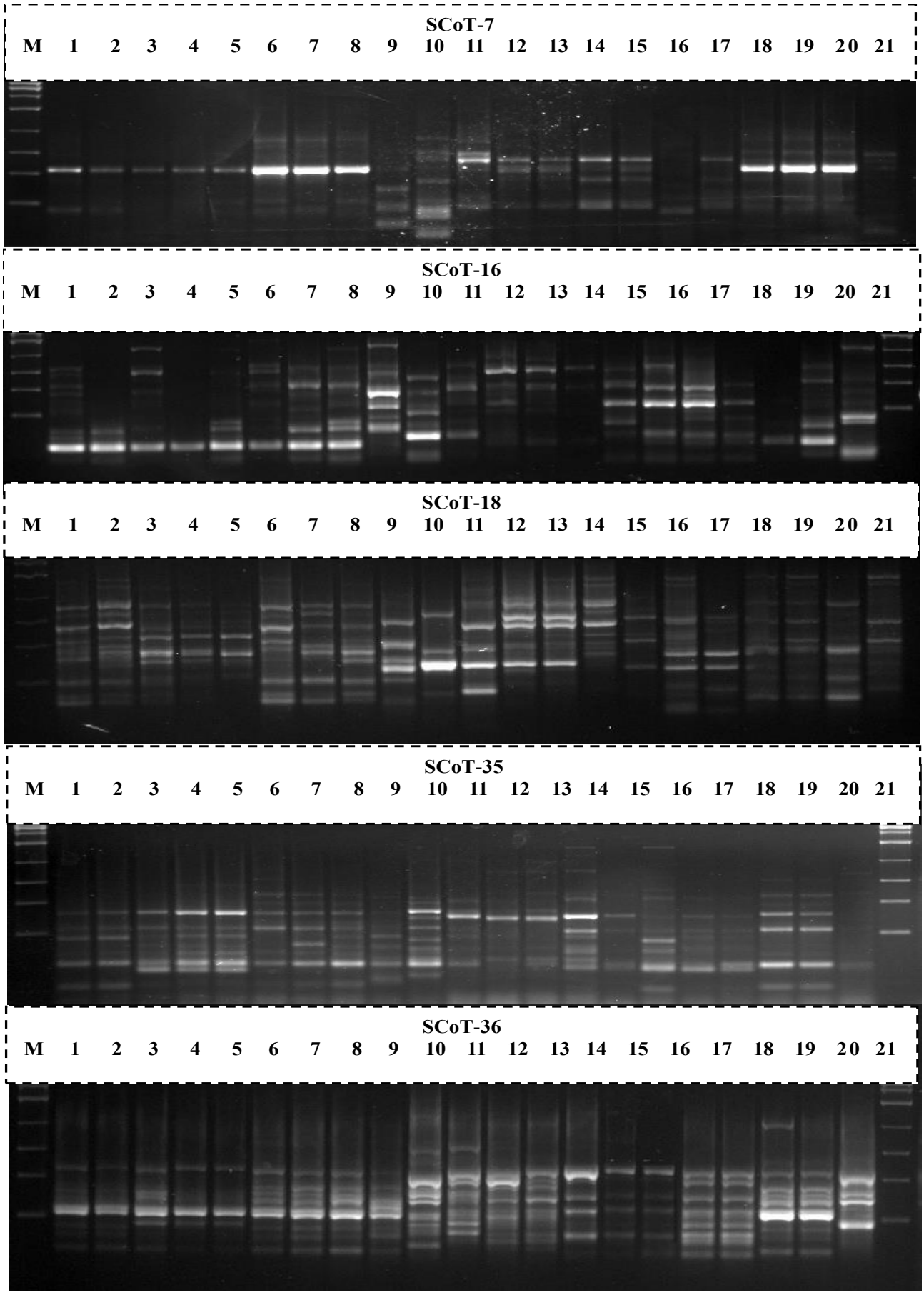

Fig. 2. DNA polymorphism generated by five SCoT primers from the genomic DNA of the investigated species of Malvoideae. Species names are arranged and numbered as in Table 1.

Table 3. Genetic similarity matrix among the studied taxa as computed according to Dice coefficient from combined of SCoT and ISSR primers. Species names from 1-21 as in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1.00																				
2	0.91	1.00																			
3	0.78	0.78	1.00																		
4	0.73	0.76	0.81	1.00																	
5	0.74	0.77	0.75	0.92	1.00																
6	0.81	0.82	0.73	0.71	0.69	1.00															
7	0.81	0.79	0.73	0.68	0.70	0.82	1.00														
8	0.81	0.78	0.78	0.71	0.72	0.78	0.83	1.00													
9	0.68	0.66	0.67	0.63	0.68	0.69	0.71	0.77	1.00												
10	0.62	0.60	0.60	0.56	0.56	0.63	0.60	0.64	0.57	1.00											
11	0.67	0.69	0.57	0.57	0.58	0.70	0.62	0.62	0.63	0.69	1.00										
12	0.63	0.59	0.56	0.54	0.53	0.59	0.58	0.60	0.62	0.53	0.65	1.00									
13	0.60	0.63	0.57	0.60	0.63	0.61	0.58	0.61	0.63	0.54	0.67	0.81	1.00								
14	0.60	0.63	0.57	0.62	0.63	0.64	0.59	0.59	0.59	0.54	0.61	0.68	0.70	1.00							
15	0.64	0.64	0.61	0.64	0.67	0.65	0.60	0.65	0.68	0.59	0.63	0.68	0.63	0.75	1.00						
16	0.61	0.66	0.62	0.64	0.66	0.62	0.57	0.60	0.59	0.59	0.57	0.51	0.56	0.68	0.71	1.00					
17	0.68	0.65	0.59	0.62	0.64	0.60	0.61	0.62	0.64	0.61	0.70	0.63	0.63	0.64	0.77	0.70	1.00				
18	0.74	0.74	0.67	0.65	0.68	0.70	0.65	0.73	0,67	0.61	0.72	0.62	0.66	0.64	0.73	0.70	0.84	1.00			
19	0.70	0.71	0.67	0.60	0.62	0.75	0.69	0.72	0.67	0.60	0.67	0.59	0.67	0.64	0.60	0.61	0.66	0.79	1.00		
20	0.71	0.70	0.70	0.68	0.66	0.75	0.79	0.76	0.68	0.61	0.62	0.59	0.58	0.64	0.65	0.62	0.62	0.71	0.80	1.00	
21	0.63	0.62	0.66	0,60	0.57	0.63	0.64	0.64	0.61	0.58	0.58	0.61	0.60	0.61	0.59	0.58	0.66	0.63	0.63	0.67	1.00

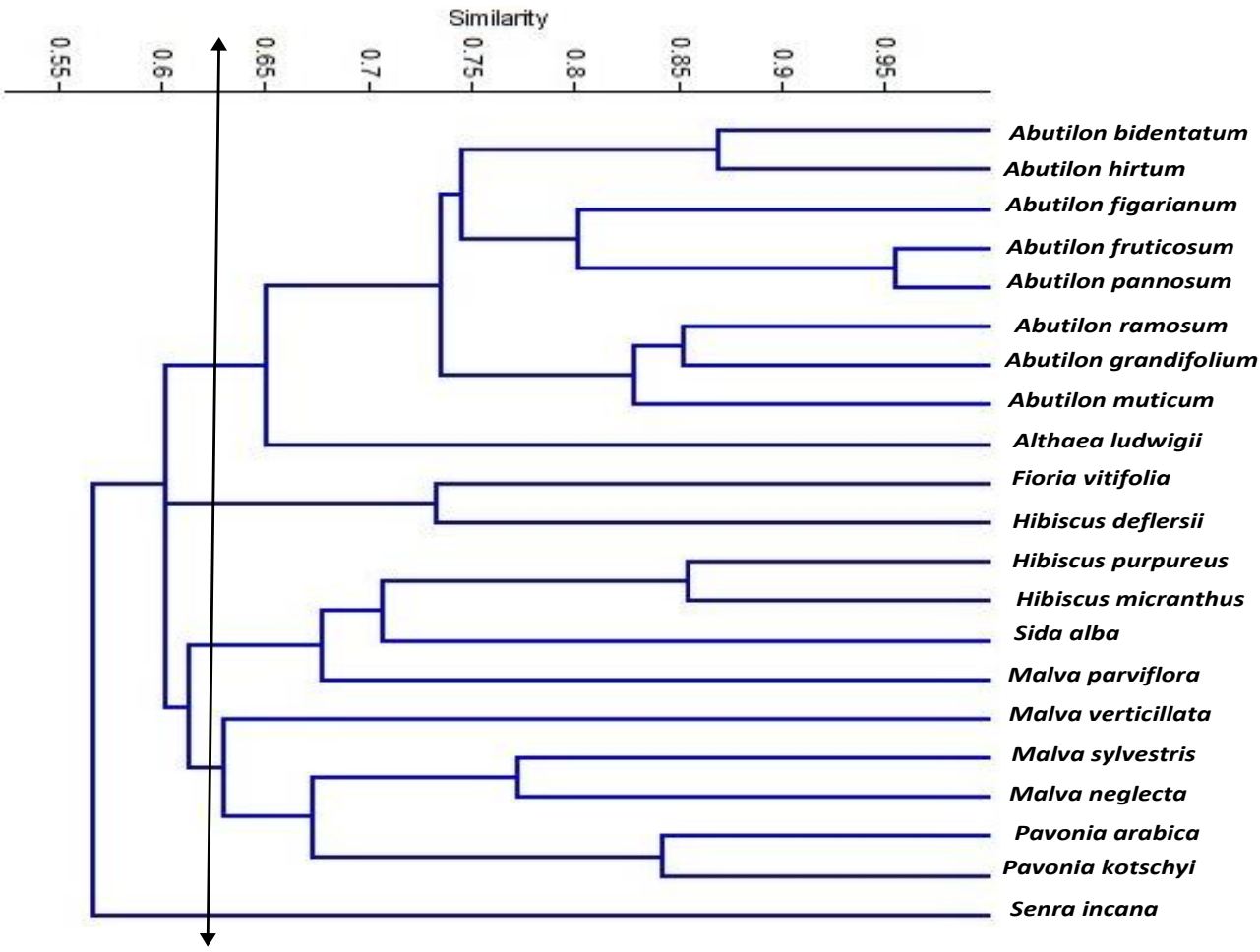

Fig. 3. UPGMA phenogram viewing the genetic diversity of the 21 species of Malvoideae based on SCoT characters.

Results

SCoT analysis: Ten SCoT primers were used to examine the outline of genetic differences among the 21 species of the family Malvaceae growing in Saudi Arabia. Each primer was verified on all samples and was selected for genotype analysis because its patterns were reproducible and constant. Five primers exhibited polymorphism. Polymorphic bands were selected for recognizing the genetic resemblance among the group of species. A total of 89 reproducible polymorphic bands were identified by using five SCoT-PCR primers. The average similarity coefficient ranged from 0.55 to 1.00. Primers SCoT-18 and SCoT-35 produced the maximum number of polymorphic amplifications of DNA fragments (20 bands). A phenogram was constructed based on the similarity coefficients to establish the relationship between the studied taxa. To calculate, 100 bands were grouped and the number of bands for each size of DNA fragments (bp) was counted for every species. One branch and four clusters sharing 0.65 similarities were noted (Fig. 3) as: (i) a branch including *Senra incana*; (ii) a cluster comprising of eight species of *Abutilon* and *Althaea ludwigii* having 0.65 genetic similarities; (iii) a cluster including *Fioria vitifolia* and *Hibiscus deflersii* with 0.75 genetic similarities; (iv) a cluster of *Hibiscus purpureus*, *Hibiscus micranthus, Sida alba,* and *Malva parviflora*; (v) a cluster including *Malva sylvestris*, *Malva neglecta*, *Pavonia arabica* and *Pavonia kotschyi* with 0.67 genetic similarities.

ISSR analysis: Five ISSR primers were used to examine the genetic variations among the species of Malvaceae growing in the wild habitat of Saudi Arabia. In total, these primers produced 60 reproducible bands (49 polymorphic bands and 11 monomorphic bands). These bands were used for studying the genetic similarity among the species. The average similarity coefficient ranged from 0.60 to 1. The results indicated that all primers were polymorphic. Primer ISSR- 4 produced the highest number (12 bands) of polymorphic DNA fragments. The results of the consensus tree from ISSR data displayed that the tree was divided into two branches and three clusters with 0.65 similarities (Fig. 4): (i) a branch including *Fioria vitifolia*; (ii) a branch including *Hibiscus deflersii*; (iii) a cluster of *Althaea ludwigii* and eight species of *Abutilon* with 0.75 genetic similarities; (iv) a cluster including *Hibiscus purpureus* and *Hibiscus micranthus* with 0.75 genetic similarities; (v) a cluster including a subgroup of *Sida alba, Malva verticillata*, *Malva parviflora, Malva neglecta, Malva sylvestris,* and *Pavonia arabica* with a similarity score of 0.75, and another subgroup of *Pavonia kotschyi* and *Senra incana* having a similarity score of 0.88.

Combined analysis of SCoT and ISSR markers: The UPGMA dendrogram achieved from the cluster analysis of SCoT and ISSR joined data exhibited almost a similar clustering pattern, and the similarity coefficient ranged from 0.60 to 0.96. The consensus tree was separated into two major branches and three clusters with a similarity

score of 0.65 (Fig. 5): (i) a branch including *Senra incana*; (ii) a branch of *Fioria vitifolia*; (iii) a cluster comprising of *Hibiscus deflersii*, *Malva neglecta*, *Malva sylvestris*, *Malva parviflora, Sida alba,* and *Malva verticillata* with a similarity score of 0.67; (iv) a cluster of three groups; the first group included *Pavonia arabica* and *Pavonia kotschyi;* the second group contained eight species of *Abutilon*; and the third group comprised of *Althaea ludwigii* with a similarity score of 0.73; (v) a cluster consisting of *Hibiscus purpureus* and *Hibiscus micranthus* with 0.85 genetic similarities.

Discussion

Several researchers have developed classification systems to categorize the family Malvaceae into subfamilies, tribes, and subtribes (Bentham & Hooker, 1862, Kearney, 1951, Schultze-Motel, 1964, Hutchinson, 1967, Bates, 1968, Krebs, 1994, Duke & Doebley, 1995). These studies were based on a few traits including life forms, fruits, seeds, carpel morphology, and number and position of ovules in each carpel. Environmental conditions change the morphological characters of the plants, which may affect the divergence during classification.

SCoT and ISSR molecular markers generate reliable and reproducible bands and are broadly used for the genetic analysis of different plant populations (Nagaoka & Ogihara, 1997, Collard & Mackill, 2009, Zhang *et al*., 2015). The present study was established that both SCoT and ISSR techniques combined with the right statistical tools can accurately evaluate the genetic diversity and analyze the phylogeny of subfamily Malvoideae. SCoT and ISSR markers depicted significant differences during the detection of polymorphism and discriminating capacity. However, both techniques are exhibited almost similar topology in dendrograms, which were generated based on the similarity matrices. A significant link between these two dendrograms suggested that both markers were similarly efficient in measuring phylogenetic relationships among the investigated taxa. The genotype scattering on the consensus tree, which was constructed based on the shared banding patterns of SCoT and ISSR, may significantly vary as each technique magnifies different parts of the genome (Abd El-Hak *et al*., 2019a**)**. The SCoT markers use longer primers and are very reproducible whereas ISSR amplifies the region between two microsatellites (Abd El-Hak *et al*., 2019b**)**. Hence, polymorphisms reveal the variety of these regions in the genome. Therefore, to generate a reliable consensus tree the banding patterns of both techniques should be used to cover expanded sites of the genome. In general, results of SCoT and ISSR analyses proposed groups and partially established the tribes, subtribes, and section classification of Malvoideae as has been reported with traditional methods (Mattei, 1915, Kearney, 1951, Schultze-Motel, 1964, Hutchinson, 1967, Bates, 1968, La Duke & Doebley, 1995), and molecular data (Fryxell, 2002, Bayer & Kubitzki, 2003, Tate *et al*., 2005, Reveal, 2012).

***Abutilon* group (G2 B):** Adaptation of *Abutilon* group under various climatic conditions and better plasticity are the main reason for its complex taxonomy. According to Hutchinson (1967), the tribe Abutileae comprises two subtribes: Abutilinae (including *Abutilon*) and Sidinae (including *Sida* and *Malvastrum*). However, the tribe Abutileae is considered within Malveae in the system of Takhtajan (2009). Reveal (2012) separated both subtribes Abutilinae and Sidinae from Malveae under tribe Sidieae by using molecular data. The infrageneric classification of *Abutilon* is not properly understood. Previously, the genus has been ordered into sections and subsections but only for the species of limited geographical areas such as Brazilian species (Schumann, 1891), East African species by using the ranks, stirps and substirps (Mattei, 1915), and Mexican species (Fryxell, 1988). Mattei (1915) grouped the East African *Abutilon* into three natural stirps (Capsulati, Cephalocarpi, and Monospermi) and seven substirps (Fruticosi, Indici, Cuispidati, Graveolenti, Mericarpi, Blepharocarpi, and Mutici) based on the seeds, carpels, and leaf morphology.

The results of a systematic revision of *Abutilon* (Mattei, 1915) species distributed in Saudi Arabia were compared with the findings of this study. Mattei (1915) treated *A. fruticosum* under stirps Capsulati substirps Frutieosi; *A. ramosum* in stirps Capsulati substirps Cuspidati; *A. figarianum* in stirps Cephalocarpi substirps Graveolenti; *A. hirtum*, *A. bidentatum*, and *A. grandifloium* in stirps Cephalocarpi substirps Mierocarpi; and *A. pannosum* and *A. muticum* in stirps Cephalocarpi substirps Mutici. Fryxell (2002) presented a nomenclature of more than 500 names at the specific rank and 25 names in the infrageneric rank of *Abutilon*. He treated *A. bidentatum* and *A. grandifloium* in section Beloere; *A. muticum*, *A. figarianum*, *A. pannosum* in section Muticum; and *A. fruticosum* in section Oligocaroae. Fuertes Aguilar *et al*., (2003) also studied the phylogenetic relationship between the members of subtribe Abutilinae. Based on the internal transcribed spacers of nuclear ribosomal DNA (ITS) the taxa in the *Sida* generic alliance from 58 species of Malvaceae were sequenced. The ITS data revealed that *Abutilon* and *Sida* were not monophyletic. Similarly, Tate *et al*., (2005) studied the phylogenetic relations within Malveae tribe based on sequence data from (ITS) regions of the 18–26S nuclear ribosomal repeat and accepted two main clades: one comprising of *Abutilon* and *Sida* (Abutilon alliance) and a second covering the rest of taxa revealing that *Abutilon*, *Sida,* and *Tetrasida* are not monophyletic. Taia (2009) investigated the morphology-based systematic revision of five *Abutilon* species from Saudi Arabia. He found a close relationship between species and classified them into two groups.

According to the combined SCoT and ISSR tree (group 2B), the studied taxa of *Abutilon* were grouped in one cluster that split into two groups. One group was comprised of *A. bidentatum*, *A. hirtum*, *A. grandifloium*, and *A. muticum* (Stirps Cephalocarpi), and *A. ramosum* (stirps Capsulati) with 0.82 genetic similarities. These species are morphologically distinguished by the dorsally dehiscent fruit, mericarps that lack wings, absence of epicalyx, and leaves that are sub-entire to serrate margins (Taia, 2009). The second group includes *A. pannosum* and *A. figarianum* (Stirps Cephalocarpi), and *A. fruticosum* (Stirps Capsulati) with 0.80 genetic similarities. All of the three species have rounded or largely ovate leaf blades with either acute or rounded apices. The results of this study propose that the species of *Abutilon* form a polyphyletic group. Our data support previous approach of distinctly treating tribe Abutileae (Sidieae sensu Reveal, 2012) and its two subtribes from Malveae. Therefore, these results are partially in line with Mattei (1915) and Fryxell (2002), and congruent with the findings of Reveal (2012), Tate *et al*., (2005) and Fuertes Aguilar *et al*., (2003).

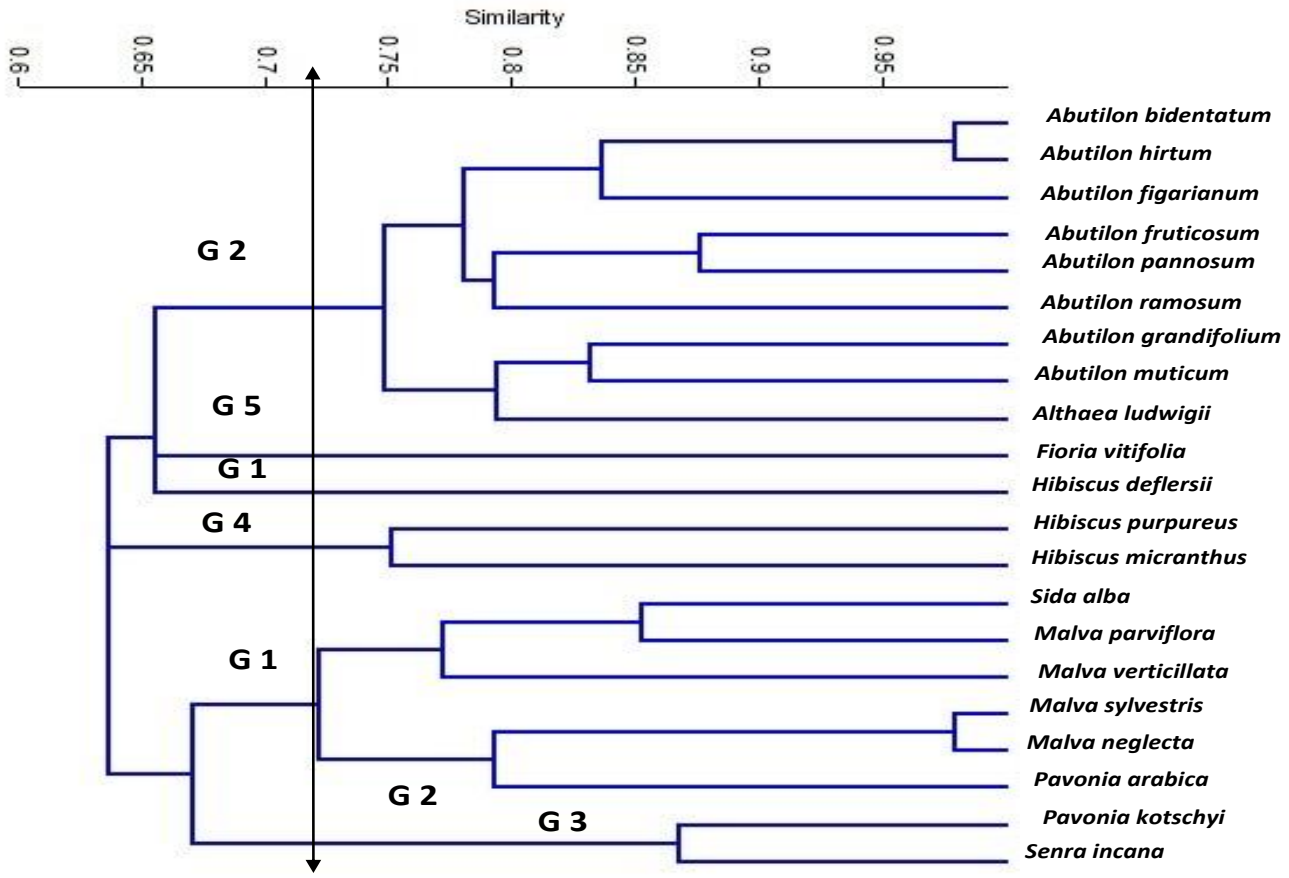

Fig. 4. UPGMA phenogram viewing the genetic diversity of the 21 species of Malvoideae based on ISSR characters.

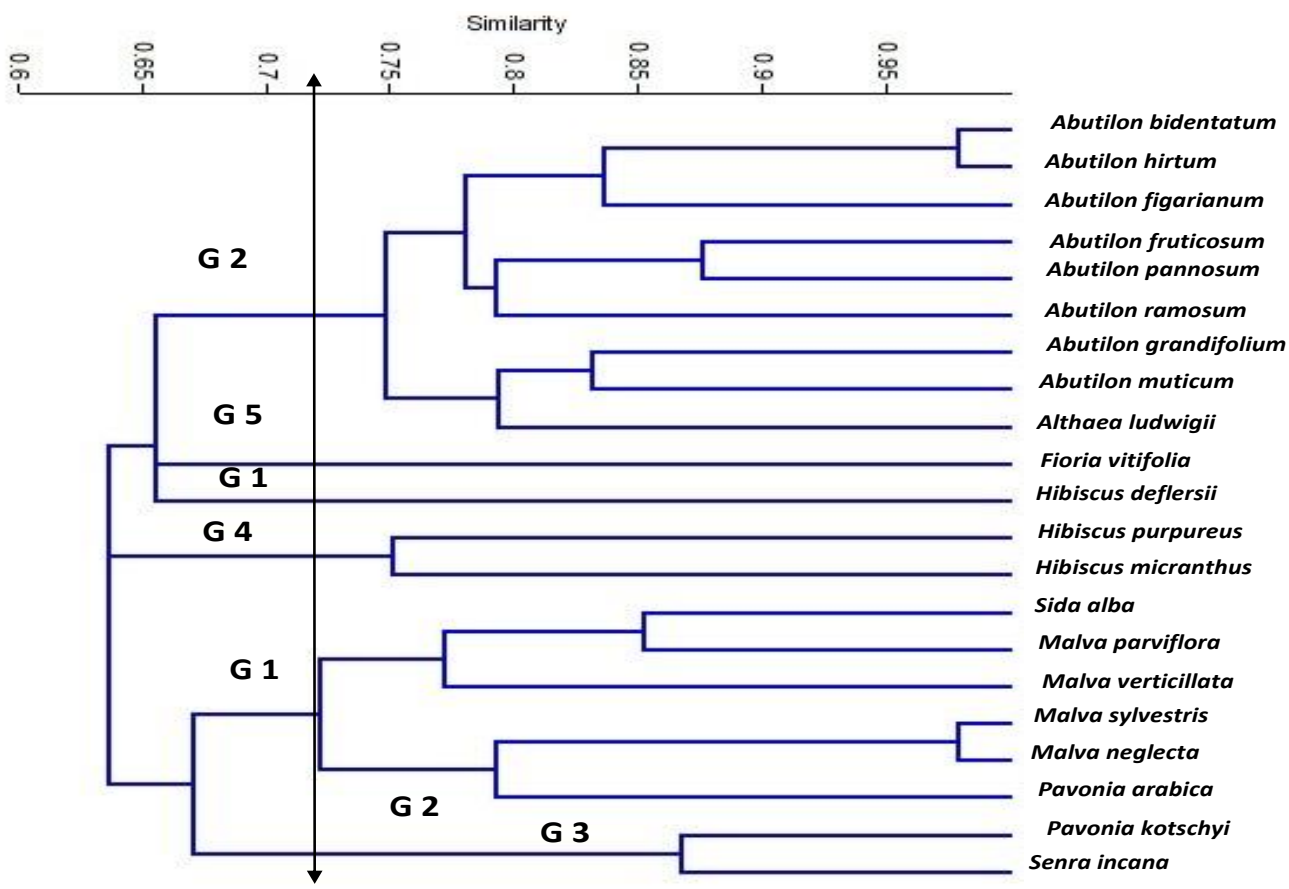

Fig. 5. UPGMA phenogram viewing the genetic diversity of the 21 species of Malvoideae based on SCoT and ISSR characters.

***Pavonia* group (G 2 A):** This group was exhibiting 0.80 genetic similarities comprises of only *P. arabica* and *P. kotschyi* and had been documented as a distinct clade. Specific features like the presence of epicalyx, perennial, leaves not lobed, and five mericarps with one seed per mericarp define these species. Fryxell (1997) investigated *Pavonia* of the new world and reported that all species of this genus contain the same base chromosome number (x=7). Most of the authors have placed *Pavonia* in a separate tribe Ureneae (Bentham & Hooker, 1862; Schultze-Motel, 1964; Hutchinson, 1967; Cheek, 2007) except Takhtajan (2009) and Reveal (2012) who placed this genus with *Hibiscus* in the tribe Hibiscieae. Our data support the previous work of treating *Pavonia* under the separate tribe Ureneae.

***Althaea* clade (G 2 C):** Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012) were treated this genus under the tribe Malveae. However, Jun Qiana *et al*., (2020) was used a complete chloroplast genome sequence for the phylogenetic analysis of *Althaea rosea* and depicted a close relationship among *Althaea*, *Gossypium,* and *Hibiscus* in Malvaceae. The results of combined SCoT and ISSR tree do not support the placement of *Althaea ludwigii* in tribes Malveae or Hibisceae as *Althaea ludwigii* was assigned to a distinct branch with high genetic similarities. This species morphologically differs from the other species by having an annual life form, indehiscent fruit, three epicalyx segments, many mericarps having a single seed in each. The results of this study disagree with Bentham & Hooker (1862), Hutchinson (1967), Reveal (2012), and Jun Qiana *et al*., (2020), for treating it as tribe Malveae or Hibisceae. Further investigations are necessary to clarify this assumption.

***Senra* clade (G 3):** This branch includes only *Senra incana* and is known as a separate clade with high genetic similarities. Several features such as spheroidal pollen shape, hairy seed, 5-branched style, capsule containing 1-locule and 1-seed, and absence of oil glands in calyx distinguish it from others (Abdel Khalik & Al-Ruzayza, 2021).

Bentham & Hooker (1862), and Hutchinson (1967) were treated this genus in the tribe Hibiscieae. However, Reveal (2012) used molecular data to place *Senra* in the tribe Gossypieae. Our data support the previous work of treating *Senra* in tribe Gossypieae (Reveal, 2012), because it has a unique characters and further support comes from the molecular data of SCoT and ISSR, which indicates that is monophyletic clade.

***Fioria* clade (G 5):** The results revealed that *Fioria vitifolia* showed the largest distance from all other groups. The characteristics such as 5-toothed persistent calyx without oil glands, five broadly winged fruits, tuberculate, and seed reniform make it distinct from others. Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012) have treated this genus in the tribe Hibiscieae. Our data support the previous work of treating *Fioria* in a tribe Hibiscieae and in congruence with those authors.

***Hibiscus* groups (G 1 & G 4):** The results of our study do not support the monophyly of the non-natural section Bombycella as the *H. purpureus, H. micranthus,* and *H. deflersii* were situated within two separate clusters and clade sharing 0.82 genetic similarities.

Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012) treated this genus in the tribe Hibisceae. Hochreutiner (1900) classified the genus *Hibiscus* into 12 sections and concluded that *Hibiscus* is very heterogeneous and needs more attention. Ulbrich (1921) divided section Bombycella into three subsections: Syriaca, Eubombycella, and Africana. Moreover, Cufodontis (1948) studied *Hibiscus* species in Africa and treated *H. purpureus, H. micranthus*, and *H. deflersii* in section Bomycella. Fryxell (1980) further concluded that section *Bombicella* is paraphyletic and the second most diverse section of *Hibiscus* after section *Furcaria*. They also reported significantly variable chromosome numbers among species such as American species x=11 diploids, African species x=16 diploids or tetraploids, and Australian species 2n= 54 allotetraploids. Based on the results we propose that species of section Bombicella subsection Eubombycella (Cufodontis, 1948, Engler, 1921) form a paraphyletic group. These results are similar to the findings of Hochreutiner (1900) and Fryxell (1980) who treated section Bombycella as a heterogeneous section.

***Malva-Sida* group (G 1):** Bentham & Hooker (1862), Hutchinson (1967), and Reveal (2012) treated *Malva* in the tribe Malveae. However, they preserved *Sida* in the tribes Malveae, Abutileae, and Sideae, respectively. Based on flower structure, Baker (1890) divided the genus *Malva* into three sections: Bismalva, Bibracteolata, and Fusciculata including *M. parviflora, M. sylvestris, M. neglecta,* and *M. verticillata*. However, Dalby (1968) categorized European species of the genus *Malva* into two sections Bismalva and Malva. The section Bismalva includes species consisting of a single flower on the leaf axils or possesses a congested terminal raceme, while the species of the section Malva have two or more flowers on each leaf axil (Dalby, 1968). Bates (1968) suggested that *Malva* might be polyphyletic as its chromosome number is in the range of 2n=14 and therefore, should be considered diploids, whereas species having chromosome number 2n=40-44 are hexaploids and this number is related to *Sida* and other taxa. Furthermore, Luque & Devesa (1986) reported hexaploids with chromosome base number 2n= 42 in *M. parviflora, M. neglecta, M. sylvestris*, and dodecaploids (chromosome count=76, 84, 112) in *M. verticillata*. Besides, Fryxell (1997) reported that *Sida* is heterogeneous and has the same base chromosome number x=7 or 8 (2n=14, 28; 2n=16, 32). Based on an ITS sequence analysis, Ray (1995) differentiated Malvoid and Lavateroid groups and placed all *Malva* species in the malvoid group. Similarly, Garcia *et al*., (2009) used five molecular markers (ITS, matK + trnK, ndhF, trnL-trnF, and psbA-trnH) to investigate a phylogenetic hypothesis of *Malva* alliance (Malvaceae), and reported that *Althaea, Malva*, and *Lavatera* are highly polyphyletic. Based on total plastid markers, they treated *M. neglecta* and *M. sylvestris* in one subgroup with 0.96 genetic similarities whereas *M. verticillata* and *M. parviflora* belong in another

subgroup having 0.82 genetic similarities. Furthermore, Celka *et al*., (2010) determined genetic relationships among eight *Malva* taxa by using ISSR and ISJ markers. The species were classified into two groups consistent with the sections *Bismalva* (*M. excisa, M. alcea*, and *M. moschata*) and *Malva* (*M. neglecta*, *M. sylvestris, M. pusilla*, and *M. verticillata*). The results of combined SCoT and ISSR tree support the placement of *M. neglecta*, *M. sylvestris, M. parviflora*, and *M. verticillata* in the section Malva but it is not monophyletic. This is due to the placement of *M. verticillata*, *M. parviflora,* and *Sida alba* within a separate sub-cluster, and *M. neglecta*, *M. sylvestris*, and *H. deflersii* in another sub-cluster with 0.71 genetic similarities. Schizocarp fruit, many mericarps, unlobed leaves, and base chromosome number x=7 (Bates 1968) morphologically differentiate this cluster from other species.

In general, morphological results are compatible with phylogenetic studies. The results of our study are largely consistent with the findings of Bates (1968), Ray (1995), Garcia *et al*., (2009), and Celka *et al*., (2010) that *Malva* is highly polyphyletic. Our results are also in line with the morphological division of *Malva* into sections as suggested by Baker (1890), and Dalby (1968).

Conclusion

Genetic investigation and phylogenetic analyses of 21 species, representing 8 genera of the subfamily Malvoideae, from Saudi Arabia were carried out by using united of ISSR and SCoT markers. Five clusters and clades can be accepted within Malvoideae, which generally verified traditional groupings but partially disagreed as well. The results of this study offer valuable data about the taxonomy of Malvoideae at tribe, infrageneric, and subgeneric levels. In general, results were largely consistent with the previous phylogenetic findings that *Abutilon, Hibiscus,* and *Malva* are polyphyletic, and that the species of sections Bombicella and *Malva* are highly heterogeneous. A remarkable result of this study was to identify *Senra incana* with distinctive characters and reported that it should be preserved as a separate tribe. Similarly, differences between the closely related genera *Fioria* and *Althaea* were also noted suggesting that they should be placed in different tribes. Molecular data of SCoT and ISSR indicated similarity among the species of *Pavonia* and supported the monophyly of this genus. Nevertheless, we believe that molecular and morphological techniques should be combined to achieve a generally acceptable phylogenetic reconstruction of Malvoideae. Moreover, a broad study covering extra species from different genera is necessary for reliable classification.

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